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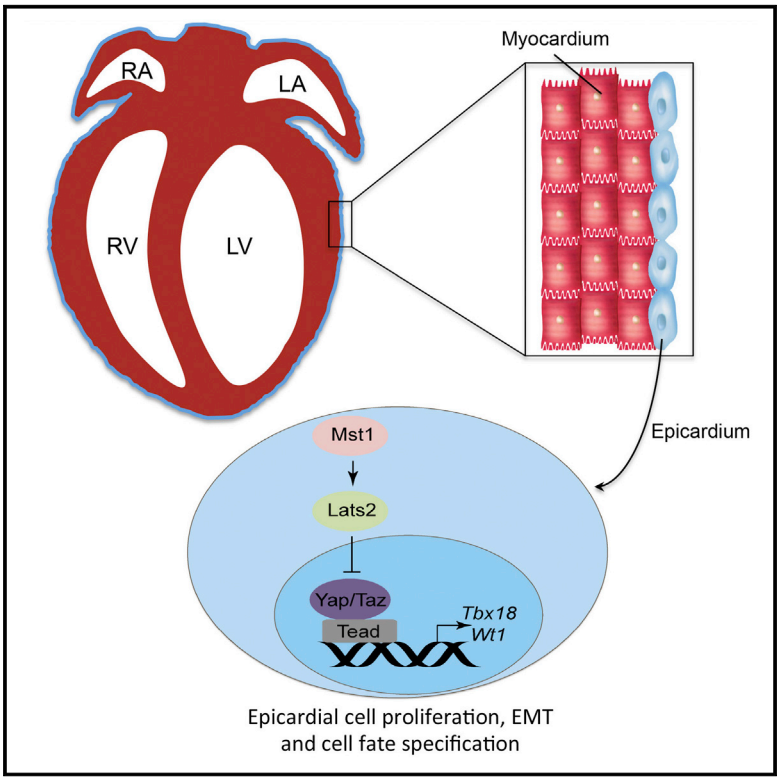
Hippo Signaling Mediators Yap and Taz Are Required in the Epicardium for Coronary Vasculature Development

Graphical Abstract



Highlights

- Hippo signaling components are expressed in the developing proepicardium and epicardium
- Genetic deletion of Yap and Taz leads to coronary vasculature defects
- Yap and Taz regulate epicardial cell proliferation, EMT, and cell fate specification

Authors

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In Brief

Singh et al. show that Hippo signaling components are expressed in proepicardial and epicardial cells and are required for coronary vasculature development. Yap and Taz regulate epicardial cell proliferation, EMT, and cell fate specification, in part by regulating *Tbx18* and *Wt1* expression.

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Hippo Signaling Mediators Yap and Taz Are Required in the Epicardium for Coronary Vasculature Development

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SUMMARY

Formation of the coronary vasculature is a complex and precisely coordinated morphogenetic process that begins with the formation of epicardium. The epicardium gives rise to many components of the coronary vasculature, including fibroblasts, smooth muscle cells, and endothelium. Hippo signaling components have been implicated in cardiac development and regeneration. However, a role of Hippo signaling in the epicardium has not been explored. Employing a combination of genetic and pharmacological approaches, we demonstrate that inhibition of Hippo signaling mediators Yap and Taz leads to impaired epicardial epithelial-to-mesenchymal transition (EMT) and a reduction in epicardial cell proliferation and differentiation into coronary endothelial cells. We provide evidence that Yap and Taz control epicardial cell behavior, in part by regulating *Tbx18* and *Wt1* expression. Our findings show a role for Hippo signaling in epicardial cell proliferation, EMT, and cell fate specification during cardiac organogenesis.

INTRODUCTION

The coronary vasculature is required for supplying oxygenated blood to the cardiac muscle. Proper coronary blood circulation is essential for embryonic and adult cardiac tissue homeostasis. Defects associated with the coronary function leads to myocardial ischemia, infarction, and heart failure. Therefore, identifying molecules and signaling pathways regulating coronary vessels morphogenesis, remodeling, and maturation is essential in understanding the etiology of coronary diseases. The incidents of coronary anomalies have been reported in up to 1% of the general population (Angelini, 2002). During embryogenesis, cells from multiple sources, including the proepicardium and epicardium, contribute to the development of coronary vasculature (Chen et al., 2014; Red-Horse et al., 2010; Wu et al., 2012). The epicardium is a single layer of epithelial cells that covers the heart. It develops from the proepicardial organ (PEO), a transient structure that arises from the mesothelium of the septum transversum (Männer, 1993; Mikawa and Gourdie, 1996). The epicardium plays a significant role in heart development and gives rise to the majority of cells, including fibroblasts, smooth muscle cells, and endothelium, of the coronary vasculature (Männer, 1993; Mikawa and Gourdie, 1996; Singh and Epstein, 2012; Singh et al., 2011). Epicardium-deficient hearts exhibit impaired cardiac function due to a thin myocardium, suggesting that factors secreted from the epicardium are required not only for coronary vasculature development but also for the proliferation and differentiation of the underlying myocardial cells (Männer, 1993; Männer et al., 2005; Pennisi et al., 2003). The role of the epicardium in cardiac homeostasis was recently explored using an epicardial injury model. Developmental gene programs were re-activated following injury, which led to epicardial cell expansion and differentiation into cardiac fibroblasts and smooth muscle cells (Zhou et al., 2011). A better understanding of embryonic epicardial biology will help to understand the pathophysiology of coronary defects and it may suggest strategies to manipulate adult epicardial cells to facilitate myocardial regrowth and angiogenesis after cardiac injury.

The Hippo signaling is an evolutionary conserved pathway that control organ size by regulating cell proliferation, cell survival, and stem cell self renewal (Zhao et al., 2011). Hippo signaling has been implicated in cardiac development as well as in cardiac repair and regeneration after myocardial injury. Genetic deletion, with a cardiac-specific Cre-recombinase, of *Mst1/2*, *Lats2*, or *Salvador* (*Salv*) leads to an expansion of ventricular myocardium due to increased cardiomyocyte proliferation (Heallen et al., 2011). Global deletion of *Yap* results in embryonic lethality around embryonic day 8.5 (E8.5) due to defects in yolk sac vasculogenesis, chorioallantonic fusion, and body axis elongation (Morin-Kensicki et al., 2006). However, *Taz* knockout mice are viable through adulthood, although some

develop glomerulocystic kidney disease and pulmonary disease (Xin et al., 2013). *Yap* and *Taz* double-null embryos die prior to the morula stage, suggesting functional redundancy during early embryonic development (Nishioka et al., 2009). Expression of a constitutively active form of Yap in the heart results in increased cardiomyocyte proliferation and heart size (von Gise et al., 2012; Xin et al., 2011). Yap has been shown to regulate cardiomyocyte proliferation by interacting with the insulin-like growth factor (IGF) and Wnt signaling pathways (Heallen et al., 2011; Xin et al., 2011). In addition, recent work by Zhang et al. demonstrates that Yap can regulate epithelial-to-mesenchymal transition (EMT) of the atrioventricular cushion by modulating transforming growth factor β (TGF-β)/Smad signaling (Zhang et al., 2014). During cardiac development, Yap and Taz are functionally redundant, but tissue-specific deletion of both molecules leads to lethal cardiomyopathy in a gene-dose-dependent manner (Xin et al., 2013). Despite the studies described above, a role for Yap and Taz in the epicardium has not been explored.

