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Hippo Pathway Inhibits Wnt Signaling to Restrain Cardiomyocyte Proliferation and Heart Size

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Abstract

Genetic regulation of mammalian heart size is poorly understood. Hippo signaling represents an organ-size control pathway in *Drosophila*, where it also inhibits cell proliferation and promotes apoptosis in imaginal discs. To determine whether Hippo signaling controls mammalian heart size, we inactivated Hippo pathway components in the developing mouse heart. Hippo-deficient embryos had overgrown hearts with elevated cardiomyocyte proliferation. Gene expression profiling and chromatin immunoprecipitation revealed that Hippo signaling negatively regulates a subset of Wnt target genes. Genetic interaction studies indicated that β-*catenin* heterozygosity suppressed the Hippo cardiomyocyte overgrowth phenotype. Furthermore, the Hippo effector Yap interacts with β-catenin on *Sox2* and *Snai2* genes. These data uncover a nuclear interaction between Hippo and Wnt signaling that restricts cardiomyocyte proliferation and controls heart size.

In mammals, organ-extrinsic influences such as nutritional status, circulating growth factors, and hormones have a large impact on organ size control (1). Several lines of evidence indicate that there are also organ-intrinsic mechanisms to modulate organ size (1, 2). Insight into genetic pathways regulating organ size has come from *Drosophila*, where two main organ-size control pathways are bone morphogenetic protein (Bmp) and Hippo signaling pathways (3, 4). Whether these growth control mechanisms are broadly conserved in mammalian organs remains unclear.

Organ size is important in cardiac development, because the heart must be large enough to generate a physiological cardiac output but not so large as to block cardiac outflow, as in obstructive cardiomyopathies. The mammalian core Hippo signaling components include Ste20 family kinases *Mst1* and *Mst2*, which are homologous to *Drosophila Hippo*. Mst kinases form an active complex with WW repeat scaffolding protein Salvador (Salv), also called WW45, that phosphorylates large tumor suppressor homolog (Lats) kinase. Mammals have two *Lats* genes, *Lats1* and *Lats2*, which are homologous to *Drosophila Warts*. Lats kinases complex with Mob to phosphorylate Yap and Taz, two related transcriptional coactivators. Upon phosphorylation, Yap and Taz, the most downstream Hippo signaling components, are excluded from the nucleus and are transcriptionally inactive.

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Supporting Online Material: www.sciencemag.org/cgi/content/full/332/6028/458/DC1, Materials and Methods, Figs. S1 to S9, Tables S1 and S2, References

To determine whether the Hippo signaling pathway functions in the determination of mammalian heart size, we inactivated the single mammalian *Salv* ortholog in the mouse heart by using a *Salv* conditional null allele and the *Nkx2.5* ^{cre} allele that directs cardiac cre activity (5–7). Yap phosphorylation at Ser^{127} (pYAP) was examined in *Nkx2.5*^{cre}:*Salv*$^{f/f}$ (*Salv CKO*) mutants via Western blot and immunofluorescence (IF) (fig. S1). IF of embryonic day 9.5 (E9.5) sagittal sections revealed pYap signal in both second heart field (SHF) progenitors and outflow tract and ventricular cardiomyocytes (fig. S1, A to D). *Salv CKO* hearts had reduced pYAP but no change in total Yap, revealing that Hippo signaling is active in the developing heart and that *Salv* deletion reduces cardiac Hippo activity (fig. S1, E and F).

Salv CKO mutants survived development, but most mutants expired postnatally with obvious heart enlargement or cardiomegaly (Fig. 1, A and B, and table S1). Histological examination of *Salv CKO* mutant hearts revealed that, although organ size is affected, arterioventricular connections and arrangement of chambers and valves were unaffected (Fig. 1, C and D), consistent with preserved patterning observed in Hippo mutant imaginal discs (4). Hearts of some *Salv CKO* mutant animals had a ventricular septal defect (VSD), indicating that Hippo signaling regulates ventricular septation (Fig. 1D). Because VSD can cause heart failure, we confined further analysis of *Salv CKO* mutants to stages before ventricular septation is completed, E14.5 and earlier stages.

