

SIRT2 deacetylase regulates the activity of GSK3 isoforms independent of inhibitory phosphorylation

Mohsen Sarikhani¹, Sneha Mishra¹, Sangeeta Maity¹, Chaithanya Kotyada², Donald Wolfgeher³, Mahesh P. Gupta⁴, Mahavir Singh², Nagalingam R. Sundaresan^{1, #}

¹ Department of Microbiology and Cell Biology, Indian Institute of Science, Bengaluru, India

² Molecular Biophysics Unit, Indian Institute of Science, Bengaluru, India

³ Department of Molecular Genetics and Cell biology, University of Chicago, Chicago, Illinois, USA

⁴ Department of Surgery, University of Chicago, Chicago, Illinois, USA

Short title: Lysine acetylation inhibits GSK3 activity

[#] Corresponding author; Mailing address: Lab # SB-02, Department of Microbiology and Cell Biology, Division of Biological Sciences, Indian Institute of Science, Bengaluru – 560012, Karnataka, India. Email: rsundaresan@iisc.ac.in
Phone: +91 80 2293 2068, Fax: +91 80 2360 2697

Highlights

- Acetylation at Lys183 reduces enzymatic activity of GSK3 β independent of Ser9 inhibitory phosphorylation.
- Acetylation of GSK3 β at Lys183 by acetyl transferase p300 might hinder both the adenosine binding as well as prevent stable interactions of negatively charged phosphates with GSK3 β .
- MS/MS analysis and molecular modelling of GSK3 α suggests that acetylation of Lys246, which is analogous to residue Lys183 of GSK3 β , might reduce the enzymatic activity of GSK3 α by reducing its ATP binding ability.
- SIRT2 deacetylase binds to and deacetylates GSK3 β at Lys183, and thus enhances binding to ATP.
- GSK3 is required for the anti-hypertrophic function of SIRT2 in cardiomyocytes.

Abstract

Glycogen synthase kinase 3 (GSK3) is a critical regulator of diverse cellular functions involved in the maintenance of structure and function. Enzymatic activity of GSK3 is inhibited by N-terminal serine phosphorylation. However, alternate post translational mechanism(s) responsible for GSK3 inactivation are not characterized. Here, we report that GSK3 α and GSK3 β are acetylated at Lys246 and Lys183 respectively. Molecular modeling and/or molecular dynamics simulations indicate that acetylation of GSK3 isoforms would hinder both the adenosine binding and prevent stable interactions of the negatively charged phosphates. We found that SIRT2 deacetylates GSK3 β , and thus enhances its binding to ATP. Interestingly, the reduced activity of GSK3 β is associated with lysine acetylation, but not with phosphorylation at Ser9 in hearts of SIRT2-deficient mice. Moreover, GSK3 is required for the anti-hypertrophic function of SIRT2 in cardiomyocytes. Overall, our study identified lysine acetylation as a novel post-translational modification regulating GSK3 activity.

Keywords: Lysine acetylation; Sirtuins; SIRT2; p300; GSK3 α ; GSK3 β

70 Introduction

71 Glycogen synthase kinase 3 (GSK3) is a highly conserved, ubiquitously expressing kinase
 72 having 2 isoforms, α and β (Woodgett, 1990). Both these GSK3 isoforms are encoded by
 73 distinct genes, which share 97% amino acid sequence identity within their catalytic domains and
 74 differ significantly outside the catalytic domain (Kaidanovich-Beilin and Woodgett, 2011). In
 75 cardiomyocytes, GSK3 α preferentially localizes inside the nucleus, while GSK3 β is present
 76 mostly in the cytoplasm (Matsuda et al., 2008). Increasing lines of evidence suggest that
 77 GSK3 α and GSK3 β have both common and non-overlapping functions (Rayasam et al., 2009).
 78 Unlike other kinases, GSK3 requires a primed substrate for its function (Cohen and Frame,
 79 2001; Doble and Woodgett, 2003). Activity of GSK3 is carefully regulated by the combination of
 80 (a) Tyrosine phosphorylation at catalytic domain (Dajani et al., 2001; Hughes et al., 1993), (b)
 81 N-terminal serine phosphorylation (Cross et al., 1995) and (c) sub-cellular localization (Beurel
 82 et al., 2015). Studies indicate that GSK3 is constitutively phosphorylated (Tyr279-GSK3 α and
 83 Tyr216-GSK3 β) and that tyrosine phosphorylation is necessary for its catalytic activity.
 84 Phosphorylation of Tyr279 in GSK3 α and Tyr216 in GSK3 β has been reported to facilitate
 85 GSK3 activity by promoting substrate accessibility (Dajani et al., 2001; Hughes et al., 1993).
 86 Interestingly, phosphorylation of GSK3 β at Tyr216 occurs through intramolecular
 87 autophosphorylation during protein folding (Cole et al., 2004). In contrast, N-terminal serine
 88 phosphorylation of GSK3 i.e. Ser21- GSK3 α and Ser9-GSK3 β by several kinases including Akt,
 89 protein kinase A, protein kinase C and p90Rsk has been shown to decrease its catalytic activity
 90 (Ali et al., 2001; Beurel et al., 2015; Cross et al., 1995; Song et al., 2015). GSK3 β is inhibited by
 91 N-terminal Ser9 phosphorylation, in which the phosphorylated N terminus binds as a
 92 competitive pseudo-substrate with p-Ser9 occupying the P+4 site (Dajani et al., 2001; Stamos
 93 et al., 2014). Previous studies have shown that GSK3 β -S9A mutation, which prevents the N-
 94 terminal Ser9 phosphorylation, causes GSK3 β to be constitutively active (MacAulay et al.,
 95 2005; Stambolic and Woodgett, 1994). GSK3 β was also observed to be inhibited by
 96 phosphorylation at Ser398 by p38 MAPK (Thornton et al., 2008). However, alternate post
 97 translational mechanism(s) responsible for the GSK3 inactivation are not yet characterized.

98 Reversible lysine acetylation is known to regulate nuclear and non-nuclear proteins in the cell
 99 (Verdin and Ott, 2015). The acetylation reaction is catalyzed by a group of enzymes called
 100 histone acetyl transferases, and is reversible by histone deacetylases (HDACs) (Haberland et
 101 al., 2009). Reversible acetylation has been shown to be responsible for activation/inactivation,
 102 sub-cellular localization, DNA binding, protein-protein interaction, membrane trafficking, stability
 103 and degradation of proteins (Choudhary et al., 2009; Jeffers and Sullivan, 2012; Yang and Seto,
 104 2008). Acetylation of a protein may enhance or repress its activity, and it has been shown that
 105 acetylation competes with phosphorylation (Kouzarides, 2000), and interferes with other lysine
 106 modifications, ubiquitination or sumoylation (Yang and Seto, 2008). HDACs are classified into 3
 107 distinct groups based on their structure and function (Class I to III) (Haigis and Guarente, 2006).
 108 The sirtuins, which are class III HDACs, are catalytically distinct, as they require NAD⁺ for their
 109 deacetylase activity. In mammals, seven different sirtuin isoforms (SIRT1 to 7), have been
 110 characterized (Guarente, 2007; Longo and Kennedy, 2006). SIRT2 is the cytoplasmic
 111 deacetylase that affects the microtubule network by deacetylating α -tubulin (North et al., 2003).
 112 In addition, SIRT2 regulates nuclear envelope dynamics, cardiac hypertrophy, metabolism and
 113 stress-induced cell death (de Oliveira et al., 2012; Kaufmann et al., 2016; Sarikhani et al.,
 114 2018a; Sarikhani et al., 2018b). In neurons, inhibition of SIRT2 salvage α -synuclein toxicity in

Parkinson's disease, and reduce sterol-mediated toxicity in Huntington's disease (Donmez and Outeiro, 2013). In contrast, SIRT2 deficient mice show increased mammary tumors, hepatocellular carcinoma and heart failure (Kim et al., 2011; Tang et al., 2017). Similarly, SIRT2 deficiency increases susceptibility to colitis and iron deficiency-induced hepatocyte cell death (Lo Sasso et al., 2014; Yang et al., 2017).

In the present work, we demonstrate that SIRT2 binds to and deacetylates GSK3 isoforms to promote ATP binding. Interestingly, reversible acetylation regulates the activity of GSK3 isoforms independent of its inhibitory phosphorylation. We also demonstrate that activity of GSK3 isoforms are essential for inhibition of cardiac hypertrophy by SIRT2 deacetylase.

Results

Acetyltransferase p300 Regulates the GSK3 β Acetylation

To test whether GSK3 β acetylation plays a role in the development of heart failure, we first created a mice model of cardiac hypertrophy. The cardiac hypertrophic agonist, isoproterenol (ISO) was infused chronically into mice by implanting osmotic mini-pumps in the peritoneal cavity (Sundaresan et al., 2009; Sundaresan et al., 2012). Chronic ISO infusion significantly increased heart weight/ tibia length (HW/TL) ratio as well as left ventricular wall thickness, which is an indicator of cardiac hypertrophy, while reducing the ejection fraction of heart (Figure 1A-1C), suggesting that ISO-infusion causes adverse cardiac remodeling and contractile dysfunction in mice. Previous studies indicate that GSK3 β activity is reduced in hypertrophic hearts (Sugden et al., 2008). To test whether GSK3 β activity is reduced in our cardiac hypertrophy model, we immunoprecipitated GSK3 β from the heart lysates and assessed *in vitro* activity of GSK3 β against the peptide of Glycogen synthase. Our results revealed that activity of GSK3 β was significantly low in ISO-infused heart samples (Figure 1D). Moreover, the levels of β -catenin, a GSK3 target transcription factor, which degrade after phosphorylation by GSK3 (Liu et al., 2002), was high in ISO-infused hearts (Figure 1E). These findings further confirm reduced activity of GSK3 β in ISO-infused heart samples. Next, we immunoprecipitated GSK3 β from heart lysates and assessed the status of its acetylation and levels of phosphorylation by Western blotting. Interestingly, we found markedly increased acetylation of GSK3 β in the heart samples of ISO-infused mice (Figure 1E, 1F). However, we did not see marked changes in Ser9 phosphorylation of GSK3 β in the chronic ISO infused heart samples (Figure 1E). Our results were consistent with previous observations, where the total protein levels and Ser9 phosphorylation of GSK3 β were not changed in the heart after pressure overload or myocardial infarction (Zhai et al., 2007). Similarly, the GSK3 β Ser9 phosphorylation was unchanged in the heart of mice-treated with ISO (Zhang et al., 2011). These findings indicate that the reduced activity of GSK3 β found in chronic ISO-infused hearts is correlated with increased lysine acetylation, but not with inhibitory Ser 9 phosphorylation. Furthermore, we observed reduced levels of Tyr216 phosphorylation of GSK3 β in ISO-infused heart samples. The expression levels of SIRT2 also reduced in the ISO-treated heart samples (Figure 1E), suggesting that acetylation of GSK3 β may be linked to reduced levels of SIRT2 deacetylase.

Acetyltransferase p300 has been shown to play a major role in the cardiac homeostasis (Yanazume et al., 2003; Yanazume et al., 2002). Our immunoprecipitation experiments showed that p300 binds to GSK3 β (Figure 1G). Further confocal microscopy analysis indicated that p300 co-localizes with GSK3 β (Figure 1H). Next, we assessed direct binding of p300 with

GSK3 β using recombinant purified proteins. Our results indicate that p300 binds to GSK3 β directly in an *in vitro* reaction (Figure 1I). In the next set of experiments, GSK3 β was immunoprecipitated from p300-depleted cells and its acetylation status and activity were analyzed. We found that the depletion of p300 resulted in reduced levels of acetylation, though the effect is not robust, it increased the activity of GSK3 β , as suggested by increase in GS phosphorylation (Figure 1J-1L). We believe that GSK3 β acetylation may be regulated by multiple acetyltransferases including p300. Interestingly, depletion of p300 markedly increased the phosphorylation of Tyr216, but not Ser9 in GSK3 β (Figure 1J). To validate our findings, we performed p300 overexpression experiments in cardiomyocytes. We found significantly increased acetylation of GSK3 β , which is associated with reduced activity of GSK3 β in p300 overexpressing cells (Figure 1M, 1N and 1O). Moreover, overexpression of p300 markedly reduced the Tyr216, but not Ser9 phosphorylation of GSK3 β (Figure 1M). Next, we tested the enzymatic activity of acetylated GSK3 β *in vitro* and found that p300 mediated acetylation significantly impairs kinase activity of GSK3 β (Figure 1P). These findings suggest that p300-mediated acetylation might influence the phosphorylation of GSK3 β at Tyr216 residue, but not Ser9, to inhibit the enzymatic activity.

Acetylation of GSK3 β Influences ATP binding: Insights from Molecular Modeling and Molecular Dynamics Simulation of GSK3 β WT and Acetylated K183 Mutant

In our previous work, we found that acetylation of GSK3 β at K15 promotes mitochondrial localization and is regulated inside the mitochondria by SIRT3 deacetylase. SIRT3 mediated acetylation of GSK3 β regulates the development of organ fibrosis (Sundaresan et al., 2016). Since SIRT2 is a cytoplasmic protein, we suspected that SIRT2 might not be regulating K15 acetylation of GSK3 β . Therefore, we performed tandem mass spectrometry (MS/MS) analysis of GSK3 β and identified two lysine residues, K150 and K183, as acetylation sites in GSK3 β (Figure 2A). While K183 is located in the vicinity of nucleotide binding pocket of GSK3 β , K150 is located on the surface away from both nucleotide binding pocket as well as substrate binding region (Figure 2B I-III). Magnesium is an important cofactor that helps in neutralizing the negative charges of the phosphate residues of nucleotide. Interestingly, the side-chain of K183 residue is proximal to the magnesium ion of the adenosine di-phosphate nucleotide (Figure 2B, 2C). The addition of the acetylated group to the NZ atom of K183 side-chain may result in destabilization of Mg²⁺ from ADP (Figure 2B II-IV). Overall, *in-silico* analysis suggests that the acetylation of K183 side-chain may reduce the affinity of adenosine tri-phosphate (ATP) nucleotide to the binding pocket by interfering with its magnesium cofactor.

To better understand the effect of acetylation of K183 on the binding of adenine nucleotide in the pocket of GSK3 β , we performed molecular dynamics simulations for the ADP loaded wild-type and acetylated K183 mutant (acK183). Initial model for the wild-type was generated from the crystal structure of GSK3 β (PDB ID 4NMO). For the acK183 mutant, the wild-type model was used as a template to generate in-silico acetylation on the K183 residue (further details in Materials and Methods). We ran five independent trajectories of 20 ns each totaling to 100 ns for each system. Multiple production runs would provide greater sampling of conformations for each system and further present an opportunity to capture the stochastic events, which otherwise might not be captured in a single long trajectory. Visual analyses of the molecular dynamics (MD) trajectories show drastic fluctuations in the ADP nucleotide with adenosine base completely destabilized in the acK183 mutant (Figure 2D). To quantify these effects, we first performed the root mean square deviation (RMSD) measurements for the C α backbone atoms

of the protein and ADP nucleotide, in both the systems. RMSD plots of the C α backbone atoms for the five individual trajectories of 20ns were plotted separately for the wild type and acK183 mutant (Figure 2E, 2H). The RMSD deviations were similar for both the plots ranging between 0.1 – 0.35nm (Figure 2F, 2I). In agreement, we found only moderate changes in the global root mean square fluctuations (RMSF) of all the backbone C α atoms of the acK183 mutant (Figure 2-figure supplement 1). Interestingly, we observed significant variations in the RMSD plots of the ADP nucleotide in the acK183 mutant compared to the wild-type (Figure 2F, 2I). The drastic deviations observed in the RMSD plots of ADP nucleotide in acK183 mutant concurs with the fluctuations seen in the snap-shots of structures during MD simulations (Figure 2D). For further analysis, we considered two trajectories with stable C α backbone RMSD's for each of the wild type (yellow, blue) (Figure 2E) and acK183 mutant (red, green) of GSK3 β (Figure 2E). We plotted time dependent distance measurements between protein-nucleotide interactions in the binding pocket of GSK3 β . We found that acetylation causes increase in distance between the side-chain amine of K85 and the phosphates of ADP nucleotide (Figure 2G, 2J). Positively charged K85 residue is essential for neutralizing the negative charge of phosphates of nucleotides. The escalation in distances between ADP and K85 suggest reduced stability for negatively charged phosphates in the acetylated mutant. Our results suggest that the acetylation would hinder both the adenosine binding as well as prevent stable interactions of the negatively charged phosphates with the protein. Collectively, we propose that the acetylation of K183 would destabilize the binding of the adenine nucleotide to the pocket of GSK3 β .

To test this possibility further, we performed ATP binding assay with wild type and mutant versions of GSK3 β , where K183 was mutated either to glutamine (Q, acetylation mimetic) or to arginine (R, deacetylation mimetic). GSK3 β -K183 mutations significantly reduced binding of ATP to GSK3 β , when compared to wild type GSK3 β (Figure 2K). These findings provided strong evidence that K183 is critically important for ATP binding to GSK3 β and any modifications, including acetylation might affect its ATP binding ability. To test whether K183 acetylation affects the catalytic activity, we performed in vitro kinase assay with mutants of GSK3 β . In this assay, kinase-dead GSK3 β -K85A was used as a negative control and GSK3 β -S9A was used as the catalytically active mutant. Our results indicate that mutation of K183 to either arginine or glutamine drastically reduced the catalytic activity towards a glycogen synthase peptide (Figure 2L). Mutation of K150, which is less conserved among species (Figure 2-figure supplement 2) and exhibited low stoichiometry of acetylation (Figure 2-figure supplement 3), had no effect on GSK3 β activity (Figure 2L). It is interesting to note that mutation of K183 to either K183R or K183Q, reduced the catalytic activity more than 4 folds, indicating that K183 is critical for the functionality of GSK3 β . Collectively, these findings suggest that K183 is an important residue for ATP binding and the catalytic activity of GSK3 β .

SIRT2 Modulates the Kinase Activity of GSK3 β by Reversible Acetylation

In our work, we found reduced levels of SIRT2 deacetylase in the ISO treated mice hearts. Since SIRT2 and GSK3 β share cytoplasmic localization, we suspected that SIRT2 may be the GSK3 β deacetylase. To test our hypothesis, we overexpressed all the sirtuin isoforms and assessed the acetylation, phosphorylation and activity of GSK3 β . Our results suggest that SIRT2 overexpression markedly reduced the acetylation of GSK3 β , while increasing its activity against glycogen synthase (Figure 3-figure supplement 1). Interestingly, we do not observe any changes in the phosphorylation of Ser9 residue of GSK3 β . However, we found increased phosphorylation of GSK3 β at Tyr216. Consistent with our previous work (Sundaresan et al., 2016), we found SIRT3 to be capable of deacetylating GSK3 β and enhancing its catalytic

activity (Figure 3-figure supplement 1). To further verify our findings, we immunoprecipitated GSK3 β from SIRT2-deficient heart lysates and tested the levels of acetylation. As expected, we found markedly increased acetylation of GSK3 β in SIRT2-KO hearts (Figure 3A, 3B). To test whether SIRT2 interacts with GSK3 β , we immunoprecipitated GSK3 β from heart samples and tested its binding with SIRT2 by Western blotting. We found that SIRT2 interacts with GSK3 β (Figure 3C). We also found that recombinant purified GSK3 β is capable of binding to SIRT2 *in vitro*, suggesting direct interaction between both proteins (Figure 3D). To test whether SIRT2 deacetylates GSK3 β , we performed an *in vitro* deacetylation assay, where the acetylated GSK3 β was incubated with either WT or SIRT2-H187Y, catalytic mutant of SIRT2 with and without NAD⁺. Western blotting analysis indicated WT, but not catalytic mutant-SIRT2 markedly reduced the acetylation status of GSK3 β in an NAD⁺ dependent manner (Figure 3E). These findings indicate that SIRT2 is a GSK3 β deacetylase. Next, we tested whether SIRT2-mediated deacetylation enhances the catalytic activity of GSK3 β by an *in vitro* kinase assay. The acetylated and deacetylated GSK3 β were incubated with GS peptide and the GSK3 β activity was monitored. Results indicated that acetylation significantly reduced the activity of GSK3 β . However, incubation of acetylated-GSK3 β with wild type, but not SIRT2-H187Y catalytic inactive mutant of SIRT2 restored the activity of GSK3 β in a NAD⁺ dependent manner (Figure 3F), suggesting that SIRT2-mediated deacetylation increases GSK3 β activity.

To further test the activity of GSK3 β , we measured the levels of phosphorylation of glycogen synthase in SIRT2 depleted cardiomyocytes. SIRT2 depletion increases the cellular global lysine acetylation and the acetylation of GSK3 β (Figure 3G). Moreover, GSK3 β -specific phosphorylation of glycogen synthase and β -catenin was reduced in SIRT2-depleted cardiomyocytes, which is associated with reduced Tyr216, but not Ser9 phosphorylation of GSK3 β (Figure 3G). To further validate the results *in vivo*, we tested the phosphorylation of glycogen synthase in SIRT2 deficient heart samples, and observed reduced phosphorylation of glycogen synthase, which is associated with reduced Tyr216, but not Ser9 phosphorylation of GSK3 β in SIRT2-KO hearts (Figure 3H). To further confirm the reduced activity of GSK3 β in SIRT2 deficient hearts, we performed an *in vitro* kinase assay. Consistent with our findings, we observed significantly reduced activity of GSK3 β towards a peptide of glycogen synthase (Figure 3I). Next, we measured the total and phosphorylated protein levels of β -catenin, a GSK3 target transcription factor, which is degraded, after phosphorylation by GSK3 (Liu et al., 2002), in the heart samples of SIRT2 deficient mice. Our results suggest that the protein levels of β -catenin are high in SIRT2-KO heart lysates, while the GSK3 target phosphorylation of β -catenin was decreased drastically (Figure 3H), indicating that SIRT2-mediated deacetylation might control the kinase activity of GSK3 β .

