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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.

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Author contributions

A.S. and S.R. equally contributed to this work as first authors. A.S., S.R., D.M.C., L.S.Y., J.L., L.L., L.J.M., M.K.S. designed and performed experiments, 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.

Disclosures

None

Keywords

Epicardium; Proepicardium; Hippo signaling; Yap; Taz; Epithelial to mesenchymal transition (EMT)

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 proepicardium/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 (Manner, 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 (Manner, 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 not only required for coronary vasculature development but also for the proliferation and differentiation of the underlying myocardial cells (Manner, 1993; Manner 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 reactivated 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 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 EMT of the atrioventricular cushion by modulating TGFβ-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 E11.5–12.5 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 controls 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 (Figure 1A–C). Yap expression is maintained in migrating proepicardial and epicardial cells from E9.5 to E12.5 (Figure 1D–I). To demonstrate that Yap is expressed specifically in epicardial cells, Yap colocalization with Wt1 was performed (Figure 1J–L). Yap colocalizes with Wt1 in the developing epicardium. Similar to Yap, Taz expression is prominent in the epicardium from E10.5 to E12.5 (Figure 1M–R). 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 (Figure 1S–U). To determine whether other Hippo signaling components are expressed during epicardium development, we performed quantitative RT-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 (Figure 1V–X) (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 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*$^{GFPCre/+}$ knock-in 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 (Figure 2B,C), 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 (Figure 2D–L). 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 (Figure 2D,E). However, the myocardium of mutant embryos at E10.5 did not display any obvious morphological defects (Figure 2F,G). 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 (Figure 2H,I). However, Ki67 staining showed a significant reduction in epicardial cell proliferation in mutant hearts compared to controls (Figure 2J–L). 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).

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*$^{flox/flox}$ post-natal pups, indicating embryonic lethality (Figure S2A). To determine if loss of *Yap* and *Taz* in the epicardium has effects on coronary vasculature development, whole-mount PECAM-1 immunostaining was performed on

control and *Wt1*$^{CreERT2/+}$*:Yap*$^{flox/flox}$*:Taz*$^{flox/flox}$ mutant E15.5 hearts. PECAM-1 staining of mutant 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).

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 hrs 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 (Figure 3E, F). To better understand the underlying molecular changes, we measured the expression of regulatory genes involved in epicardial development and EMT and detected significant down-regulation 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* (Figure 3G, H).

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 (vehicle alone, i.e. DMSO) treated explants, epicardial cells migrated away from the explant. Many fewer migrating epicardial cells were evident in explants treated with either of the Hippo inhibitors (Figure S3A, B). 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 (Figure S3C, D).

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 (TBS) in each promoter (Figure 4A,B). 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 (5μM) was added 24 hours after transfection. Activation of *Tbx18* and *Wt1* promoters by Yap was completely abolished by verteporfin (Figure 4F). We further examined whether 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 (Figure 4G,H). 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 not only are Hippo signaling components strongly expressed in the epicardium but they are 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.

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 epicardail 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 (Martinez-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 TGFβ-Smad signaling (Zhang et al., 2014). Wt1 is expressed by tumor vessels, regulates *PECAM-1* 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., 2013; Xin et al., 2011; 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 of 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., 2013; Xin et al., 2011). The $Yap^{flox/flox}$ and $Taz^{flox/flox}$ alleles were generated in the laboratory of Prof. Eric N. Olson at the University of Texas Southwestern Medical Center (Dallas, TX). All mice were maintained on a mixed genetic background.

Histology and immunohistochemistry

Histology and immunohistochemistry were performed as described previously (Singh et al., 2011). Briefly, embryos were dissected in phosphate-buffered saline (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 PFA-fixed hearts. Primary antibodies used for immunohistochemistry were, anti-Yap rabbit polyclonal (Cell Signaling, Cat. no. 4912S), anti-Taz (V386) rabbit polyclonal (Cell Signaling, Cat. no. 4883), anti-GFP antibody goat polyclonal (Abcam, Cat. no. ab6673), anti-Tbx18 goat polyclonal (Santa Cruz, Cat. no. sc-17869) and anti-Wt1 rabbit polyclonal (Santa Cruz, Cat. no. sc-192). Whole-mount immunostaining for PECAM-1 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 PECAM-1 primary antibody (BD Pharmingen, Cat. no. 553370) was applied overnight at 4°C at a dilution of 1:200. The secondary antibody goat anti-rat IgG-HRP (Abcam, Cat. no. ab6120) was applied overnight at 4°C at a dilution of 1:500 color development was performed using a DAB kit (Vector lab, SK-4100).