Here, we show that Hippo signaling components are expressed during epicardium formation. To determine the significance of Yap and Taz in the developing epicardium, we generated epicardium-specific *Yap*/*Taz* double-knockout mice. Genetic deletion of *Yap* and *Taz* using $Sema3d^{GFPCre/+}$ mice leads to embryonic lethality between E11.5 and E12.5 due to cardiac defects. Furthermore, the inducible genetic deletion of *Yap* and *Taz* using $Wt1^{CreERT2/+}$ mice reveals impaired coronary vasculature development. Pharmacological and genetic experiments suggest that the impaired coronary vasculature development observed in Yap/Taz mutants is due to defects in epicardial cell proliferation, EMT, and fate determination. We provide further evidence that Yap/Taz control epicardial cell proliferation, EMT, and fate determination, in part by regulating *Tbx18* and *Wt1* expression.

RESULTS

Hippo Signaling Components Are Expressed in the Murine Proepicardium and Epicardium during Development

To establish the pattern of Yap expression during epicardium development, we performed Yap immunohistochemistry on embryonic hearts from E9.5 to E12.5. At E9.5, Yap expression was noted in the PEO, where it colocalizes with Tbx18 (Figures 1A–1C). Yap expression is maintained in migrating proepicardial and epicardial cells from E9.5 to E12.5 (Figures 1D–1I). To demonstrate that Yap is expressed specifically in epicardial cells, Yap colocalization with Wt1 was performed (Figures 1J–1L). Yap colocalizes with Wt1 in the developing epicardium. Similar to Yap, Taz expression is prominent in the epicardium from E10.5 to E12.5 (Figures 1M–1R). In addition, we utilized heart sections from $Sema3d^{GFPCre/+}$ mice and assayed for colocalization of Yap and GFP. At E12.5, we observed Yap and GFP colocalization in epicardial cells (Figures 1S–1U). To determine whether other Hippo signaling components are expressed during epicardium development, we performed qRT-PCR gene expression analysis on RNA harvested from epicardial explants. To first establish the robustness of the epicardial explant system, we generated epicardial explants from $Sema3d^{GFPCre/+}$;$R26^{Tom/+}$ embryos to determine the relative percentage of fate-mapped epicardial cells within a sample. Consistent with previous reports, the majority of migrating cells are RFP positive, demonstrating epicardial identity (Figures 1V–1X) (Grieskamp et al., 2011; Takeichi et al., 2013). Utilization of this explant system revealed that *Yap*, *Taz*, and *Tead1-3* are expressed by epicardial cells (Figure 1Y). *Tead4* expression was barely detectable in epicardial explant cells. Western blot analysis demonstrated that the Hippo kinases Lats1 and Lats2 are also expressed in epicardial cells (Figure 1Z).

$Sema3d^{GFPCre/+}$-Mediated Epicardial Deletion of Yap and Taz Leads to Embryonic Lethality

To determine a potential role for Yap and Taz in the epicardium during coronary vasculature development, conditional $Yap^{flox/flox}$ and $Taz^{flox/flox}$ alleles were crossed with a $Sema3d^{GFPCre/+}$ knockin mouse, thereby targeting Cre-recombinase to the PEO and epicardium (Figure S1) (Katz et al., 2012). *Sema3d* is expressed by many, but not all, PEO progenitors (Katz et al., 2012). We did not recover any $Sema3d^{GFPCre/+}$:$Yap^{flox/flox}$:$Taz^{flox/+}$ or $Sema3d^{GFPCre/+}$:$Yap^{flox/flox}$:$Taz^{flox/flox}$ neonates from the breeding of $Sema3d^{GFPCre/+}$:$Yap^{flox/+}$:$Taz^{flox/+}$ and $Yap^{flox/flox}$:$Taz^{flox/flox}$ mice, demonstrating that epicardial inactivation of *Yap and Taz* is embryonic lethal (Figure 2A). Yap plays a dominant role compared to Taz in Sema3d-expressing cells as loss of both alleles of *Yap* in a *Taz* heterozygous background leads to postnatal lethality, while loss of *Taz* in a *Yap* heterozygous background produced viable mice (Figure 2A). Genotyping of embryos from timed matings showed that the loss of *Yap* and *Taz* resulted in embryonic lethality between E11.5 and E12.5 (Figure 2A). At E11.5, double-null embryos were smaller than control littermates and occasionally showed hemorrhage, consistent with embryonic lethality due to cardiovascular insufficiency (Figures 2B and 2C), although loss of *Yap*/*Taz* due to Sema3d-Cre expression in other tissues could also be responsible or contributory. To better understand the cardiac defects caused by epicardial deletion of *Yap and Taz*, we performed a detailed histological examination of both mutant and control embryos at E10.5 and E11.5 (Figures 2D–2L). At E11.5, immunostaining for the cardiac marker MF-20 showed thin and fragmented myocardium in mutant hearts, but not in controls, suggesting that Yap and Taz may play a role in modulating paracrine effects of epicardial cells on adjacent myocardiaum (Figures 2D and 2E). However, the myocardium of mutant embryos at E10.5 did not display any obvious morphological defects (Figures 2F, and 2G). Fate-mapping analysis using $Sema3d^{GFPCre/+}$ mice showed that epicardial formation was grossly intact in mutants compared to controls, suggesting that Yap and Taz are not required for the initial migration of $Sema3d^{GFPCre/+}$ proepicardial cells over the heart (Figures 2H and 2I). However, Ki67 staining showed a significant reduction in epicardial cell proliferation in mutant hearts compared to controls (Figures 2J–2L). To determine whether the reduced myocardial thickness in mutants could be due to impaired epicardial-myocardial signaling, we measured expression of paracrine factors known to regulate myocardial growth and observed significantly lower expression of *Fgf9*, *Raldh2*, and *Wnt5a* in mutant explants. There was no significant difference in the expression of *Fgf16*, *Fgf20*, *EPO*, *Igf2*, and *Wnt9b* (Figure 2M).