To further understand the mechanism involved in acetylation-mediated regulation of GSK3 β activity, we examined the subcellular localization of GSK3 β . Treatment of cells with a SIRT2 inhibitor (AGK2) enhanced the acetylation of tubulin, a well-accepted target of SIRT2, and the acetylation of GSK3 β , while decreasing its activity against glycogen synthase (Figure 3-figure supplement 2). Consistent with previous results, we did not observe any changes in the phosphorylation of Ser9 residue of GSK3 β following AGK2 treatment. However, we found decreased phosphorylation of GSK3 β at Tyr216 (Figure 3-figure supplement 2). Interestingly, AGK2 treatment had no effect on subcellular distribution of the GSK3 β (Figure 3-figure supplement 3). Next, we tested the localization of K183 mutants of GSK3 β . As expected, the results suggest that the WT and K183 mutants of GSK3 β exhibited cytoplasmic localization (Figure 3-figure supplement 4). These results indicate that change in the activity of GSK3 β is

not associated with its localization. To test whether SIRT2 deficiency is compensated by other sirtuin isoforms, we checked for the levels of all the sirtuins in SIRT2 deficient mice hearts. However, we found no difference in expression levels of any other sirtuin isoforms in SIRT2 deficient mice heart samples (Figure 3-figure supplement 5).

Our MS/MS analysis indicated that GSK3 β K150 and K183 residues are acetylated and GSK3 β K183 is involved in the regulation of its kinase activity. To test whether SIRT2 deacetylates K183, we performed an *in vitro* assay, where wild type and GSK3 β K183R mutants were incubated with SIRT2. Our results revealed that GSK3 β -K183R showed basal level acetylation, which is appreciably lower than the acetylation of wild type GSK3 β . These findings confirm our observations that GSK3 β is acetylated at multiple residues endogenously, and K183 residue is one among them. SIRT2 deacetylates the wild type GSK3 β , but not GSK3 β -K183R in the presence of NAD⁺, indicating that K183R may be the key target residue for SIRT2 deacetylase (Figure 3J, 3K). Collectively, these findings suggest that catalytic activity of GSK3 β is governed by acetylation at GSK3 β K183 residue. To test whether SIRT2 mediated deacetylation enhances the ATP binding to GSK3 β , we analyzed the ATP binding ability of acetylated and deacetylated GSK3 β . As expected, we found that acetylation inhibits the ATP binding, while SIRT2-mediated deacetylation restores the ATP binding ability of GSK3 β (Figure 3L).

GSK3 β kinase activity is known to be inhibited by N-terminal Ser9 phosphorylation (Ali et al., 2001; Song et al., 2015). Therefore, we tested the phosphorylation of GSK3 β in SIRT2-deficient heart samples with an antibody that recognizes the phosphorylation at Ser9. Surprisingly, we did not observe any differences in the phosphorylation at Ser9 of GSK3 β in control and SIRT2 deficient cardiomyocytes or hearts (Figure 3G and 3H), suggesting that the reduced activity of GSK3 β is associated with acetylation, but not the phosphorylation at Ser9 residue. We next tested whether the activity of constitutively active GSK3 β S9A mutant is inhibited by acetylation mimetic mutation (GSK3 β -S9A/K183Q). Our results suggest that the activity of GSK3 β -S9A mutant, which is not inhibited by the Ser9 phosphorylation, was reduced almost 3 folds when K183 was mutated to K183Q (Figure 3M). These findings suggest that K183 residue is critical for ATP binding in constitutively active GSK3 β S9A mutant. We presume that acetylation might inhibit the activity of constitutively active GSK3 β S9A mutant independent of inhibitory phosphorylation, but further experiments are required to prove this assumption.

Next we tested the phosphorylation of GSK3 β at Tyr216 residue, which is known to enhance GSK3 activity by promoting substrate accessibility (Dajani et al., 2001; Hughes et al., 1993). Surprisingly, we found markedly reduced phosphorylation of GSK3 β at Tyr216 residue in the cardiomyocytes or heart lysates of SIRT2-deficient mice (Figure 3G and 3H). These results suggest that acetylation might have negative impact on the activating tyrosine phosphorylation of GSK3 β .

Molecular Modeling of GSK3 α Suggests that the Acetylation of Residue K246 would have Similar Consequences as K183 of GSK3 β

GSK3 has two isoforms, α and β , which have overlapping cellular substrates and functions (Rayasam et al., 2009). In the heart, both α and β isoforms of GSK3 are expressed (Sugden et al., 2008). To test whether both isoforms of GSK3 are acetylated in SIRT2 depleted cells, we immunoprecipitated GSK3 isoforms and acetylation status was tested by Western blotting. We found both GSK3 α and GSK3 β to be significantly acetylated in SIRT2 depleted cells as compared to control cells (Figure 4A, 4B). Next, we tested whether SIRT2-mediated

deacetylation increases the catalytic activity of GSK3 α . The acetylated and deacetylated GSK3 α was incubated with GS peptide and the GSK3 α activity was measured. Results suggest that acetylation significantly reduced the activity of GSK3 α and incubation of acetylated- GSK3 α with wild type SIRT2 restored its activity in an NAD⁺ dependent manner (Figure 4C), suggesting that SIRT2-mediated deacetylation enhances the activity of GSK3 α . Further, our MS/MS analysis indicate that K99 and K246 are the acetylation sites in GSK3 α (Figure 4D). The protein sequence alignment of GSK3 β and GSK3 α showed that K246 of GSK3 α is analogous to residue K183 of GSK3 β (Figure 4E). To further strengthen our assumption, we generated the homology model of GSK3 α using the online Swiss-Modeler tool (Biasini et al., 2014). The modeled GSK3 α further confirmed that the residue K246 would occupy the same position proximal to the adenine nucleotide as K183 of GSK3 β (Figure 4F). We therefore assume that acetylation of residue K246 would play a similar role in de-stabilizing adenine nucleotide in the pocket of GSK3 α .

GSK3 is required for the Anti-Hypertrophic Role of SIRT2 deacetylase

Activation of GSK3 β antagonizes the development of cardiac hypertrophy (Antos et al., 2002; Kerkela et al., 2008; Sugden et al., 2008). Cardiac myocyte-specific deletion of GSK3 α and GSK3 β , together, results in severe dilated cardiomyopathy (Zhou et al., 2016). Recently, SIRT2 was demonstrated to be an anti-hypertrophic molecule (Tang et al., 2017). In our recent work, we also found that SIRT2 deficiency induces spontaneous cardiac hypertrophy in mice by hyperactivation of NFAT transcription factors (Sarikhani et al., 2018a). To understand the contribution of GSK3 as a key molecule in the anti-hypertrophic signaling regulated by SIRT2, we treated SIRT2 overexpressing cardiomyocytes with GSK3 inhibitors, LiCl (Bertsch et al., 2011) and GSK3 inhibitor X (Li et al., 2014; Meijer et al., 2003), and studied the ISO-induced cardiac hypertrophy. Treatment with GSK3 inhibitors markedly reduced the activity of GSK3 as measured by phosphorylation of GS (Figure 5A). In cardiomyocytes, SIRT2 overexpression reduces ISO-induced protein synthesis, cardiac myocyte size and the expression of ANP, a fetal gene that is considered as a marker of cardiac hypertrophy. However, treatment of GSK3 inhibitors abrogated the anti-hypertrophic role of SIRT2 in cardiomyocytes treated with ISO (Figure 5B-D, Figure 5-figure supplement 1), suggesting that both GSK3 isoforms are required for the anti-hypertrophic function of SIRT2 deacetylase.

The acetyl transferase p300 enhances the acetylation of GSK3 and thus inhibits its catalytic activity. Therefore, we tested whether depletion of p300 could reduce the acetylation of GSK3 and thus rescue the effect of SIRT2 deficiency. Our *in vitro* global protein synthesis experiment, which measures the incorporation of puromycin (Schmidt et al., 2009), suggest that depletion of p300 reduces the abnormal protein synthesis in SIRT2 depleted cardiomyocytes (Figure 5E, 5F). To validate our findings *in vivo*, we performed rescue experiments with anacardic acid, a p300 inhibitor. Western blotting analysis suggested that treatment with anacardic acid markedly reduces GSK3 β acetylation in SIRT2 deficient mice (Figure 5G, 5H). Furthermore, we found that treatment of anacardic acid partially restores the activity of GSK3 in SIRT2 deficient hearts, as measured by phosphorylation of GS (Figure 5G), indicating that p300 inhibition could rescue the impaired activity of GSK3 β caused by SIRT2 deficiency. However, the change in acetylation status of GSK3 β after p300 inhibitor treatment is not correlated well with the GSK3 β activity on glycogen synthase. We believe that glycogen synthase might have been post-translationally modified in SIRT2-KO hearts. It is possible that SIRT2-KO hearts might have increased expression of phosphatases that could regulate glycogen synthase phosphorylation. In our

recent work, we demonstrated the development of spontaneous cardiac hypertrophy in SIRT2-KO mice (Sarikhani et al., 2018a). In the present study, we found similar results as SIRT2 deficiency induces spontaneous cardiac hypertrophy in mice (Figure 5I-5J). Interestingly, inhibition of p300 significantly reduced HW/TL ratio, and wall thickness, while increasing ejection fraction in SIRT2 deficient mice (Figure 5I-5K), indicating that p300 inhibition rescues cardiac hypertrophy and contractile dysfunction resulting from SIRT2 deficiency, which could be due to the restoration of GSK3 activity.

Discussion

We identified acetylation of GSK3 isoforms as novel posttranslational modification, which hinders the ATP binding, and thus inhibits its kinase activity independent of its inhibitory phosphorylation during heart failure. We found that SIRT2 binds to and deacetylates GSK3 β at Lys183, which enhances ATP binding and its kinase activity.

The highly conserved lysine residues in the ATP binding pocket i.e. Lys183 in GSK3 β and Lys246 in GSK3 α , are acetylated. The acetylated lysine residues interfere with the binding of ATP to GSK3 due to charge repulsion and thus reduce the catalytic activity. We believe that the mechanism of inhibition of GSK3 isoforms by acetylation is unique when compared to inhibitory phosphorylation, where the phosphorylation of GSK3 α at Ser21 or GSK3 β at Ser9 causes the N-terminal tail of GSK3 to act as a pseudo substrate (Dajani et al., 2001; Stamos et al., 2014). The phosphorylated serine tail hinders the binding of primed substrates by self-associating with the primed-substrate binding pocket of GSK3, and thus reduce the kinase activity of GSK3 (Dajani et al., 2001; Stamos et al., 2014). It is worth mentioning that acetylation might even inhibit the activity of non-phosphorylated GSK3, which is capable of binding to the primed-substrates. On the other hand, deacetylated GSK3 can also be inhibited by Ser9 phosphorylation, since both acetylation and phosphorylation are two independent events regulating the activity of GSK3 isoforms. In contrast to our findings, it has previously been demonstrated that treatment of AGK2 in platelets decreases Ser9 phosphorylation of GSK3 β , due to enhanced acetylation and reduced activity of the Akt, an upstream kinase capable of phosphorylating GSK3 β at Ser9 residue (Moscardo et al., 2015), suggesting that the relationship between SIRT2 and Ser9 phosphorylation of GSK3 β is context-dependent. In our previous work, we found that deacetylation of Akt by SIRT1 enhances its kinase activity and thus SIRT1 indirectly inhibits GSK3 activity by increasing Ser9 phosphorylation (Sundaresan et al., 2011). Our current work demonstrates that deacetylation of GSK3 by SIRT2 promotes its activity. Consequently, acetylation is not only involved with direct regulation of GSK3, but also by an indirect mechanism via PDK1/AKT signaling.

In our previous work, we identified acetylation of GSK3 β at Lys15, which influences its mitochondrial localization (Sundaresan et al., 2016). Since Lys15 is away from the ATP binding pocket of GSK3 β , we assume that Lys15 will not regulate the ATP binding. Similarly, it has previously been shown that K189 in the Arabidopsis GSK3 orthologue BIN2 (equivalent to K205 in human GSK3 β) is targeted by the deacetylase HDAC6 and the deacetylation of K189 in BIN2 was shown to decrease its kinase activity (Hao et al., 2016). Our study suggests that the acetylation of K183 decreases GSK3 β kinase activity by directly interfering with its adenine nucleotide binding. Since, K189 in the Arabidopsis GSK3 orthologue BIN2 in activation loop located away from the nucleotide-binding site (data not shown), it would certainly follow a distinct mechanism in influencing the kinase activity of BIN2. Therefore, we believe that distinct

acetylation events within the kinase domain of GSK3 might have opposing effects on its kinase activity.

Previous studies indicate that Tyr216 phosphorylation of GSK3 β is dynamic and mirrors its catalytic activity. Tyr216 phosphorylation of GSK3 β has been shown to decrease immediately after reoxygenation in cultured cortical neurons (Chen et al., 2016). In contrast, the phosphorylation of GSK3 β at Tyr216 residue increases during ischemic injury of heart (Abdillahi et al., 2012) and after IFN- γ treatment in RAW264.7 cells (Tsai et al., 2009). Interestingly, phosphorylation of GSK3 β at Tyr216 was frequently increased and was predictive of better prognosis in early-stage gastric carcinoma (Cho et al., 2010). Studies indicate that Tyr216 phosphorylation of GSK3 β is required for its maximal activity and is considered as an autophosphorylation event that occurs during protein translation (Dajani et al., 2001; Hughes et al., 1993). Moreover, the phosphorylation of the endogenous GSK3 β at Tyr216 residue was suppressed by small molecule inhibitors of GSK3, which are known to block autophosphorylation at Tyr216 residue and catalytic activity of GSK3 β (Cole et al., 2004). Our work demonstrates that the phosphorylation of GSK3 β at Tyr216 residue was reduced in SIRT2-deficient mice. Since acetylation hinders the binding of ATP to GSK3 β , it is possible that autophosphorylation of GSK3 β might also be reduced in SIRT2 deficient conditions, when GSK3 β is mostly acetylated. Conventionally, GSK3 β is considered to be a cytoplasmic protein, though it is found inside nucleus and mitochondria (Sundaresan et al., 2016). However, the p300 is mostly nuclear protein and SIRT2 is majorly a cytoplasmic protein. Since, GSK3 shuttles between cytoplasm and nucleus, it is possible that p300 and SIRT2 may function as acetyl transferase and deacetylase respectively, though they are localized in different subcellular compartments. During cellular stress, GSK3 β localizes to mitochondria and this localization is required for cellular apoptosis (Maurer et al., 2006). In our previous work, we identified SIRT3 deacetylase mediated regulation of Lys15 to be critical for its mitochondrial localization (Sundaresan et al., 2016). However, the current work suggests that SIRT2 dependent regulation of GSK3 β Lys183 acetylation do not influence its cytoplasmic localization. Since Sirtuins are localized to sub-cellular compartments, SIRT2 might regulate the GSK3 β activity in the cytoplasm, while SIRT3 is controlling the GSK3 β activity inside mitochondria.

GSK3 plays a key physiological roles in cellular protein synthesis, cell survival, cell proliferation, cell differentiation, cellular metabolism, microtubule dynamics and cell motility (Beurel et al., 2015; Cohen and Frame, 2001; Doble and Woodgett, 2003; Rayasam et al., 2009). Impaired GSK3 activity is involved in the development of diverse pathological conditions like cardiovascular disease, obesity, inflammation, diabetes, Alzheimer disease and cancer (Cohen and Frame, 2001). GSK3 is one of the few signaling mediators that organize diverse signaling pathways, including those activated by Wnts, growth factors, cytokines, G protein-coupled, and α and β adrenoceptor ligands (Sugden et al., 2008). GSK3 isoforms play a key role in antagonizing the development of cardiac hypertrophy. GSK3 also blocks cellular protein synthesis necessary for the development of cardiac hypertrophy (Sugden et al., 2008). In particular, active GSK3 prevents cardiac hypertrophic growth and fibrosis through inhibition of transcription factors such as NFAT, GATA4, β -Catenin, myocardin, c-Myc, and c-Jun, and the translational regulator, eIF2Be (Sugden et al., 2008). There are reports suggesting that endothelin-1, GPCR, and β -adrenoceptor ligands induce N-terminal serine phosphorylation of GSK3 in cardiomyocytes, that might be one of the possible mechanism of GSK3 inhibition in the heart (Matsuda et al., 2008; Sugden et al., 2008). On the other hand, studies conducted on mice models suggest that N-terminal Ser9 phosphorylation of GSK-3 β was neither changed ,

nor is associated with the reduced activity in the heart after pressure overload or myocardial infarction (Zhai et al., 2007). On similar lines, GSK3 S21A/S9A knock-in mice, which is resistant to inhibitory N-terminal phosphorylation is not protected from cardiac hypertrophy, suggesting that inhibitory N-terminal phosphorylation is not the only mechanism that regulates the activity of GSK3 (Matsuda et al., 2008).

Our work suggests that GSK3 activity is critically regulated by phosphorylation-independent mechanisms such as acetylation during the heart failure. Interestingly, inhibition of the acetyl transferase p300 reduced the acetylation and partially restored the activity of GSK3 β in SIRT2 deficient cells. Furthermore, treatment of GSK3 inhibitors attenuate the beneficial effects of SIRT2 overexpression or p300 depletion, indicating the importance of GSK3 in SIRT2-mediated regulation of cardiac hypertrophy. A recent work suggests that SIRT2 deacetylates LKB1 to promote AMPK activity and regulates the development of cardiac hypertrophy (Tang et al., 2017). In our recent work, we also found that SIRT2 deficiency induces spontaneous cardiac hypertrophy in mice through hyperactivation of NFAT transcription factors (Sarikhani et al., 2018a). In the current work, we found that GSK3 β activity is required for the protective functions of SIRT2 in cardiomyocytes. Previous studies indicate that GSK3 β is a master regulator of divergent signaling pathways. Notably, GSK3 β phosphorylates and represses NFAT transcription factors (Neal and Clipstone, 2001). Similarly, GSK3 β and AMPK are known to regulate each other (Horike et al., 2008; Suzuki et al., 2013). It is possible that SIRT2 might have multiple and redundant targets to regulate cardiac hypertrophy, as observed in members of sirtuin family (Martinez-Redondo and Vaquero, 2013).

Overall, our work identified GSK3 isoforms as a novel target of SIRT2 deacetylase. SIRT2-dependent regulation critically modulates the activity of GSK3 β independent of Ser9 inhibitory phosphorylation.

Materials and Methods

Key resources table

Reagent type (species) or resources	Designation	Source or reference	Identifiers	Additional information
Strain, A2:A128 strain background (Mus musculus, C57BL/6J)	Sirt2 knockout mice, JAX Stock #012772 - B6.129-Sirt2 ^{tm1.1Fwa} /J	Jackson Laboratories, USA		
Strain, strain background (Mus musculus, 129/SvJ)	WT, JAX stock # 000691	Jackson Laboratories, USA		
Cell line (human)	HeLa	ATCC		
Cell line (human)	HEK 293	ATCC		
Cell line (mouse)	GSK3 β -KO fibroblasts	James Woodgett, Mount Sinai Hospital, Toronto, Canada		

Strain, (Wistar rats)	WT	Central Animal Facility, Indian Institute of Science, India		P1-P3 pups used for primary cardiomyocytes culture
Antibody	anti-GSK3 β	Cell Signaling Technology	9315	1:1000 diluted in 5% BSA
Antibody	anti-GSK3 β	Santa Cruz Biotechnology	sc-9166	1:1000 diluted in 5% milk for Western blotting 1:200 diluted in 1% BSA for immuno-fluorescence
Antibody	anti-Acetylated-Lysine	Cell Signaling Technology	9681	1:1000 diluted in 5% BSA
Antibody	anti-Acetylated-Lysine	Cell Signaling Technology	9441	1:1000 diluted in 5% BSA
Antibody	anti-GSK3 β Ser-9	Cell Signaling Technology	9336	1:1000 diluted in 5% BSA
Antibody	anti-GSK3 β Tyr 279/216	Merck Millipore	05-413	1:250 diluted in 5% BSA
Antibody	anti-Phospho- β -Catenin	Cell Signaling Technology	9561	1:2000 diluted in 5% BSA
Antibody	anti- β -Catenin	Cell Signaling Technology	8480	1:1000 diluted in 5% BSA
Antibody	anti-SIRT2	Sigma-Aldrich	S8447	1:2000 diluted in 5% BSA
Antibody	anti-SIRT2	Merck Millipore	09-843	1:1000 diluted in 5% BSA
Antibody	anti-SIRT2	Cell Signaling Technology	12650	1:1000 diluted in 5% BSA
Antibody	anti-ANP	Abcam	14348	1:250 diluted in 5% BSA
Antibody	anti-ANP	Cloud-Clone Corporation	PAA225Ra03	1:200 diluted in 1% BSA
Antibody	anti-GAPDH	Santa Cruz Biotechnology	sc-25778	1:1000 diluted in 5% milk
Antibody	anti-p300	Merck Millipore	05-257	1:1000 diluted in 5% BSA for Western blotting, 1:200 diluted in 1% BSA for immuno-fluorescence
Antibody	anti-phospho-Glycogen Synthase	Merck Millipore	07-817	1:1000 diluted in 5% BSA
Antibody	anti-Glycogen Synthase	Cell Signaling Technology	3893	1:1000 diluted in 5% BSA
Antibody	anti- β -actin (HRP-conjugate)	Cell Signaling Technology	12262	1:3000 diluted in 5% BSA

Antibody	anti- β -actin (HRP-conjugate)	Sigma-Aldrich	A3854	1:3000 diluted in 5% BSA
Antibody	anti-GSK3 α/β	Merck Millipore	04-903	1:1000 diluted in 5% BSA
Antibody	anti-puromycin	Developmental Studies Hybridoma Bank	PMY-2A4	1:500 diluted in 5% BSA
Antibody	anti-NFATc2	Thermo Fisher Scientific	MA1-025	1:100 diluted in 5% BSA
Antibody	anti-Flag	Sigma-Aldrich	F2555	1:2000 diluted in 5% BSA
Antibody	anti- α -Tubulin	Cell Signaling Technology	2144	1:2000 diluted in 5% BSA
Antibody	anti-Acetyl- α -Tubulin (Lys40)	Cell Signaling Technology	5335	1:1000 diluted in 5% BSA
Antibody	anti-SIRT1	Santa Cruz Biotechnology	sc-15404	1:1000 diluted in 5% milk
Antibody	anti-SIRT3	Cell Signaling Technology	5490	1:1000 diluted in 5% BSA
Antibody	anti-SIRT4	Cloud-Clone Corporation	PAE914Hu01	1:500 diluted in 5% BSA
Antibody	anti-SIRT5	Cloud-Clone Corporation	PAE915Mu01	1:500 diluted in 5% BSA
Antibody	anti-SIRT6	Cell Signaling Technology	12486	1:1000 diluted in 5% BSA
Antibody	anti-SIRT7	Cloud-Clone Corporation	PAE917Hu01	1:500 diluted in 5% BSA
Antibody	anti-HA	Sigma-Aldrich	H9658	1:2000 diluted in 5% BSA
Antibody	anti-HA	Santa Cruz Biotechnology	sc-805	1:100 diluted in 1% BSA for immuno-fluorescence
Antibody	anti-p300	Merck Millipore	05-257	1:1000 diluted in 5% BSA for Western, 1:100 diluted in 1% BSA for immuno-fluorescence
Antibody	anti-SOD2	Santa Cruz Biotechnology	sc-515068	1:200 diluted in 5% milk
Antibody	anti- α -Actinin	Sigma-Aldrich	A5044	1:200 diluted in 5% BSA
Antibody	Clean-Blot IP Detection Reagent	Thermo Fisher Scientific	21230	1:2000-5000 diluted in 5% milk
Antibody	anti-rabbit HRP	Santa Cruz Biotechnology	sc-2004	1:5000 diluted in 1% milk
Antibody	anti-mouse HRP	Santa Cruz Biotechnology	sc-2005	1:5000 diluted in 1% milk

