Developmental Cell, Volume *39*

Supplemental Information

Acetylation of VGLL4 Regulates Hippo-YAP Signaling and Postnatal Cardiac Growth

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Supporting Information for:

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Supporting Information

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A. Detailed Materials and Methods.

Animal experiments

All animal procedures were approved by the Boston Children's Hospital Animal Care and Use Committee. Rosa26^{BirA} and Rosa26^{mTmG} mice were previously described (Driegen et al., 2005; Muzumdar et al., 2007) and were obtained from Jackson Labs. Tead1^{fb} knock-in mice were generated by targeting the C-terminus of *Tead1* in murine embryonic stem cells to introduce FLAG and Bio epitope tags, followed by embryonic stem cell blastocyst injection. After establishing germline transmission, the Frt-neo-Frt resistance cassette was removed using FLP expressing mice. These mice are available through the mutant mouse resource (MMRRC: 037514-JAX). Echocardiography was performed in conscious mice by investigators blinded to genotype or treatment group on a VisualSonics Vevo 2100 with Vevostrain software.

Human myocardium

Human left ventricular myocardium was obtained from unused donor hearts without known heart disease, under protocols approved by the Institutional Review Boards of the University of Sydney and St. Vincent's Hospital. Myocardial samples from the left ventricle were snap frozen in liquid nitrogen within 2 hours of organ harvest.

Cardiomyocyte proliferation Clonal analysis

1-day-old Rosa26 Confetti/+ mouse (Livet et al., 2007) pups were administrated with AAV9.Cre together with AAV.Luciferase, AAV9. Vgll4 or AAV9. Vgll4[M], respectively. 7 days after virus injection, hearts were collected and processed for cryosectioning. To quantify the different color clones for each heart, whole heart cross-section images were taken using the Nikon TE2000 epifluorescent microscope equipped with Velocity stitching program. Clone numbers were counted virus type blinded.

Histology and measurement of cardiomyocyte proliferation, apoptosis, and necrosis

Hearts were fixed in 4% PFA, washed in PBS, equilibrated with 30% sucrose, and embedded in OCT. 10 μm cryosections were used for H&E staining, Sirius Red-Fast Green staining and immunostaining.

Antibodies used for immunostaining are listed below:

Antigen	Company (catalog #)	Origin	Working dilution
Primary antibodies			
Cardiac troponin I (TNNI3)	Abcam (ab56357)	Goat	1:200 for IF
Flag	Sigma (F3165)	Rabbit	1:1000 for western blot
GAPDH	Sigma (WH0002597M1)	Mouse	1:200,000 for WB
WGA-647	Life technology (W32466)	NA	1:250 for IF
YAP	Sigma (Y4770)	Rabbit	1:1000 for WB
HA tag	CST(2367)	Mouse	1:1000 for WB
His tag	Life technology (R93025)	Mouse	1:1000 for WB
GFP	Memorial-Sloan Kettering Monoclonal Ab Facility	Mouse	IP 1:100 for Co-IP
Phospho Histone 3	Upstate (06-570)	Rabbit	1:200 for IF

Tead1 (Tef1)	BD biosciences (610923)	mouse	1:1000 for WB
GFP	Rockland (600-101-215)	Goat	1:1000 for WB
V5	Life technology (R960-25)	Mouse	1:1000 for WB
p300	Santa Cruz (sc-585x)	Rabbit	1:500 for Co-IP and 1:2000 for WB
VGLL4	Bioss Inc.(bs-9185R)	Rabbit	1:1000 for WB
Myh1e	The Developmental Studies Hybridoma Bank (MF20)	Mouse	NA
Secondary antibodies			
Donkey Anti-Goat Alexa488	Life technology(A11055)	Donkey	1:500 for IF
Clear blot IP detection regent	Thermo fisher (21230)		1:400 for WB
Donkey Anti-Rabbit HRP	Jaskson lab (705-035-147)	Donkey	1:10000 for WB
Donkey Anti-Rabbit Alexa555	Life technology (A21206)	Donkey	1:500 for IF

CM apoptosis was detected on cryosections using the Roche in situ death detection kit.