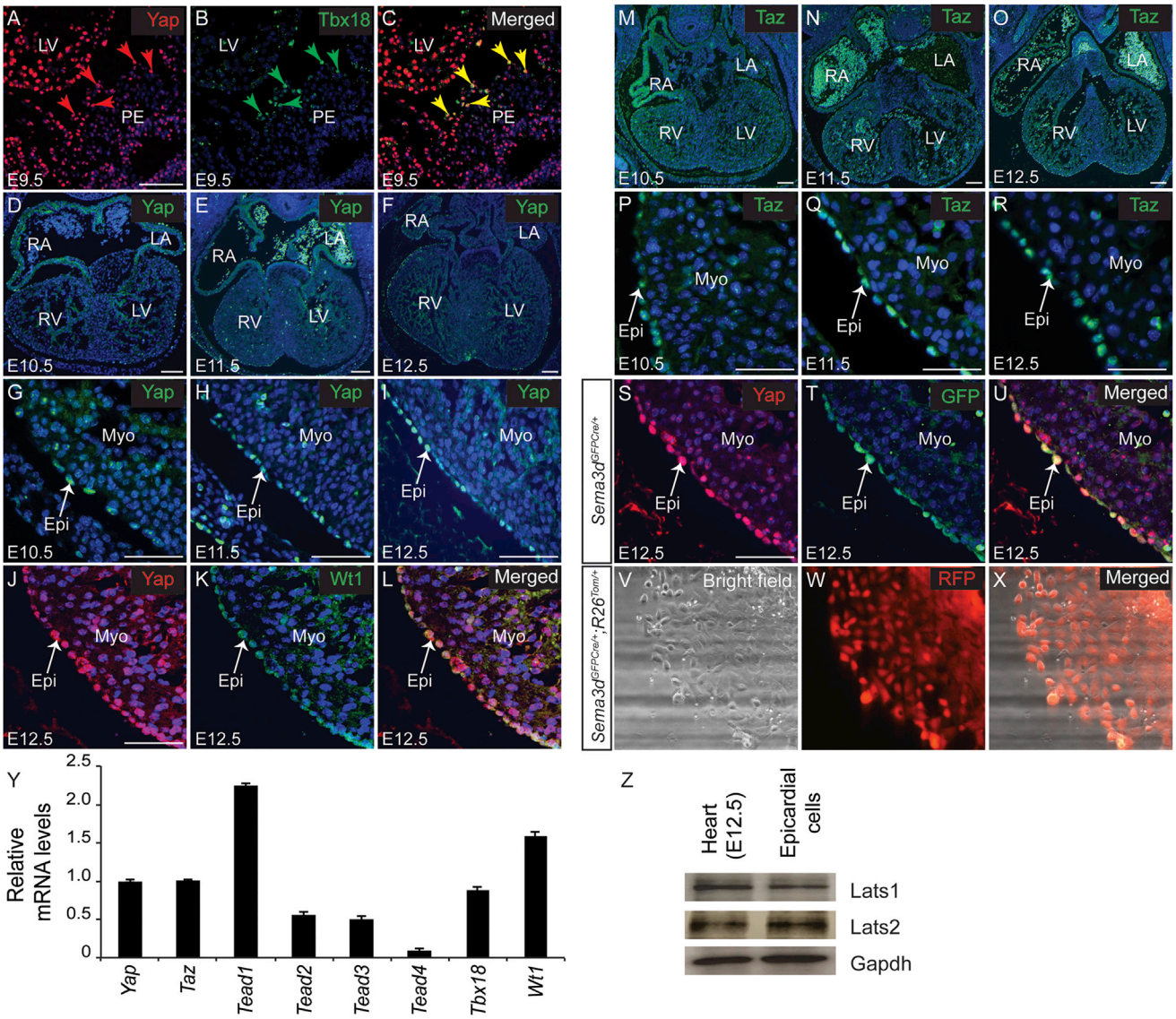

Figure 1. Hippo Signaling Mediators Are Expressed in Proepicardial and Migrating Epicardial Cells during Embryonic Development

(A–C) Immunohistochemistry for Yap and Tbx18 was performed on E9.5 mouse heart sections. (A) Magnified view of the PEO shows Yap expression in the PEO (red arrows). (B and C) Merged image shows that Yap and Tbx18 (green arrows) are co-localized within PEO (yellow arrows). Nuclei were visualized by DAPI staining (blue).

(D–O) Immunohistochemistry for Yap was performed on E10.5–E12.5 mouse heart sections. (D–I) Yap is expressed in the developing epicardium of E10.5–E12.5 hearts. (J–L) Yap and Wt1 expression are co-localized in the epicardium.

(M–R) Immunohistochemistry for Taz was performed on E10.5 to E12.5 mouse heart sections.

(S–U) Immunohistochemistry with an anti-GFP antibody on *Sema3d*$^{GFPCre/+}$ embryos. Yap and GFP expression are co-localized in the epicardium.

(V–X) Epicardial explants from *Sema3d*$^{GFPCre/+}$;*R26*$^{Tom/+}$ embryos. The majority of the epicardial cells in the explant are RFP positive.

(Y) Real-time qPCR for the Hippo signaling mediators *Yap*, *Taz*, *Tead1*, *Tead2*, *Tead3*, and *Tead4* using RNA isolated from epicardial explants. *Tbx18* and *Wt1* are presented as controls.

(Z) Western blot analysis for Lats1 and Lats2 was performed using total lysates from wild-type E12.5 hearts and epicardial explants. Gapdh is shown as a loading control.

RA, right atrium; LA, left atrium; LV, left ventricle; RV, right ventricle; PE, proepicardium; Epi, epicardium; Myo, myocardium. Scale bars, 100 μm.

Genetic Deletion and Pharmacological Inhibition of *Yap* and *Taz* Leads to Impaired Epicardial EMT and Differentiation into Coronary Endothelial Cells

To allow for the assessment of older *Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ epicardial-deleted embryos, we deleted *Yap* and *Taz* in the epicardium using an inducible *Wt1*$^{CreERT2/+}$ mouse (Zhou et al., 2008). Cre-recombinase activity was induced at E11.5, and we did not recover any *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ postnatal pups, indicating embryonic lethality (Figure S2A). To determine if loss of *Yap* and *Taz* in the epicardium has effects on coronary

A Viability of *Sema3d*$^{GFPCre/+}$;*Yap*$^{flox/flox}$;*Taz*$^{flox/flox}$ mice at different developmental stages from *Sema3d*$^{GFPCre/+}$;*Yap*$^{flox/+}$;*Taz*$^{flox/+}$ X *Yap*$^{flox/flox}$;*Taz*$^{flox/flox}$ crosses. (* Statistically significant)