Antibody	anti-mouse HRP	Thermo Fisher Scientific	31430	1:5000 diluted in 1% milk
Antibody	anti-rabbit HRP	Thermo Fisher Scientific	31460	1:5000 diluted in 1% milk
Antibody	anti-rabbit IgG light chain HRP	Abcam,	ab99697	1:5000 diluted in 1% milk
Antibody	Donkey anti-mouse, Alexa Fluor 488	Thermo Fisher Scientific	A-21202	1:200 diluted in 5% BSA
Antibody	Goat anti-rabbit, Alexa Fluor 546	Thermo Fisher Scientific	A-11035	1:200 diluted in 5% BSA
Antibody	Ni-NTA Agarose	Qiagen	30230	
Antibody	ANTI-FLAG M2 Affinity Agarose Gel	Sigma-Aldrich	A2220	
Antibody	Glutathione Sepharose® 4B	GE healthcare	17-0756-01	
Antibody	Monoclonal Anti-HA-Agarose antibody produced in mouse	Sigma-Aldrich	A2095	
Antibody	Protein A/G Agarose	Santa Cruz Biotechnology	sc-2003	
transfected construct	pcDNA3 Flag HA	Addgene	Plasmid 10792	1436 pcDNA3 Flag HA plasmid DNA was a gift from William Sellers
transfected construct (human)	HA GSK3 beta wt pcDNA3	Addgene	Plasmid 14753	<u>PMID: 7715701</u>
transfected construct (human)	HA GSK3 alpha wt	Modified Addgene, plasmid 15896	This paper	
transfected construct (human)	HA GSK3 beta S9A pcDNA3	Addgene	Plasmid 14754	<u>PMID: 7980435</u>
transfected construct (human)	HA GSK3 beta K85A pcDNA3	Addgene	Plasmid 14755	HA GSK3 beta K85A pcDNA3 was a gift from Jim Woodgett
transfected construct (human)	HA GSK3 beta K150Q pcDNA3	Modified Addgene, plasmid 14753	This paper	For, caccggcagggtct gctgcgcgcggctat aatg; Rev, cattatagccgcgcg cagcagaccctgcc ggtg;
transfected construct (human)	HA GSK3 beta K150R pcDNA3	Modified Addgene, plasmid 14754	This paper	For, attatagccgcgcga gacagaccctgccg ; Rev, cggcagggtctgtct cgcgcggtataat;
transfected construct (human)	HA GSK3 beta K183Q pcDNA3	Modified Addgene,	This paper	For, gcaggttctgcggct

		plasmid 14755		gaatatcgcatggc aaatgcaaag; Rev, cttggcatttgccatc gcatattcagccgc agaacctgc;
transfected construct (human)	HA GSK3 beta K183R pcDNA3	Modified Addgene, plasmid 14756	This paper	For, aggttctgcggcttaa tatcgcatggcaaa tgcca; Rev, tggcatttgccatcgc gatattagaccgcag aacct.
transfected construct (human)	GST-GSK3 β		This paper	
transfected construct (human)	HIS- GSK3 β		This paper	
transfected construct (human)	HIS- GSK3 β -K183R		This paper	
transfected construct (human)	HIS- GSK3 β -K183Q		This paper	
transfected construct (human)	SIRT1 Flag	Addgene	Plasmid 13812	<u>PMID:</u> <u>12620231</u>
transfected construct (human)	SIRT2 Flag	Addgene	Plasmid 13813	<u>PMID:</u> <u>12620231</u>
transfected construct (human)	SIRT3 Flag	Addgene	Plasmid 13814	<u>PMID:</u> <u>12620231</u>
transfected construct (human)	SIRT4 Flag	Addgene	Plasmid 13815	<u>PMID:</u> <u>12620231</u>
transfected construct (human)	SIRT5 Flag	Addgene	Plasmid 13816	<u>PMID:</u> <u>12620231</u>
transfected construct (human)	SIRT6 Flag	Addgene	Plasmid 13817	<u>PMID:</u> <u>12620231</u>
transfected construct (human)	SIRT7 Flag	Addgene	Plasmid 13818	<u>PMID:</u> <u>12620231</u>
transfected construct (human)	SIRT2-H187Y Flag	Modified from Addgene, plasmid 13818		For 5'atgtgtagaagggtg ccatacgcctccacc aagtcc3'- Rev 5'ggacttggtggag gcgtatggcaccttct acacat3'.
Infected construct (human)	Ad-Null	Vector Biolabs	Adenovirus 1300	
Infected construct (human)	Ad-GFP	Vector Biolabs	Adenovirus 1060	
Infected construct (human)	Ad-SIRT2	Vector Biolabs	Adenovirus 1519	
Infected construct (human)	Ad-h-EP300	Vector Biolabs	Adenovirus ADV- 207954	

Infected construct (human)	Ad-luc-shRNA	B. Thimmapaya, Northwestern University, Chicago, IL, USA		<u>PMID: 26667039</u>
Infected construct (human)	Ad-h-EP300-shRNA	B. Thimmapaya, Northwestern University, Chicago, IL, USA		<u>PMID: 26667039</u>
Recombinant protein	p300	Merck Millipore	14-418	http://dx.doi.org/10.1038/s41418-018-0069-8
sequence-based reagent	SMART pool: siGENOME Non-Targeting siRNA 1	Dharmacon	D-001206-13-50	100 nM siRNA transfected by Lipofectamine™ RNAiMAX Transfection Reagent
sequence-based reagent	SMART pool: siGENOME Rat Sirt2 siRNA	Dharmacon	M-082072-01-0010	100 nM SMARTpool siRNA transfected by Lipofectamine™ RNAiMAX Transfection Reagent
commercial assay or kit	GSK-3 Activity Assay Kit	Sigma-Aldrich	CS0990	<u>PMID: 26667039</u>
commercial assay or kit	QuikChange Site-Directed Mutagenesis Kit	Agilent Technologies,	200518	<u>PMID: 26667039</u> Sequences verified by sequencing, SciGenom Labs
commercial assay or kit	GenElute™ HP Plasmid Midiprep Kit	Sigma-Aldrich	NA0200	
commercial assay or kit	GenElute™ HP Plasmid Miniprep Kit	Sigma-Aldrich	PLN70	
commercial assay or kit	Qubit dsDNA HS assay kit	Thermo Fisher Scientific	Q32851	<u>PMID: 25871545</u>
chemical compound, drug	Lipofectamine™ 2000 Transfection Reagent	Thermo Fisher Scientific	11668019	
chemical compound, drug	Lipofectamine™ RNAiMAX Transfection Reagent	Thermo Fisher Scientific	13778150	
chemical compound, drug	Horse serum, heat inactivated	Thermo Fisher Scientific	26050088	
chemical compound, drug	Fetal Bovine Serum	Thermo Fisher Scientific	10500064	
chemical compound, drug	Penicillin-Streptomycin	Thermo Fisher Scientific	15070063	

chemical compound, drug	Gelatin, Type B	Sigma-Aldrich	G9382	0.2% w/v
chemical compound, drug	D-glucose	Sigma-Aldrich	G8270	0.01 M prepared in PBS
chemical compound, drug	Collagenase, Type II	Thermo Fisher Scientific	17101015	0.4 mg/ml prepared in Trypsin-PBS-Glucose
chemical compound, drug	Trypsin	Thermo Fisher Scientific	15050057	0.2% prepared in PBS-Glucose
chemical compound, drug	Trypsin-EDTA	Thermo Fisher Scientific	25200056	0.1% prepared in PBS
chemical compound, drug	Isoproterenol	Sigma-Aldrich	I6504	https://doi.org/10.1172/JCI39162
chemical compound, drug	AGK2	Cayman Chemical	13145	http://dx.doi.org/10.1038/s41418-018-0069-8
chemical compound, drug	Lithium chloride	Sigma-Aldrich	203637	PMID: 20926980
chemical compound, drug	GSK-3 Inhibitor X	Calbiochem	CAS 740841-15-0 -	PMID: 16984885
chemical compound, drug	Anacardic Acid	Cayman Chemical	CAS16611840	PMID: 28513807
chemical compound, drug	Puromycin	VWR	J593	
chemical compound, drug	Fluoromount-G®	Southern Biotech	0100-01	
chemical compound, drug	Hoechst 33342	Thermo Fisher Scientific	H3570	
chemical compound, drug	Dulbecco's Modified Eagle's Medium- High glucose	Sigma-Aldrich	D5648	
chemical compound, drug	Isoflurane	Sosrane Neon Laboratories Ltd		
chemical compound, drug	Nicotinamide	Sigma-Aldrich	N3376	
chemical compound, drug	Trichostatin A	Sigma-Aldrich	T8552	
chemical compound, drug	ProLong Gold Antifade Mounting medium with DAPI	Thermo Fisher Scientific	P36931	
chemical compound, drug	Acrylamide	Sigma-Aldrich	A9099	
chemical compound, drug	Tris	Sigma-Aldrich	T6066	
chemical	Hydrochloric acid	Fischer	29505	

compound, drug		Scientific		
chemical compound, drug	Sodium-dodecyl sulphate	VWR	0227	
chemical compound, drug	Ammonium persulphate	Sigma-Aldrich	A3678	
chemical compound, drug	TEMED	Sigma-Aldrich	T7024	
chemical compound, drug	Sodium chloride	Merck Millipore	106404	
chemical compound, drug	Triton X-100	Sigma-Aldrich	T8787	
chemical compound, drug	EDTA	Sigma-Aldrich	E5134	
chemical compound, drug	EGTA	Sigma-Aldrich	324626	
chemical compound, drug	sodium pyrophosphate	Sigma-Aldrich	221368	
chemical compound, drug	sodium orthovanadate	Sigma-Aldrich	450243	
chemical compound, drug	Tween-20	Sigma-Aldrich	P9416	
chemical compound, drug	cOmplete™, Mini Protease Inhibitor Cocktail	Sigma-Aldrich	118361530 01 ROCHE	
chemical compound, drug	PMSF	Sigma-Aldrich	P7626	
chemical compound, drug	2X Laemmli Sample Buffer	Bio-Rad	161-0737	
chemical compound, drug	β-mercapto ethanol	VWR	0482	
chemical compound, drug	DMSO	Sigma-Aldrich	D8418	
chemical compound, drug	Clarity ECL Western Blotting Substrate	BioRad	5060	
chemical compound, drug	SuperSignal West Pico chemiluminescent Substrate	Thermo Fisher Scientific	34080	
chemical compound, drug	IPTG	Sigma-Aldrich	I6758	
chemical compound, drug	Glycerol	PUREGENE	PG-4580	
chemical compound, drug	Sodium hydroxide	Sigma-Aldrich	221465	
chemical compound, drug	Calcium chloride	Sisco Research Laboratories	70650	
chemical compound, drug	formaldehyde solution	Sigma-Aldrich	F1635	
chemical compound, drug	Bovine serum albumin	HIMEDIA	MB083	
chemical compound, drug	Glycine	Fischer Scientific	12835	
chemical	Non-fat-milk	HIMEDIA	GRM1254	

compound, drug				
chemical compound, drug	Methanol	Honeywell	230-4	
chemical compound, drug	Bis-acrylamide	Sigma-Aldrich	M7279	
chemical compound, drug	Sodium deoxycholate	Sigma-Aldrich	D6750	
chemical compound, drug	Sodium bicarbonate	Sigma-Aldrich	S6014	
chemical compound, drug	Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	5000006	
chemical compound, drug	Ampicillin	VWR	0339	
chemical compound, drug	Leucine-free minimal essential medium	Thermo fisher Scientific	30030	
chemical compound, drug	DTT	Sigma-Aldrich	DTT-RO	
chemical compound, drug	Sodium butyrate	Sigma-Aldrich	567430	
chemical compound, drug	Magnesium chloride	Sigma-Aldrich	M8266	
chemical compound, drug	Sodium fluoride	Sigma-Aldrich	450022	
chemical compound, drug	Glutathione-reduced	Sigma-Aldrich	G4251	
chemical compound, drug	Bromophenol blue	Sigma-Aldrich	B8026	
chemical compound, drug	HEPES	Sigma Aldrich	H3784	
chemical compound, drug	ATP	Cell Signaling Technology	9804	
chemical compound, drug	γ - ³² P-ATP	Bhabha Atomic Research Centre, India		
chemical compound, drug	[³ H]leucine	Amersham Biosciences	TRK510	
chemical compound, drug	NAD ⁺	Sigma Aldrich	NAD100-RO-Roche	
Equipment	VisualSonics high-frequency ultrasound system	Vevo 1100		
Equipment	SDS-PAGE Gel running apparatus	Bio-Rad		
Equipment	Western blotting apparatus	Bio-Rad		
Equipment	Scintillation counter	Beckman		
Equipment	Chemiluminescence imager	Chemidoc Touch, Biorad, USA		
Equipment	ThermoMixer C	Eppendorf		
Equipment	Power-pack	Bio-Rad		

Equipment	LSM 880 confocal microscope	Zeiss		
Equipment	Tissue-culture ware	Eppendorf		
Software, algorithm	GraphPad Prism 5	GraphPad Software		
Software, algorithm	QuickChange Primer Design	Agilent Genomics		
Software, algorithm	ImageJ	National Institutes of Health		
Software, algorithm	ZEN 5	Zeiss		
Software, algorithm	Image Lab	Bio-Rad		
Software, algorithm	Mascot data explorer software	Matrix Science, London, United Kingdom		
Software, algorithm	Scaffold_2.1.03	Proteome Software, Inc., Portland, OR		
Software, algorithm	Swiss-model tool	ExPASy web server		PMID:24782522
Software, algorithm	UCSF Chimera software package	Resource for Biocomputing, Visualization, and Informatics, NHI		PMID:15264254
Software, algorithm	GROMACS simulation package, version 5.0.4			
Software, algorithm	PyTMs plugin of PyMOL			https://doi.org/10.1186/s12859-014-0370-6
Miscellaneous	Osmotic Minipumps	ALZET	Models 2002, 2001	PMID: 19652361
Miscellaneous	Cover-slip 18mm	Blue Star Slides		
Miscellaneous	PVDF membrane Amersham Hybond P	GE Healthcare	10600023	
Miscellaneous	Nitrocellulose paper	Biorad		
Miscellaneous	Cell culture wares	Eppendorf		
Miscellaneous	Sigma® cell scraper	Sigma-Aldrich	SIAL0010	

500 **Animal experiments**

501 All animal experiments were performed with the approval of Institutional animal ethics
502 committee of Indian Institute of Science, Bengaluru, India. All the animal experiments were
503 carried out as per the strict accordance with the recommendations of the Committee for the

Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The protocols were approved by the Institutional Animal Ethics Committee of the Indian Institute of Science (Permit Numbers: 559/2017, 568/2017, 376/2014). Mice were sacrificed using CO₂ before harvesting and every effort was made to minimize suffering. 129/SvJ mice (renamed as 129X1/SvJ, JAX stock # 000691) and Sirt2 knockout mice (JAX stock #012772 - B6.129-Sirt2^{tm1.1Fwa}/J) were procured from the Jackson Laboratories, USA. Mice were housed in individually ventilated cages (IVC) under 12 hours light/dark cycle in the clean air facility of Central Animal Facility, Indian Institute of Science. Chow diet and water were given *ad libitum* to animals.

Induction of cardiac hypertrophy in mice

Cardiac hypertrophy was induced in 8 weeks old 129/Sv mice by surgically implanting Isoproterenol (ISO)-filled osmotic minipumps (models 2002, 2001; ALZET), into the peritoneal cavity of the mice. ISO (Sigma-Aldrich) was dissolved in buffer containing 150 mM NaCl and 1 mM acetic acid and delivered chronically at the dose of 10 mg/kg/day for 7 days. Control mice underwent similar procedure, except that the pumps were filled with vehicle solution containing 150 mM NaCl and 1 mM acetic acid. The induction of hypertrophy was noninvasively assessed by use of echocardiography. Mice were sacrificed, the heart was quickly collected and soaked in ice cold PBS. Heart was squeezed to remove excess blood and PBS, and heart weight was measured by a weighing balance. Tibia length of mice was measured by a Vernier caliper. The heart was sectioned into smaller pieces, immediately snap-frozen in liquid nitrogen and stored at -80° till further processed.

Echocardiography of mice

Mice were anaesthetized by continuously infusing ~1% Isoflurane via nasal cone. A commercial topical depilatory was used to remove chest hair of mice. Body temperature of the mice was maintained by a heated imaging platform. Electrocardiogram leads were attached to limb of mice for gating. FUJIFILM VisualSonic Vevo 1100 high-frequency ultrasound equipped with 30 MHz high -frequency transducer was used to image the animals in the left lateral decubitus position. Two-dimensional echocardiographic images were recorded in parasternal long- and short-axis projections, with guided M-mode recordings at the midventricular level in both views. Left ventricular cavity size and left ventricular wall thickness were measured in at least 3 beats from each projection and averaged for data analysis. Left ventricular fractional shortening was calculated from the M-mode measurements by an in-built software.

Cell lines, plasmids and transfection

HeLa and HEK 293 cell lines were purchased from ATCC. The identity of the cell lines has been authenticated by short tandem repeat (STR) analysis by the supplier. These cells were tested mycoplasma free by PCR based screening in the laboratory. GSK3 β -Knock-out (GSK3 β -KO) mouse embryonic fibroblasts were kindly provided by James Woodgett, Mount Sinai Hospital, Toronto, Canada. HeLa, HEK 293 and GSK3 β -KO embryonic fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin at 37°C and 5% CO₂. Refer Key resources table for source and description of plasmids used. Site directed mutagenesis was carried out as per previously described protocol (Zheng et al., 2004). QuickChange Primer Design tool was used to design primers for site directed mutagenesis of Sirt2 and Gsk3b. The site-directed

mutation was verified by sequencing (SciGenom). Cells were transfected with plasmids harboring gene of interest using Lipofectamine™ 2000 transfection reagent as per manufacturer's protocols. Briefly, plasmid encoding control, or the desired construct were diluted in serum free media and incubated for 5 mins. Similarly, Lipofectamine was diluted in serum free media, and incubated for 5 mins. Mixtures were vortexed and centrifuged. Post-incubation, diluted plasmid and lipofectamine were pooled together, vortexed, centrifuged, and incubated at room temperature for 30 min. Equal volume of the mixture was added to the cells in serum free media for 6 hr.

Culture of cardiomyocytes

Primary rat neonatal cardiomyocytes were isolated from 1- or 2-day-old Wistar rats as described earlier (Jain et al., 2017). Rat pups were anaesthetized using 1-2 % isoflurane and were sacrificed by decapitation. The excised hearts were placed in sterile ice-cold PBS containing D-glucose (0.01 M), minced into small pieces and further enzymatically digested by mixture of 0.2% trypsin, Collagenase Type II (0.4 mg/ml) and 0.01M D-glucose containing PBS in a 1.5 ml microcentrifuge tube. Approximately 65–75 µl of the digestion mixture was used per heart. Enzymatic digestion was carried out for 5 min in 37 °C with shaking at 250 rpm. The unwanted erythrocytes and the debris were discarded from the first round of digestion. A total of eight to ten rounds of digestion was carried out and the supernatant containing a single cell suspension from each digestion was collected in a 15 ml falcon tube containing 100% horse serum. The cell suspension was maintained at 37 °C throughout the digestion process. Isolated neonatal rat cardiomyocytes were pre-plated on uncoated tissue culture plates to remove the adherent fibroblasts. After 1 hour of pre-plating, the non-adherent cell population enriched with cardiomyocytes were collected and centrifuged at 1000rpm for 10 min. The supernatant was discarded, and cell pellet suspended in high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cardiomyocytes were seeded onto gelatin (0.2% w/v) coated sterile tissue culture dishes for further experiments.

Adenovirus infection

Adenovirus vectors encoding SIRT2 or p300 were purchased from Vector Biolabs, USA. Adenovirus vectors synthesizing shRNA against Luc or p300 were kindly gifted by B. Thimmapaya, Northwestern University, Chicago, IL, USA. After 36 hours of plating, cardiomyocytes were infected with adenoviruses; Ad-null, Ad-GFP, Ad-p300, Ad-SIRT2, Ad-Luc-shRNA or Ad-p300-shRNA at multiplicity of infection (MOI) of 10. All adenovirus infection experiments were carried out in high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin.

siRNA transfection

Smart pool SIRT2 siRNA and non-targeting siRNA were purchased from Dharmacon, USA. For the siRNA experiments, cardiomyocytes were transfected with 100 nM SMARTpool siRNA targeting rat SIRT2 using Lipofectamine™ RNAiMAX Transfection Reagent as per manufacturer's protocol. Non-targeting siRNA was used as control. Briefly, non-targeting siRNA or SMARTpool siRNA targeting rat SIRT2 were diluted in serum free media and incubated for 5 mins. Similarly, Lipofectamine™ RNAiMAX Transfection Reagent was diluted in serum free media and incubated for 5 mins. Mixtures were vortexed and centrifuged. Post-incubation, diluted plasmid and lipofectamine were pooled together, vortexed, centrifuged, and incubated at

room temperature for 30 min. Equal volume of the mixture was added to the cells in serum free media for 6 hr.