Plasmids

Mouse *Tbx18* and *Wt1* promoters (approximately 2kb) were amplified and cloned into pGL4.27 vector (Promega) for use in luciferase assays. For mutation of Tead binding sequences (TBS) in *Tbx18* and *Wt1* promoters, ~300bp DNA fragment (150bp on each side of 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 CGATAC and TCATAC respectively. In *Wt1* promoter, mutant TBS-1 and TBS-2 represent the sequence TCATAC. Mouse Yap and Taz expression vectors were previously described (Murakami et al., 2005) and provided by Prof. 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).

Luciferase Assay

For luciferase reporter assay, HEK293T cells were seeded in 12-well plates the day before transfection. *Tbx18* or *Wt1* luciferase reporter plasmid, with other indicated plasmids were co-transfected into HEK293T cells using FuGENE6 reagent (Promega, Cat. 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 hours posttransfection using reporter lysis buffer (Promega, Cat. No. E3971). Luciferase activities were assayed in the cell lysates (20μl) using Luciferase Reporter Assay System kit (Promega, Cat. no. E1500). Lysates were also assayed for β-galactosidase activity using 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 the experiments were performed in duplicate, and the representative data are shown in the figures. During all the experiments, HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. In case of VP treatment, inhibitor was added into the medium 24 hours posttransfection.

Statistical Analysis

Statistical analyses were performed using the 2-tailed Student's *t* test. Data were expressed as mean ± SD. Differences were considered significant when the *P* value was <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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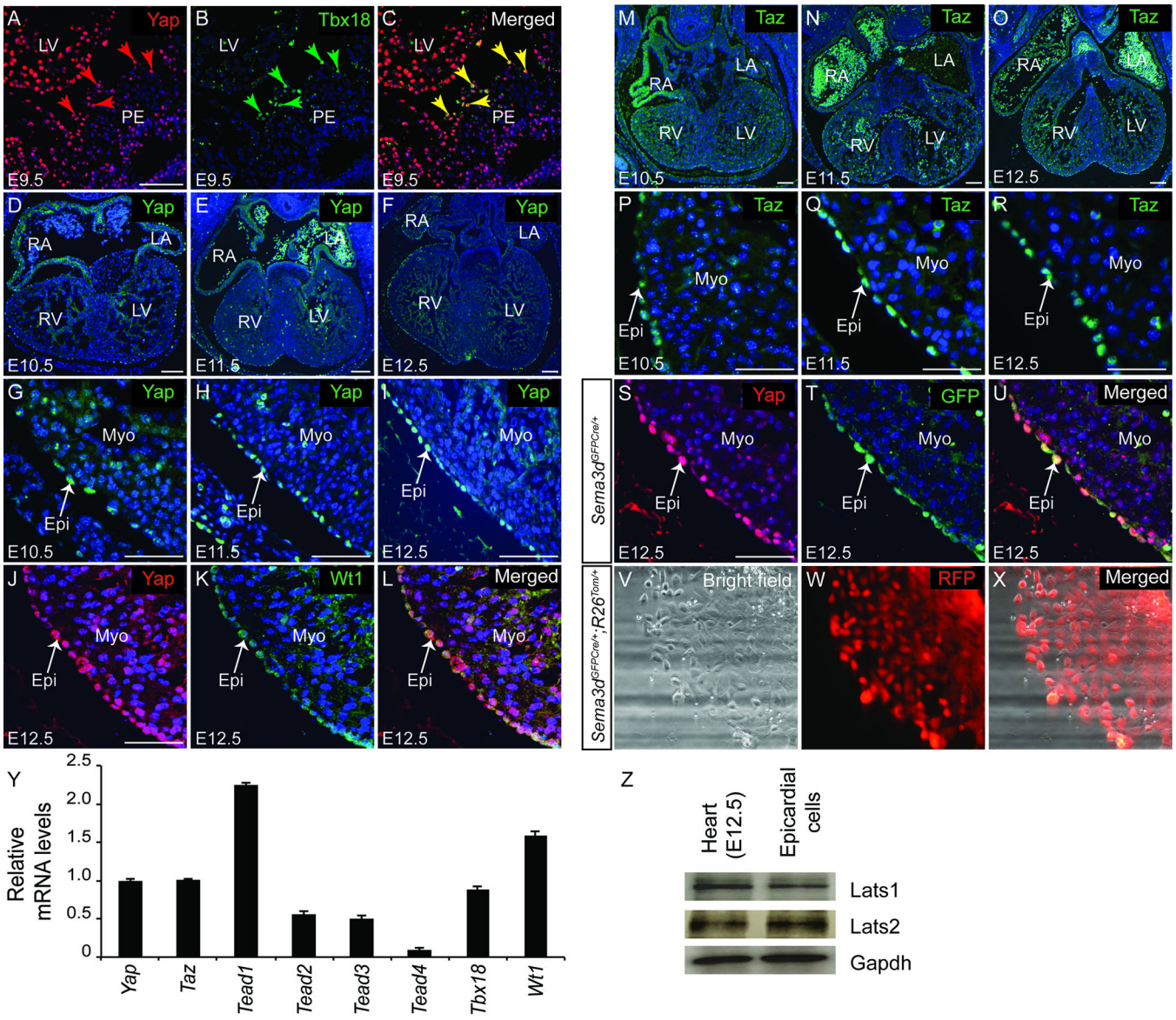