Genotypes	Age				
	E10.5 (n=72)	E11.5 (n=55)	E12.5 (n=41)	E15.5 (n=49)	P14 (n=56)
Yap$^{flox/+}$;*Taz*$^{flox/+}$	8	10	6	9	11
Yap$^{flox/flox}$;*Taz*$^{flox/+}$	9	5	4	8	10
Yap$^{flox/+}$;*Taz*$^{flox/flox}$	10	8	7	7	8
Yap$^{flox/flox}$;*Taz*$^{flox/flox}$	9	5	8	6	9
Sema3d$^{GFPCre/+}$;*Yap*$^{flox/+}$;*Taz*$^{flox/+}$	7	9	5	9	10
Sema3d$^{GFPCre/+}$;*Yap*$^{flox/flox}$;*Taz*$^{flox/+}$	11	7	4	4	0*
Sema3d$^{GFPCre/+}$;*Yap*$^{flox/+}$;*Taz*$^{flox/flox}$	8	8	7	7	8
Sema3d$^{GFPCre/+}$;*Yap*$^{flox/flox}$;*Taz*$^{flox/flox}$	10	3	0*	0*	0*

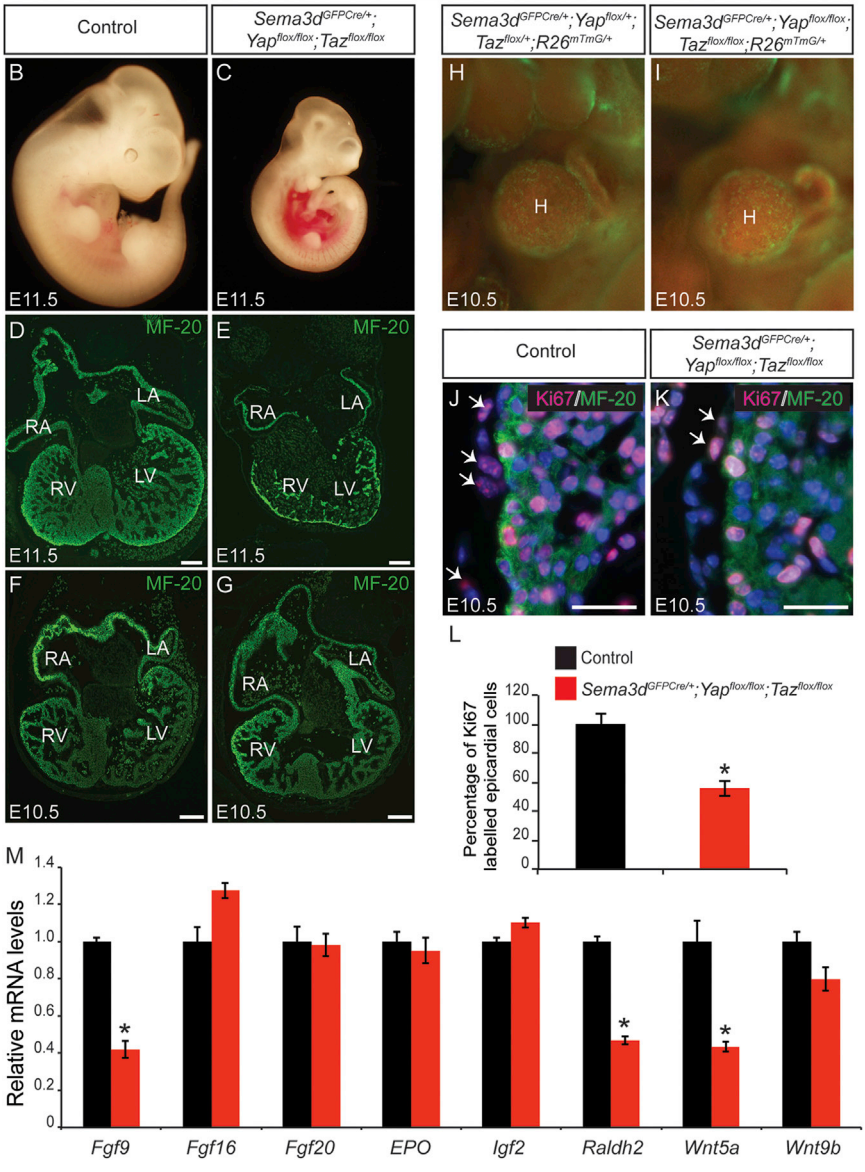

Figure 2. *Sema3d*$^{GFPCre/+}$-Mediated Deletion of *Yap* and *Taz* Leads to Embryonic Lethality

(A) Genotyping result of embryos and pups from *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/+}$:*Taz*$^{flox/+}$ and *Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ cross.

(B and C) Compared with control (B), Yap/Taz mutant embryos show reduced body size and hemorrhage (C).

(D–G) MF-20 immunohistochemistry of transverse sections from E10.5 and E11.5 control and *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ embryos.

(H–I) Whole mount fluorescence view of E10.5 *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/+}$:*Taz*$^{flox/+}$:*R26*$^{mTmG/+}$ and *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$:*R26*$^{mTmG/+}$ hearts.

(J–L) Immunostaining for Ki67, MF-20 and DAPI were performed on heart sections from E10.5 control (J) and *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ (K) embryos. Quantification of Ki67-positive cells was performed on four to six sections each from four individual hearts and averaged (L).

(M) Real-time qPCR for *Fgf9, Fgf16*, *Fgf20*, *EPO*, *Igf2*, *Raldh2*, *Wnt5a*, and *Wnt9b* on RNA isolated from control and *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ explants. Significant differences were defined by *$p < 0.05$. Scale bars, 100 μm.

hearts revealed severely disrupted coronary development compared to littermate controls. Control hearts showed an extensive network of mature coronary vessels largely on the posterior side of the heart, whereas mutant hearts showed fewer primitive vessels, suggesting that Yap and Taz are required for patterning and/or remodeling of the coronary vasculature (Figure 3A). As with the *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ cross, epicardial cell proliferation was significantly reduced in *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ hearts (Figure 3B). To determine whether epicardial cell migration into the underlying myocardium is affected, we generated *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/+}$:*Taz*$^{flox/+}$;*R26*$^{LacZ/+}$ and *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$;*R26*$^{LacZ/+}$ embryos. To trace epicardial-derived cells lacking Yap and Taz, we induced Cre-mediated recombination just prior to EMT (E11.5) and analyzed migration at E15.5. Using β-galactosidase activity to follow the epicardial-derived cells, we observed a significant reduction in the number of β-gal$^+$ cells in Yap/Taz mutants compared to littermate controls hearts (Figure 3C).

vasculature development, whole-mount PECAM1 immunostaining was performed on control and *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ mutant E15.5 hearts. PECAM1 staining of mutant