To measure CM necrosis, we adapted the protocol of Nakayama (Nakayama et al., 2007). 1-day-old Rosa26^{mTmG} mouse pups were treated with AAV9. 6 days later, 100 µl MF20 antibody (22 µg/ml) was IP injected into the mouse pups. On day 7 after virus transduction, hearts were collected, fixed, and cryosectioned as described above. To visualize cardiomyocytes which had taken up MF20 antibody in vivo, sections were stained with Alexa 647 conjugated Donkey anti mouse IgG.

Imaging was performed on a Fluoview 1000 confocal microscope, or a Nikon TE2000 epifluorescent microscope. Quantitation was performed blinded to AAV treatment group by randomly acquiring ten 20x fields per heart.

AAV9 packaging

Cardiomyocyte specific AAV9. Vgll4 or Vgll4[M] were cloned into ITR-containing AAV plasmid (Penn Vector Core P1967) harboring the chicken cardiac TNT promoter, to obtain pAAV.cTnT::Vgll4-GFP and pAAV.cTnT::Vgll4[M]-GFP, respectively. AAV9 was packaged in 293T cells with AAV9:Rep-Cap and pHelper (pAd deltaF6, Penn Vector Core) and purified and concentrated by gradient centrifugation (Lin et al., 2014). AAV9 titer was determined by quantitative PCR. The standard AAV9 dose used for neonatal mice was 2.5x10^10 GC/g. At this dose, we routinely transduce over 90% of CMs.

His-TEAD1[211-427] expression and purification

Murine TEAD1 residues 211-427, containing the YAP and VGLL4 binding domain, was cloned into the pET28a (Novagen) in frame with polyhistidine tag (His) using BamHI and NotI (NEB). The recombinant plasmid was transformed into E. coli BL21(DE3) cells. A single colony was then used to inoculate 200 ml of LB with 25 µg/ml kanamycin at 37 °C. At OD600 ~ 0.5, expression was induced with 0.67 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was further shaken at 18 °C for 16-20 h. Cells were pelleted, then suspended in 10 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 10 mM β-mercaptoethanol, and 10 mM imidazole), followed by the addition of 1 mg/ml lysozyme. After incubating on ice for

30 min, the suspension was sonicated (5 reps, 10 sec on, 30 sec off, amplitude 75) with Branson Digital Sonifier equipped with a microtip and subsequently centrifuged at 20,000g for 20 min at 4°C. The supernatant was incubated with 2 ml Ni-NTA Agarose (Qiagen) for 3 h at 4°C. The resin was washed with 20 column volumes of wash buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM PMSF, 10 mM β-mercaptoethanol and 20 mM imidazole). The protein was eluted with 4-column volumes of elution buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM PMSF, 10 mM β-mercaptoethanol and 300 mM imidazole). His-TEAD1[211-427] was concentrated and further purified by size exclusion chromatography with a Superdex 200 increase 10/300 GL column (GE Health Sciences) pre-equilibrated in a buffer of 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM PMSF, 10 mM β-mercaptoethanol.

Synthetic VGLL4 TDU domain peptides

Wild type and acetylated peptide containing VGLL4 Tdu domain and V5 epitope were synthesized in LifeTein LLC. The synthesized peptides were purified with HPLC to reach 95% purity and their molecular weight analyzed by electrospray ionization (ESI) mass spectrometry. The sequences are shown in Figure S3.

Affinity measurement by photonic crystal nanobeam sensor.

Affinity measurement was performed using nanobeam photonic sensors consisting of photonic crystal nanobeam cavities for protein sensing and polymer spot-size converters for efficient on-and-off chip light coupling (Liang et al., 2013; Quan et al., 2010). Polydimethylsiloxane (PDMS) microfluidic channels were integrated on the sensor chip for sample delivery. The photonic crystal nanobeam cavities confine the optical energy into nanoscale dimensions, and build up high quality factor (Q-factor) resonances. The nanobeam cavity consists of a tapered array of holes with periodicity 330 nm along a 600 nm wide ridge waveguide. The radii of the holes were tapered from 240 nm in the center of the cavity to 100 nm to both ends of the cavity, designed by the deterministic method described in (Quan and Loncar, 2011). The device was fabricated as described (Quan and Loncar, 2011). Protein binding was measured by monitoring the resonance shift of the nanobeam cavity.

