

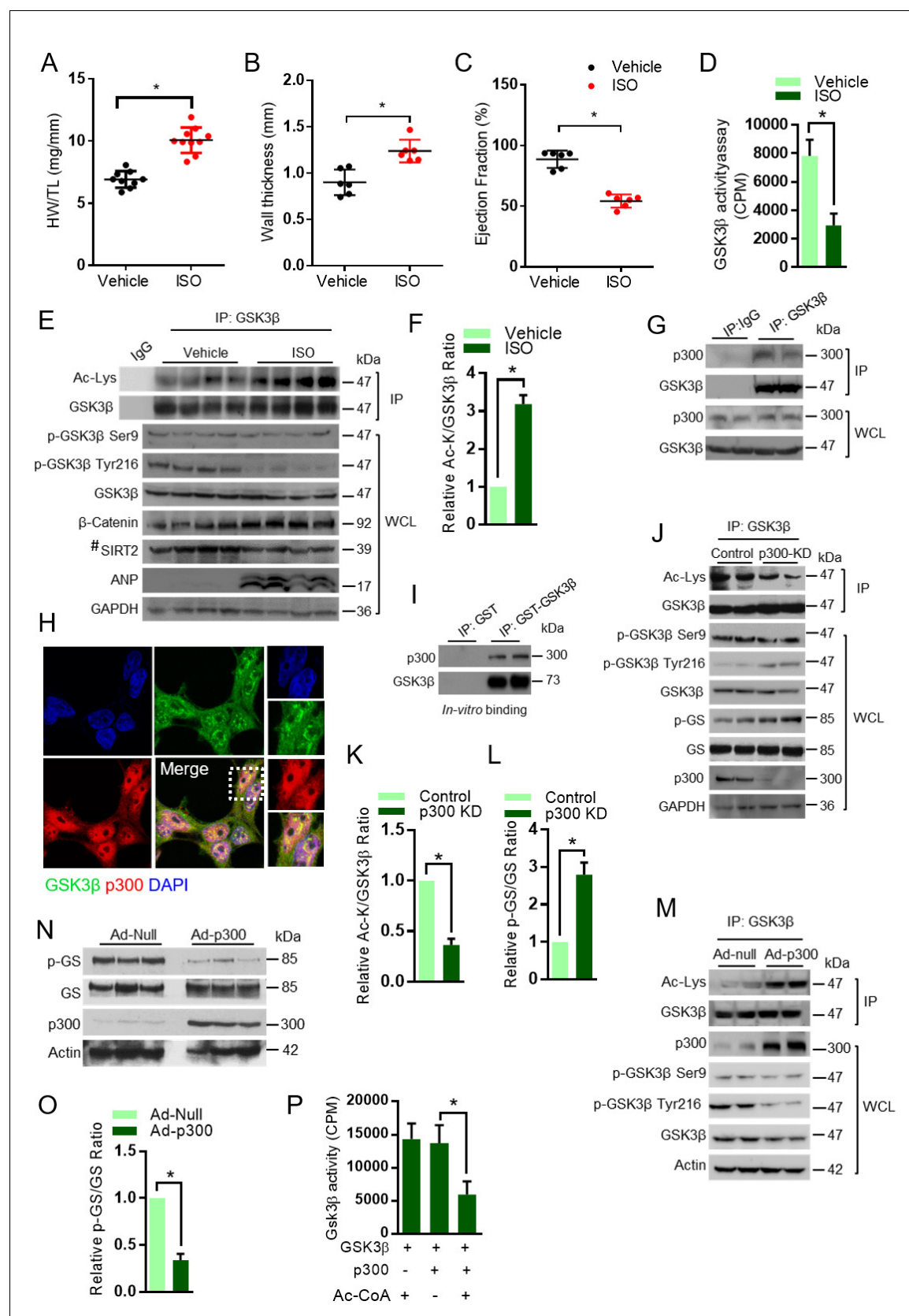


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## Figures and figure supplements

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

**Mohsen Sarikhani et al**



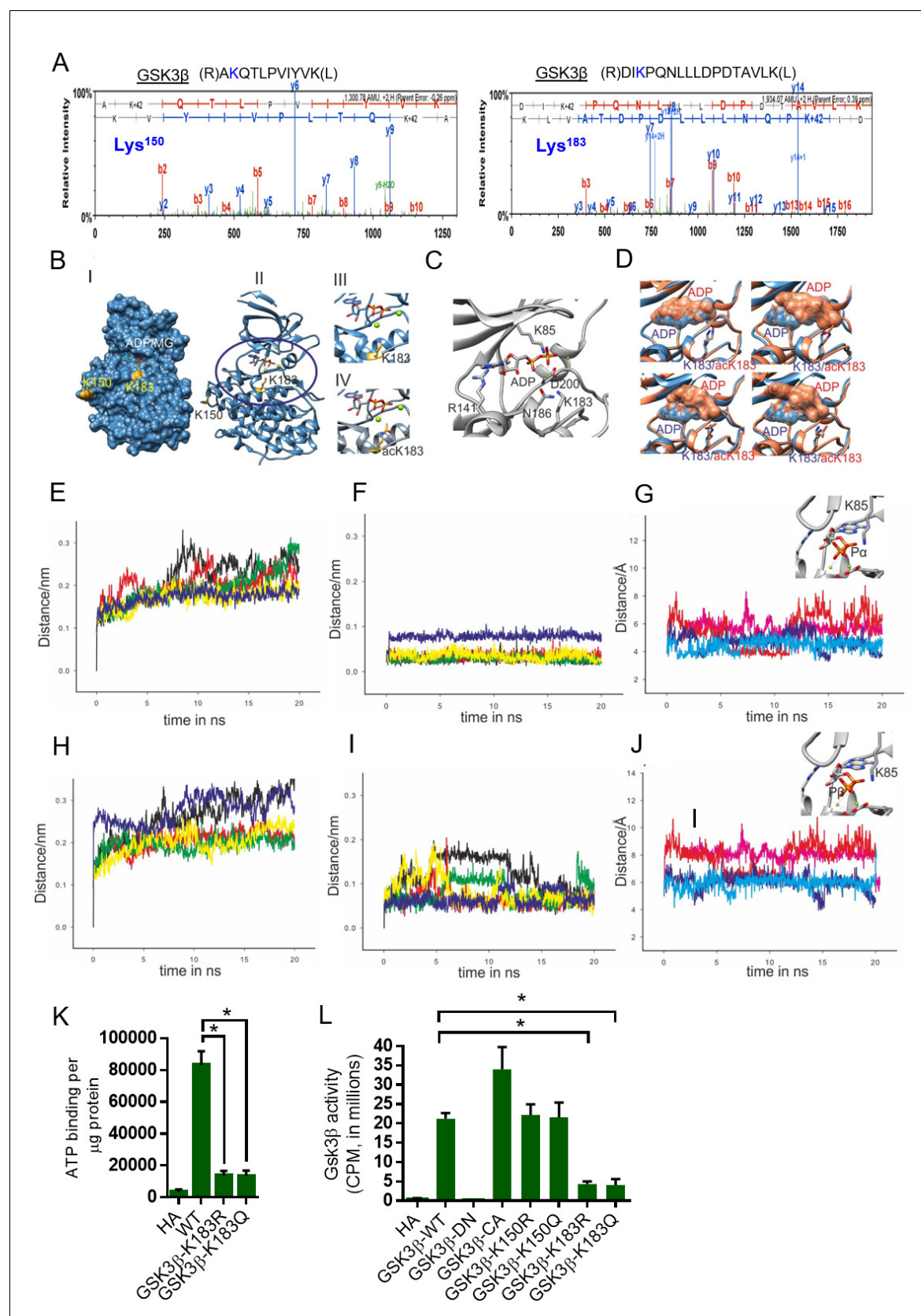
**Figure 1.** Acetylation of GSK3β increased in pathological cardiac hypertrophy. (A) Scatter plot showing cardiac hypertrophy, as measured by Heart weight/Tibia Length (HW/TL) ratio of 8 weeks old 129/Sv mice treated with either vehicle or isoproterenol (ISO) at the dose of 10 mg/kg/day. ISO was