Figure 1. Hippo signaling mediators are expressed in proepicardial and migrating epicardial cells during embryonic development

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–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 *Sema3dGFPCre/+* 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**, Quantitative real-time

PCR for 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.

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*

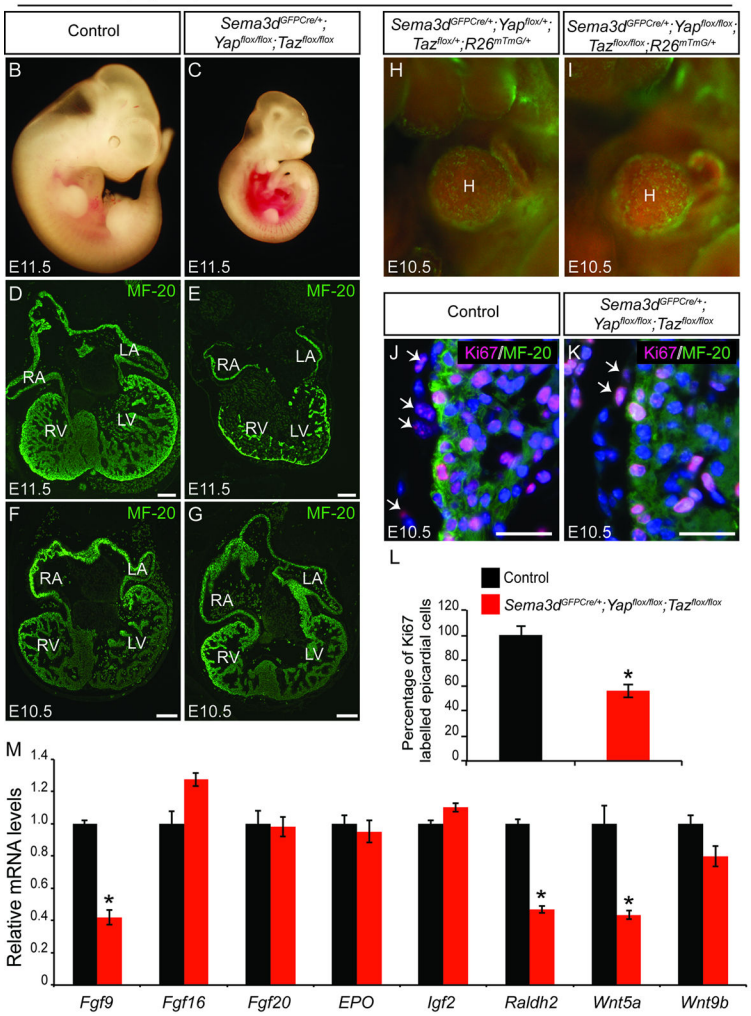

Figure 2. *Sema3d*$^{GFPCre/+}$ mediated deletion of *Yap* and *Taz* leads to embryonic lethality

A, Genotyping result of embryos and pups from *Sema3d*$^{GFPCre/+}$:*Yap*$^{flox/+}$:*Taz*$^{flox/+}$ and *Yap*$^{flox/flox}$:*Taz*$^{flox/flox}$ cross. **B–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 4–6 sections each from four individual hearts and averaged **(L)**. **M**, Quantitative real-time PCR 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.