Additionally, we performed an ex vivo collagen gel invasion assay using epicardial explants from control and *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ E12.5 embryos. Tamoxifen was administered

for 72 hr to induce Cre-recombinase activity. Epicardium-derived cells were visualized by phalloidin staining. In contrast to control, fewer epicardial cells migrated into the collagen gel from *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ explants (Figure S2B). To determine whether Hippo signaling components are required for coronary endothelial cell formation, we performed β-galactosidase/PECAM1 double staining on cryosections from *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/+}$:*Taz*$^{flox/+}$;*R26*$^{LacZ/+}$ and *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$;*R26*$^{LacZ/+}$ hearts and quantified the number of epicardial-derived endothelial cells (PECAM1^{+}; β-gal^{+}) as a percentage of the total number of β-gal^{+} cells in the myocardium. Deletion of Yap and Taz resulted in a significant reduction of epicardial-derived endothelial cells, suggesting a defect or delay in fate determination (Figure 3D). Epicardial contribution to smooth muscle cells and fibroblasts was also significantly reduced (Figures 3E and 3F). To better understand the underlying molecular changes, we measured the expression of regulatory genes involved in epicardial development and EMT and detected significant downregulation of *Tbx18*, *Wt1*, *Twist1*, *Snail1*, *Slug*, *Zeb1*, and *N-cadherin* in mutants compared to controls. *E-cadherin* levels were elevated in mutants compared to controls. There was no significant difference in the expression of *α4-integrin*, *β-catenin*, or *Cnn1* (Figures 3G and 3H).

To further validate our results regarding defective epicardial cell migration in the *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ animals, a collagen gel invasion assay was performed using epicardial explants from wild-type E12.5 embryos in the presence or absence of the chemical inhibitor verteporfin or protoporphyrin, drugs known to disrupt the physical interaction between Yap and Tead factors (Liu-Chittenden et al., 2012). In control explants (treated with vehicle alone, i.e., DMSO), epicardial cells migrated away from the explant. Fewer migrating epicardial cells were evident in explants treated with either of the Hippo inhibitors (Figures S3A and S3B). Interestingly, verteporfin displayed stronger inhibitory effects in our explant assays compared to protoporphyrin IX; therefore, we used verteporfin in subsequent experiments. The impaired migration of the verteporfin or protoporphyrin-IX-treated epicardial cells was associated with decreased expression of genes regulating epicardial EMT (Figures S3C and S3D).

Hippo Signaling Components Regulate *Tbx18* and *Wt1* Promoters

We next assessed whether Yap and Taz can regulate epicardial cell proliferation, EMT, and fate specification, at least in part, by modulating *Tbx18* and *Wt1* promoter activity. Analysis of the 2 kb of genomic sequence upstream of the transcriptional start sites of *Tbx18* and *Wt1* revealed two consensus Tead binding sequences (TBSs) in each promoter (Figures 4A and 4B). Promoters were PCR-amplified, cloned into a luciferase reporter plasmid, and tested in luciferase reporter assays. Yap expression strongly activates both *Tbx18* and *Wt1* promoter-luciferase activity. In contrast, Taz activates the *Tbx18* promoter but fails to activate the *Wt1* promoter (Figure 4C). Mutation of the TBS sites within the *Tbx18* and *Wt1* promoters significantly reduced the ability of Yap to activate these constructs (Figure 4D). Chromatin immunoprecipitation (ChIP) assays demonstrated direct binding of Yap/Taz to the *Tbx18* and *Wt1* promoters (Figure 4E).

Further evidence to indicate that Tead binding is necessary for Yap activation of these promoters derives from experiments in which verteporfin (5 μM) was added 24 hr after transfection. Activation of *Tbx18* and *Wt1* promoters by Yap was completely abolished by verteporfin (Figure 4F). We further examined whether the upstream Hippo kinases Mst1 and Lats2 could modulate reporter activity in response to Yap. Co-expression of Yap with Mst1 or Lats2, but not the kinase-inactive mutants (Mst1-K59R or LATS2-D809A), abrogated Yap-induced activation of the *Tbx18* and *Wt1* luciferase reporters (Figures 4G and 4H). Mst2 and its kinase-inactive mutant (Mst2-K56R) failed to modulate either the *Tbx18* or the *Wt1* promoter (Figure S4). These results suggest that Yap regulates coronary vascular formation in part by directly regulating *Tbx18* and *Wt1* expression in association with Tead factors.

DISCUSSION

Recent studies have implicated Hippo signaling in cardiac development and regeneration. However, a role for Hippo signaling in coronary vasculature formation has not been explored. In the present study, we have demonstrated that Hippo signaling components are not only strongly expressed in the epicardium but also required for coronary vasculature development and remodeling and for expression of some epicardial-derived growth factors, including Fgf9, Raldh2, and Wnt5a. Hippo signaling mediators *Yap* and *Taz* regulate epicardial EMT and epicardial cell proliferation and differentiation into coronary endothelial cells. We provide evidence that Yap and Taz control epicardial cell behavior, in part by regulating *Tbx18* and *Wt1* expression. Both *Tbx18* and *Wt1* promoters are more strongly activated by Yap than by Taz, and our genetic data support a dominant role for Yap compared to Taz during coronary vasculature formation.

Proepicardial cells migrate and adhere to the embryonic myocardial surface and spread over the myocardium enveloping the entire heart. The molecular mechanisms that regulate adhesion and migration of the proepicardial cells as they migrate over the myocardium appear to be intact in *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ hearts. The formation of epicardium is similarly initially normal in other mouse models engineered with other mutated epicardial genes (Greulich et al., 2012; von Gise et al., 2011; Wu et al., 2013). The formation of the epicardium requires extensive proliferation and migration of proepicardial cells. Consistent with the role of Yap and Taz as regulators of proliferation in other systems, a significant decrease in the proliferation rate of *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ epicardial cells was observed when compared to controls. Proliferation of epicardial cells is required for EMT as epicardial cells undergoing cell-cycle arrest fail to invade the myocardium (Wu et al., 2010). Reduced proliferation of epicardial cells seen in *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ mice may therefore contribute to deficient EMT and subsequent migration of epicardial derivatives into underlying myocardium. Paracrine signals from the epicardium are required for myocardial expansion. The observed decrease in *Fgf9*, *Raldh2*, and *Wnt5a* expression suggests that signaling between the epicardium and myocardium is affected in *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ embryos, which likely contributes to the myocardial defects.