Figure 1 continued on next page

## Figure 1 continued

continuously infused for 7 days using osmotic mini-pumps.  $n = 9\text{--}10$  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. 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 $\beta$  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 $\beta$  was immunoprecipitated from the heart lysates of vehicle or ISO infused mice using anti-GSK3 $\beta$  antibody, clone GSK-4B (Sigma). The immunoprecipitated GSK3 $\beta$  was incubated with the peptide substrate in the presence of  $\gamma\text{--}^{32}\text{P}$ -ATP. The incorporation of  $^{32}\text{P}$  into the GSK3 $\beta$  peptide substrate, which contains specific phosphorylation residues of GSK3 $\beta$  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 $\beta$  was immunoprecipitated from the heart lysates of vehicle or ISO infused mice using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz Biotechnology) and the affinity resin immobilized with protein A/G. Western blotting analysis was performed to detect the levels of GSK3 $\beta$  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. (F) Histogram showing relative acetylated GSK3 $\beta$  in vehicle and ISO-treated mice heart tissues, as measured from **Figure 1E**. Signal intensities of acetylated GSK3 $\beta$  and GSK3 $\beta$  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. (G) GSK3 $\beta$  was immunoprecipitated from heart tissues of 8 weeks old 129/Sv mice using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz Biotechnology), and the affinity resin with protein A/G immobilized. Western blotting was performed to detect GSK3 $\beta$  interaction with p300 using anti-p300 antibody. IgG was used as a negative control. Whole cell lysates (WCL) were probed for the presence of GSK3 $\beta$  and p300 by western blotting. (H) Co-localization of GSK3 $\beta$  with p300 was assessed in 293 T cells by confocal microscopy. The antibodies used are anti-GSK3 $\beta$  (sc-9166, Santacruz), and p300 (05–257, Millipore). DAPI was used to stain the nucleus. Expanded images (right small boxes) show yellow color in the merge image, indicating the co-localization of GSK3 $\beta$  (Green) and p300 (Red) in the nucleus. (I) In vitro binding assay to test the direct interaction between GSK3 $\beta$  and p300. Recombinant p300 (Millipore # 2273152) was incubated with recombinant GST or GST-GSK3 $\beta$ , purified from *E. coli* BL21 (DE3) by affinity chromatography using Glutathione Sepharose 4B. (J) Western blotting analysis showing the acetylation and activity of GSK3 $\beta$  in rat neonatal cardiomyocytes infected with adenovirus expressing either luciferase shRNA (control) or p300 shRNA (p300-KD) for 72 hr. Depletion of p300 was confirmed by western blotting. GSK3 $\beta$  was immunoprecipitated from control and p300-KD cells using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz Biotechnology) and the affinity resin immobilized with protein A/G. Western blotting was performed to detect acetylation of GSK3 $\beta$  using the anti Ac-Lysine antibody. GSK3 $\beta$  activity was measured by assessing the phosphorylation of glycogen synthase (p-GS). Site-specific antibodies were used to detect the phosphorylation of GSK3 $\beta$  at indicated residues in cardiomyocyte lysates (WCL). (K) Histogram showing the quantification of relative acetylated GSK3 $\beta$  in control and p300 depleted (p300-KD) rat neonatal cardiomyocytes, as measured from **Figure 1J**. Rat neonatal cardiomyocytes were infected with adenovirus expressing either luciferase shRNA (control) or p300 shRNA (p300-KD) for 72 hr. Signal intensities of acetylated GSK3 $\beta$  and GSK3 $\beta$  were quantified 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. (L) Histogram depicting the activity of GSK3 $\beta$  in control and p300 depleted (p300-KD) rat neonatal cardiomyocytes, as measured by the ratio of phosphorylation of glycogen synthase vs total glycogen synthase from **Figure 1J**. Rat neonatal cardiomyocytes were infected with adenovirus expressing either luciferase shRNA (control) or p300 shRNA (p300-KD) for 72 hr. Signal intensities of phospho-glycogen synthase and glycogen synthase were measured 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. (M) Western blotting analysis showing the acetylation of GSK3 $\beta$  in rat neonatal cardiomyocytes infected with either control (Ad-null) or p300 overexpressing adenovirus (Ad-p300) for 24 hr. Overexpression of p300 was confirmed by western blotting. GSK3 $\beta$  was immunoprecipitated using anti-GSK3 $\beta$  antibody (sc-9166, Santacruz) and the affinity resin with protein A/G immobilized. Site-specific antibodies were used to detect the phosphorylation of GSK3 $\beta$  at indicated residues in cell lysates (WCL). (N) Western blotting analysis showing the activity of GSK3 $\beta$  in rat neonatal cardiomyocytes infected with control (Ad-null) or p300 expressing adenovirus (Ad-p300) for 24 hr. Overexpression of p300 was confirmed by western blotting and the activity of GSK3 $\beta$  was probed by assessing the levels of p-GS and GS by western blotting. (O) Histogram showing the activity of GSK3 $\beta$  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 $\beta$ . Human GSK3 $\beta$  with HA tag was overexpressed in HeLa cells by transfection of the plasmid pcDNA3-HA-GSK3 $\beta$ . HA-GSK3 $\beta$  was immunoprecipitated using HA-coupled agarose beads (Sigma-Aldrich) and the HA-GSK3 $\beta$  was acetylated by recombinant p300 (Millipore), in the presence or absence of Acetyl-CoA (Ac-CoA) in HAT buffer. The enzymatic activity of GSK3 $\beta$  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.