100 ng/mL His-TEAD1[211-427] was first flowed to the nanobeam sensor via PDMS microfluidic channels, together with 4 mM sodium cyanoborohydride (Sigma) in PBS. After 2 hour-incubation at room temperature, the nanobeam sensor was washed by PBS flow for 10 min. Different concentrations of VGLL4 or acetylated VGLL4 were consecutively injected into the channel. We used the tunable laser (Santec) to scan the input wavelength and collected the signal transmitted through the cavity. We obtained the resonance shift by fitting the resonance with Lorentz curve.

mVGLL4 K216 acetylation-specific antibody generation.

The antigen design and antibody generation was carried out by Yenzym antibodies, LLC. The antigen used to raise VGLL4 antibody is synthesized mouse VGLL4 (209-222, EHFRRSLGKNYKEPE) peptide, in which K216 was acetylated. Rabbits were given four immunizations and acetylation-specific antibodies were isolated by affinity purification.

TEAD1-Dendra2 merge protein time lapse imaging

293T cells were cultured on glass bottom 35mm dishes. One day after plasmid transfection, cells were treated with Dox in the absence or presence of E64. Green Dendra2 protein was partially converted into red fluosrecenese protein with 30 seconds 405nm light illuminating. Images were taken 3 minutes after illuninating. Time lapse imaging was carried out with Nikon TE2000 epifluorescent microscope at a

speed of 1 image/min. For each group, 6 different regions of interest were used for quantifying red fluorescence intensity (RFI).

Gene Expression

Real time PCR was performed with Syber Green or Taqman detection using Bio-Rad CFX96 Real time system. PCR primers are listed below:

Primers		
Gene*	Forward	Reverse
mCTGF	CCACCCGAGTTACCAATGAC	GACAGGCTTGGCGATTTTAG
mCCNA2	GCCTTCACCATTCATGTGGAT	TTGCTCCGGGTAAAGAGACAG
mCDC20	TTCGTGTTCGAGAGCGATTTG	ACCTTGGAACTAGATTTGCCAG
mAurka	GGGTGGTCGGTGCATGCTCCA	GCCTCGAAAGGAGGCATCCCCACTA
mMyh6	CTCTGGATTGGTCTCCCAGC	GTCATTCTGTCACTCAAACTCTGG
mGapdh	CAGGTTGTCTCCTGCGACTT	GGCCTCTCTTGCTCAGTGTC
mTead1	TACTGCCATCCACAACAAGC	TGCTGCACAAAGGGCTTGAC
ABI Taqman assays	Gene Assay Number	
mNppa	PN4453320	
mGapdh	4352339E	

Statistics

Values are expressed as mean ± SEM. For two group comparisons, Student's t-test was used to test for statistical significance. To analyze data containing more than two groups, we used ANOVA with the Tukey HSD post-hoc test. Both tests were performed using JMP 10.0 (SAS).

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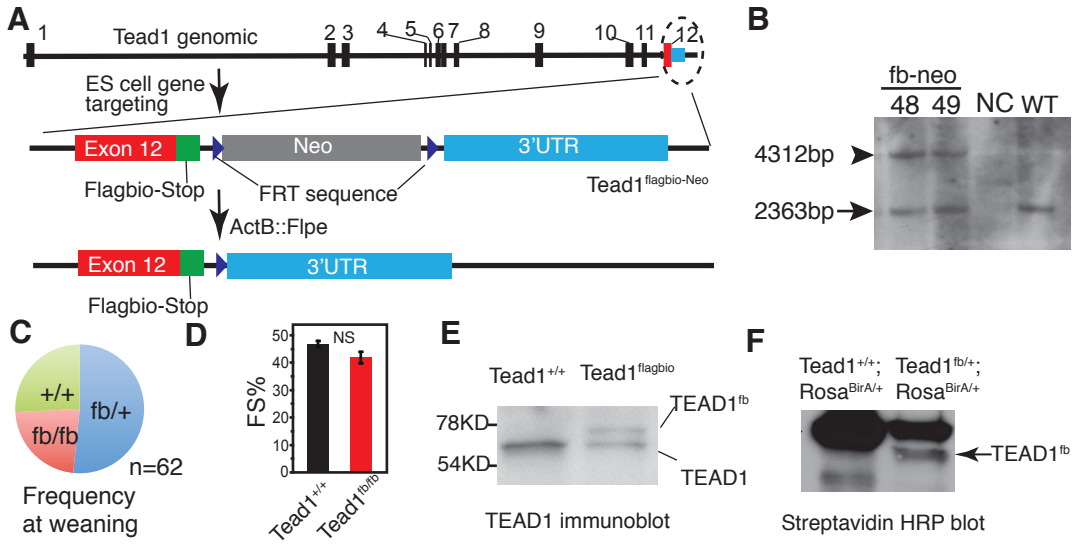