Cardiomyocyte hypertrophy experiments

Neonatal rat cardiomyocytes were exposed to ISO for inducing cardiomyocyte hypertrophy. Changes in morphology of cardiomyocytes were assessed by observing the sarcomere reorganization by immunostaining of cardiomyocytes with antibodies specific for α -actinin or myomesin. Image J software was used to measure the cardiomyocyte surface area in actinin or myomesin positive cells in an experimental group. To confirm cardiomyocyte hypertrophy, perinuclear expression of ANP was assessed by confocal microscopy. Briefly, cells were fixed with 3.7% formaldehyde for 15 min at room temperature. Fixed cells were washed thrice with PBS and permeabilized for 5 min with PBS containing 0.25% Triton X-100. After washing thrice with PBS, fixed cells were blocked with 5% BSA prepared in PBST (PBS with 1% Tween 20) containing glycine (22.52 mg/ml) for 1h. Following blocking, cells were incubated with anti-ANP (Cloud-clone corp. or abcam) and myomesin (DSHB) or actinin (abcam) antibodies prepared in 1% BSA containing PBST at 4°C overnight. Further, cells were incubated with secondary antibody conjugated with Alexa fluor 488 and Alexa fluor 546 at room temperature for 1 hr. After washing thrice with PBS for 2 min, cells were incubated with Hoechst 33342 for 10 min to stain nucleus. After nuclear staining, cells were washed thrice with PBS. and mounted on slides using Fluoromount G. Zeiss LSM 880 confocal microscope was used for image acquisition and ZEN-Black software was used for image analysis.

[³H]-Leucine incorporation assay

Leucine incorporation assay was performed as previously described (Pillai et al., 2015). Briefly, cardiomyocytes treated with either vehicle or ISO were incubated with [³H]-leucine (1.0 mCi/ml, 163 Ci/mmol specific activity, Amersham Biosciences) in leucine-free minimal essential medium (Invitrogen) for 24 h. Cells were then washed with phosphate-buffered saline, and 10% trichloroacetic acid was added to the cells to precipitate total proteins. The resultant protein pellet was solubilized using 0.2 N NaOH and further diluted with one-sixth volume of scintillation fluid. The radioactivity was measured in a scintillation counter and the values were normalized with DNA content measured by the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, USA).

Non-radioactive SUnSET Assay

To measure *in-vitro* protein synthesis, Surface sensing of translation (SUnSET), a non-radioactive method to monitor protein synthesis was employed (Schmidt et al., 2009). Cardiomyocytes seeded on 6 well plates were pulsed with puromycin (1 μ M) for 30 min prior to harvesting. Cells were washed twice with ice-cold PBS and lysed. Bradford assay was performed for protein quantification and 80 μ g of protein was boiled in Laemmli Sample Buffer (Bio-Rad) supplemented with 5% β -mercaptoethanol for 5 min at 96°C. SDS-PAGE was performed, and the proteins were then transferred onto a 0.45 μ m PVDF membrane (Amersham Hybond P, GE) by Western blotting for 16 h at 25V at 4°C. After 1 hr of blocking with 5% non-fat milk at room temperature, the membrane was incubated with anti-puromycin antibody (DSHB, University of Iowa) overnight at 4°C. Membrane was washed thrice with 1X TBST and incubated with anti-mouse secondary antibody for 1 hr at RT. Blot was washed thrice with 1X TBST and chemiluminescent signals were captured using BioRad Clarity ECL Western Blotting Substrate in a chemiluminescence imager (Chemidoc Touch, Biorad, USA).

Inhibitor experiments

Anacardic acid was dissolved in peanut oil and injected into the peritoneal cavity of mice at a dose of 5 mg/kg/day for 10 days. LiCl (Sigma, Cat. No. 203637) or GSK3 inhibitor X (Cat. No. 361551) were used to inhibit the endogenous activity of GSK3 isoforms in cardiomyocytes. For inhibition of SIRT2 activity, cardiomyocytes were treated with either vehicle or AGK2, a SIRT2 inhibitor in high glucose DMEM with 100 units/ml penicillin and 100 µg/ml streptomycin after 36 h of seeding.

Cells and tissue harvesting

Cells were washed twice with ice-cold phosphate buffered saline (PBS, 1X) before harvesting. Cells were lysed in ice-cold lysis buffer [20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1mM Na₃VO₄, 1 mM PMSF, 1X protease inhibitor cocktail (Roche)]. Heart tissue was homogenized in lysis buffer [50 mM Tris-Cl, pH 7.4 150 mM NaCl, 1% Triton-X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10mM Sodium fluoride, 2.5 mM sodium pyrophosphate, 1mM Na₃VO₄, 1 mM PMSF, 1X protease inhibitor cocktail (Roche)]. Homogenates were centrifuged at 12,000 rpm at 4 °C for 10 min and the supernatant was collected in fresh micro-centrifuge tubes for Western blotting.

Western blotting

Western blotting was performed as per the standard protocols. Protein quantification was done by Bradford reagent using a spectrophotometer. Cell or tissue lysates was mixed with laemmli buffer (2X, Bio-rad) supplemented with 5% β-mercaptoethanol in 1:1 ratio. Samples were boiled for 5 min at 95 °C for 5 min. SDS-PAGE was performed and the proteins were transferred to PVDF membrane by cold transfer (25V at 4° overnight). Protein bound membrane was blocked by a solution of 5% non-fat dried milk in TBST buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 hr at RT. Membrane was washed thrice with TBST buffer and probed with primary antibody at 4 °C overnight. Membrane was further washed thrice with TBST buffer and probed with HRP- conjugated secondary antibody at RT for 1h. Membrane was washed thrice with TBST and the chemiluminescent signals recorded using either Clarity ECL Western Blotting Substrate (BioRad) or SuperSignal West Pico chemiluminescent Substrate (ThermoScientific) in a chemiluminescence imager (Chemidoc Touch, Biorad, USA).

Immunoprecipitation assays

Immunoprecipitation assay was performed as per the standard protocols. For immunoprecipitation, 0.5-1 mg of protein from lysate was incubated with 2 µg of appropriate antibody or control IgG antibody overnight. Protein A/G conjugated agarose beads were used to capture the immune complexes. After a brief centrifugation, supernatant was discarded, and beads were washed thrice with ice-cold PBS (1X) 1000 rpm at 4 °C for 1 min. The immunoprecipitated protein was resolved by SDS-PAGE after boiling the beads at 95 °C for 5 min in 2X laemmli buffer (Bio-rad) and transferred to a PVDF membrane (GE, Cat#10600023). The membrane was blocked by a solution of 5% non-fat dried milk in TBST buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 hr at RT. Membrane was washed thrice with TBST buffer and probed with primary antibody at 4° for overnight. After washing thrice with TBST buffer and the membrane was probed with HRP conjugated secondary antibody (Clean-Blot IP Detection Reagent, Thermo Scientific or Mouse monoclonal SB62a Anti-Rabbit IgG light chain (HRP) (ab99697) prepared in 1% milk at RT for 1hr. Blot was washed thrice with TBST

(1X) and chemiluminescent signals were captured using Clarity ECL Western Blotting Substrate (BioRad) in a chemiluminescence imager (Chemidoc Touch, Biorad, USA).

Co-localization experiments

To study the co-localization of p300 with GSK3 β , confocal microscopy was performed in HEK 293 cells using antibodies specific to p300 and GSK3 β . Similarly, to test the localization of HA-tagged GSK3 β -WT or GSK3 β -K183R or GSK3 β -K183Q mutants, plasmid encoding HA-tagged GSK3 β -WT or GSK3 β -K183R or GSK3 β -K183Q mutants were transiently overexpressed in GSK3 β deficient mouse embryonic fibroblasts by Lipofectamine 2000 as per manufacturers protocols. After 48 hours of transfection, confocal microscopy was performed by an HA antibody to localize WT and mutants of GSK3 β . To test the effect of AGK2 on GSK3 β localization, confocal microscopy was performed with an antibody specific to GSK3 β and/or anti-MnSOD.

For performing confocal microscopy, cells were washed twice with PBS and fixed with 3.7% formaldehyde at room temperature for 15 min. Fixed cells were washed thrice with PBS and incubated for 5 min with 0.25% Triton X-100 prepared in PBS. After washing thrice with PBS, cells were blocked with 5% BSA prepared in PBST (PBS with 1% Tween 20) containing glycine (22.52 mg/ml) for 1hr. Following blocking, cells were incubated with respective primary antibodies prepared in 1% BSA in PBST at 4 °C overnight. Further, cells were incubated with secondary antibody conjugated with Alexa fluor 488 and/or Alexa fluor 546. After 1 hr of incubation at room temperature, antibody was discarded and washed thrice with PBS. Hoechst 33342 prepared in PBS was added for 10 min to stain nucleus. After nuclear staining, cells were washed thrice with PBS and Fluoromount G was used to mount slides. Confocal images were captured by Zeiss LSM 880 confocal microscope

GST-GSK3 β purification

Recombinant GST-GSK3 β encoding expression plasmid was transformed into competent *E.coli* BL21 (DE3) cells. Culture from single colony was grown in LB medium supplemented with ampicillin (100 μ g/ml) at 37°C in orbital shaker incubator till mid log phase. 50 μ M IPTG was added to the culture and incubated at 18 °C for 16 hr. The culture was centrifuged at 5000 rpm at 4 °C for 20 min, and the pellet resuspended in binding buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM PMSF (Sigma)). The resuspended cell pellet was lysed by sonication and centrifuged at 17000 rpm at 4 °C for 30 min. After centrifugation, supernatant was collected and incubated with Glutathione Sepharose 4B beads. Beads were washed thrice with wash buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% Triton-X 100), GST-GSK3 β eluted with elution buffer (20 mM glutathione-SH), and stored in sterile glycerol (30%) at -80 °C.

Purification of HIS-GSK3 β and its mutants

Recombinant HIS-GSK3 β or its mutants HIS-GSK3 β -K183R, HIS-GSK3 β -K183Q encoding expression plasmid were transformed into competent *E. coli* BL21 (DE3) cells. Culture from single colony was grown in LB medium supplemented with ampicillin (100 μ g/ml) at 37 °C in orbital shaker incubator till mid log phase. 50 μ M IPTG was added to the culture and incubated at 18 °C for 18 hr. The culture was centrifuged at 5000 rpm at 4 °C for 20 min, and cell pellet was lysed by sonication in lysis buffer (20 mM Tris-Cl, pH 7.4, 500 mM NaCl, 20 μ M imidazole,

1% Triton-X 100). Sonicated lysate was centrifuged at 17,000 rpm at 4 °C for 30 min. Supernatant fraction was passed through pre-washed Ni-NTA column three times. Column was washed 5 column volume wash buffer (50 mM Tris-Cl (pH 7.4), 500 mM NaCl, 20 mM imidazole, 0.5% Triton-X-100). Protein was eluted by elution buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 500 mM imidazole, 0.5% Triton-X-100) and stored in sterile glycerol (30%) at -80 °C.

GSK3 acetylation assay

His-GSK3 and HA-GSK3 was incubated with 1 µg of recombinant p300 acetyltransferase (Millipore # 2273152) in 50 µl of HAT buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 10 mM Na-butyrate, 5 mM DTT, 10mM NaCl and 10% glycerol) supplemented with 100 µM acetyl CoA. Samples were incubated at 30 °C for 2 hr. Beads were washed thrice with TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) and the protein eluted by adding 50 µl of sample buffer (125 mM Tris-Cl, pH 6.8, with 4% SDS, 20% (v/v) glycerol, and 0.004% bromophenol blue). Samples were heated for 5 min at 95 °C followed by centrifugation at 13,400 g, 30 sec, and supernatant subjected to SDS-PAGE. GSK3β acetylation was detected by a pan anti-acetyl Lysine antibody (Cell Signaling #9681).

GSK3 deacetylation assay

Plasmid encoding flag-tagged WT SIRT2 or SIRT2-H187Y was overexpressed in 293 cells by transfection with lipofectamine 2000. Cells were harvested after washing with ice-cold PBS and lysed in lysis buffer [50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1mM PMSF and protease inhibitor cocktail (Sigma Aldrich)] followed by vortexing for 15 s at 5 min interval for 4 -5 times. Cell homogenates were centrifuged at 12,000rpm at 4 °C for 10 min and the supernatant was collected. 500 µg of total protein was incubated with agarose beads conjugated to Anti-Flag antibody (Sigma A2220) and kept for end-over-end mixing for 2 hr at 4°C. Beads were washed thrice with TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) and the SIRT2 protein eluted. His-tagged or HA-tagged acetylated GSK3 was deacetylated by recombinant SIRT2 in a deacetylation buffer (250 mM Tris-Cl, pH 9.0, 20 mM MgCl₂, 250 mM NaCl, 2.5 mM DTT, 5 mM NAD⁺, 2.5 µM TSA) for 2 hr at 30 °C. The acetylation of GSK3 isoforms was analyzed by Western blotting.

ATP Binding Assay

His-tagged wild type and mutants of GSK3 or His-tagged acetylated and deacetylated GSK3 bound to Ni-NTA beads were incubated with [γ ³²P] ATP (2 µCi) at 30 °C for 10 min in a binding buffer containing 20 mM HEPES pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 200 µM ATP in a final volume of 50 µL. After 30 minutes, the beads were washed 5 times with 50 mM Tris (pH 7.5) and subjected to scintillation counting using a counter (Beckman).

Activity assay for GSK3 isoforms

Commercially available GSK3 activity assay kit (CS0990; Sigma) was used for performing GSK3 activity assay as per manufacturer's instructions. Briefly, HA-tagged GSK3β or GSK3α was immunoprecipitated using a specific anti-HA antibody conjugated agarose beads (Sigma-Aldrich). Similarly, endogenous GSK3β was immunoprecipitated from heart tissue lysates or cell lysates by a specific anti-GSK3β antibody bound protein A/G affinity gel. The immunoprecipitated kinase was incubated with γ -³²P-ATP and the incorporation of ³²P into glycogen synthase peptide, which contains specific phosphorylation residues of GSK3 was measured.

Activity assay for acetylated and deacetylated-GSK3 isoforms

HA-GSK3 β or HA-GSK3 α was overexpressed in HeLa cells by transfection of the respective plasmid encoding pcDNA3-HA-GSK3 β or pcDNA3-HA-GSK3 α . HeLa cell lysates were incubated with monoclonal Anti-HA-agarose antibody-conjugated agarose beads for 2 hr at 4 °C with end-over-end mixing. HA-GSK3 β or HA-GSK3 α bound to beads was incubated with recombinant p300 (Millipore, 1 μ g) in 50 μ l of HAT buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 10 mM Na-butyrate, 5 mM DTT, 10mM NaCl and 10% glycerol) supplemented with 100 μ M acetyl CoA for 30 °C for 60 min. Beads were washed thrice with TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) and further deacetylated by either SIRT2 or SIRT2-catalytic mutant in a deacetylation buffer (250 mM Tris-Cl, pH 9.0, 20 mM MgCl₂, 250 mM NaCl, 2.5 mM DTT, 5 mM NAD⁺, 2.5 μ M TSA) for 60 min at 30 °C. The acetylated and deacetylated HA-GSK3 β or HA-GSK3 α was incubated with γ -³²P-ATP in a kinase buffer and the incorporation of ³²P into glycogen synthase peptide, containing specific phosphorylation residues of GSK3 was measured as per the protocols of GSK3 activity assay kit (CS0990; Sigma).

Mass spectrometry analysis

GSK3 isoforms were resolved by SDS-PAGE, stained with colloidal Coomassie and the desired protein bands were excised from the gel by the use of a razor blade and divided into ~1-mm³ pieces. The gel pieces containing protein were destained using 100 mM ammonium bicarbonate (pH 8.9) in 50% acetonitrile. The destained bands were further treated with 100 μ l of 50 mM ammonium bicarbonate (pH 8.0) and 10 μ l of 10 mM TCEP [Tris (2-carboxyethyl)phosphine HCl] at 37 °C for 30 min. Subsequently, protein digestion was carried out by 1:50 sequencing-grade trypsin in 50 mM ammonium bicarbonate (pH 7.5) solution. Digested peptide samples were desalted in a C₈ OptiPak column (Optimize Technologies) and then analyzed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI/MS/MS) on a Thermo LTQ Orbitrap Hybrid FT mass spectrometer. Reflectron mode was used to acquire positive-ion mass spectra. Ions selected for MS/MS were subsequently placed on an exclusion list using an isolation width of 1.6 Da, a low-mass exclusion of 0.8 Da, and a high-mass exclusion of 0.8 Da. Tandem mass spectra were extracted by Readw.exe version 3.0. All MS/MS samples were analyzed using Mascot data explorer software (Matrix Science, London, United Kingdom). The Scaffold (version Scaffold_2.1.03; Proteome Software, Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications.

Modeling of GSK3 α

Swiss-model tool was used to generate the homology model of GSK3 α (Biasini et al., 2014) . The sequence-based template search results showed ~82% MS sequence identity with the crystal structure of GSK3 β (PDB ID 1PYX). Further, the crystal structure with PDB ID 1PYX was used to generate the final model of GSK3 α ; the final model of GSK3 α encompasses the residues 98 to 448. UCSF Chimera software package was used for visualization and generation of the final images (Pettersen et al., 2004).

Modeling of ac183 GSK3 β Molecular Dynamics Simulations and Analysis of Trajectories

Models of GSK3 β protein were generated from the crystal structure (PDB ID 4NM0 A). Computer aided acetylated lysine (acK183) mutant was generated over the crystal structure (PDB ID 4NM0 A), using the PyTMs plugin of PyMOL (Warnecke et al., 2014). UCSF Chimera

software package was used for visualization and generation of the final images (Pettersen et al., 2004).

MD Simulations on the initial models of GSK3 β wild-type and acetylated GSK3 β were carried out with the GROMACS simulation package, version 5.0.4. The systems were parameterized using CHARMM36 force field and TIP3P rigid water model (Jorgensen et al., 1983). The atomic charges for terminal acetyl group available in the CHARMM36 force field were used to build the parameters for side-chain acetylated lysine. Each model was immersed in a dodecahedron box containing TIP3P water with 10Å distance between the box surface and the protein atoms. Charge neutralization for the systems was achieved by adding Na⁺ and Cl⁻ counter ions. Steepest descent energy minimization was used until the system converged with Fmax no greater than 1000 kJ mol⁻¹ nm⁻¹. Equilibration was performed for 600ps under NVT and for 1200ps under NPT ensemble, while restraining the protein atomic positions. The coupling constant for the temperature of the bath was set to 0.1ps using v-rescale thermostat with a constant temperature of 300K. Berendsen thermostat was used to maintain the pressure a 1 bar with a coupling constant of 1ps. The electrostatic interactions were evaluated using the Particle Mesh Ewald (PME) method (Darden et al., 1993). Production run totaling to 100 ns each for the GSK3 β wild-type and GSK3 β acK183 systems were performed on the equilibrated systems using leap-frog algorithm.

GROMACS tools were used to calculate the root mean square deviation (RMSD) of C α atoms, root mean square deviation (RMSD) of ADP nucleotide, resultant root mean square fluctuations (RMSF) of C α atoms on the five MD trajectories of 20 ns each for both the wild type and acK183 mutant of GSK3 β . UCSF Chimera software tool was used for visualization and calculation of distances between the atoms (Pettersen et al., 2004).

Quantification and Statistical analysis

Statistical analysis and graph preparation was done by Graph-pad prism version 6.04. t-test was used for pair-wise comparisons. One-way ANOVA, and two-way ANOVA were used for multiple comparisons. ZEN-Black software was used for confocal image analysis and ImageJ was used for quantification. Densitometric analysis was performed using ImageJ. Western blotting images were processed by using Image-lab software (Bio-Rad).

Acknowledgements

We thank James Woodgett, Mount Sinai Hospital, Toronto, Canada for providing GSK3 β -KO mouse embryonic fibroblasts. We thank B. Thimmapaya, Northwestern University, Chicago, IL, USA for providing adenovirus vectors synthesizing shRNA against p300. We thank Naren Ramanan, Centre for Neuroscience, Indian Institute of Science for kindly sharing the GSK3 inhibitor X.

Source of funding

N.R.S. is supported by the Ramalingaswami Re-entry Fellowship and the Innovative Young Biotechnologist Award (IYBA) from the Department of Biotechnology, Government of India. S.M. is supported by the SERB-National Post-Doctoral Fellowship (N-PDF). N.R.S laboratory is supported by research funding from the Department of Science and Technology Extra Mural Research Funding (EMR/2014/000065), The Department of Biotechnology Extramural

Research Grants (BRB/10/1294/2014, MED/30/1454/2014), and The Council for Scientific and Industrial Research extramural research support (37(1646)/15/EMR-II).

Conflicts of interests: None

FIGURES Legends

Figure 1. Acetylation of GSK3 β Increased in Pathological Cardiac Hypertrophy

(A) Scatter plot showing cardiac hypertrophy, as measured by Heart weight/ Tibia Length (HW/TL) ratio of 8 weeks old 129/Sv mice treated with either vehicle or isoproterenol (ISO) at the dose of 10 mg/kg/day. ISO was continuously infused for 7 days using osmotic mini-pumps. $n = 9$ -10 mice per group. Data is presented as mean \pm s.d, $*p < 0.05$. Student's t test was used to calculate the p values.

(B) Scatter plot representing left ventricular posterior wall thickness of 8 weeks old 129/Sv mice treated with either vehicle or ISO at the dose of 10 mg/kg/day. ISO was continuously infused for 7 days using osmotic mini-pumps. $n = 6$ mice per group. Data is presented as mean \pm s.d, $*p < 0.05$. Student's t test was used to calculate the p values.

(C) Scatter plot indicating the contractile functions of heart as represented by ejection fraction of 8 weeks old 129/Sv mice treated with either vehicle or ISO at the dose of 10 mg/kg/day. ISO was continuously infused for 7 days using osmotic mini-pumps. $n = 6$ mice per group. Data is presented as mean \pm s.d, $*p < 0.05$. Student's t test was used to calculate the p values.

(D) Histogram showing GSK3 β activity assay in heart lysates of vehicle or ISO treated 8 weeks old 129/Sv mice. Mice were treated with either vehicle or ISO at the dose of 10 mg/kg/day for 7 days using osmotic mini-pumps. GSK3 β was immunoprecipitated from the heart lysates of vehicle or ISO infused mice using anti-GSK3 β antibody, clone GSK-4B (Sigma). The immunoprecipitated GSK3 β was incubated with the peptide substrate in the presence of γ - ^{32}P -ATP. The incorporation of ^{32}P into the GSK3 β peptide substrate, which contains specific phosphorylation residues of GSK3 β was measured. $n = 10$ mice per group. Data is presented as mean \pm s.d, $*p < 0.05$. Student's t test was used to calculate the p values.