DOI: <https://doi.org/10.7554/eLife.32952.002>



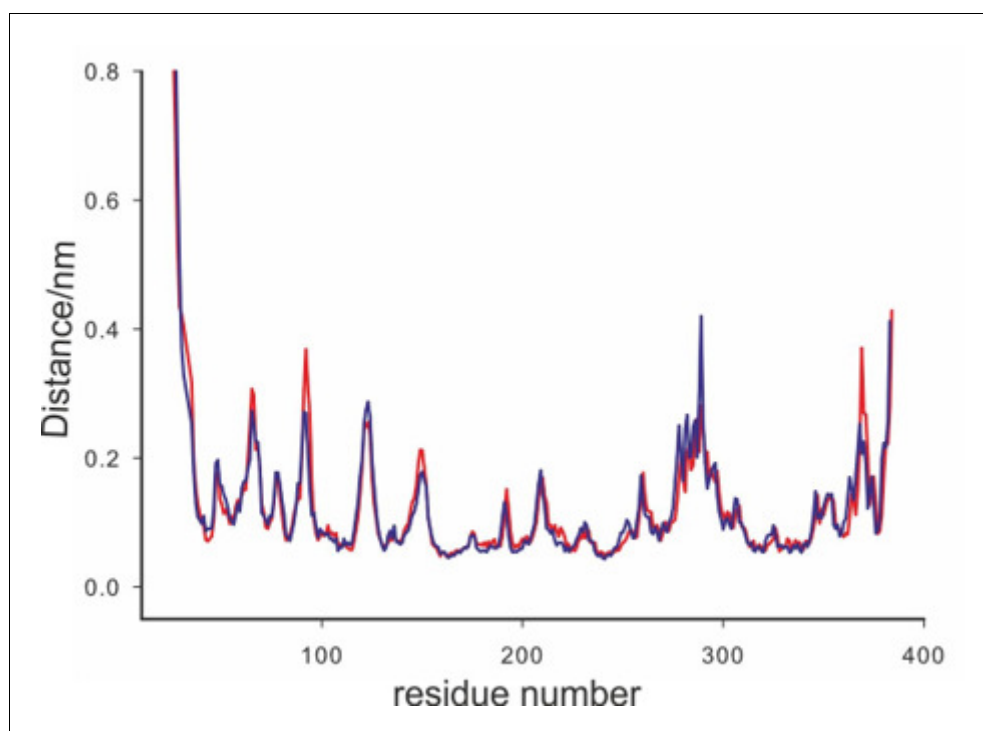
**Figure 2.** Molecular modeling and molecular dynamics simulations of GSK3β wild-type and K183 acetylated mutant. (A) Annotation of representative tandem mass spectra of trypsin-digested GSK3β, depicting K150 and K183 acetylation. (B) Representation of the acetylation sites on the crystal structure. (C) Molecular dynamics simulation of GSK3β. (D) Molecular dynamics simulation of GSK3β. (E) Distance (nm) vs time (ns) for GSK3β. (F) Distance (nm) vs time (ns) for GSK3β. (G) Distance (Å) vs time (ns) for GSK3β. (H) Distance (nm) vs time (ns) for GSK3β. (I) Distance (nm) vs time (ns) for GSK3β. (J) Distance (Å) vs time (ns) for GSK3β. (K) ATP binding per μg protein. (L) GSK3β activity (CPM, in millions).

Figure 2 continued on next page

## Figure 2 continued

structure of GSK3 $\beta$  (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 $\beta$  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 $\beta$  representing the surface of ADP nucleotide, at random snapshots in the MD trajectory. (E) Overlay of protein backbone C $\alpha$  RMSD plots of the five 20 ns MD trajectories in wild type. (F) Overlay of ADP nucleotide RMSD plots of the five 20 ns MD trajectories in wild type. (G) Overlay of the distance between the NZ atom of K85 and  $\alpha$ -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 $\alpha$  RMSD plots of the five 20 ns MD trajectories in acK183 mutant. (I) Overlay of ADP nucleotide RMSD plots of the five 20 ns MD trajectories in acK183 mutant. (J) Overlay of the distance between the NZ atom of K85 and  $\beta$ -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  $\gamma$ -<sup>32</sup>P-ATP to recombinant wild type and mutants of His-GSK3 $\beta$ . Plasmids encoding wild type and mutants of His-GSK3 $\beta$  were transformed into *E. coli* BL21 (DE3). His-GSK3 $\beta$  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 $\beta$ . HA-tagged human GSK3 $\beta$  or its mutants were overexpressed in HeLa cells by transfection of their respective plasmids. HA-GSK3 $\beta$  or its mutants were immunoprecipitated using HA-coupled agarose beads (Sigma-Aldrich). The enzymatic activity of GSK3 $\beta$  was measured against glycogen synthase (GS)-peptide as described in the Materials and methods section. GSK3 $\beta$ -DN - GSK3 $\beta$ -K85A; Dominant negative. GSK3 $\beta$ -CA- GSK3 $\beta$  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.

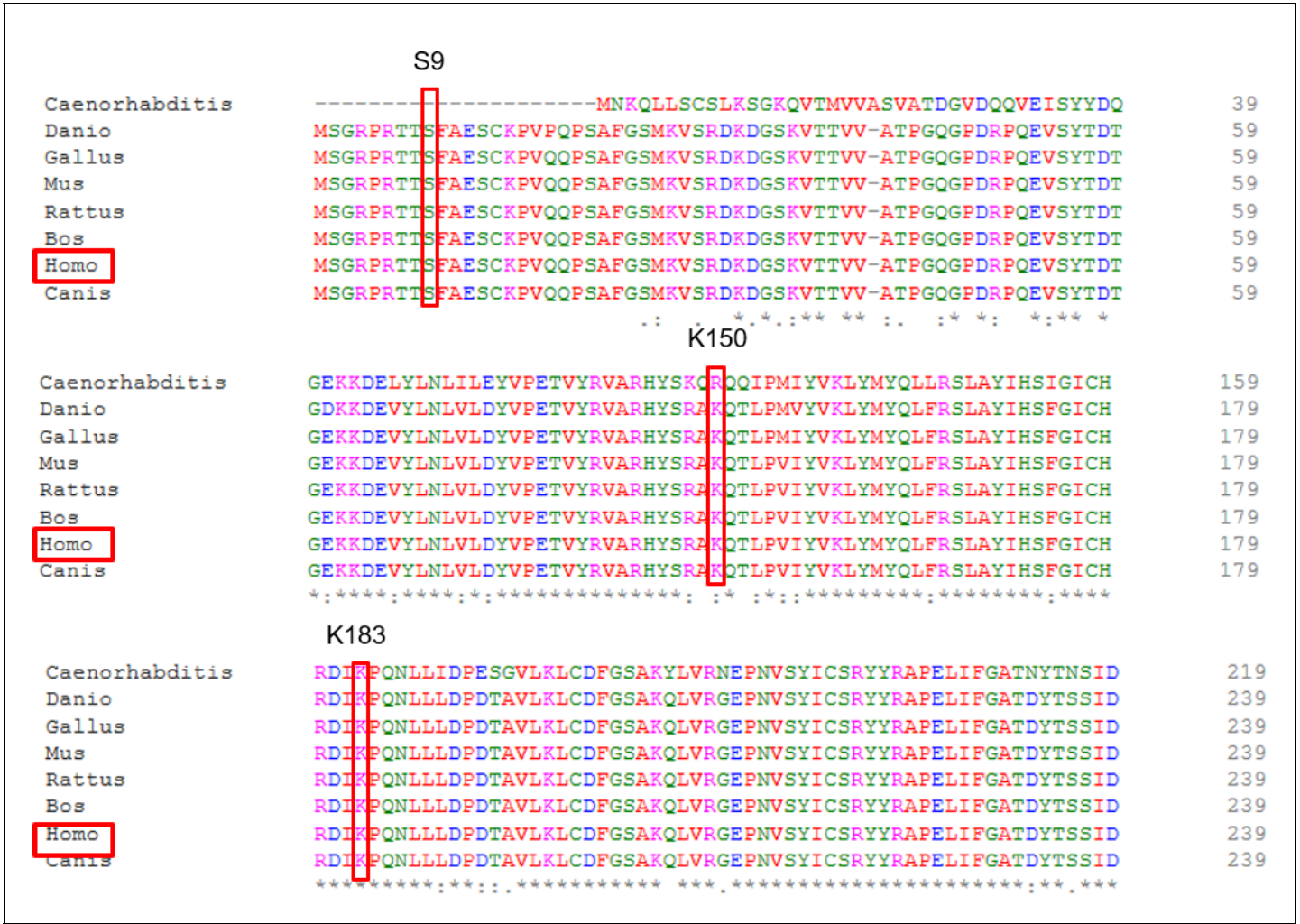
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**Figure 2—figure supplement 1.** Protein backbone C $\alpha$  RMSF plots of the wild type (dark blue) and Ac-K183 mutant (red).