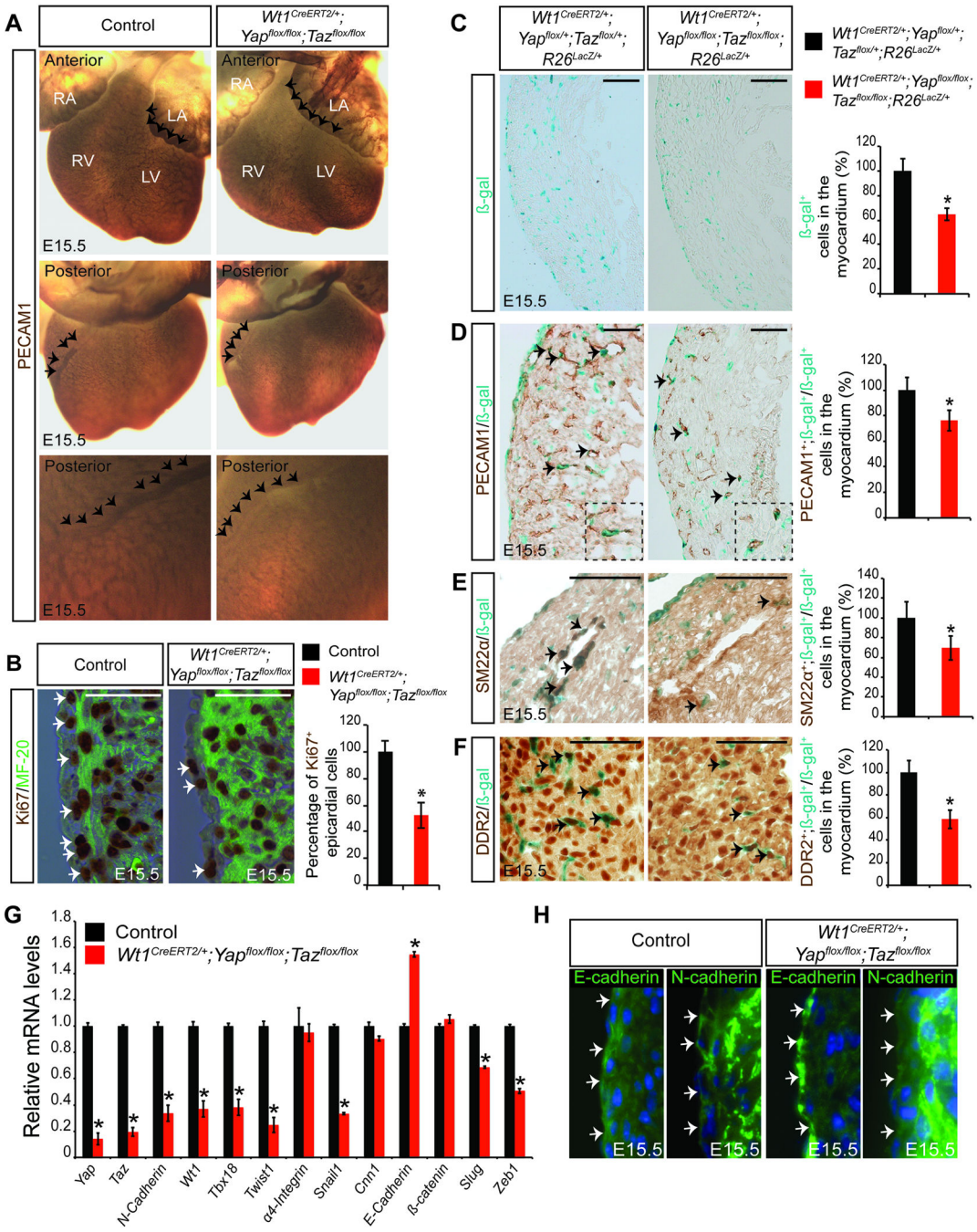

Figure 3. Genetic targeting of Hippo signaling leads to coronary vasculature defects due to impaired epicardial cell proliferation, EMT and fate determination

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 PECAM-1 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 6–8 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 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. **D**, Immunohistochemistry of 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**, Quantitative real-time PCR 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.