Salv CKO mutant hearts had expansion of trabecular and subcompact ventricular myocardial layers, thickened ventricular walls, and enlarged ventricular chambers without a change in myocardial cell size (Fig. 1, E and F, and fig. S2). *Lats2* and *Mst1/2 CKO* E11.5 mutant hearts had similar myocardial expansion phenotypes (fig. S3).

We investigated cardiomyocyte proliferation by double immunostaining with phosphorylated Ser^{10} histone H3 (pHH3) antibodies to detect mitotic cells and sarcomeric myosin (α-MF20) (Fig. 2, top and middle). Less than 1% of control ventricular cardiomyocytes were pHH3-positive, whereas about 4.5% of *Salv CKO* mutant cardiomyocytes were pHH3-positive, indicating excessive cardiac proliferation in cardiomyocytes (Fig. 2, bottom). Cardiomyocyte proliferation was elevated in both left and right *Salv CKO* ventricles. Cell counting indicated elevated ventricular cardiomyocte number in *Salv CKO* mutants but no changes in ventricular smooth muscle (α-SMA) or cardiac fibroblasts (α-DDR2) (fig. S4).

SHF cardiac progenitors that contribute to right ventricle had normal cellular proliferation levels in *Salv CKO, Lats2 CKO*, and *Mst1/2 CKO* mutants, indicating that *Salv CKO* mutant cardiomegaly was unlikely to be due to elevated cardiac progenitor number (fig. S5).

Microarray revealed that known canonical Wnt target genes were up-regulated in *Salv CKO* embryos (Fig. 3A). This Wnt-Hippo signature included *Sox2*, which has been implicated in cardiac repair and cell reprogramming (8, 9); *Snai2/Slug*, a tumorigenesis factor having roles in epithelial-mesenchymal transition and cell growth (10); and the anti-apoptosis gene *Birc5/ Survivin* (11).

Reverse transcription quantitative polymerase chain reaction (qRT-PCR) validated up-regulated expression of *Sox2* and *Snai2*, as well as *cdc20, l-Myc, Birc2*, and *Birc5*, in *Salv CKO* embryonic hearts (Fig. 3B). With the exception of *l-Myc*, reduced expression was observed in *Mef2c*^{cre}; β-*catenin*$^{f/f}$ mutants (Fig. 3C). Other β-catenin–regulated genes, such as *Fgf10* and *Isl1*, were unchanged in *Salv CKO* hearts, indicating incomplete overlap between Yap targets and β-catenin targets (fig. S6). In situ hybridization revealed *Sox2* and *Snai2* up-regulation in *Salv CKO* E12.5 hearts (fig. S6).

E12.5 hearts were immunostained with β-catenin–specific antibodies to assay the nuclear β-catenin index, a readout for cells receiving a Wnt signal. Whereas β-catenin localized to plasma membrane junctions and cytosol of control myocardium, *Salv CKO* mutants had a fourfold increase in nuclear β-catenin staining (Fig. 3, D to F). These findings indicate that canonical Wnt signaling in cardiomyocytes is derepressed upon *Salv* deletion and support the notion that Hippo signaling inhibits Wnt/β-catenin to regulate heart size.

To obtain genetic evidence that Hippo signaling negatively regulates Wnt/ β-catenin–dependent cardiac growth, we crossed *Salv CKO* mice to a β-*catenin* conditional null allele to generate *Nkx2.5*^{cre}*; Salv*$^{f/f}$;β*cat*$^{f/+}$ (*Salv*/β*cat*$^{f/+}$ *CKO*) embryos that are Hippo-deficient with reduced β-*catenin* dosage.