DOI: <https://doi.org/10.7554/eLife.32952.004>





**Figure 2—figure supplement 2.** Homology alignment of GSK3β between different species.

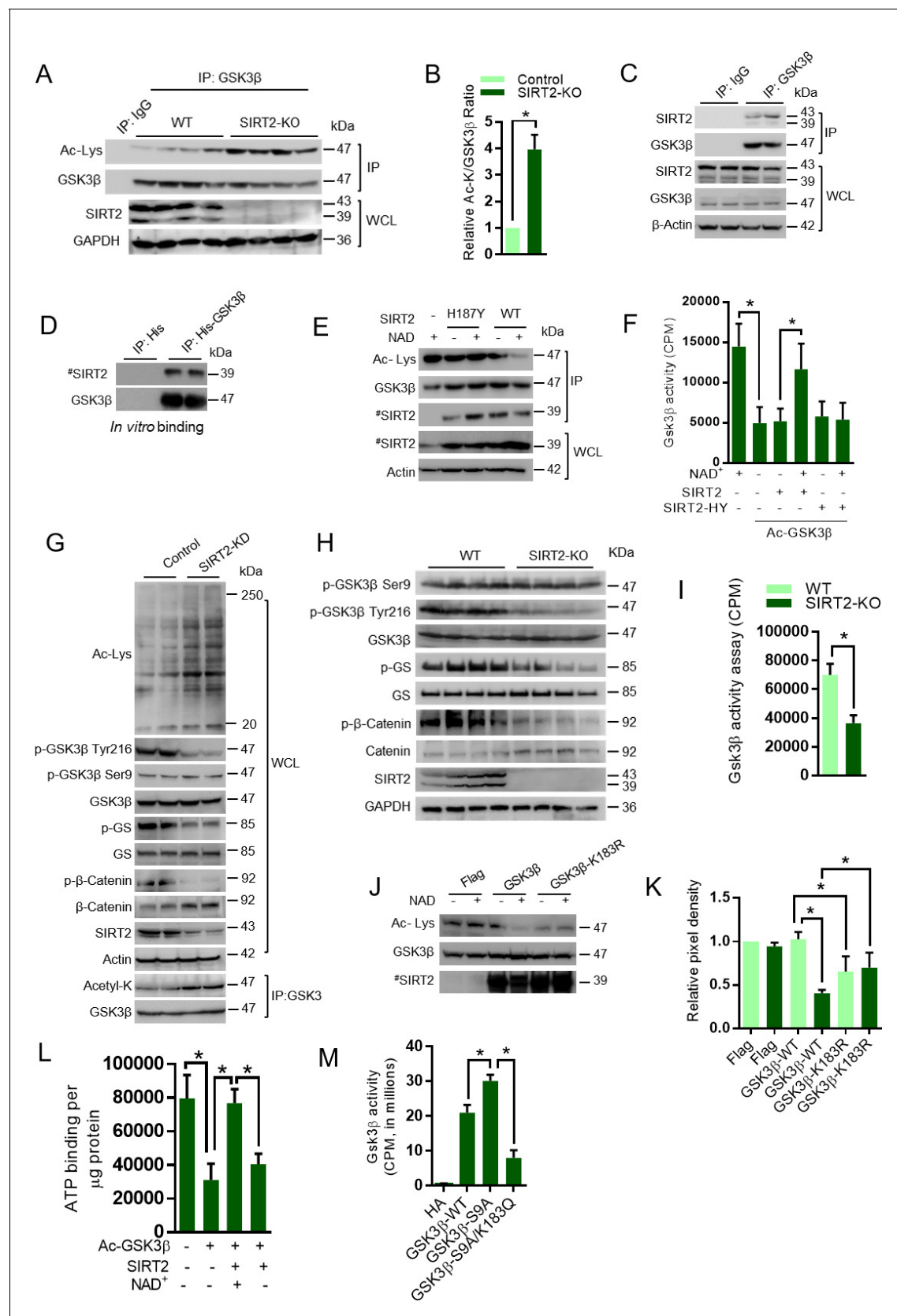
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K150	Charge	Scans	Intensity	Stoichiometry
AKQTLPIYVK	2+			
Non-Ac Mass	630.38	1538-1736	582000	<b>0.59%</b>
Ac Mass	651.39	1538-1736	3480	
K183	Charge	Scans	Intensity	Stoichiometry
DIKPQNLLDPDTAVLK	2+			
Non-Ac Mass	947.04	5751-5833	16200	<b>25.03%</b>
Ac Mass	968.04	5751-5833	5410	

**Figure 2—figure supplement 3.** Stoichiometry for GSK3 $\beta$ -K150, -K183 acetylation.

DOI: <https://doi.org/10.7554/eLife.32952.006>



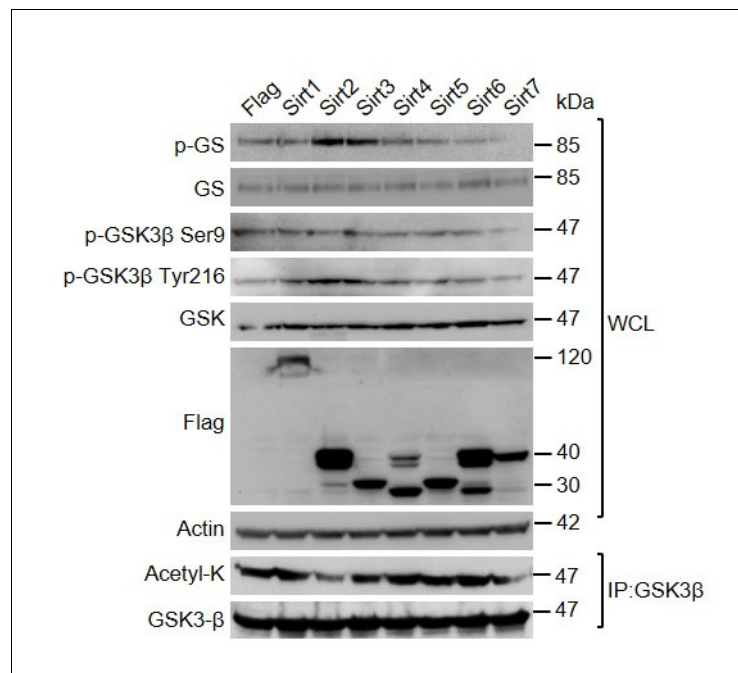


**Figure 3.** SIRT2 binds to, deacetylates and activates GSK3 $\beta$ . (A) Western blot analysis of acetylated GSK3 $\beta$  in heart samples of 9 months old WT and SIRT2-KO littermates. GSK3 $\beta$  was immunoprecipitated from heart tissue lysates of WT and SIRT2-KO mice using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz). Figure 3 continued on next page