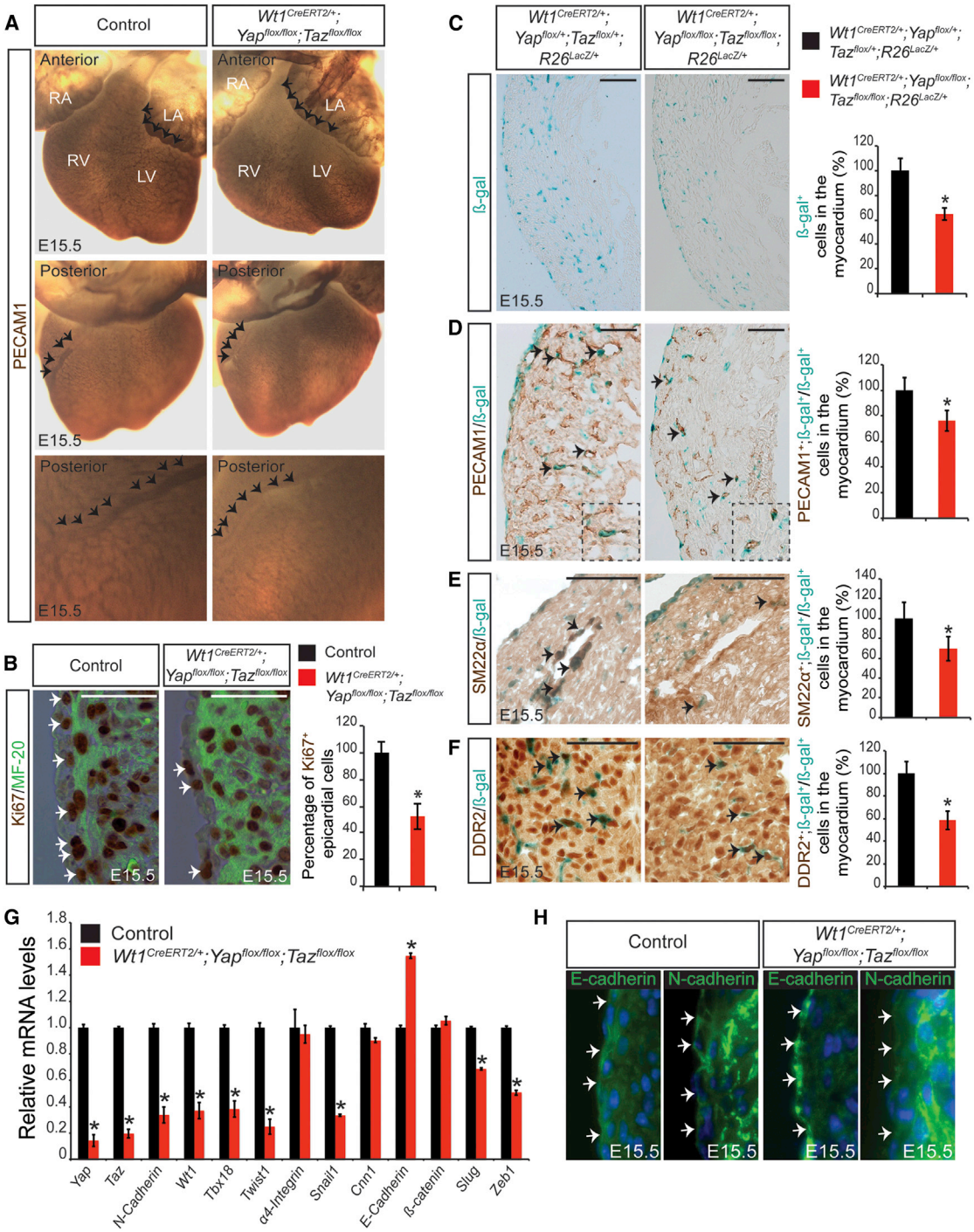

Figure 3. Genetic Targeting of Hippo Signaling Leads to Coronary Vasculature Defects due to Impaired Epicardial Cell Proliferation, EMT, and Fate Determination

Epicardial-specific deletion of Hippo signaling components Yap and Taz was achieved using an inducible Cre line ($Wt1^{CreERT2/+}$). Cre activity was induced at E11.5 and hearts were harvested at E15.5.

(A) Whole-mount PECAM1 staining was performed on E15.5 hearts from control and $Wt1^{CreERT2/+}$:$Yap^{flox/flox}$:$Taz^{flox/flox}$ embryos to analyze coronary vasculature development. Arrows highlight the area where differences are most obvious.

(B) Immunostaining for Ki67 and MF-20 were performed on heart sections from control and $Wt1^{CreERT2/+}$:$Yap^{flox/flox}$:$Taz^{flox/flox}$ embryos. Quantification was performed on six to eight sections each from three individual hearts and averaged.

(C) Cryosections from $Wt1^{CreERT2/+}$:$Yap^{flox/+}$:$Taz^{flox/+}$;$R26^{LacZ/+}$ and $Wt1^{CreERT2/+}$:$Yap^{flox/flox}$:$Taz^{flox/flox}$;$R26^{LacZ/+}$ were stained for β-galactosidase (β-gal) activity, and the number of β-gal$^+$ cells in the myocardium of mutant hearts was quantified and expressed as a percent of the number of cells quantified in controls.

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Hippo signaling has been recently implicated in cell fate determination in multiple organs (Imajo et al., 2015; Yimlamai et al., 2014). However, the role of Hippo signaling components in epicardial cell fate determination has not been explored. Recently, we demonstrated that *Sema3d/Scx*-positive proepicardial cells are competent to differentiate into coronary endothelial cells in vivo and in vitro (Katz et al., 2012). We were unable to definitively determine if cell fate determination is affected in *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ embryos due to early embryonic lethality. We observed decreased endothelial, smooth muscle, and fibroblast cells as a percentage of total epicardial derivatives in mutant hearts, but embryonic lethality precluded us from determining if these deficiencies resulted from delayed differentiation or a failure of fate specification.

Yap and Taz may regulate epicardial cell proliferation, EMT, and fate determination via multiple mechanisms. In this study, we provide evidence that Yap/Taz function in part by directly modulating *Tbx18* and *Wt1* expression. Members of the T-box family of transcription factors regulate a variety of developmental processes, including coronary vasculature development. *Tbx18*-deficient hearts show abnormal coronary vascular plexus formation due to impaired epicardial signaling, cell proliferation, and cell fate determination (Greulich et al., 2012; Wu et al., 2013). *Tbx5*, a gene mutated in Holt-Oram syndrome, is expressed in the PEO and epicardium. *Tbx5* mutant hearts show abnormal coronary vasculature due to impaired production of epicardial-derived cells and their migration into the underlying myocardium (Diman et al., 2014; Hatcher et al., 2004). Interestingly, Yap and Taz interact with Tbx5 and regulate Tbx5-dependent gene programs (Murakami et al., 2005). Wt1 regulates epicardial EMT and myocardial growth by controlling *Snail* and *E-cadherin* expression (Martínez-Estrada et al., 2010), promoting Wnt/β-catenin signaling and expression of *Wnt5a* and *Raldh2* (Guadix et al., 2011; von Gise et al., 2011). Yap can also regulate endocardial cell proliferation and EMT through modulation of TGF-β/Smad signaling (Zhang et al., 2014). Wt1 is expressed by tumor vessels, regulates *PECAM1* expression and conditional deletion of *Wt1* in endothelial cells results regression of tumor vascularization (Wagner et al., 2014). Recent studies have implicated Wt1 in cell fate determination in other tissues (Wen et al., 2014; Zhang et al., 2015). Since Yap and Wt1 are co-expressed in many tissues during development and disease, our findings may have broad relevance (Hiemer et al., 2014; Loeb et al., 2001).