(E) Eight weeks old 129/Sv mice were treated with either vehicle or ISO at the dose of 10 mg/kg/day for 7 days using osmotic mini-pumps. GSK3 β was immunoprecipitated from the heart lysates of vehicle or ISO infused mice using anti-GSK3 β antibody (sc-9166, Santacruz) and the affinity resin immobilized with protein A/G. Western blotting analysis was performed to detect the levels of GSK3 β acetylation (Ac-Lys) by anti-acetyl-lysine antibody. IgG was used as negative control in this assay. Heart tissue lysates (WCL) were probed for indicated proteins by Western blotting. ANP was used as a positive control to assess cardiac hypertrophy in ISO infused mice. $n = 4$ mice per group. # marked Western blotting images denotes SIRT2 antibody (#12650; Cell Signaling), used in this assay detects single band.

882 (F) Histogram showing relative acetylated GSK3 β in vehicle and ISO treated mice heart tissues,
 883 as measured from figure 1E. Signal intensities of acetylated GSK3 β and GSK3 β were
 884 measured by densitometry analysis (ImageJ software). $n = 4$ mice per group. Data is presented
 885 as mean \pm s.d. * $p < 0.05$. Student's t test was used to calculate the p values.

886 (G) GSK3 β was immunoprecipitated from heart tissues of 8 weeks old 129/Sv mice using anti-
 887 GSK3 β antibody (sc-9166, Santacruz), and the affinity resin with protein A/G immobilized.
 888 Western blotting was performed to detect GSK3 β interaction with p300 using anti-p300
 889 antibody. IgG was used as a negative control. Whole cell lysates (WCL) were probed for the
 890 presence of GSK3 β and p300 by Western blotting.

891 (H) Co-localization of GSK3 β with p300 was assessed in 293T cells by confocal microscopy.
 892 The antibodies used are anti-GSK3 β (sc-9166, Santacruz), and p300 (05-257, Millipore). DAPI
 893 was used to stain the nucleus. Expanded images (right small boxes) show yellow color in the
 894 merge image, indicating the co-localization of GSK3 β (Green) and p300 (Red) in the nucleus.

895 (I) *In vitro* binding assay to test the direct interaction between GSK3 β and p300. Recombinant
 896 p300 (Millipore # 2273152) was incubated with recombinant GST or GST-GSK3 β , purified from
 897 *E. coli* BL21 (DE3) by affinity chromatography using Glutathione Sepharose 4B.

898 (J) Western blotting analysis showing the acetylation and activity of GSK3 β in rat neonatal
 899 cardiomyocytes infected with adenovirus expressing either luciferase shRNA (control) or p300
 900 shRNA (p300-KD) for 72 hours. Depletion of p300 was confirmed by Western blotting. GSK3 β
 901 was immunoprecipitated from control and p300-KD cells using anti-GSK3 β antibody (sc-9166,
 902 Santacruz) and the affinity resin immobilized with protein A/G. Western blotting was performed
 903 to detect acetylation of GSK3 β using the anti Ac-Lysine antibody. GSK3 β activity was measured
 904 by assessing the phosphorylation of glycogen synthase (p-GS). Site specific antibodies were
 905 used to detect the phosphorylation of GSK3 β at indicated residues in cardiomyocyte lysates
 906 (WCL).

907 (K) Histogram showing the quantification of relative acetylated GSK3 β in control and p300
 908 depleted (p300-KD) rat neonatal cardiomyocytes, as measured from Figure 1J. Rat neonatal
 909 cardiomyocytes were infected with adenovirus expressing either luciferase shRNA (control) or
 910 p300 shRNA (p300-KD) for 72 hours. Signal intensities of acetylated GSK3 β and GSK3 β were
 911 quantified by densitometry analysis (ImageJ software). $n = 3$ independent experiments. Data is
 912 presented as mean \pm s.d. * $p < 0.05$. Student's t test was used to calculate the p values.

913 (L) Histogram depicting the activity of GSK3 β in control and p300 depleted (p300-KD) rat
 914 neonatal cardiomyocytes, as measured by the ratio of phosphorylation of glycogen synthase vs
 915 total glycogen synthase from Figure 1J. Rat neonatal cardiomyocytes were infected with
 916 adenovirus expressing either luciferase shRNA (control) or p300 shRNA (p300-KD) for 72
 917 hours. Signal intensities of phospho-glycogen synthase and glycogen synthase were measured
 918 by densitometry analysis (ImageJ software). $n = 3$ independent experiments. Data is presented
 919 as mean \pm s.d. * $p < 0.05$. Student's t test was used to calculate the p values.

920 (M) Western blotting analysis showing the acetylation of GSK3 β in rat neonatal cardiomyocytes
 921 infected with either control (Ad-null) or p300 overexpressing adenovirus (Ad-p300) for 24 hours.
 922 Overexpression of p300 was confirmed by Western blotting. GSK3 β was immunoprecipitated
 923 using anti-GSK3 β antibody (sc-9166, Santacruz) and the affinity resin with protein A/G
 924 immobilized. Site specific antibodies were used to detect the phosphorylation of GSK3 β at
 925 indicated residues in cell lysates (WCL).

(N) Western blotting analysis showing the activity of GSK3 β in rat neonatal cardiomyocytes infected with control (Ad-null) or p300 expressing adenovirus (Ad-p300) for 24 hours. Overexpression of p300 was confirmed by Western blotting and the activity of GSK3 β was probed by assessing the levels of p-GS and GS by Western blotting.

(O) Histogram showing the activity of GSK3 β in control (Ad-Null) or p300 overexpressing (Ad-p300) rat neonatal cardiomyocytes, as measured by the ratio of phosphorylation of glycogen synthase vs total glycogen synthase from Figure 1N. Signal intensities of phospho-glycogen synthase and glycogen synthase were assessed by densitometry analysis (ImageJ software). $n = 3$ independent experiments. Data is presented as mean \pm s.d. $*p < 0.05$. Student's t test was used to calculate the p values.

(P) *In vitro* kinase assay showing the activity of acetylated and non-acetylated GSK3 β . Human GSK3 β with HA tag was overexpressed in HeLa cells by transfection of the plasmid pcDNA3-HA-GSK3 β . HA-GSK3 β was immunoprecipitated using HA-coupled agarose beads (Sigma-Aldrich) and the HA-GSK3 β was acetylated by recombinant p300 (Millipore), in the presence or absence of Acetyl-CoA (Ac-CoA) in HAT buffer. The enzymatic activity of GSK3 β was measured against Glycogen Synthase (GS)-peptide. $n = 6$ independent experiments. Data is presented as mean \pm s.d. $*p < 0.05$. One-way ANOVA was used to calculate the p values.

Figure 2. Molecular Modelling and Molecular Dynamics Simulations of GSK3 β Wild-type and K183 Acetylated Mutant

(A) Annotation of representative tandem mass spectra of trypsin-digested GSK3 β , depicting K150, K183 acetylation.

(B) Representation of the acetylation sites on the crystal structure of GSK3 β (PDB ID 4NM0): (i) surface (ii) cartoon representation. (iii) Magnified active site representing position of K183 and (iv) magnified active site representing position of acetylated K183 (acK183).

(C) Nucleotide binding site in GSK3 β crystal structure (PDB ID 4NM0) representing ADP, nucleotide interacting residues and K183.

(D) Overlay of the wild-type (blue) and acK183 mutant (orange) of GSK3 β representing the surface of ADP nucleotide, at random snapshots in the MD trajectory.

(E) Overlay of protein backbone C α RMSD plots of the five 20ns MD trajectories in wild-type.

(F) Overlay of ADP nucleotide RMSD plots of the five 20ns MD trajectories in wild-type.

(G) Overlay of the distance between the NZ atom of K85 and α -phosphate of ADP as a function of time for two stable trajectories (dark blue/cyan – wild-type, pink/red – acK183).

(H) Overlay of protein backbone C α RMSD plots of the five 20ns MD trajectories in acK183 mutant.

(I) Overlay of ADP nucleotide RMSD plots of the five 20ns MD trajectories in acK183 mutant.

(J) Overlay of the distance between the NZ atom of K85 and β -phosphate of ADP as a function of time for two stable trajectories (dark blue/cyan – wild-type, pink/red – acK183).

(K) Histogram showing binding of γ -³²P-ATP to recombinant wild type and mutants of His-GSK3 β . Plasmids encoding wild type and mutants of His-GSK3 β were transformed into *E. coli* BL21 (DE3). His-GSK3 β and its mutants were purified by Ni-NTA affinity chromatography. $n = 4$ independent experiments. Data is presented as mean \pm s.d. * $p < 0.05$. One-way ANOVA was used to calculate the p values.

(L) Histogram showing activity of HA-tagged WT or mutants of GSK3 β . HA-tagged human GSK3 β or its mutants were overexpressed in HeLa cells by transfection of their respective plasmids. HA-GSK3 β or its mutants were immunoprecipitated using HA-coupled agarose beads (Sigma-Aldrich). The enzymatic activity of GSK3 β was measured against Glycogen Synthase (GS)-peptide as described in the methods section. GSK3 β -DN - GSK3 β -K85A; Dominant negative. GSK3 β -CA- GSK3 β S9A; catalytically active. $n = 4$ independent experiments. Data is presented as mean \pm s.d. * $p < 0.05$. One-way ANOVA was used to calculate the p values.

Figure 2-figure supplement 1

Protein backbone C α RMSF plots of the wild-type (dark blue) and Ac-K183 mutant (red).

Figure 2-figure supplement 2

Homology alignment of GSK3 β between different species.

Figure 2-figure supplement 3

Stoichiometry for GSK3 β -K150, -K183 acetylation.

Figure 3. SIRT2 Binds to, Deacetylates and Activates GSK3 β

(A) Western blot analysis of acetylated GSK3 β in heart samples of 9 months old WT and SIRT2-KO littermates. GSK3 β was immunoprecipitated from heart tissue lysates of WT and SIRT2-KO mice using anti-GSK3 β antibody (sc-9166, Santacruz), and the affinity resin immobilized with protein A/G. Western blotting was performed to detect GSK3 β acetylation by anti-Ac-Lysine antibody. IgG was used as a negative control. Whole cell lysates (WCL) were probed for the SIRT2 and GAPDH by Western blotting. $n = 4$ mice per group.

(B) Histogram showing relative acetylated GSK3 β in 9 months old WT and SIRT2-KO mice heart tissues, as measured from figure 3A. Signal intensities of acetylated GSK3 β and GSK3 β were measured by densitometry analysis (ImageJ software). $n = 4$ mice per group. Data is presented as mean \pm s.d, * $p < 0.05$. Student's t test was used to calculate the p values.

(C) GSK3 β was immunoprecipitated from heart tissue lysates of 8 weeks old 129/Sv mice using anti-GSK3 β antibody (sc-9166, Santacruz), and the affinity resin immobilized with protein A/G. GSK3 β interaction with SIRT2 was tested by Western blotting using anti-SIRT2 antibody. IgG was used as negative control. Heart lysates was probed for indicated proteins by Western blotting.

(D) *In vitro* binding assay to test the interaction between GSK3 β and SIRT2. Flag-SIRT2 was overexpressed in 293 cells by a plasmid encoding human Flag-SIRT2. Recombinant His or His-GSK3 β was purified from *E. coli* BL21 (DE3) by Ni-NTA affinity chromatography and were

incubated with 293T cell lysates overexpressing human Flag-SIRT2. Interaction between GSK3 β and SIRT2 was tested by western blotting. # marked western images denotes SIRT2 antibody used in this assay detects single band.

(E) *In vitro* deacetylation assay showing SIRT2 as GSK3 β deacetylase. Human HA-GSK3 β was overexpressed in HeLa cells by transfection of the plasmid pcDNA3-HA-GSK3 β . HA-GSK3 β was immunoprecipitated using HA-coupled agarose beads (Sigma-Aldrich) and the HA-GSK3 β was acetylated by recombinant p300 (Millipore), in the presence or absence of Acetyl-CoA (Ac-CoA) in HAT buffer. The acetylated HA-GSK3 β was further incubated with either Flag-tagged SIRT2 or SIRT2-H187Y, which were immunoprecipitated from HEK 293 cell lysates overexpressing respective plasmids encoding Flag-tagged WT or SIRT2-H187Y using agarose beads conjugated to Anti-Flag antibody (Sigma A2220). The deacetylation reaction was carried out in the presence or absence of NAD⁺ in a HDAC buffer. GSK3 β acetylation was analyzed by western blotting using anti-Ac-Lysine antibody. # marked western images denotes SIRT2 antibody used in this assay detects single band.

(F) *In vitro* kinase assay depicting the activity of acetylated and deacetylated GSK3 β . Human HA-GSK3 β was overexpressed in HeLa cells by transfection of the plasmid pcDNA3-HA-GSK3 β . Recombinant HA-GSK3 β was immunoprecipitated using HA-coupled beads and was acetylated by recombinant p300 in the presence or absence of Acetyl-CoA (Ac-CoA) in HAT buffer. Acetylated GSK3 β was further deacetylated by either Flag-tagged WT or SIRT2-H187Y (SIRT2-HY), a catalytic inactive mutant of SIRT2, which was immunoprecipitated from HEK 293 cells, overexpressed with plasmid encoding Flag-tagged WT or SIRT2-H187Y using agarose beads conjugated to Anti-Flag antibody (Sigma A2220). The deacetylation reaction was carried out in the presence or absence of NAD⁺ in a HDAC buffer and further enzymatic activity of GSK3 β was measured against Glycogen Synthase (GS)-peptide, as described in the methods section. $n = 5$. Data is presented as mean \pm s.d. * $p < 0.05$. One-way ANOVA was used to calculate the p values.

(G) Western blot analysis of acetylated GSK3 β from control or SIRT2 depleted (SIRT2-KD) cardiomyocytes. Neonatal rat cardiomyocytes were transfected with either non-targeting (control) or SIRT2 siRNA targeting SIRT2 using Lipofectamine RNAiMAX reagent for 72 hours. SIRT2 depletion was confirmed by Western blotting. Total cellular acetylation was probed by anti-Ac-Lysine antibody to test the effect of SIRT2 depletion in cardiomyocytes. GSK3 β was immunoprecipitated from these cell lysates using anti-GSK3 β antibody (sc-9166, SantaCruz), and the affinity resin immobilized with protein A/G. Western blotting was performed to detect acetylation of GSK3 β by anti-Ac-Lysine antibody. Cell lysates (WCL) from control and SIRT2-KD cardiomyocytes were probed for indicated proteins by Western blotting.

(H) Western blotting analysis of hearts lysates from 9 months old WT and SIRT2-KO mice littermates for indicated proteins. $n = 4$ mice per group.

(I) Histogram showing activity of GSK3 β in WT and SIRT2-KO mice hearts at 9 months of age. GSK3 β was immunoprecipitated from the heart lysates of WT and SIRT2-KO mice using anti-GSK3 β antibody, clone GSK-4B (Sigma). The immunoprecipitated GSK3 β was incubated with the peptide substrate in the presence of γ -³²P-ATP. The incorporation of ³²P into the GSK3 β Peptide Substrate, which contains specific phosphorylation residues of GSK3 β was measured. $n = 6$ mice per group. Data is presented as mean \pm s.d. * $p < 0.05$. Student's t test was used to calculate the p values.

(J) *In vitro* deacetylation assay to test whether SIRT2 deacetylates K183 residue of GSK3 β . HA-tagged GSK3 β or GSK3 β -K183R was overexpressed in HeLa cells and was immunoprecipitated using HA-coupled beads. HA-tagged WT-GSK3 β or GSK3 β -K183R were incubated with Flag-SIRT2 immunoprecipitated from HEK 293T cells using agarose beads conjugated to Anti-Flag antibody (Sigma A2220). The deacetylation reaction was carried out in the presence or absence of NAD⁺ in a deacetylation buffer. Acetylation status of GSK3 β was analyzed by western blotting. # marked western images denotes SIRT2 antibody used in this assay detects single band.

(K) Histogram showing relative acetylation of HA-tagged GSK3 β or GSK3 β -K183R, which was incubated with Flag-SIRT2. The data is generated from Figure 3J. Signal intensities of acetylated-GSK3 β and GSK3 β were measured by densitometry analysis (ImageJ software). $n = 4$ independent experiments. Data is presented as mean \pm s.d. * $p < 0.05$. One-way ANOVA was used to calculate the p values.

(L) Histogram showing binding of γ -³²P-ATP to acetylated and deacetylated His-GSK3 β . Recombinant His-GSK3 β was purified from *E. coli* BL 21 (DE3) by Ni-NTA affinity chromatography. Purified His-GSK3 β was acetylated by recombinant p300 in the presence of Ac-CoA in HAT buffer. Acetylated His-GSK3 β was further deacetylated by Flag-SIRT2 immunoprecipitated from HEK 293T cells. The binding of γ -³²P-ATP to acetylated and deacetylated His-GSK3 β was assessed by the protocol described in methods section. $n = 4$. Data is presented as mean \pm s.d. * $p < 0.05$. One-way ANOVA was used to calculate the p values.

(M) Histogram showing activity of WT or mutants of GSK3 β . HA-tagged WT or mutants of GSK3 β was immunoprecipitated from HeLa cells transfected with respective plasmids using HA-coupled agarose beads. The enzymatic activity of GSK3 β was measured against Glycogen Synthase (GS)-peptide, as described in the methods section. $n = 4$. Data is presented as mean \pm s.d. * $p < 0.05$. One-way ANOVA was used to calculate the p values.

Figure 3-figure supplement 1

Western blotting analysis showing acetylation and phosphorylation of GSK3 β and its downstream target GS in HeLa cells overexpressing the Sirtuin isoforms, SIRT1-SIRT7. Cells were transiently overexpressed with either pcDNA-Flag or pcDNA-Flag-SIRT1-7 plasmid for 48 hours, and western blotting was performed to detect the indicated proteins. HeLa cell lysates (WCL) were probed with Flag-antibody for detecting the expression of Sirtuins. GSK3 β was immunoprecipitated from these overexpressed lysates using anti-GSK3 β antibody (sc-9166, Santacruz) and tested for its acetylation by western blotting using anti-Ac-Lys antibody.

Figure 3-figure supplement 2

Western blotting analysis of vehicle or SIRT2 inhibitor, AGK-2 (10 μ M, 12 hrs) treated rat neonatal cardiomyocytes for indicated proteins. Acetylation of tubulin at K40, which is a specific deacetylation target of SIRT2 was probed to test the efficacy of SIRT2 inhibition. GSK3 β was immunoprecipitated from these cell lysates using anti-GSK3 β antibody, and the affinity resin immobilized with protein A/G. GSK3 β acetylation was detected by Western blotting.

Figure 3-figure supplement 3

Representative confocal images of vehicle or AGK2 (10 μ M, 12 hrs) treated HeLa cells stained with GSK3 β (Green). MnSOD was used to localize the mitochondria (Red). DAPI was used to stain the nucleus (Blue).

Figure 3-figure supplement 4

Representative confocal images of HA-tagged WT or mutants of GSK3 β transiently overexpressed in GSK3 β deficient mouse embryonic fibroblasts. Immunostaining was performed using HA antibody to localize WT and mutants of GSK3 β (Red).

Figure 3-figure supplement 5

Western blot analysis of 9 months old WT and SIRT2-KO mice heart samples for indicated proteins. n=4 mice per group.

Figure 4. Molecular Modeling of Acetylated GSK3 α

(A) Western blot analysis showing acetylation status of both isoforms of GSK3 in control or SIRT2 depleted (SIRT2-KD) cardiomyocytes. Neonatal rat cardiomyocytes were transfected with either non-targeting or siRNA pool targeting SIRT2 using Lipofectamine RNAiMAX reagent for 72 hours. SIRT2 depletion was confirmed by western blotting. GSK3 was immunoprecipitated from cell lysates using anti-GSK3 antibody and the affinity resin immobilized with protein A/G. Western blotting was performed to detect GSK3 α/β acetylation by anti-Ac-Lysine antibody. Cell lysates was probed for SIRT2 and actin antibodies by Western blotting.

(B) Histogram showing relative acetylated-GSK3 α and GSK3 β in control and SIRT2 depleted (SIRT2-KD) cardiomyocytes, as measured from figure 4A. Signal intensities of acetylated-GSK3 α and acetylated-GSK3 β were measured by densitometry analysis (ImageJ software). $n = 3$. Data is presented as mean \pm s.d. $*p < 0.05$. Student's t test was used to calculate the p values.

(C) Histogram showing enzymatic activity of acetylated and deacetylated GSK3 α . Recombinant HA- GSK3 α was immunoprecipitated from HeLa cells overexpressing pcDNA-HA-GSK3 α using HA-coupled agarose beads. Immunoprecipitated HA-GSK3 α was acetylated by p300 in the presence of Acetyl-CoA (Ac-CoA) in HAT buffer. Acetylated GSK3 α was further deacetylated by Flag-SIRT2 immunoprecipitated from HEK 293T cells overexpressing plasmid encoding Flag-tagged SIRT2-WT using agarose beads conjugated to Anti-Flag antibody (Sigma A2220). The enzymatic activity of GSK3 α was measured against Glycogen Synthase (GS)-peptide. $n = 5$. Data is presented as mean \pm s.d. $*p < 0.05$. One-way ANOVA was used to calculate the p values.

(D) Annotation of representative tandem mass spectra of trypsin-digested GSK3 α , depicting K99, K246 acetylation.

(E) Protein sequence alignment of the modeled region of GSK3 α and the structure of GSK3 β .

(F) Cartoon, surface representation of the homology model of GSK3 α (highlighted is the adenine nucleotide binding pocket and position of K246 residue).

Figure 5. GSK3 β is Required for the Anti-Hypertrophic Role of SIRT2 deacetylase

(A) Western blotting analysis depicting the activity of GSK3 inhibitor X (GSK3-X). Neonatal rat cardiomyocytes were treated with vehicle or 500nM GSK3-X for 48 hours and the activity of GSK3 was assessed by monitoring the phosphorylation of GS by specific antibody.

(B) [3 H]-leucine incorporation into total cellular protein of control (Ad-GFP) or SIRT2-overexpressing (Ad-SIRT2) rat neonatal cardiomyocytes treated with either vehicle or 500nM GSK3 inhibitor X (GSK3-X) for 48 hours. Cardiomyocytes were infected with adenoviral vectors encoding either GFP or SIRT2 for 24 hours prior to GSK3-X treatment. After the GSK3-X treatment, cardiomyocytes were stimulated with either vehicle or 20 μ M ISO for 24 hours and the [3 H]-leucine incorporation was monitored. c.p.m. counts per minute. $n = 10$. Data is presented as mean \pm s.d. * $p < 0.05$. Two-way ANOVA was used to calculate the p values.