## Figure 3 continued

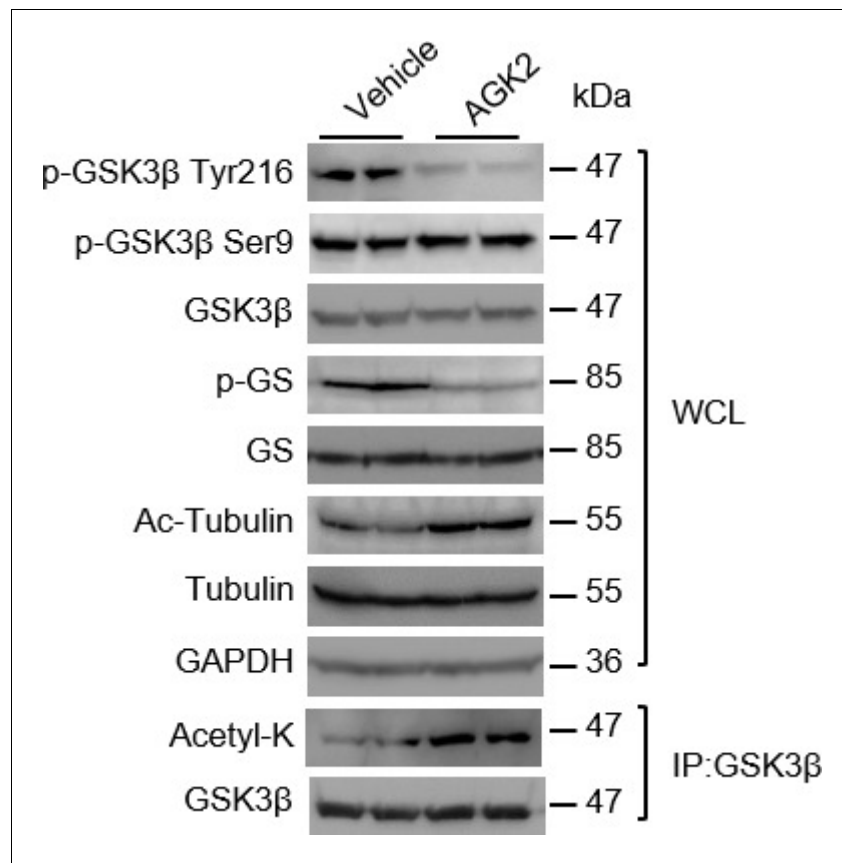
Cruz Biotechnology), and the affinity resin immobilized with protein A/G. Western blotting was performed to detect GSK3 $\beta$  acetylation by anti-Ac-Lysine antibody. IgG was used as a negative control. Whole cell lysates (WCL) were probed for the SIRT2 and GAPDH by western blotting. *n* = 4 mice per group. (B) Histogram showing relative acetylated GSK3 $\beta$  in 9 months old WT and SIRT2-KO mice heart tissues, as measured from **Figure 3A**. Signal intensities of acetylated GSK3 $\beta$  and GSK3 $\beta$  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 $\beta$  was immunoprecipitated from heart tissue lysates of 8 weeks old 129/Sv mice using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz Biotechnology), and the affinity resin immobilized with protein A/G. GSK3 $\beta$  interaction with SIRT2 was tested by western blotting using anti-SIRT2 antibody. IgG was used as negative control. Heart lysates were probed for indicated proteins by western blotting. (D) In vitro binding assay to test the interaction between GSK3 $\beta$  and SIRT2. Flag-SIRT2 was overexpressed in 293 cells by a plasmid encoding human Flag-SIRT2. Recombinant His or His-GSK3 $\beta$  was purified from *E. coli* BL21 (DE3) by Ni-NTA affinity chromatography and were incubated with 293 T cell lysates overexpressing human Flag-SIRT2. Interaction between GSK3 $\beta$  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 $\beta$  deacetylase. Human HA-GSK3 $\beta$  was overexpressed in HeLa cells by transfection of the plasmid pcDNA3-HA-GSK3 $\beta$ . HA-GSK3 $\beta$  was immunoprecipitated using HA-coupled agarose beads (Sigma-Aldrich) and the HA-GSK3 $\beta$  was acetylated by recombinant p300 (Millipore), in the presence or absence of Acetyl-CoA (Ac-CoA) in HAT buffer. The acetylated HA-GSK3 $\beta$  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<sup>+</sup> in a HDAC buffer. GSK3 $\beta$  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 $\beta$ . Human HA-GSK3 $\beta$  was overexpressed in HeLa cells by transfection of the plasmid pcDNA3-HA-GSK3 $\beta$ . Recombinant HA-GSK3 $\beta$  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 $\beta$  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<sup>+</sup> in a HDAC buffer and further enzymatic activity of GSK3 $\beta$  was measured against glycogen synthase (GS)-peptide, as described in the Materials and 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 $\beta$  from control or SIRT2-depleted (SIRT2-KD) cardiomyocytes. Neonatal rat cardiomyocytes were transfected with either non-targeting (control) or siRNA targeting SIRT2 using Lipofectamine RNAiMAX reagent for 72 hr. 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 $\beta$  was immunoprecipitated from these cell lysates using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz Biotechnology), and the affinity resin immobilized with protein A/G. Western blotting was performed to detect acetylation of GSK3 $\beta$  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 $\beta$  in WT and SIRT2-KO mice hearts at 9 months of age. GSK3 $\beta$  was immunoprecipitated from the heart lysates of WT and SIRT2-KO mice using anti-GSK3 $\beta$  antibody, clone GSK-4B (Sigma). The immunoprecipitated GSK3 $\beta$  was incubated with the peptide substrate in the presence of  $\gamma$ -<sup>32</sup>P-ATP. The incorporation of <sup>32</sup>P into the GSK3 $\beta$  Peptide Substrate, which contains specific phosphorylation residue of GSK3 $\beta$  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 $\beta$ . HA-tagged GSK3 $\beta$  or GSK3 $\beta$ -K183R was overexpressed in HeLa cells and was immunoprecipitated using HA-coupled beads. HA-tagged WT-GSK3 $\beta$  or GSK3 $\beta$ -K183R were incubated with Flag-SIRT2 immunoprecipitated from HEK 293 T cells using agarose beads conjugated to Anti-Flag antibody (Sigma A2220). The deacetylation reaction was carried out in the presence or absence of NAD<sup>+</sup> in a deacetylation buffer. Acetylation status of GSK3 $\beta$  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 $\beta$  or GSK3 $\beta$ -K183R, which was incubated with Flag-SIRT2. The data is generated from **Figure 3J**. Signal intensities of acetylated-GSK3 $\beta$  and GSK3 $\beta$  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  $\gamma$ -<sup>32</sup>P-ATP to acetylated and deacetylated His-GSK3 $\beta$ . Recombinant His-GSK3 $\beta$  was purified from *E. coli* BL 21 (DE3) by Ni-NTA affinity chromatography. Purified His-GSK3 $\beta$  was acetylated by recombinant p300 in the presence of Ac-CoA in HAT buffer. Acetylated His-GSK3 $\beta$  was further deacetylated by Flag-SIRT2 immunoprecipitated from HEK 293 T cells. The binding of  $\gamma$ -<sup>32</sup>P-ATP to acetylated and deacetylated His-GSK3 $\beta$  was assessed by the protocol described in Materials and 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 $\beta$ . HA-tagged WT or mutants of GSK3 $\beta$  was immunoprecipitated from HeLa cells transfected with respective plasmids using HA-coupled agarose beads. The enzymatic activity of GSK3 $\beta$  was measured against glycogen synthase (GS)-peptide, as described in the Materials and 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.