Figure S1. Construction and validation of Tead1^{fb} allele (supporting Figure 1). A. Gene targeting strategy for generation of Tead1^{flagbio} knock-in mice (Tead1$^{fb/+}$). Flag and bio epitope tags were placed on the Tead1 C-terminus. Tead1$^{flagbio-Neo}$ mouse was mated to ActB::Flpe mouse to removed Neomycin. **B**. Homologous recombination in embryonic stem (ES) cells was confirmed by Southern Blotting. Arrow indicates the wildtype allele, and arrowhead indicates the targeted allele. Two independent Tead1$^{flagbio-Neo}$ ES clones(#48 and #49) were tested. NC, negative control, no gemomic DNA included. WT, wild type ES genomic DNA. **C**. After removal of the Frt-neo-Frt cassette by ActB::Flpe, Tead1$^{fb/+}$ mice were intercrossed. Tead1$^{fb/fb}$ mice survived normally. **D**. Echocardiography measurement of Tead1$^{fb/fb}$ mice heart function. 4 months old willd type mice and Tead1$^{fb/fb}$ mice were used for heart function test. NS, no significant difference. N=4. **E**. Western blot with adult heart tissue from indicated mice. **F**. Western blot with E14.5 heart tissue. Sreptavidin HRP was used to detect biotinylated Tead1^{fb}, demonstrating in vivo biotinylation.

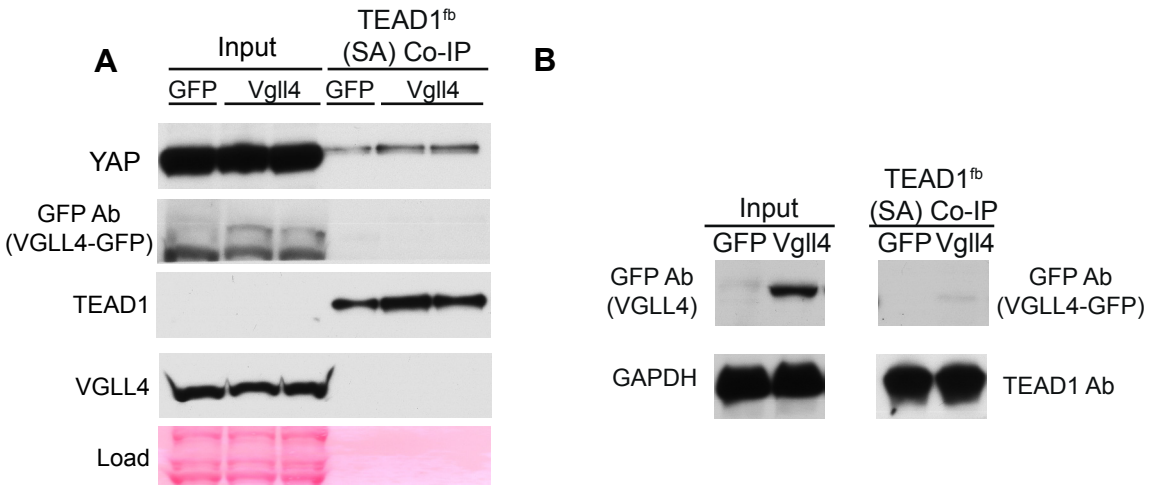

Figure S2. TEAD1 interacts with VGLL4-GFP in the adult heart (supporting Figure 2). AAV-GFP (GFP) or AAV-VGLL4-GFP (Vgll4) were administered to 1 day old Tead1$^{fb/+}$;R26$^{BirA/+}$ pups. Mouse hearts were collected at either P8 (A) or at 1 month after AAV administration (B). Tead1^{fb} was pulled down on SA beads, and co-precipitated VGLL4-GFP was analyzed by western blotting. Ponceau S (A) or GAPDH (B) were used as loading controls.