Myocardial thickness of *Salv*/β*cat*$^{f/+}$ *CKO* hearts was significantly reduced by comparison to *Salv CKO* (figs. S7, B and C, and S2). Ventricular proliferation rates, as well as subcompact and trabecular myocardial thickness, in *Salv*/β*cat*$^{f/+}$ *CKO*; mutants resemble those of control (Fig. 4A and figs. S2 and S7, A and C). qRT-PCR indicated that *Sox2, Snai2, Birc2*, and *Cdc20* expression levels reverted to control or were lower in hearts with reduced β-catenin dosage (Fig. 4B). Suppression of the Hippo myocardial overgrowth phenotype by reduced β-*catenin* indicates that Wnt signaling is required for up-regulated cardiomyocyte proliferation and cardiomegaly in Hippo mutants.

To investigate whether Yap and β-catenin are in the same molecular complex, we immunoprecipitated protein extracts from E14.5 hearts with Yap and pYAP-specific antibodies (Fig. 4C). Western blotting with antibodies against β-catenin revealed that β-catenin forms a complex only with nuclear, nonphosphorylated Yap, indicating that the Yap/β-catenin molecular interaction is nuclear (Fig. 4C).

Because Yap associates with DNA-binding Tead/Tef transcription factors whereas β-catenin binds to Lef/Tcf factors (12, 13), we examined surrounding genomic sequence of genes within the Wnt-Hippo expression signature for Tead/Tef and Lef/Tcf binding sites. For *Sox2* and *Snai2*, candidate Yap/Tead binding sites were identified both upstream and downstream of open reading frames (fig. S7D). Conserved Tcf/Lef binding elements (CTTTG) were in close proximity to *Sox2* and *Snai2* downstream candidate Yap/Tead sites (fig. S7D).

Chromatin immunoprecipitation (ChIP) revealed that Yap and β-catenin were recruited to *Sox2* and *Snai2* downstream regions but not upstream sites (Fig. 4D). Sequential ChIP revealed that Yap and β-catenin concurrently occupied *Sox2* and *Snai2*, suggesting that Yap and β-catenin are contained within a common regulatory complex on *Sox2* and *Snai2* (Fig. 4D).

Transfection experiments indicated that Yap and β-catenin transfection along with their DNA binding co-factors induced luciferase expression from both *Sox2* and *Snai2* reporter plasmids. Luciferase reporter activity was significantly reduced by preventing Yap or β-catenin recruitment to the reporter either individually or concurrently (Fig. 4E). These findings support the model that Yap and β-catenin recruitment to *Sox2* and *Snai2* chromatin through their respective DNA binding partners potentiates transcriptional activity.

The Yap and β-catenin interaction on genes such as *Snai2* and *Sox2* uncovers a nuclear mechanism for antagonistic control of cardiomyocyte growth by Hippo and canonical Wnt signaling. Our model is supported by genetic suppression of Hippo-enlarged hearts by reduced β-*catenin*. Hippo signaling inhibits a pro-growth Wnt/β-catenin-Yap interaction in differentiating cardiomyocytes as the heart transitions from rapid progenitor cell growth to more measured growth in the maturing heart.

Although our pYap data indicate that there is some Hippo activity in E9.5 SHF cardiac progenitors, cell proliferation in Hippo mutant SHF was unchanged from control. This may reflect *Salv*-independent Hippo activity and perhaps overlapping functions with other Hippo pathway kinases (i.e., *Lats1*). Our data support the previous observation that Hippo signaling was low in E10.5 myocardium (14). Although *Salv CKO* cardiomyocyte cell size was unchanged, this question requires further study under stressed conditions (15).

Hippo regulates growth and progenitor genes like *Sox2, Snai2, Ccdn1, Cdc20*, and *l-Myc* in cardiomyocytes. In livers overexpressing Yap and *Hippo* loss-of-function mutants, expression of *c-Myc* and *Ccdn1* is up-regulated, suggesting shared mechanisms between liver and heart (4). Although apoptosis inhibitors *Birc2* and *Birc5* were up-regulated in *Salv CKO* mutant hearts, apoptosis was unchanged.