In summary, we have provided evidence for the role of Hippo signaling in epicardial biology and coronary vascular development. Future work will focus on the role of Hippo signaling in the adult epicardium and its role in myocardial regeneration after injury.

EXPERIMENTAL PROCEDURES

Generation of *Yap/Taz* Mutant Mice

Epicardium-specific *Yap/Taz* mutant mice were generated by crossing the *Sema3d*$^{GFPCre/+}$ or *Wt1*$^{CreERT2/+}$ transgenic line with *Yap*$^{flox/flox}$,*Taz*$^{flox/flox}$ mice (Katz et al., 2012; Xin et al., 2011, 2013; Zhou et al., 2008). Resulting *Sema3d*$^{GFPCre/+}$;*Yap*$^{flox/+}$,*Taz*$^{flox/+}$ or *Wt1*$^{CreERT2/+}$;*Yap*$^{flox/+}$,*Taz*$^{flox/+}$ offspring were then backcrossed to *Yap*$^{flox/flox}$,*Taz*$^{flox/flox}$ mice to obtain either *Wt1*$^{CreERT2/+}$;*Yap*$^{flox/flox}$,*Taz*$^{flox/flox}$ or *Sema3d*$^{GFPCre/+}$;*Yap*$^{flox/flox}$,*Taz*$^{flox/flox}$ mice. Cre activity in *Wt1*$^{CreERT2/+}$ mice was induced at E11.5 by oral administration of 3.5 mg tamoxifen (Sigma, T5648). Tamoxifen was dissolved in ethanol and then emulsified with corn oil to a final concentration of 12.5 mg/ml before administration. Yap and Taz floxed animals were genotyped as described previously (Xin et al., 2011, 2013). The *Yap*$^{flox/flox}$ and *Taz*$^{flox/flox}$ alleles were generated in the laboratory of Professor Eric N. Olson at the University of Texas Southwestern Medical Center (Dallas, TX). All mice were maintained on a mixed genetic background. All animal protocols were approved either by the University of Pennsylvania institutional animal care and use committee or the SingHealth institutional animal care and use committee.