(C) Histogram showing quantification of relative cardiomyocyte area in control (Ad-Null) and SIRT2-overexpressing (Ad-SIRT2) rat neonatal cardiomyocytes treated with either vehicle or 500nM GSK3 inhibitor X (GSK3-X) for 48 hours. Cardiomyocytes were infected with adenoviral vectors encoding either control SIRT2 for 24 hours prior to GSK3-X treatment. After the GSK3-X treatment, cardiomyocytes were stimulated with either vehicle or 20 μ M ISO for 24 hours and the relative cardiomyocyte area is quantified as described in methods section. Data is presented as mean \pm s.d. * $p < 0.05$. Two-way ANOVA was used to calculate the p values.

(D) Representative confocal images depicting perinuclear expression of ANP in control (Ad-Null) or SIRT2-overexpressing (Ad-SIRT2) cardiomyocytes treated with either vehicle or ISO (20 μ M, 24 hrs), with or without GSK3 inhibitor X (GSK3-X, 500nM, 48 hr). Scale bar = 20 μ m. ANP (Green), Myomesin (Red), Hoechst (Blue).

(E) Western blotting analysis for puromycin incorporation in control or SIRT2 depleted (SIRT2-KD) neonatal rat cardiomyocytes infected with adenovirus expressing either control (Ad-luc shRNA) or p300 shRNA (Ad-p300-shRNA) 48 hours. p300 depletion was confirmed by western blotting. Pulse of puromycin was given 30 minutes prior to harvesting of cardiomyocytes and puromycin incorporation into nascent proteins was tested using anti-puromycin antibody. # marked Western images denotes SIRT2 antibody used in this assay detects single band.

(F) Histogram showing relative puromycin levels in control or SIRT2 depleted (SIRT2-KD) cardiomyocytes infected with adenovirus expressing either control (Ad-luc shRNA) or p300 shRNA (Ad-p300-shRNA). The data is generated from Figure 5E. Signal intensities of puromycin and actin were measured by densitometry analysis using ImageJ software. $n = 3$ independent experiments. Data is presented as mean \pm s.d. * $p < 0.05$. Two-way ANOVA was used to calculate the p values.

(G) Western blotting analysis of GSK3 β acetylation and activity in heart lysates of vehicle or anacardic acid (p300 inhibitor) treated 9 months old WT and SIRT2-KO mice littermates. Anacardic acid was injected intraperitoneal at the dose of 5mg/kg/day for 10 days in mice. Peanut oil was used as vehicle. GSK3 β was immunoprecipitated from heart lysates of WT and SIRT2-KO mice using anti-GSK3 β antibody (sc-9166, Santacruz), and the affinity resin with protein A/G immobilized. Western blotting was performed to detect GSK3 β acetylation by anti-Ac-Lysine antibody. GSK3 β activity was measured by detecting the phosphorylation of GS. SIRT2 depletion was confirmed by Western blotting. Whole cell lysates (WCL) was probed for indicated proteins by Western blotting.

(H) Histogram showing relative GSK3 β acetylation in heart lysates of vehicle or anacardic acid (5mg/kg/day for 10 days) treated 9 months old WT and SIRT2-KO mice from figure 5G. $n = 3$. Signal intensities of GSK3 β and acetylated-GSK3 β was measured by densitometry analysis using ImageJ software. Data is presented as mean \pm s.d. $*p < 0.05$. Two-way ANOVA was used to calculate the p values.

(I) Scatter plot depicting HW/TL ratio of 9 months old WT and SIRT2-KO mice treated with either vehicle or anacardic acid, (p300-INH), at the dose of 5mg/kg/day for 10 days. $n = 5$ mice per group. Data is presented as mean \pm s.d. $*p < 0.05$. Two-way ANOVA was used to calculate the p values.

(J) Scatter plot showing left ventricular posterior wall thickness of 9 months old WT and SIRT2-KO mice treated with either vehicle or anacardic acid, (p300-INH), at the dose of 5mg/kg/day for 10 days. $n = 6-8$ mice per group. Data is presented as mean \pm s.d. $*p < 0.05$. Two-way ANOVA was used to calculate the p values.

(K) Scatter plot depicting cardiac contractile functions, as measured by ejection fraction of 9 months old WT and SIRT2-KO mice treated with either vehicle or anacardic acid, (p300-INH), at the dose of 5mg/kg/day for 10 days. $n = 6-8$ mice per group. Data is presented as mean \pm s.d. $*p < 0.05$. Two-way ANOVA was used to calculate the p values.

Figure 5-figure supplement 1

(A) Western blotting analysis to confirm the activity of LiCl. Neonatal rat cardiomyocytes were treated with 20mM LiCl for 48 hr and the activity of GSK3 β was assessed by monitoring the phosphorylation of p-GS.

(B) [3 H]-leucine incorporation into total cellular protein of control (Ad-GFP) or SIRT2-overexpressing (Ad-SIRT2) rat neonatal cardiomyocytes treated either with vehicle or ISO (20 μ M, 24 hrs), with or without LiCl (20 mM, 48 hr). c.p.m. counts per minute. $n = 10$. Data is presented as mean \pm s.d. $*p < 0.05$. Two-way ANOVA was used to calculate the p values.

(C) Histogram showing quantification of relative cardiomyocyte area in control (Ad-Null) or SIRT2-overexpressing (Ad-SIRT2) rat neonatal cardiomyocytes treated either with vehicle or ISO (20 μ M, 24 hrs), with or without LiCl (20 mM, 48 hr). Data is presented as mean \pm s.d. $*p < 0.05$. Two-way ANOVA was used to calculate the p values.

(D) Representative confocal images depicting perinuclear expression ANP in control (Ad-Null) or SIRT2-overexpressing (Ad-SIRT2) rat neonatal cardiomyocytes treated either with vehicle or ISO (20 μ M, 24 hrs), with or without 20 mM LiCl. Scale bar = 20 μ m. α - Actinin (Red), ANP (Red) and Hoechst (Blue).

References

- Abdillahi, M., Ananthakrishnan, R., Vedantham, S., Shang, L., Zhu, Z., Rosario, R., Zirpoli, H., Bohren, K.M., Gabbay, K.H., and Ramasamy, R. (2012). Aldose reductase modulates cardiac glycogen synthase kinase-3 β phosphorylation during ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* 303, H297-308.
- Ali, A., Hoeflich, K.P., and Woodgett, J.R. (2001). Glycogen synthase kinase-3: properties, functions, and regulation. *Chemical reviews* 101, 2527-2540.

1211 Antos, C.L., McKinsey, T.A., Frey, N., Kutschke, W., McAnally, J., Shelton, J.M., Richardson, J.A., Hill, J.A., and Olson,
1212 E.N. (2002). Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. *Proceedings of the*
1213 *National Academy of Sciences of the United States of America* 99, 907-912.

1214 Bertsch, S., Lang, C.H., and Vary, T.C. (2011). Inhibition of glycogen synthase kinase 3[beta] activity with lithium in
1215 vitro attenuates sepsis-induced changes in muscle protein turnover. *Shock* 35, 266-274.

1216 Beurel, E., Grieco, S.F., and Jope, R.S. (2015). Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases.
1217 *Pharmacol Therapeut* 148, 114-131.

1218 Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T.G., Bertoni, M.,
1219 Bordoli, L., *et al.* (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary
1220 information. *Nucleic Acids Res* 42, W252-W258.

1221 Chen, X., Liu, Y., Zhu, J., Lei, S., Dong, Y., Li, L., Jiang, B., Tan, L., Wu, J., Yu, S., *et al.* (2016). GSK-3beta
1222 downregulates Nrf2 in cultured cortical neurons and in a rat model of cerebral ischemia-reperfusion. *Sci Rep* 6,
1223 20196.

1224 Cho, Y.J., Kim, J.H., Yoon, J., Cho, S.J., Ko, Y.S., Park, J.W., Lee, H.S., Lee, H.E., Kim, W.H., and Lee, B.L. (2010).
1225 Constitutive activation of glycogen synthase kinase-3beta correlates with better prognosis and cyclin-dependent
1226 kinase inhibitors in human gastric cancer. *BMC Gastroenterol* 10, 91.

1227 Choudhary, C., Kumar, C., Gnad, F., Nielsen, M.L., Rehman, M., Walther, T.C., Olsen, J.V., and Mann, M. (2009).
1228 Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions. *Science* 325, 834-840.

1229 Cohen, P., and Frame, S. (2001). The renaissance of GSK3. *Nat Rev Mol Cell Biol* 2, 769-776.

1230 Cole, A., Frame, S., and Cohen, P. (2004). Further evidence that the tyrosine phosphorylation of glycogen synthase
1231 kinase-3 (GSK3) in mammalian cells is an autophosphorylation event. *Biochemical Journal* 377, 249-255.

1232 Cross, D.A.E., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of Glycogen-Synthase
1233 Kinase-3 by Insulin-Mediated by Protein-Kinase-B. *Nature* 378, 785-789.

1234 Dajani, R., Fraser, E., Roe, S.M., Young, N., Good, V., Dale, T.C., and Pearl, L.H. (2001). Crystal structure of glycogen
1235 synthase kinase 3 beta: Structural basis for phosphate-primed substrate specificity and autoinhibition. *Cell* 105,
1236 721-732.

1237 Darden, T., York, D., and Pedersen, L. (1993). Particle Mesh Ewald - an N.Log(N) Method for Ewald Sums in Large
1238 Systems. *Journal of Chemical Physics* 98, 10089-10092.

1239 de Oliveira, R.M., Sarkander, J., Kazantsev, A.G., and Outeiro, T.F. (2012). SIRT2 as a therapeutic target for age-
1240 related disorders. *Front Pharmacol* 3.

1241 Doble, B.W., and Woodgett, J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116, 1175-
1242 1186.

1243 Donmez, G., and Outeiro, T.F. (2013). SIRT1 and SIRT2: emerging targets in neurodegeneration. *Embo Mol Med* 5,
1244 344-352.

1245 Guarente, L. (2007). Sirtuins in aging and disease. *Cold Spring Harb Sym* 72, 483-488.

1246 Haberland, M., Montgomery, R.L., and Olson, E.N. (2009). The many roles of histone deacetylases in development
1247 and physiology: implications for disease and therapy. *Nature Reviews Genetics* 10, 32-42.

1248 Haigis, M.C., and Guarente, L.P. (2006). Mammalian sirtuins-emerging roles in physiology, aging, and calorie
1249 restriction. *Genes & Development* 20, 2913-2921.

1250 Hao, Y., Wang, H., Qiao, S., Leng, L., and Wang, X. (2016). Histone deacetylase HDA6 enhances brassinosteroid
1251 signaling by inhibiting the BIN2 kinase. *Proc Natl Acad Sci U S A* 113, 10418-10423.

1252 Horike, N., Sakoda, H., Kushiya, A., Ono, H., Fujishiro, M., Kamata, H., Nishiyama, K., Uchijima, Y., Kurihara, Y.,
1253 Kurihara, H., *et al.* (2008). AMP-activated protein kinase activation increases phosphorylation of glycogen synthase
1254 kinase 3beta and thereby reduces cAMP-responsive element transcriptional activity and phosphoenolpyruvate
1255 carboxykinase C gene expression in the liver. *J Biol Chem* 283, 33902-33910.

1256 Hughes, K., Nikolakaki, E., Plyte, S.E., Totty, N.F., and Woodgett, J.R. (1993). Modulation of the Glycogen-Synthase
1257 Kinase-3 Family by Tyrosine Phosphorylation. *Embo J* 12, 803-808.

1258 Jain, A., Ravi, V., Muhamed, J., Chatterjee, K., and Sundaresan, N.R. (2017). A simplified protocol for culture
1259 of murine neonatal cardiomyocytes on nanoscale keratin coated surfaces. *Int J Cardiol* 232, 160-170.

1260 Jeffers, V., and Sullivan, W.J. (2012). Lysine Acetylation Is Widespread on Proteins of Diverse Function and
1261 Localization in the Protozoan Parasite *Toxoplasma gondii*. *Eukaryot Cell* 11, 735-742.

1262 Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W., and Klein, M.L. (1983). Comparison of Simple
1263 Potential Functions for Simulating Liquid Water. *Journal of Chemical Physics* 79, 926-935.

1264 Kaidanovich-Beilin, O., and Woodgett, J.R. (2011). GSK-3: Functional Insights from Cell Biology and Animal Models.
1265 *Front Mol Neurosci* 4, 40.

1266 Kaufmann, T., Kukolj, E., Brachner, A., Beltzung, E., Bruno, M., Kostrhon, S., Opravil, S., Hudecz, O., Mechtler, K.,
1267 Warren, G., *et al.* (2016). SIRT2 regulates nuclear envelope reassembly through ANKLE2 deacetylation. *Journal of*
1268 *Cell Science* 129, 4607-4621.

1269 Kerkela, R., Kockeritz, L., MacAulay, K., Zhou, J., Doble, B.W., Beahm, C., Greytak, S., Woulfe, K., Trivedi, C.M.,
1270 Woodgett, J.R., *et al.* (2008). Deletion of GSK-3 beta in mice leads to hypertrophic cardiomyopathy secondary to
1271 cardiomyoblast hyperproliferation. *Journal of Clinical Investigation* 118, 3609-3618.

1272 Kim, H.S., Vassilopoulos, A., Wang, R.H., Lahusen, T., Xiao, Z., Xu, X., Li, C., Veenstra, T.D., Li, B., Yu, H., *et al.* (2011).
1273 SIRT2 maintains genome integrity and suppresses tumorigenesis through regulating APC/C activity. *Cancer Cell* 20,
1274 487-499.

1275 Kouzarides, T. (2000). Acetylation: a regulatory modification to rival phosphorylation? *The EMBO journal* 19, 1176-
1276 1179.

1277 Li, C.L., Sathyamurthy, A., Oldenborg, A., Tank, D., and Ramanan, N. (2014). SRF Phosphorylation by Glycogen
1278 Synthase Kinase-3 Promotes Axon Growth in Hippocampal Neurons. *Journal of Neuroscience* 34, 4027-4042.

1279 Liu, C.M., Li, Y.M., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z.H., Lin, X.H., and He, X. (2002). Control of
1280 beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108, 837-847.

1281 Lo Sasso, G., Menzies, K.J., Mottis, A., Piersigilli, A., Perino, A., Yamamoto, H., Schoonjans, K., and Auwerx, J.
1282 (2014). SIRT2 deficiency modulates macrophage polarization and susceptibility to experimental colitis. *PLoS One* 9,
1283 e103573.

1284 Longo, V.D., and Kennedy, B.K. (2006). Sirtuins in aging and age-related disease. *Cell* 126, 257-268.

1285 MacAulay, K., Blair, A.S., Hajdich, E., Terashima, T., Baba, O., Sutherland, C., and Hundal, H.S. (2005). Constitutive
1286 activation of GSK3 down-regulates glycogen synthase abundance and glycogen deposition in rat skeletal muscle
1287 cells. *Journal of Biological Chemistry* 280, 9509-9518.

1288 Martinez-Redondo, P., and Vaquero, A. (2013). The diversity of histone versus nonhistone sirtuin substrates.
1289 *Genes & cancer* 4, 148-163.

1290 Matsuda, T., Zhai, P., Maejima, Y., Hong, C., Gao, S.M., Tian, B., Goto, K., Takagi, H., Tamamori-Adachi, M.,
1291 Kitajima, S., *et al.* (2008). Distinct roles of GSK-3 alpha and GSK-3 beta phosphorylation in the heart under pressure
1292 overload. *Proceedings of the National Academy of Sciences of the United States of America* 105, 20900-20905.

1293 Maurer, U., Charvet, C., Wagman, A.S., Dejardin, E., and Green, D.R. (2006). Glycogen synthase kinase-3 regulates
1294 mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 21, 749-760.

1295 Meijer, L., Skaltsounis, A.L., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X.Z.P., Vonica, C.A.,
1296 Brivanlou, A., Dajani, R., *et al.* (2003). GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chemistry &*
1297 *Biology* 10, 1255-1266.

1298 Moscardo, A., Valles, J., Latorre, A., Jover, R., and Santos, M.T. (2015). The histone deacetylase sirtuin 2 is a new
1299 player in the regulation of platelet function. *J Thromb Haemost* 13, 1335-1344.

1300 Neal, J.W., and Clipstone, N.A. (2001). Glycogen synthase kinase-3 inhibits the DNA binding activity of NFATc. *J Biol*
1301 *Chem* 276, 3666-3673.

1302 North, B.J., Marshall, B.L., Borra, M.T., Denu, J.M., and Verdin, E. (2003). The human Sir2 ortholog, SIRT2, is an
1303 NAD(+)-dependent tubulin deacetylase. *Mol Cell* 11, 437-444.

1304 Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF
1305 chimera - A visualization system for exploratory research and analysis. *J Comput Chem* 25, 1605-1612.

1306 Pillai, V.B., Samant, S., Sundaresan, N.R., Raghuraman, H., Kim, G., Bonner, M.Y., Arbiser, J.L., Walker, D.I., Jones,
1307 D.P., Gius, D., *et al.* (2015). Honokiol blocks and reverses cardiac hypertrophy in mice by activating mitochondrial
1308 Sirt3. *Nat Commun* 6, 6656.

1309 Rayasam, G.V., Tulasi, V.K., Sodhi, R., Davis, J.A., and Ray, A. (2009). Glycogen synthase kinase 3: more than a
1310 namesake. *Brit J Pharmacol* 156, 885-898.

1311 Sarikhani, M., Maity, S., Mishra, S., Jain, A., Tamta, A.K., Ravi, V., Spurthi, K.M., Desingu, P.A., Khan, D., Kumar, S.,
1312 *et al.* (2018a). SIRT2 deacetylase represses NFAT transcription factor to maintain cardiac homeostasis. *J Biol Chem.*
1313 Published online. doi: 10.1074/jbc.RA117.000915. PMID: 29440391

1314 Sarikhani, M., Mishra, S., Desingu, P.A., Kotyada, C., Wolfgeher, D., Gupta, M.P., Singh, M., and Sundaresan, N.R.
1315 (2018b). SIRT2 regulates oxidative stress-induced cell death through deacetylation of c-Jun NH2-terminal kinase.
1316 *Cell Death Differ.* Published online. doi: 10.1038/s41418-018-0069-8. PMID: 29449643

1317 Schmidt, E.K., Clavarino, G., Ceppi, M., and Pierre, P. (2009). SUNSET, a nonradioactive method to monitor protein
1318 synthesis. *Nature methods* 6, 275-277.

1319 Song, W.J., Song, E.A.C., Jung, M.S., Choi, S.H., Baik, H.H., Jin, B.K., Kim, J.H., and Chung, S.H. (2015).
1320 Phosphorylation and Inactivation of Glycogen Synthase Kinase 3 beta (GSK3 beta) by Dual-specificity Tyrosine
1321 Phosphorylation-regulated Kinase 1A (Dyrk1A). *J Biol Chem* 290, 2321-2333.

1322 Stambolic, V., and Woodgett, J.R. (1994). Mitogen Inactivation of Glycogen-Synthase Kinase-3-Beta in Intact-Cells
1323 Via Serine-9 Phosphorylation. *Biochemical Journal* 303, 701-704.

1324 Stamos, J.L., Chu, M.L., Enos, M.D., Shah, N., and Weis, W.I. (2014). Structural basis of GSK-3 inhibition by N-
1325 terminal phosphorylation and by the Wnt receptor LRP6. *Elife* 3, e01998.

1326 Sugden, P.H., Fuller, S.J., Weiss, S.C., and Clerk, A. (2008). Glycogen synthase kinase 3 (GSK3) in the heart: a point
1327 of integration in hypertrophic signalling and a therapeutic target? A critical analysis. *Brit J Pharmacol* 153, S137-
1328 S153.

1329 Sundaresan, N.R., Bindu, S., Pillai, V.B., Samant, S., Pan, Y., Huang, J.Y., Gupta, M., Nagalingam, R.S., Wolfgeher, D.,
1330 Verdin, E., *et al.* (2016). SIRT3 Blocks Aging-Associated Tissue Fibrosis in Mice by Deacetylating and Activating
1331 Glycogen Synthase Kinase 3 beta. *Molecular and Cellular Biology* 36, 678-692.

1332 Sundaresan, N.R., Gupta, M., Kim, G., Rajamohan, S.B., Isbatan, A., and Gupta, M.P. (2009). Sirt3 blocks the cardiac
1333 hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest*
1334 119, 2758-2771.

1335 Sundaresan, N.R., Pillai, V.B., Wolfgeher, D., Samant, S., Vasudevan, P., Parekh, V., Raghuraman, H., Cunningham,
1336 J.M., Gupta, M., and Gupta, M.P. (2011). The deacetylase SIRT1 promotes membrane localization and activation of
1337 Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Sci Signal* 4, ra46.

1338 Sundaresan, N.R., Vasudevan, P., Zhong, L., Kim, G., Samant, S., Parekh, V., Pillai, V.B., Ravindra, P.V., Gupta, M.,
1339 Jeevanandam, V., *et al.* (2012). The sirtuin SIRT6 blocks IGF-Akt signaling and development of cardiac hypertrophy
1340 by targeting c-Jun. *Nature medicine* 18, 1643-+.

1341 Suzuki, T., Bridges, D., Nakada, D., Skiniotis, G., Morrison, S.J., Lin, J.D., Saltiel, A.R., and Inoki, K. (2013). Inhibition
1342 of AMPK catabolic action by GSK3. *Mol Cell* 50, 407-419.

1343 Tang, X., Chen, X.F., Wang, N.Y., Wang, X.M., Liang, S.T., Zheng, W., Lu, Y.B., Zhao, X., Hao, D.L., Zhang, Z.Q., *et al.*
1344 (2017). SIRT2 Acts as a Cardioprotective Deacetylase in Pathological Cardiac Hypertrophy. *Circulation* 136, 2051-
1345 2067.

1346 Thornton, T.M., Pedraza-Alva, G., Deng, B., Wood, C.D., Aronshtam, A., Clements, J.L., Sabio, G., Davis, R.J.,
1347 Matthews, D.E., Doble, B., *et al.* (2008). Phosphorylation by p38 MAPK as an alternative pathway for GSK3 beta
1348 inactivation. *Science* 320, 667-670.

1349 Tsai, C.C., Kai, J.I., Huang, W.C., Wang, C.Y., Wang, Y., Chen, C.L., Fang, Y.T., Lin, Y.S., Anderson, R., Chen, S.H., *et al.*
1350 (2009). Glycogen synthase kinase-3beta facilitates IFN-gamma-induced STAT1 activation by regulating Src
1351 homology-2 domain-containing phosphatase 2. *J Immunol* 183, 856-864.