Important recent work uncovered a repressive cytoplasmic interaction between pTaz and Dvl in kidney (16). Our findings, uncovering a nuclear interaction between Wnt and Hippo, suggest a two-tiered mechanism by which Hippo negatively modulates Wnt signaling in multiple contexts (fig. S8).

Wnt signaling has distinct functions in the two cardiac progenitor fields (13). Nonetheless, we find no proliferation difference between first heart field–derived left ventricle and SHF-derived right ventricle in *Salv CKO* hearts, indicating shared organ size-control mechanisms for the two myocardial lineages. Also Wnt signaling is low in myocardium even though Wnt ligands are expressed (17). Our findings suggest that, in Hippo-low cardiac progenitors, progrowth genes regulated by Hippo and Wnt are actively transcribed. In cardiomyocytes, Hippo signaling restricts Yap from the nucleus, resulting in diminution of Hippo-Wnt–regulated genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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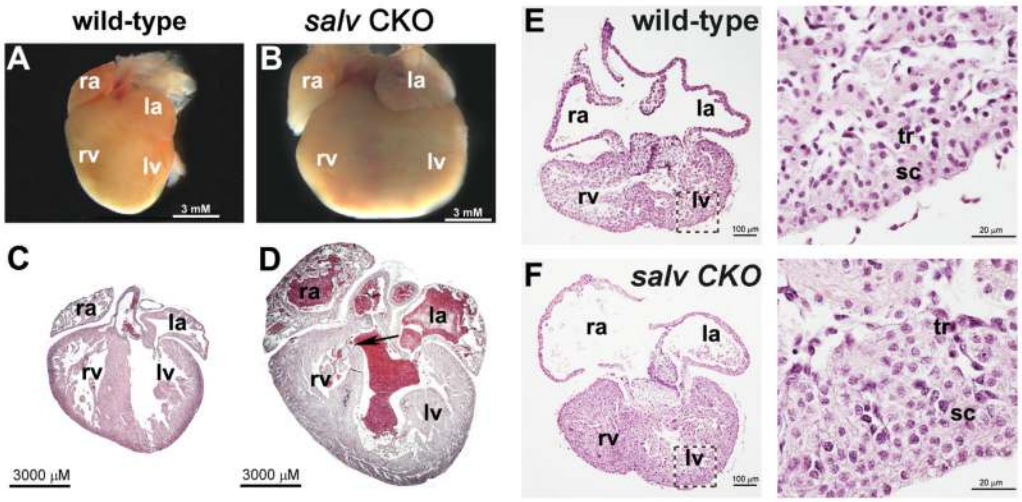

Fig. 1.

Salvador mutant cardiomegaly. (**A** to **D**) Control [(A) and (C)] and *Salv CKO* [(B) and (D)] P2 neonate hearts. ra indicates right atrium; la, left atrium; rv, right ventricle; lv, left ventricle. Hearts in (A) and (B) were sectioned and stained with hematoxylin and eosin (H&E), as shown in (C) and (D). Arrow, ventricular septum defect. (**E** and **F**) H&E-stained control (E) and *Salv CKO* (F) hearts. High magnification of (E) and (F) are shown in the right-hand images; subcompact, sc; trabecular, tr myocardium. Control genotype is $Nkx2.5^{cre}; Salv^{f/+}$.