DOI: <https://doi.org/10.7554/eLife.32952.007>



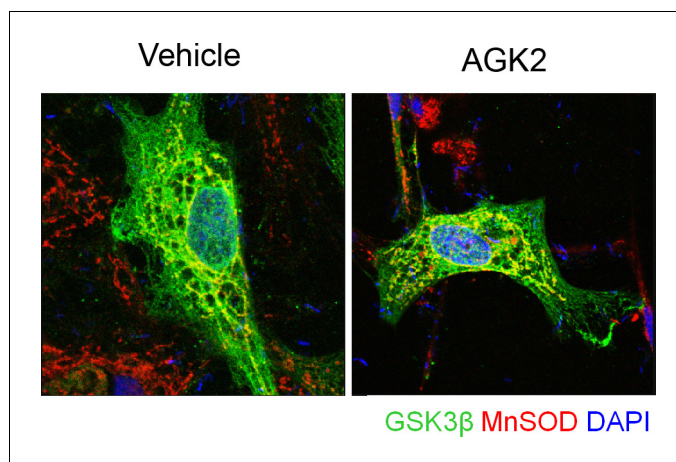
**Figure 3—figure supplement 1.** Western blotting analysis showing acetylation and phosphorylation of GSK3 $\beta$  and its downstream target GS in HeLa cells overexpressing the Sirtuin isoforms, SIRT1-SIRT7. Cells were transiently overexpressed with either pcDNA-Flag or pcDNA-Flag-SIRT1-7 plasmid for 48 hr, 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 $\beta$  was immunoprecipitated from these overexpressed lysates using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz Biotechnology) and tested for its acetylation by western blotting using anti-Ac-Lys antibody.

DOI: <https://doi.org/10.7554/eLife.32952.008>



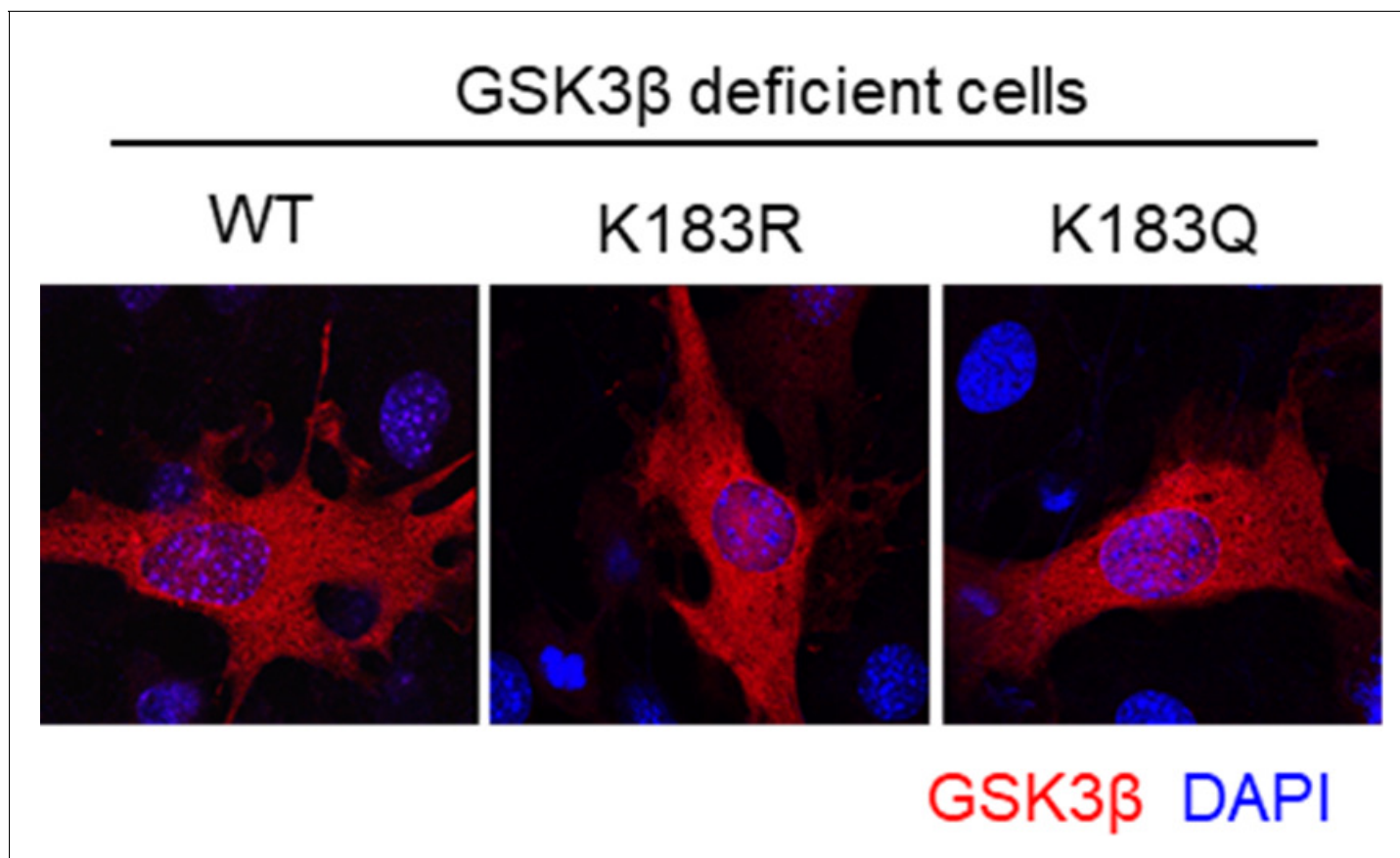
**Figure 3—figure supplement 2.** Western blotting analysis of vehicle or SIRT2 inhibitor, AGK2-(10  $\mu$ M, 12 hr) treated rat neonatal cardiomyocytes for indicated proteins. Acetylation of tubulin at K40, which is a specific deacetylation target of SIRT2 was probed to test the efficacy of SIRT2 inhibition. GSK3 $\beta$  was immunoprecipitated from these cell lysates using anti-GSK3 $\beta$  antibody, and the affinity resin immobilized with protein A/G. GSK3 $\beta$  acetylation was detected by Western blotting.