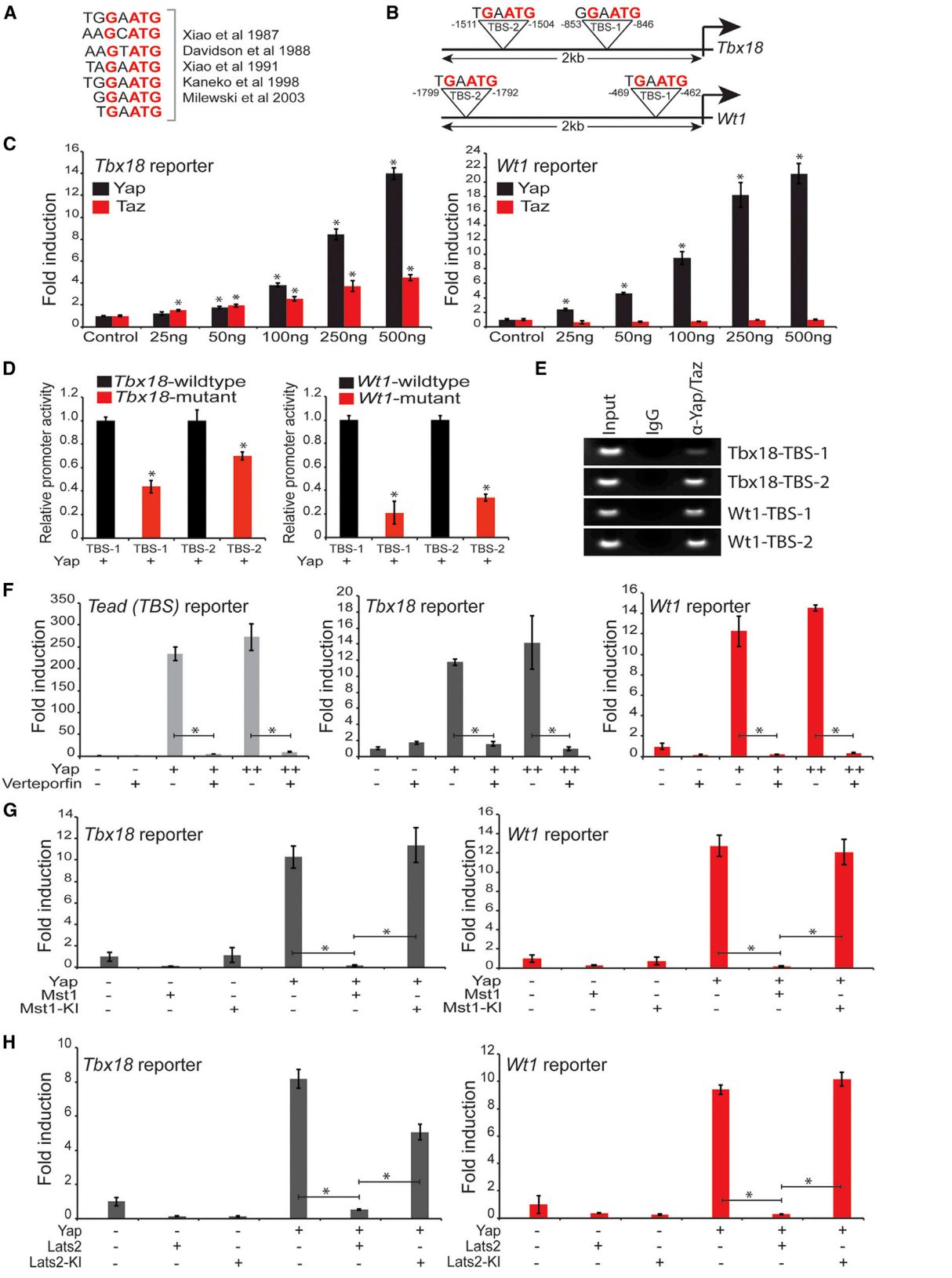
Histology and Immunohistochemistry

Histology and immunohistochemistry were performed as described previously (Singh et al., 2011). Briefly, embryos were dissected in PBS and fixed in 4% paraformaldehyde overnight at 4°C. Embryos were washed with PBS, dehydrated in an ethanol series, and stored in 100% ethanol at −20°C. Immunohistochemical detection was performed on paraffin sections of paraformaldehyde (PFA)-fixed hearts. Primary antibodies used for immunohistochemistry were anti-Yap rabbit polyclonal (Cell Signaling Technology, catalog no. 4912S), anti-Taz (V386) rabbit polyclonal (Cell Signaling Technology, catalog no. 4883), anti-GFP antibody goat polyclonal (Abcam, catalog no. ab6673), anti-Tbx18 goat polyclonal (Santa Cruz Biotechnology, catalog no. sc-17869), and anti-Wt1 rabbit polyclonal (Santa Cruz Biotechnology, catalog no. sc-192). Whole-mount immunostaining for PECAM1 was carried out as described previously (Singh et al., 2011). Briefly, endogenous peroxidase activity was blocked with 5% H_2O_2/methanol for 60 min at room temperature. The rat anti-mouse PECAM1 primary antibody (BD PharMingen, catalog no. 553370) was applied overnight at 4°C at a dilution of 1:200. The secondary antibody goat anti-rat IgG-HRP (Abcam, catalog no. ab6120) was applied overnight at 4°C at a dilution of 1:500 color development was performed using a DAB kit (Vector Laboratories, SK-4100).

Plasmids

Mouse *Tbx18* and *Wt1* promoters (~2 kb) were amplified and cloned into pGL4.27 vector (Promega) for use in luciferase assays. For mutation of TBSs in *Tbx18* and *Wt1* promoters, an ~300-bp DNA fragment (150 bp on each side of the TBS) was amplified and cloned into pGL4.27 vector and used as control. Mini genes for same fragment differing only by the presence of a mutated TBS were purchased from Integrated DNA Technologies (IDT) and subsequently cloned into pGL4.27 vector. In *Tbx18* promoter, mutant TBS-1 and TBS-2 represent the sequence 5′-CGATAC-3′ and 5′-TCATAC-3′, respectively. In *Wt1* promoter, mutant TBS-1 and TBS-2 represent the sequence 5′-TCATAC-3′. Mouse Yap and Taz expression vectors were previously described (Murakami et al., 2005) and provided by Professor Eric Olson's lab. Murine hippo kinases Mst1 and Mst1-KI were previously described (Lin et al., 2002) and obtained from Addgene (Addgene plasmids 1965 and 1966). Expression plasmids for human hippo kinases LATS2 and LATS2-KI were provided by Dr. D. Pan (Dong et al., 2007).

(D) Immunohistochemistry of X-galactosidase (X-gal)-stained cryosections with PECAM1 to identify epicardial-derived endothelial cells and quantification as a percent of total X-gal stained cells.
(E) Immunohistochemistry of X-gal-stained cryosections with SM22α to visualize epicardial-derived smooth muscle cells and quantification.
(F) Immunohistochemistry of X-gal-stained cryosections with DDR2 to visualize epicardial-derived fibroblast cells and quantification.
(G) Real-time qPCR for *Yap*, *Taz*, *N-cadherin*, *Wt1*, *Tbx18*, *Twist1*, *α4-integrin*, *Snail1*, *Cnn1*, *E-cadherin*, *β-catenin*, *Slug*, and Zeb1 on RNA isolated from control and *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ explants treated with 4-hydroxytamoxifen.
(H) Immunostaining for E-cadherin and N-cadherin on heart sections from control and *Wt1*$^{CreERT2/+}$:*Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ embryos. Significant differences were defined by *p < 0.05. Scale bars, 100 μm.



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Luciferase Assay

For luciferase reporter assay, HEK293T cells were seeded in 12-well plates the day before transfection. The *Tbx18* or *Wt1* luciferase reporter plasmid along with other indicated plasmids was co-transfected into HEK293T cells using FuGENE6 reagent (Promega, catalog no. E2691). For transfection control 50 ng of lacZ expression plasmid was utilized in all the wells. All transfections maintained an equal concentration of total DNA with the inclusion of the pcDNA3.1 empty vector (Invitrogen). Cells were subjected to lysis 60 hr posttransfection using reporter lysis buffer (Promega, catalog No. E3971). Luciferase activities were assayed in the cell lysates (20 μl) using the Luciferase Reporter Assay System kit (Promega, catalog no. E1500). Lysates were also assayed for β-galactosidase activity using the β-Galactosidase Enzyme Assay System (Promega, Cat. no. E2000). The measured luciferase reporter activity was normalized to β-galactosidase activity. The luciferase assay results were reproduced in at least three independent experiments. All experiments were performed in duplicate, and the representative data are shown in the figures. During all the experiments, HEK293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. In case of verteporfin treatment, inhibitor was added into the medium 24 hr posttransfection.

Statistical Analysis

Statistical analyses were performed using the two-tailed Student's t test. Data are expressed as mean ± SD. Differences were considered significant when the p value was < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.027.

AUTHOR CONTRIBUTIONS

A.S., S.R., D.M.C., L.S.Y., J.L., L.L., L.J.M., and M.K.S. designed and performed experiments and analyzed data. E.N.O. provided the Yap and Taz floxed alleles. M.K.S. and J.A.E. oversaw the entire project, designed experiments, analyzed data, and wrote the paper.

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Figure 4. Yap and Taz and Their Upstream Kinases Mst1 and Lats2 Regulate *Tbx18* and *Wt1* Expression

(A) Reported Tead binding sequences (TBSs).
(B) Predicted TBSs in *Tbx18* and *Wt1* promoters.
(C) Results of normalized luciferase reporter assays in HEK293T cells with *Tbx18* or *Wt1*-luciferase reporters in the presence of Yap or Taz.
(D) Promoter fragments containing wild-type TBSs driving luciferase were compared to mutant fragments with mutated TBSs for each site (TBS-1 and TBS-2). Luciferase reporter activity obtained after addition of Yap was normalized to the activity observed with reporter alone. The normalized reporter activity for wild-type constructs was set at 1.
(E) ChIP assay using chromatin from E12.5 hearts and Yap/Taz antibody. Predicted binding sites in *Tbx18* and *Wt1* promoters were tested.
(F) *Tead* (*TBS*), *Tbx18*, or *Wt1*-luciferase reporters were transfected in HEK293T cells with or without Yap in presence or absence of verteporfin (5 μM).
(G) *Tbx18* or *Wt1*-luciferase reporters were transfected in HEK293T cells with or without Yap, Mst1, or kinase inactive form of Mst1 (Mst1-KI).
(H) *Tbx18* or *Wt1*-luciferase reporters were transfected in HEK293T cells with or without Yap, Lats2, or kinase inactive form of Lats2 (Lats2-KI). Significant differences are indicated by $^*p < 0.05$.

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