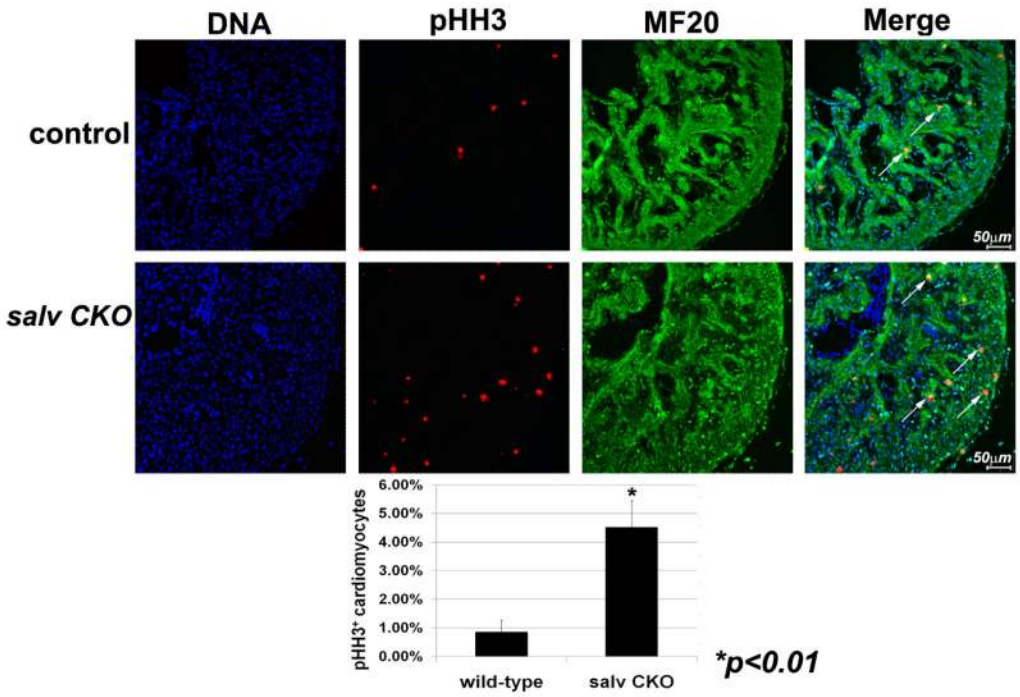

Fig. 2.

Cardiomyocyte proliferation in *Salvador* mutant ventricles. E12.5 control (**top**) and *Salv CKO* (**middle**) coronal sections of left ventricles: stain with TO-PRO-3, blue; α-pHH3, red; and α-MF20, green. Arrows, pHH3/MF20-positive cells. (**Bottom**) pHH3-positive cardiomyocytes quantification. Control genotype is $Nkx2.5^{cre}; Salv^{f/+}$.

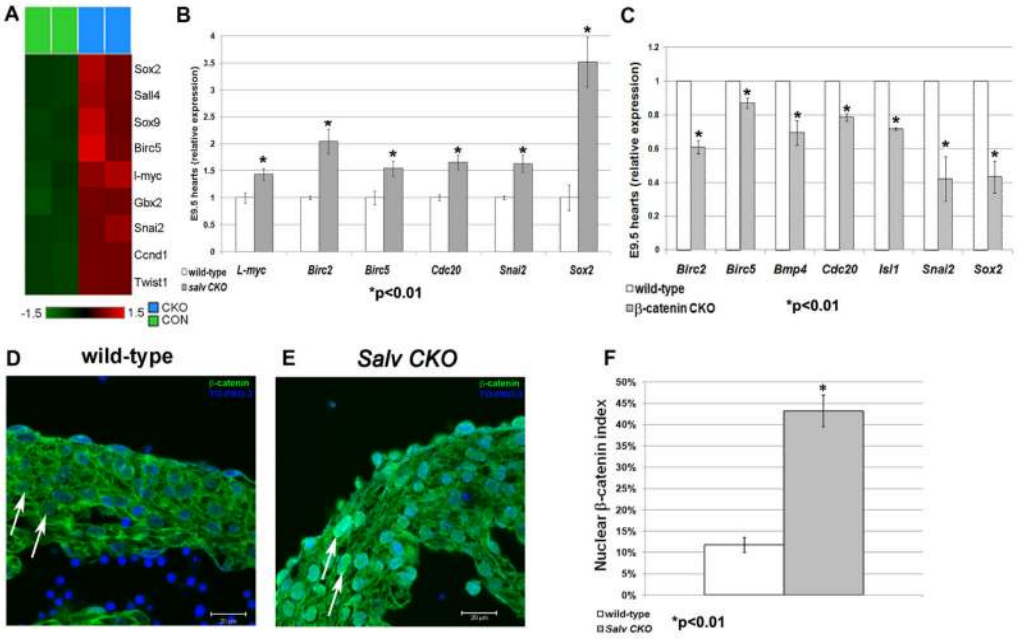

Fig. 3.