DOI: <https://doi.org/10.7554/eLife.32952.009>



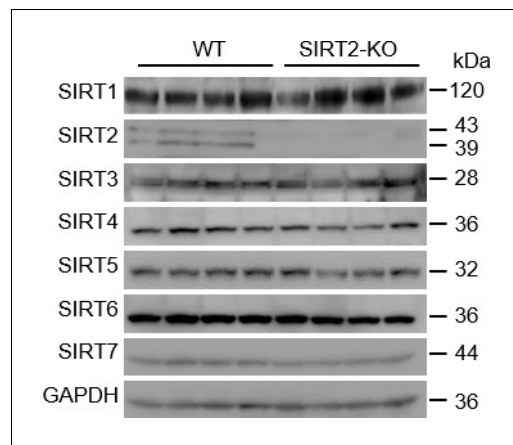
**Figure 3—figure supplement 3.** Representative confocal images of vehicle or AGK2 (10  $\mu$ M, 12 hr) treated HeLa cells stained with GSK3 $\beta$  (Green). MnSOD was used to localize the mitochondria (Red). DAPI was used to stain the nucleus (Blue).

DOI: <https://doi.org/10.7554/eLife.32952.010>



**Figure 3—figure supplement 4.** Representative confocal images of HA-tagged WT or mutants of GSK3 $\beta$  transiently overexpressed in GSK3 $\beta$ -deficient mouse embryonic fibroblasts. Immunostaining was performed using HA antibody to localize WT and mutants of GSK3 $\beta$  (Red).

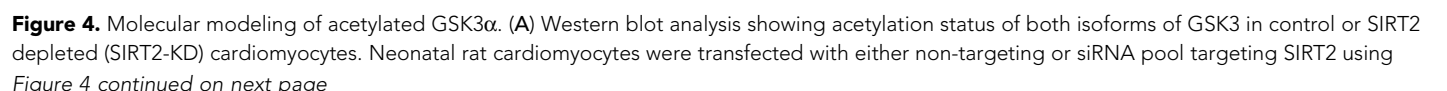
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**Figure 3—figure supplement 5.** Western blot analysis of 9 months old WT and SIRT2-KO mice heart samples for indicated proteins. n = 4 mice per group.

DOI: <https://doi.org/10.7554/eLife.32952.012>

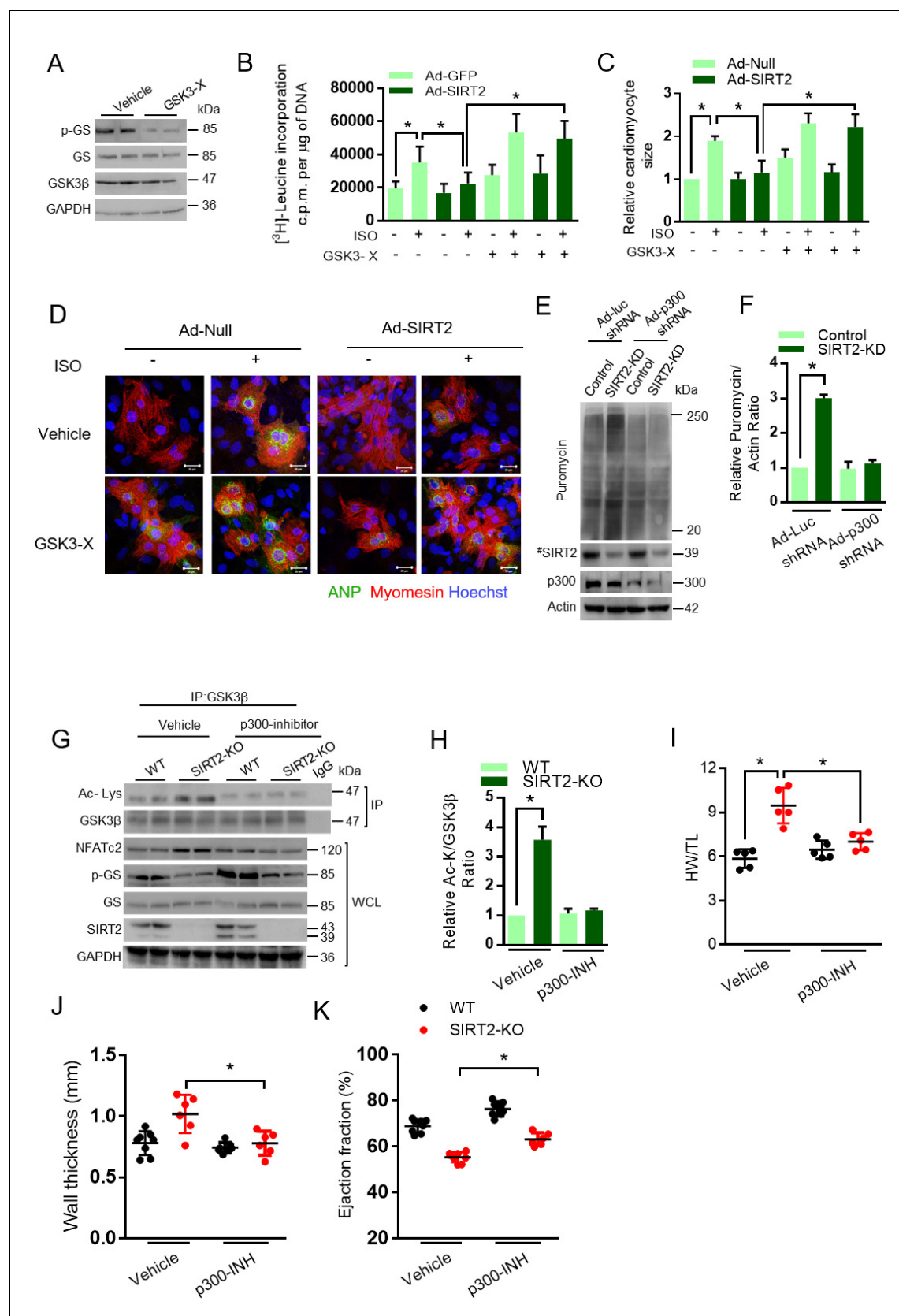




## Figure 4 continued

Lipofectamine RNAiMAX reagent for 72 hr. 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 $\alpha/\beta$  acetylation by anti-Ac-Lysine antibody. Cell lysates were probed for SIRT2 and actin antibodies by western blotting. (B) Histogram showing relative acetylated-GSK3 $\alpha$  and GSK3 $\beta$  in control and SIRT2-depleted (SIRT2-KD) cardiomyocytes, as measured from **Figure 4A**. Signal intensities of acetylated-GSK3 $\alpha$  and acetylated-GSK3 $\beta$  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 $\alpha$ . Recombinant HA-GSK3 $\alpha$  was immunoprecipitated from HeLa cells overexpressing pcDNA-HA-GSK3 $\alpha$  using HA-coupled agarose beads. Immunoprecipitated HA-GSK3 $\alpha$  was acetylated by p300 in the presence of Acetyl-CoA (Ac-CoA) in HAT buffer. Acetylated GSK3 $\alpha$  was further deacetylated by Flag-SIRT2 immunoprecipitated from HEK 293 T cells overexpressing plasmid encoding Flag-tagged SIRT2-WT using agarose beads conjugated to anti-Flag antibody (Sigma A2220). The enzymatic activity of GSK3 $\alpha$  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 $\alpha$ , depicting K99, K246 acetylation. (E) Protein sequence alignment of the modeled region of GSK3 $\alpha$  and the structure of GSK3 $\beta$ . (F) Cartoon, surface representation of the homology model of GSK3 $\alpha$  (highlighted is the adenine nucleotide-binding pocket and position of K246 residue).