1352 Verdin, E., and Ott, M. (2015). 50 years of protein acetylation: from gene regulation to epigenetics, metabolism
1353 and beyond. *Nat Rev Mol Cell Bio* 16, 258-264.

1354 Warnecke, A., Sandalova, T., Achour, A., and Harris, R.A. (2014). PyTMs: a useful PyMOL plugin for modeling
1355 common post-translational modifications. *Bmc Bioinformatics* 15, 370.

1356 Woodgett, J.R. (1990). Molecular-Cloning and Expression of Glycogen-Synthase Kinase-3 Factor-A. *Embo J* 9, 2431-
1357 2438.

1358 Yanazume, T., Hasegawa, K., Morimoto, T., Kawamura, T., Wada, H., Matsumori, A., Kawase, Y., Hirai, M., and Kita,
1359 T. (2003). Cardiac p300 is involved in myocyte growth with decompensated heart failure. *Mol Cell Biol* 23, 3593-
1360 3606.

1361 Yanazume, T., Morimoto, T., Wada, H., Kawamura, T., Kawase, Y., and Hasegawa, K. (2002). Cardiac nuclear
1362 acetylation by p300 is involved in myocyte growth with decompensated heart failure. *Circulation* 106, 142-142.

1363 Yang, X., Park, S.H., Chang, H.C., Shapiro, J.S., Vassilopoulos, A., Sawicki, K.T., Chen, C., Shang, M., Burrridge, P.W.,
1364 Epting, C.L., *et al.* (2017). Sirtuin 2 regulates cellular iron homeostasis via deacetylation of transcription factor
1365 NRF2. *The Journal of clinical investigation* 127, 1505-1516.

1366 Yang, X.J., and Seto, E. (2008). Lysine acetylation: Codified crosstalk with other posttranslational modifications.
1367 *Molecular Cell* 31, 449-461.

1368 Zhai, P., Gao, S., Holle, E., Yu, X., Yatani, A., Wagner, T., and Sadoshima, J. (2007). Glycogen synthase kinase-3alpha
1369 reduces cardiac growth and pressure overload-induced cardiac hypertrophy by inhibition of extracellular signal-
1370 regulated kinases. *J Biol Chem* 282, 33181-33191.

1371 Zhang, W., Yano, N., Deng, M., Mao, Q., Shaw, S.K., and Tseng, Y.T. (2011). beta-Adrenergic receptor-PI3K signaling
1372 crosstalk in mouse heart: elucidation of immediate downstream signaling cascades. *PLoS One* 6, e26581.

1373 Zheng, L., Baumann, U., and Reymond, J.L. (2004). An efficient one-step site-directed and site-saturation
1374 mutagenesis protocol. *Nucleic acids research* 32, e115.

1375 Zhou, J., Ahmad, F., Parikh, S., Hoffman, N.E., Rajan, S., Verma, V.K., Song, J., Yuan, A., Shanmughapriya, S., Guo, Y.,
1376 *et al.* (2016). Loss of Adult Cardiac Myocyte GSK-3 Leads to Mitotic Catastrophe Resulting in Fatal Dilated
1377 Cardiomyopathy. *Circulation research* 118, 1208-1222.

1378

1379

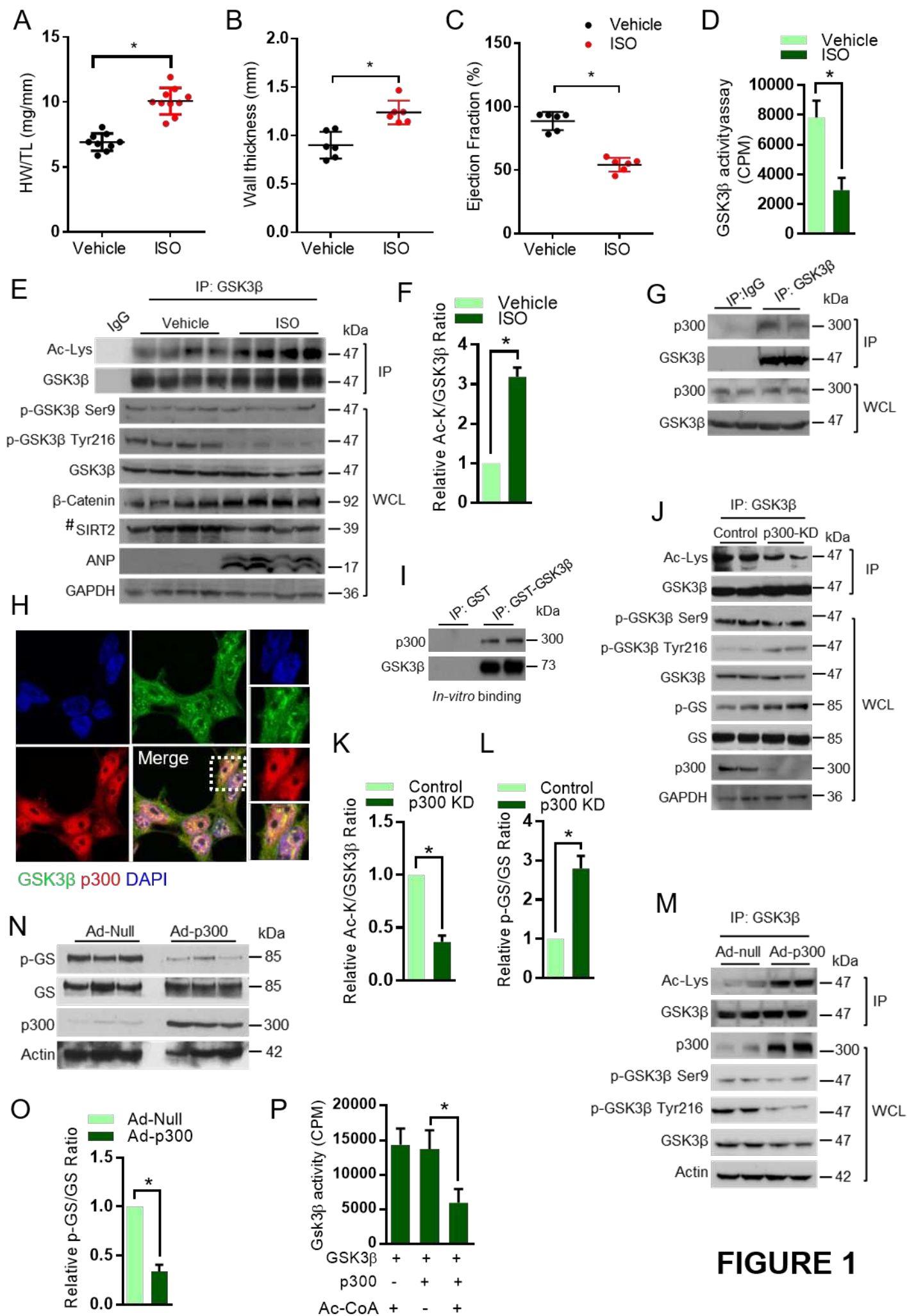


FIGURE 1

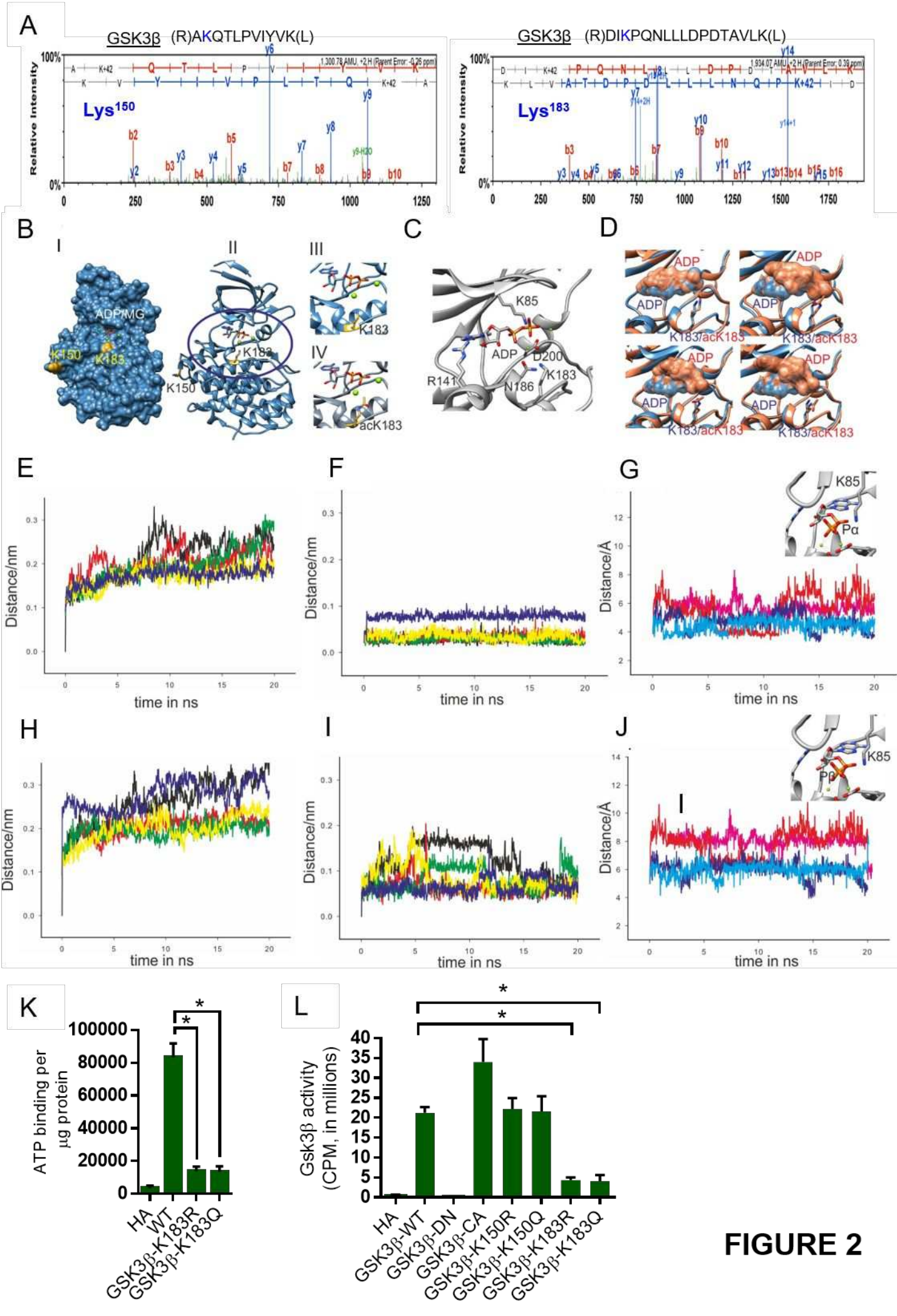
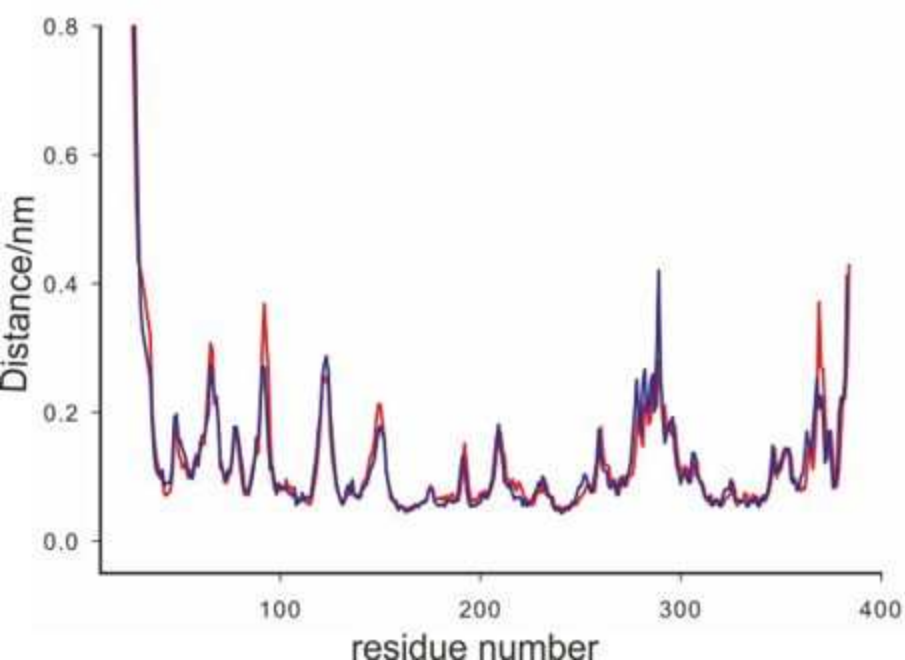


FIGURE 2



S9

Caenorhabditis	-----MNKQLLSCSLKSGKQVTMVASVATDGVDDQQVEISYYDQ	39
Danio	MSGRPRTTSFAESC KPVQP S AFGSMKVS RD KDGSKVTTVV-ATPGQGPD RPQEVSYTDT	59
Gallus	MSGRPRTTSFAESC KPVQP S AFGSMKVS RD KDGSKVTTVV-ATPGQGPD RPQEVSYTDT	59
Mus	MSGRPRTTSFAESC KPVQP S AFGSMKVS RD KDGSKVTTVV-ATPGQGPD RPQEVSYTDT	59
Rattus	MSGRPRTTSFAESC KPVQP S AFGSMKVS RD KDGSKVTTVV-ATPGQGPD RPQEVSYTDT	59
Bos	MSGRPRTTSFAESC KPVQP S AFGSMKVS RD KDGSKVTTVV-ATPGQGPD RPQEVSYTDT	59
Homo	MSGRPRTTSFAESC KPVQP S AFGSMKVS RD KDGSKVTTVV-ATPGQGPD RPQEVSYTDT	59
Canis	MSGRPRTTSFAESC KPVQP S AFGSMKVS RD KDGSKVTTVV-ATPGQGPD RPQEVSYTDT	59
.: *.:** ** :. :* *: *:* *		
K150		

Caenorhabditis	GEKKDELYLNLILEYVPETVYRVARHYSKQRQQIPMIYVKLYMYQLLRS LAYIHSIGICH	159
Danio	GDKKDEVYLNVLVDYVPETVYRVARHYSRAKQTLP MVYVKLYMYQLFRSLAYIHSFGICH	179
Gallus	GEKKDEVYLNVLVDYVPETVYRVARHYSRAKQTLP MIYVKLYMYQLFRSLAYIHSFGICH	179
Mus	GEKKDEVYLNVLVDYVPETVYRVARHYSRAKQTLP VIYVKLYMYQLFRSLAYIHSFGICH	179
Rattus	GEKKDEVYLNVLVDYVPETVYRVARHYSRAKQTLP VIYVKLYMYQLFRSLAYIHSFGICH	179
Bos	GEKKDEVYLNVLVDYVPETVYRVARHYSRAKQTLP VIYVKLYMYQLFRSLAYIHSFGICH	179
Homo	GEKKDEVYLNVLVDYVPETVYRVARHYSRAKQTLP VIYVKLYMYQLFRSLAYIHSFGICH	179
Canis	GEKKDEVYLNVLVDYVPETVYRVARHYSRAKQTLP VIYVKLYMYQLFRSLAYIHSFGICH	179
*:****:*****:*:*****: :* :*:*****:*****:****		

K183

Caenorhabditis	RDIKPQNLLIDPESGVLKLCDFGSAKYLV RN EPNVSYICSRYYRAPELIFGATNYTNSID	219
Danio	RDIKPQNLLLD PDTAVLKLCD FGS AKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSID	239
Gallus	RDIKPQNLLLD PDTAVLKLCD FGS AKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSID	239
Mus	RDIKPQNLLLD PDTAVLKLCD FGS AKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSID	239
Rattus	RDIKPQNLLLD PDTAVLKLCD FGS AKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSID	239
Bos	RDIKPQNLLLD PDTAVLKLCD FGS AKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSID	239
Homo	RDIKPQNLLLD PDTAVLKLCD FGS AKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSID	239
Canis	RDIKPQNLLLD PDTAVLKLCD FGS AKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSID	239
*****:*****:***** *****:****		

K150	Charge	Scans	Intensity	Stoichiometry
AKQTLPIYVK	2+			
Non-Ac Mass	630.38	1538-1736	582000	0.59%
Ac Mass	651.39	1538-1736	3480	

K183	Charge	Scans	Intensity	Stoichiometry
DIKPQNLLDPDTAVLK	2+			
Non-Ac Mass	947.04	5751-5833	16200	25.03%
Ac Mass	968.04	5751-5833	5410	

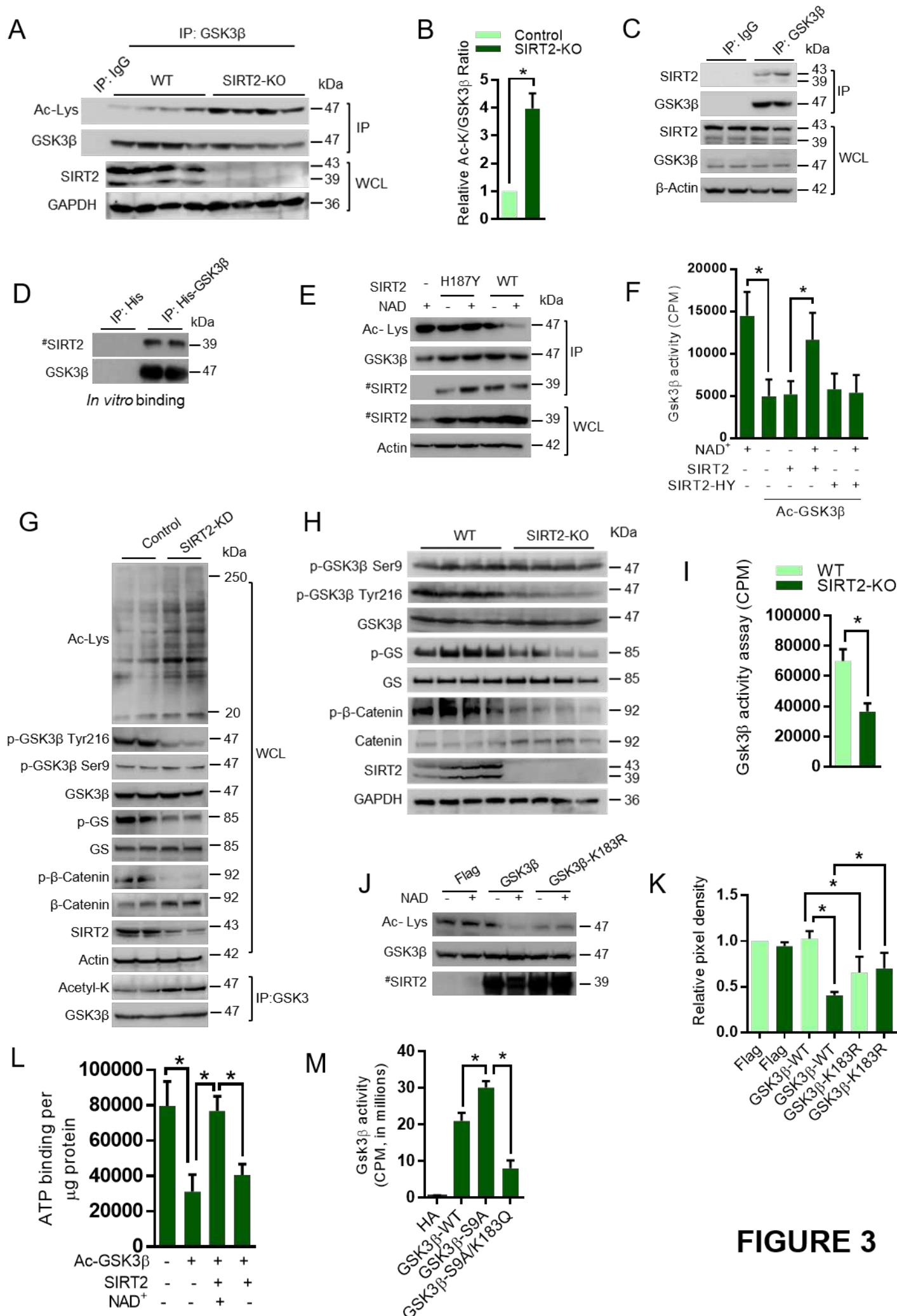
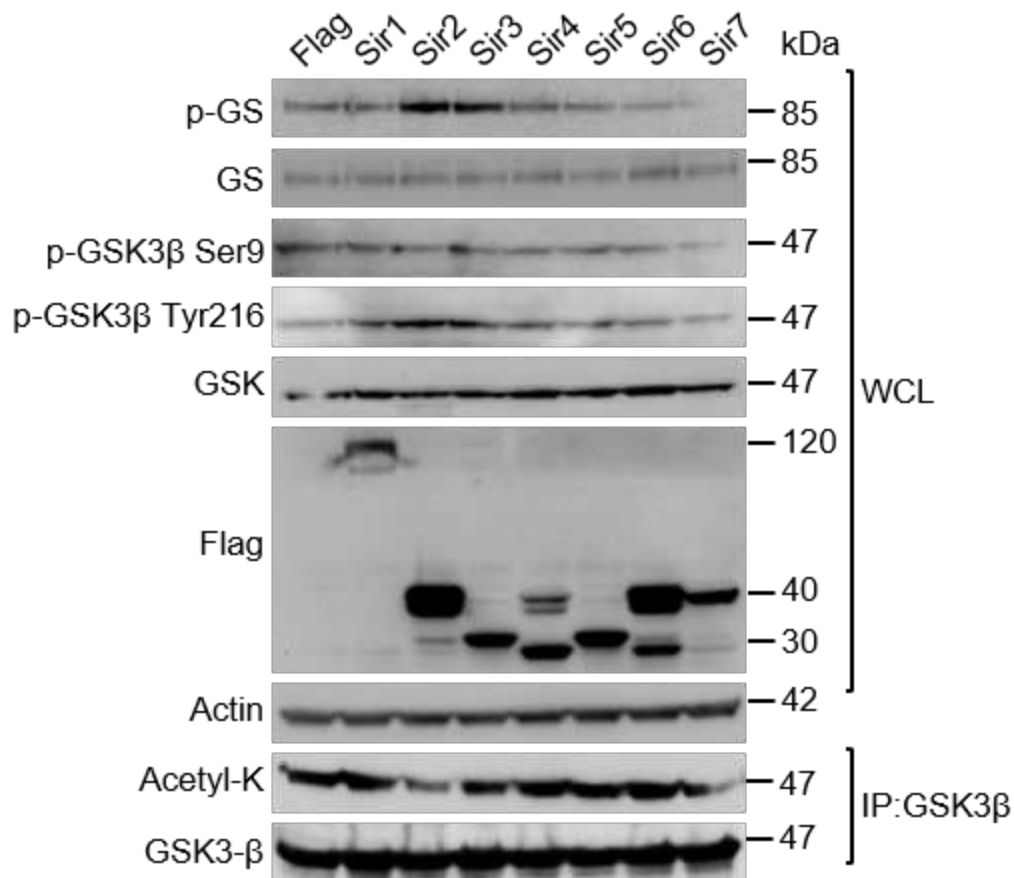
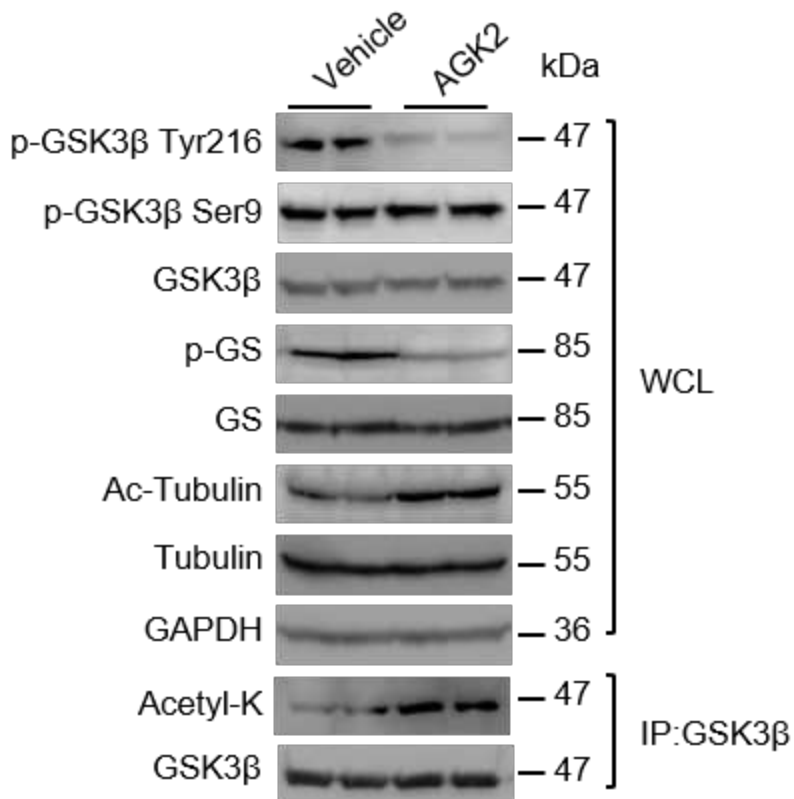
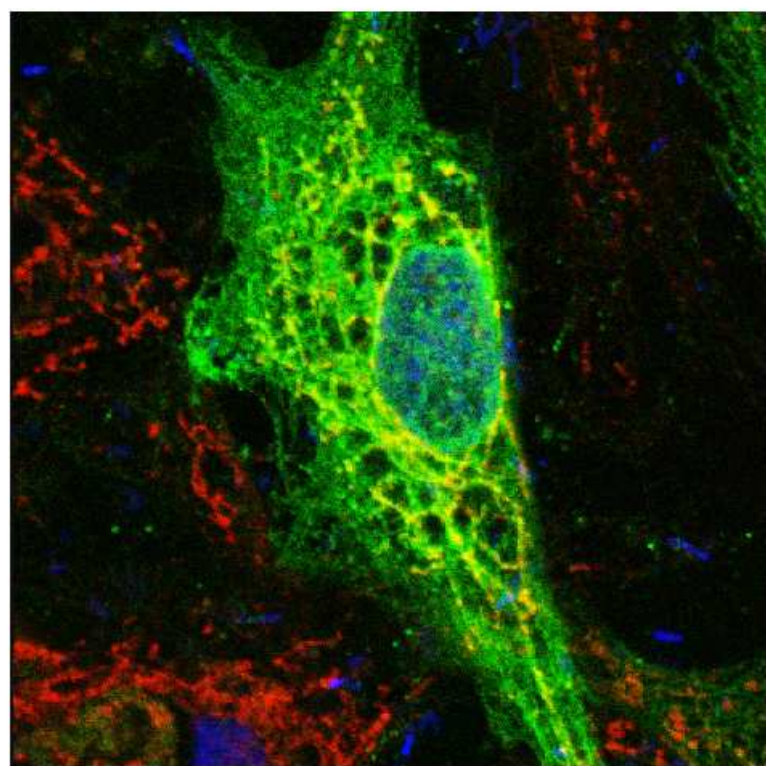