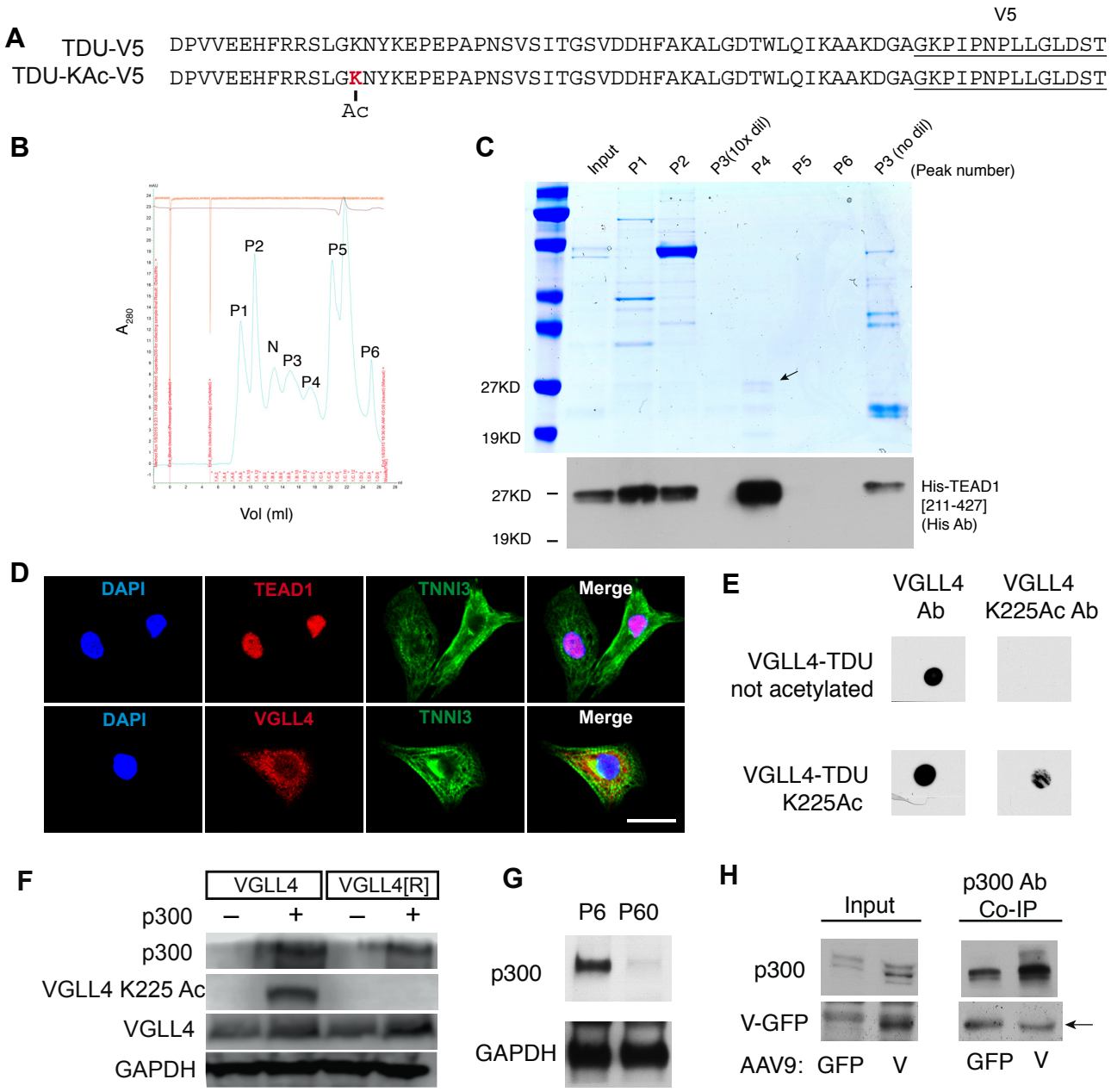

Figure S3. **Recombinant TEAD1 and synthetic VGLL4-TDU peptides; Validation of VGLL4 and TEAD1 antibody for immunofluorescence staining;p300 and VGLL4 interaction analysis in adult heart. Related to Figure 3.**