DOI: <https://doi.org/10.7554/eLife.32952.013>



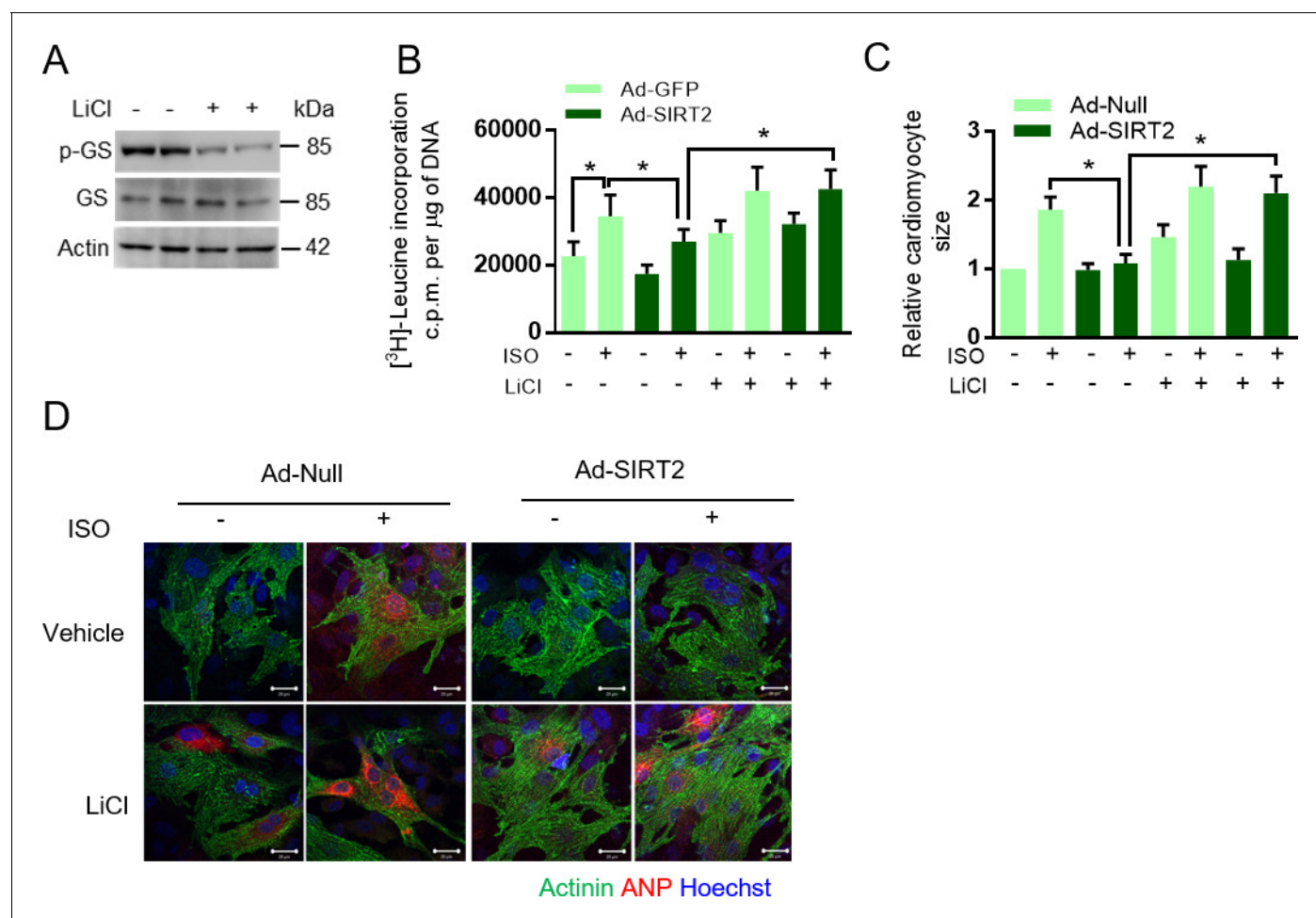
**Figure 5.** GSK3 $\beta$  is required for the anti-hypertrophic role of SIRT2 deacetylase. (A) Western blotting analysis depicting the activity of GSK3 inhibitor X (GSK3-X). Neonatal rat cardiomyocytes were treated with vehicle or 500 nM GSK3-X for 48 hr and the activity of GSK3 was assessed by monitoring the

Figure 5 continued on next page

## Figure 5 continued

phosphorylation of GS by specific antibody. **(B)** [ $^3\text{H}$ ]-leucine incorporation into total cellular protein of control (Ad-GFP) or SIRT2-overexpressing (Ad-SIRT2) rat neonatal cardiomyocytes treated with either vehicle or 500 nM GSK3 inhibitor X (GSK3-X) for 48 hr. Cardiomyocytes were infected with adenoviral vectors encoding either GFP or SIRT2 for 24 hr prior to GSK3-X treatment. After the GSK3-X treatment, cardiomyocytes were stimulated with either vehicle or 20  $\mu\text{M}$  ISO for 24 hr and the [ $^3\text{H}$ ]-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 500 nM GSK3 inhibitor X (GSK3-X) for 48 hr. Cardiomyocytes were infected with adenoviral vectors encoding either control or SIRT2 for 24 hr prior to GSK3-X treatment. After the GSK3-X treatment, cardiomyocytes were stimulated with either vehicle or 20  $\mu\text{M}$  ISO for 24 hr and the relative cardiomyocyte area is quantified as described in Materials and 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  $\mu\text{M}$ , 24 hr), with or without GSK3 inhibitor X (GSK3-X, 500 nM, 48 hr). Scale bar = 20  $\mu\text{m}$ . 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 hr. p300 depletion was confirmed by western blotting. Pulse of puromycin was given 30 min 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 $\beta$  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 5 mg/kg/day for 10 days in mice. Peanut oil was used as vehicle. GSK3 $\beta$  was immunoprecipitated from heart lysates of WT and SIRT2-KO mice using anti-GSK3 $\beta$  antibody (sc-9166, Santa Cruz Biotechnology), and the affinity resin with protein A/G immobilized. Western blotting was performed to detect GSK3 $\beta$  acetylation by anti-Ac-Lysine antibody. GSK3 $\beta$  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 $\beta$  acetylation in heart lysates of vehicle or anacardic acid (5 mg/kg/day for 10 days) treated 9 months old WT and SIRT2-KO mice from **Figure 5G**.  $n = 3$ . Signal intensities of GSK3 $\beta$  and acetylated-GSK3 $\beta$  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 5 mg/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 5 mg/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 5 mg/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.

DOI: <https://doi.org/10.7554/eLife.32952.014>



**Figure 5—figure supplement 1.** GSK3 inhibition abrogates anti-hypertrophic role of SIRT2 deacetylase. (A) Western blotting analysis to confirm the activity of LiCl. Neonatal rat cardiomyocytes were treated with 20 mM LiCl for 48 hr and the activity of GSK3 $\beta$  was assessed by monitoring the phosphorylation of p-GS. (B) [<sup>3</sup>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  $\mu$ M, 24 hr), 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  $\mu$ M, 24 hr), 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  $\mu$ M, 24 hr), with or without 20 mM LiCl. Scale bar = 20  $\mu$ m.  $\alpha$ - Actinin (Green), ANP (Red) and Hoechst (Blue).

DOI: <https://doi.org/10.7554/eLife.32952.015>