FIGURE 3

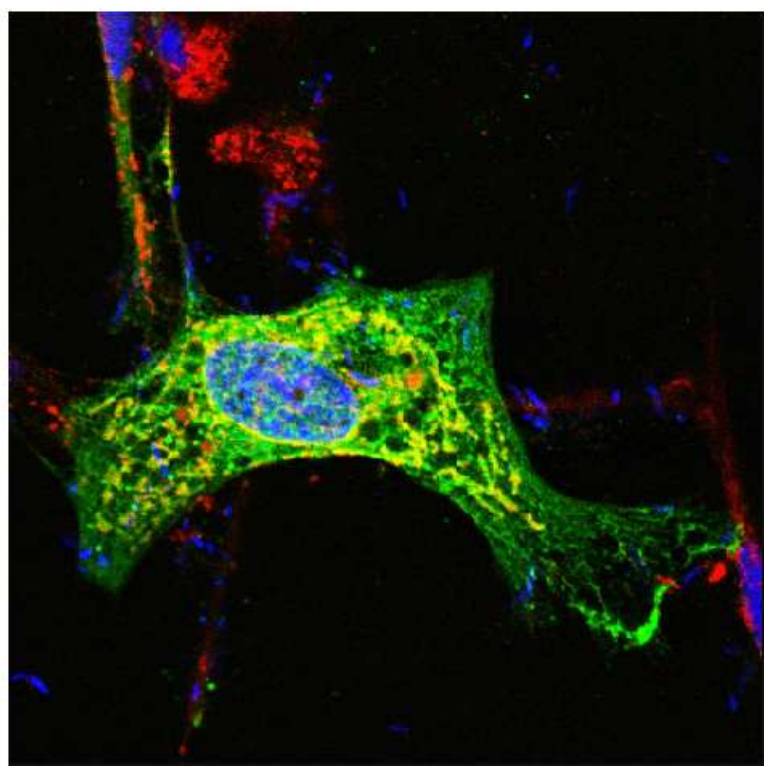




Vehicle



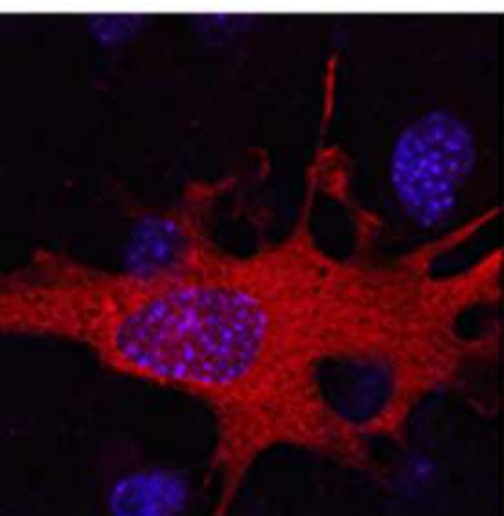
AGK2



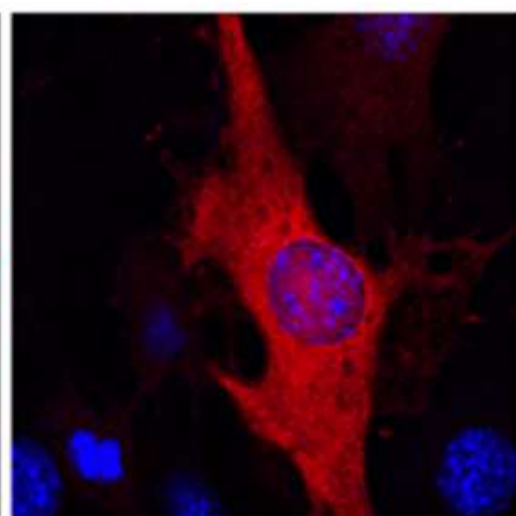
GSK3 β MnSOD DAPI

GSK3 β deficient cells

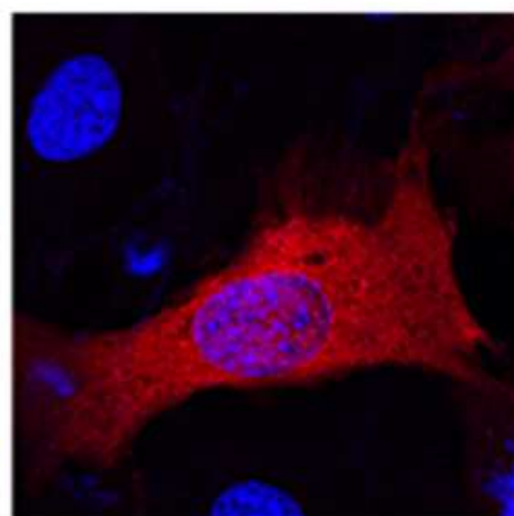
WT



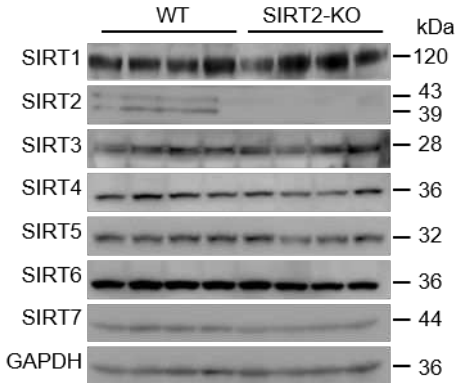
K183R



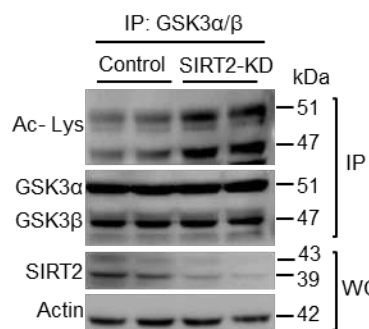
K183Q



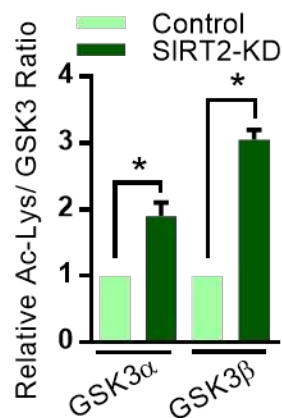
GSK3 β DAPI



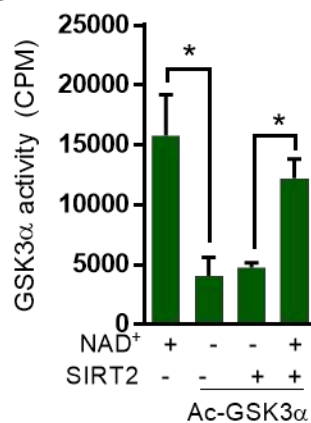
A



B



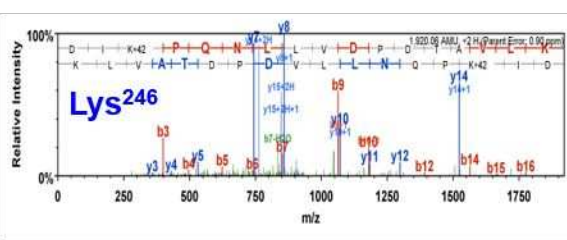
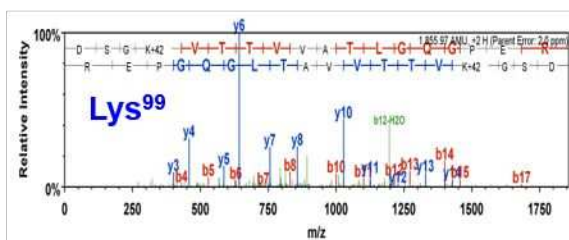
C



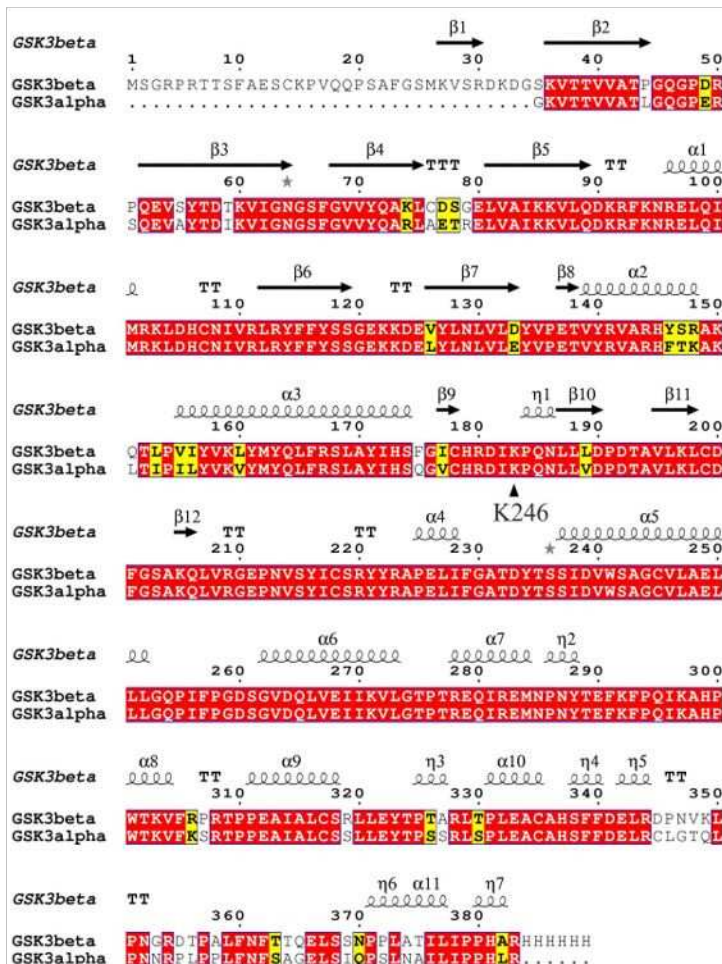
D

GSK3 α (R)DSGKVTTVVATLGQGPER(S)

GSK3 α (R)DIKPQNLLVDPDTAVLK(L)



E



F

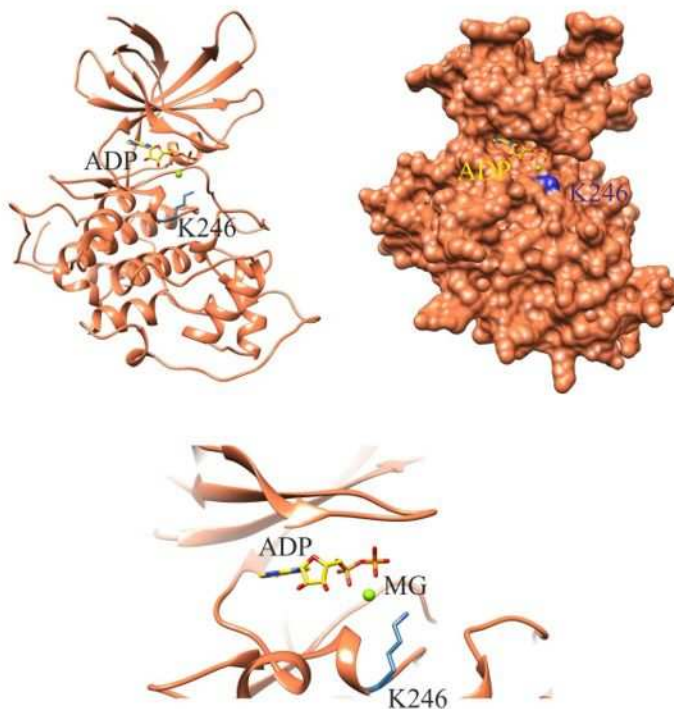


FIGURE 4

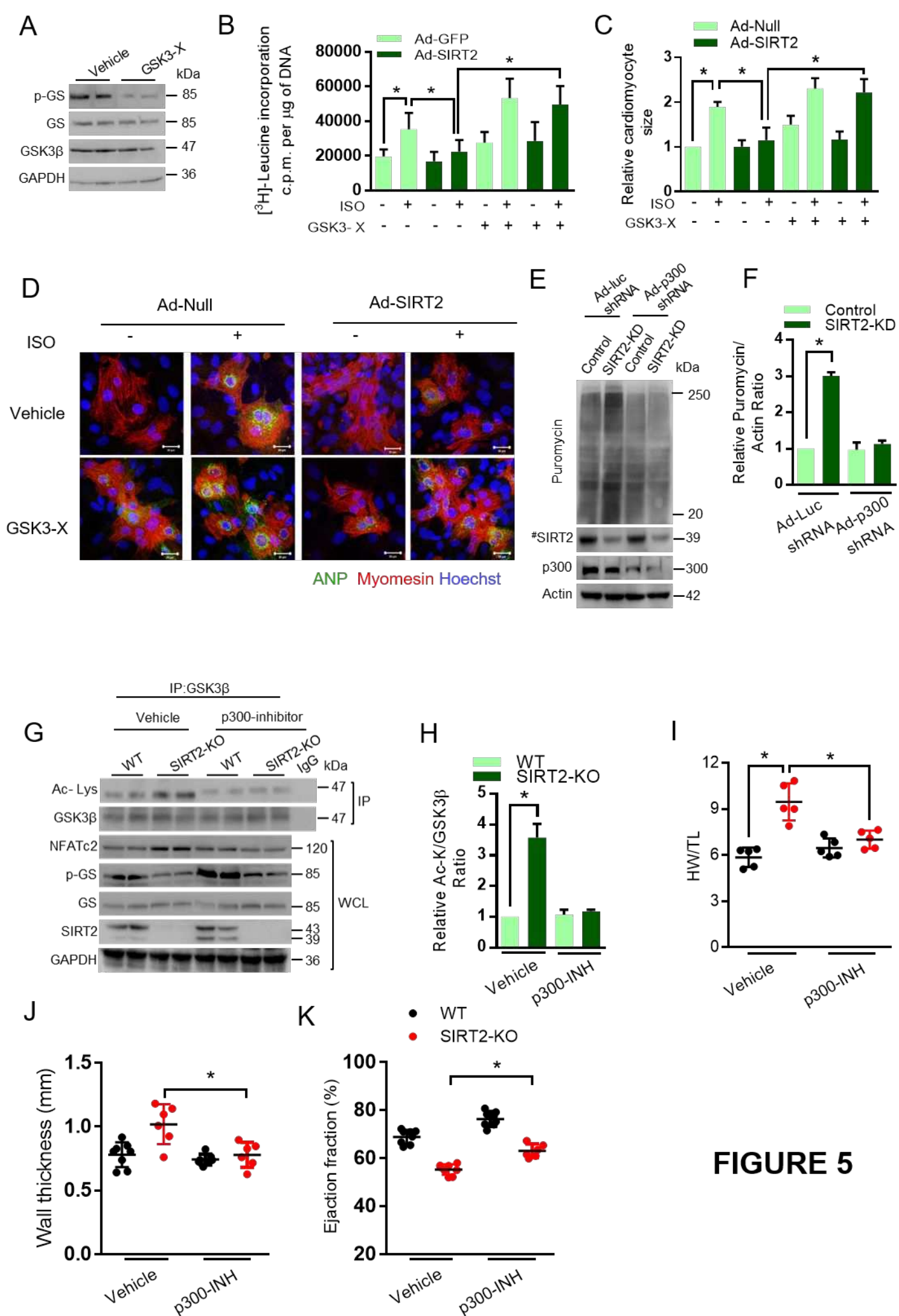
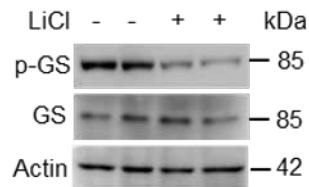
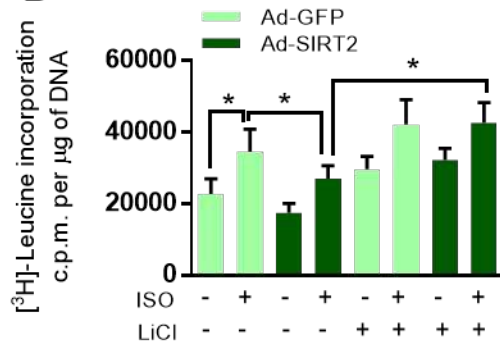


FIGURE 5

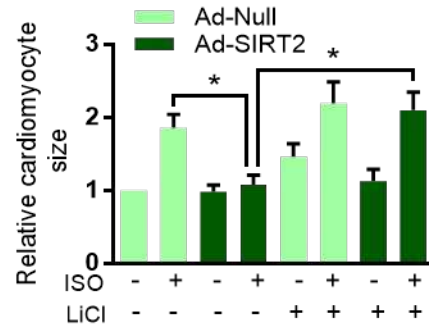
A



B



C



D

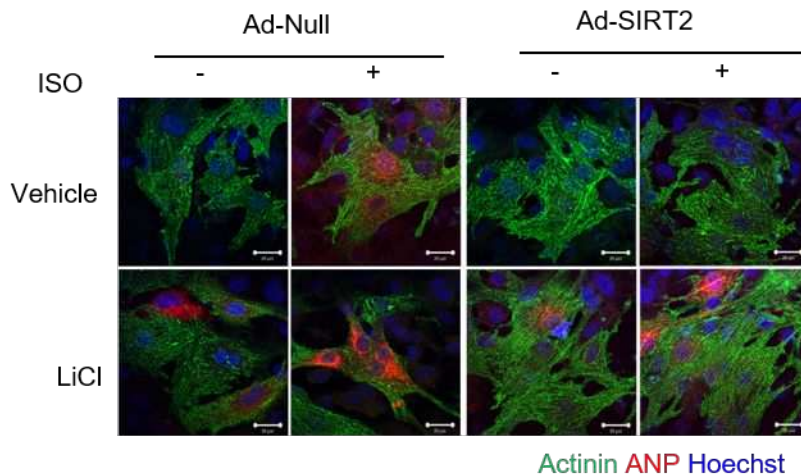


Figure 5-figure supplements 1