A. Sequence of synthesized VGLL4 TDU domain peptide. Underlined characters indicate V5 peptide sequence. The acetylated lysine is shown in red. **B**. TEAD1 YBD domain was fused to His tag, and expressed in E.Coli. Soluble proteins were run through Ni resin to purify TEAD1-YBD-His(T-YBD-His). FPLC peaks are labeled with number. The elution volume for peaks 1, 2, 3, 4, 5, 6, are 8.8, 10.6, 15, 17, 20, 21ml, respectively. Peak "N" not included in the western blot. **C**. Commassie blue staining and western blot. Arrow indicate the T-YBD-His protein. His tag antibody was used to detect His-TEAD1 in the western blot. Peak 3 was run in two lanes, lane 5 and lane 9. In lane 5, samples from peak 3 were diluted 10 times. **D**. Validation of TEAD1 and VGLL4 antibodies for immunofluoresence staining. NRVMs were fixed with PFA and stained with the indicated anitbodies. TNNI3 was used as a cardiomyocyte marker. Bar = 20 μm. **E**. Validation of VGLL4-K225Ac antibody. Acetylated or non-acetylated synthetic VGLL4 peptides were bound to PVDF membranes and then probed with antibody directed against total or K225Ac VGLL4. Bound antibody was visualized with HRP-conjugated secondary antibody. **F**. Validation of VGLL4-K255Ac antibody in cell lysates. 293T cells were co-transfected with p300 and VGLL4 or VGLL4[R] expression constructs. Lysates were immunoblotted wtih total VGLL4 and VGLL4-K225-Ac antibodies. **G**. Expression of p300 in neonatal and adult heart. **H**, p300 does not interact with VGLL4 in the adult heart. AAV9-GFP (GFP) and AAV9-V-GFP (V) were delivered into the P1 mouse pups, respectively. At P60, hearts were collected for p300 Co-IP assay. Arrow indicates non-specific IgG band. VGLL4-GFP did not detectably co-immunoprecipitate with p300.