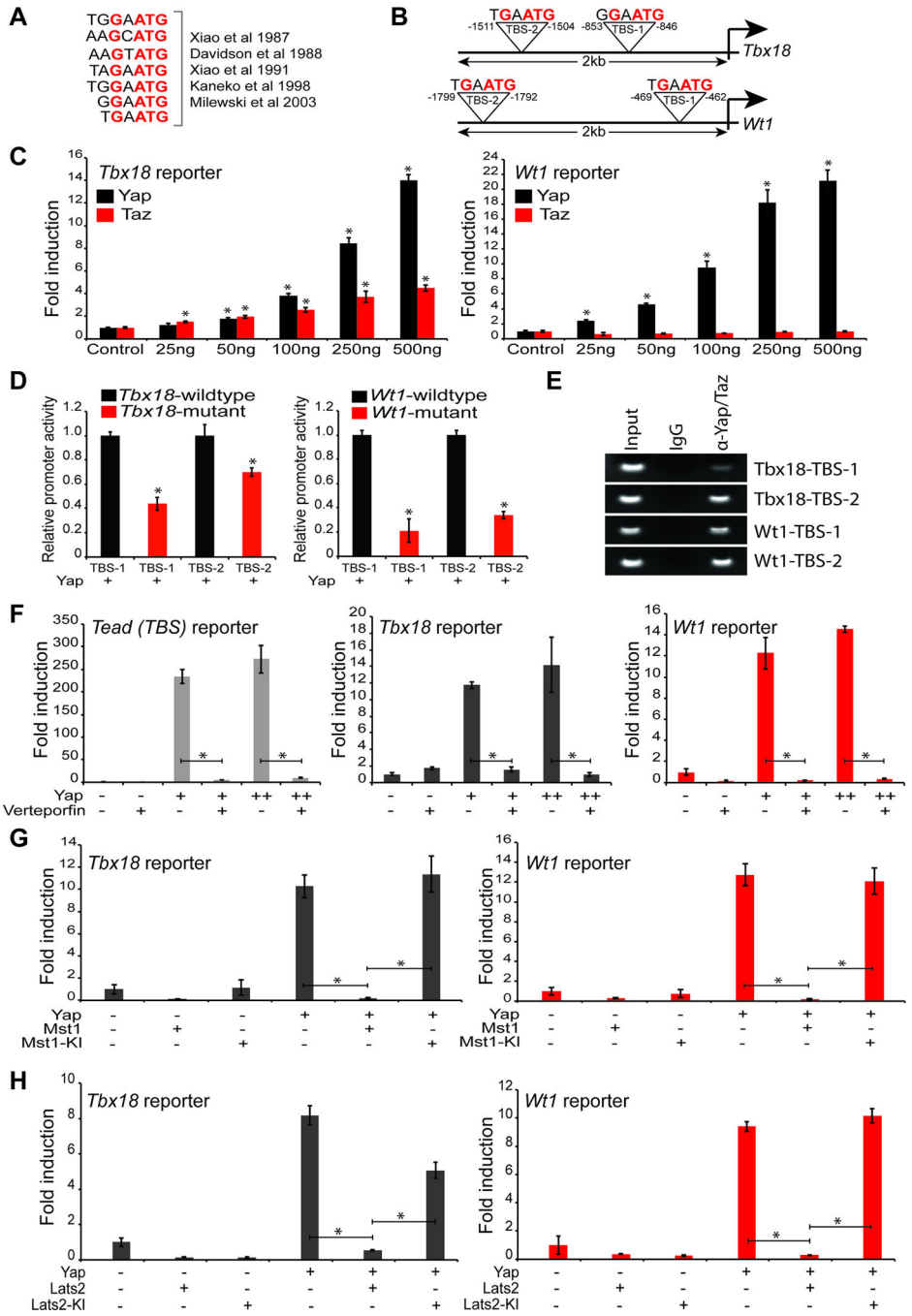

Figure 4. Yap and Taz and their upstream kinases Mst1 and Lats2 regulate *Tbx18* and *Wt1* expression

A, Reported Tead binding sequences. **B**, Predicted Tead binding sequences (TBS) 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 wildtype Tead binding sequences driving luciferase were compared to mutant fragments with mutated Tead binding sequences 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 wildtype 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 (5μM). **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 *, $p < 0.05$.