Salvador deletion potentiates canonical Wnt signaling. Heat map (**A**) and qRT-PCR validation (**B**) showing relative transcript levels of Wnt/β-catenin target genes. Values were determined as a mean of three samples ± SD with glyceraldehyde-3-phosphate dehydrogenase control. (**C**) qRT-PCR of Wnt/β-catenin target genes in β-*catenin CKO* hearts. (**D** and **E**) IF images with quantification (**F**) of E12.5 heart sections: stain with TO-PRO-3, blue, and α-β-catenin, green. Nuclear β-catenin identified by β-catenin/TO-PRO-3 signal overlap (arrows). Control genotype is *Nkx2.5*^{cre}*; Salv*$^{f/+}$.

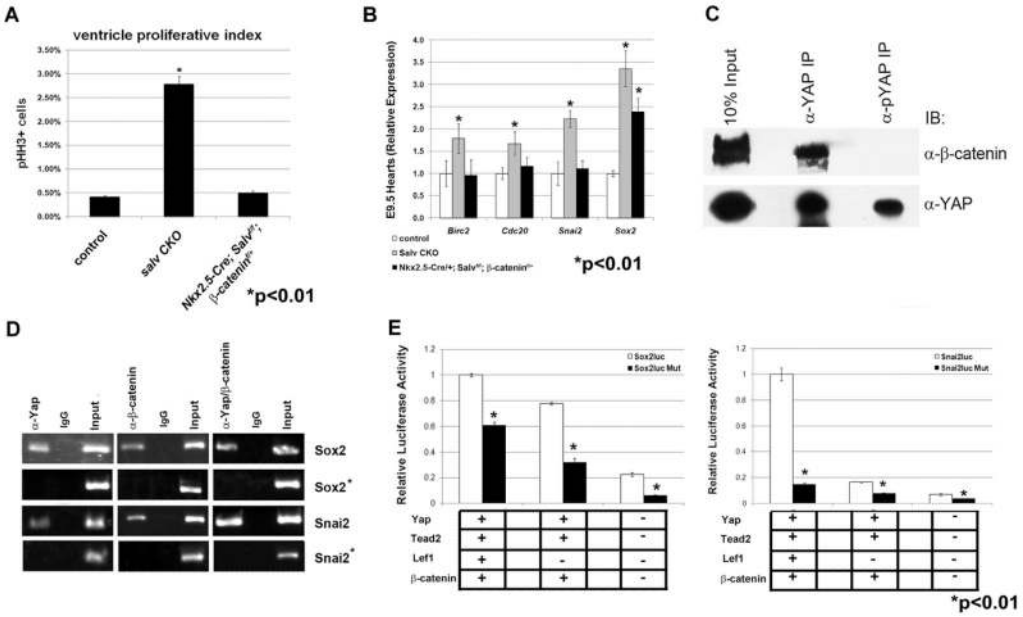

Fig. 4.

Yap interacts with β-catenin. (**A**) Quantification of pHH3 indices in control, *salv CKO*, and *Nkx2.5*^{cre}*; Salv*$^{f/f}$; β-*catenin* $^{F/+}$ E12.5 hearts. (**B**) qRT-PCR with indicated genes. (**C**) Immunoprecipitation/Western with indicated antibodies. IB, immunoblot. (**D**) ChIP and sequential ChIP with indicated antibodies. Control ChIP sites proximal to *Sox2* and *Snai2* loci were tested (asterisks). Refer to fig. S7D for ChIP assay design. IgG, immunoglobulin G. (**E**) Luciferase reporter assays with co-transfected factors.