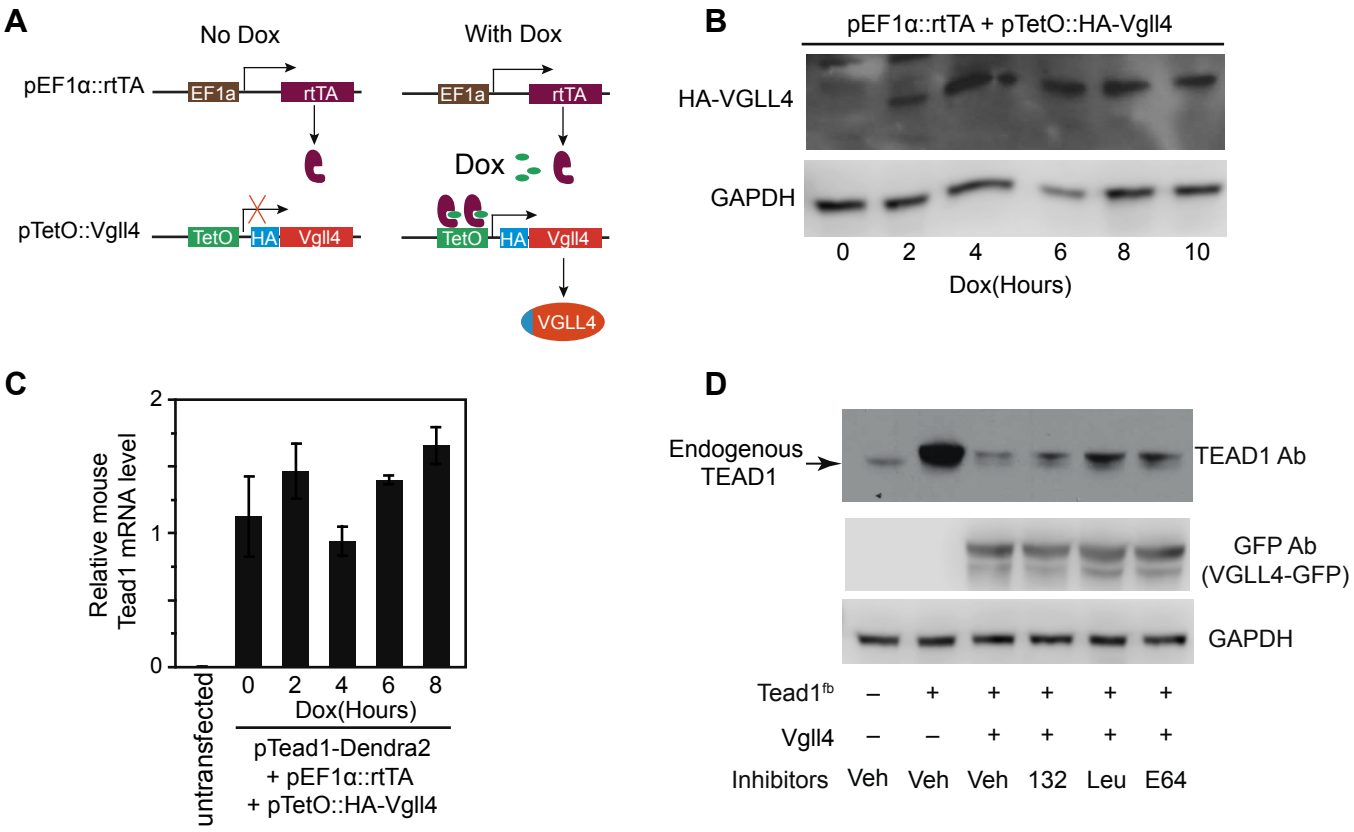

Figure S4. VGLL4 induces TEAD1 degradation (supporting Figure 4).

A. Schematic view of the Doxycycline (Dox) inducible HA-VGLL4 expresison system. HA-VGLL4 was cloned downstream of TetO promoter. pEF1α::rtTA was used to express rtTA. The expression of HA-VGLL4 will be activated in the presence of both Dox and rtTA.

B. Validation of Dox inducible expression of HA-VGLL4. 293T cells were co-transfected with pEF1α::rtTA and pTetO::HA-Vgll4. 24 hours after transfection, cells were treated with 1mg/ml Dox for different hours. Immuno blot was carried out to detect the expression of HA-VGLL4.

C. Measurement of Tead1-Dendra2 mRNA level following VGLL4 induction. Mouse Tead1 specific primers were used to measure relative Tead1-Dendra2 mRNA level by qRT-PCR. Bars indicate standard error of the mean. n=3.

D. TEAD1 degradation is dependent on cysteine proteases and is independent of the proteasome. 293T cells were first co-transfected with TEAD1^{fb} and Vgll4-GFP plasmids. 1 day after transfection, cells were treated with indicated inhibitors for 6 hours. 0.1% DMSO was used as control vechicle. β-tubulin was used as loading control. 132: Mg132. Leu: Leupeptin.

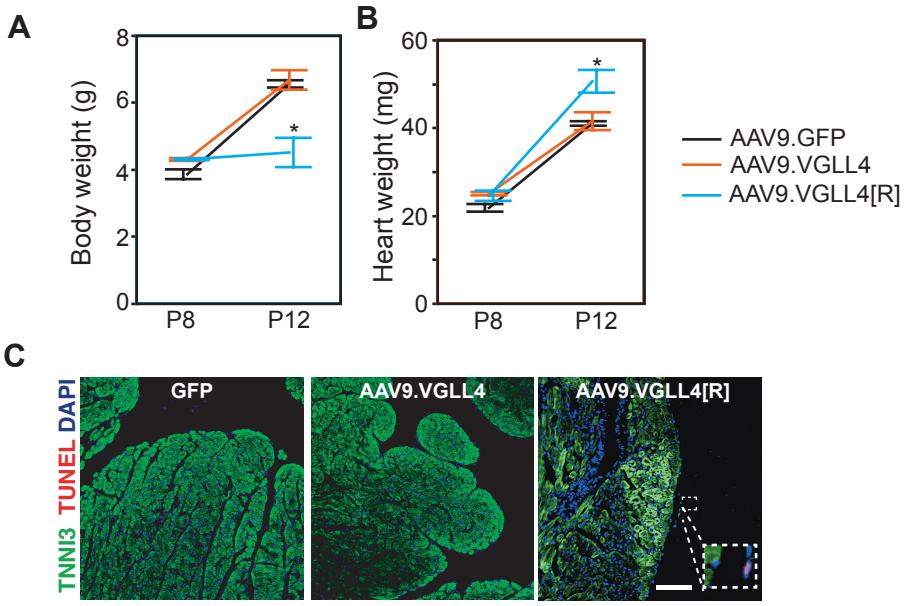

Figure S5, Related to Figure 5. A-B, Measurement of AAV transduced mouse heart and body weight at different age. **A**. Body weight measurment. **B**. Heart weight measurement. A-B, *, P<0.05. Bars indicate standard error of the mean. P8, n=3. P12, n=4. **C**, VGLL4[R] overexpression caused heart failure without affecting cardiomyocyte apoptosis. Mouse pups were transduced with indicated virus at P1, and hearts were collected for analysis at P12. TUNEL assay on sections of hearts treated with the indicated AAV9 vector. In the AAV9. VGLL4[R] group, TUNEL positive non-cardiomyocyte was shown in the zoomed in rectangle. Bar=100 μm.

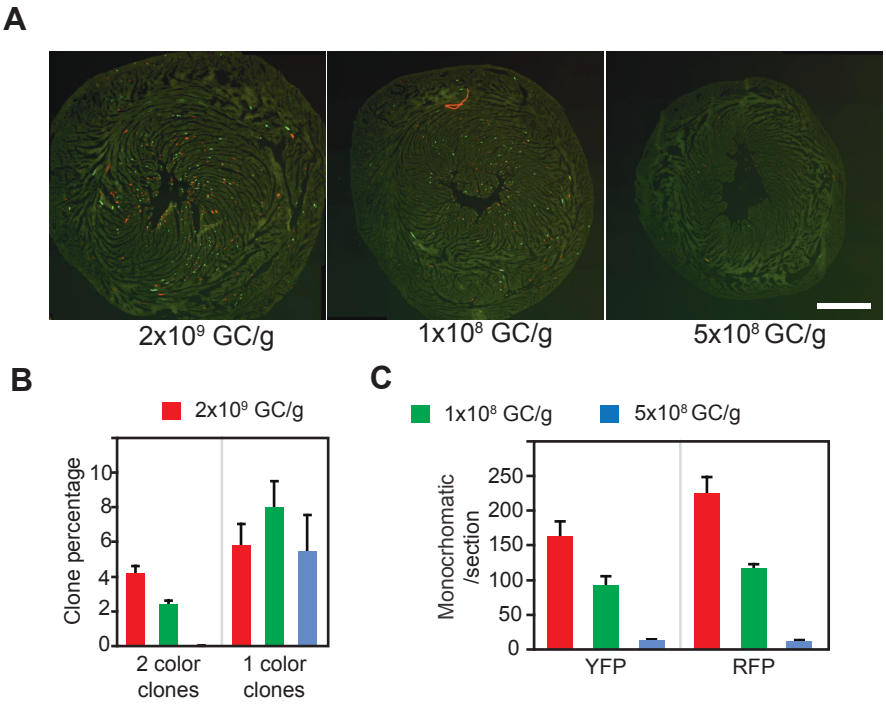

Figure S6. Titration of AAV9.cTNT::Cre in neonatal Rosa26 Brainbow mice (supporting Figure 6). Different dosase of AAV9.cTNT::Cre was administered to P1 Rosa26$^{Brainbow/+}$ neonatal mice. Hearts were collected for analysis at P8. Cre recombination activates GFP, YFP and RFP expression. **A**. Immunofluorescent images of heart cryosections. Bar = 500 μm. **B-C**. Quantifcation of clones. Bars represent standard error of the mean. n=3.