

RESEARCH ARTICLE

Yap and Taz play a crucial role in neural crest-derived craniofacial development

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ABSTRACT

The role of the Hippo signaling pathway in cranial neural crest (CNC) development is poorly understood. We used the *Wnt1^{Cre}* and *Wnt1^{Cre2SOR}* drivers to conditionally ablate both *Yap* and *Taz* in the CNC of mice. When using either Cre driver, *Yap* and *Taz* deficiency in the CNC resulted in enlarged, hemorrhaging branchial arch blood vessels and hydrocephalus. However, *Wnt1^{Cre2SOR}* mutants had an open cranial neural tube phenotype that was not evident in *Wnt1^{Cre}* mutants. In O9-1 CNC cells, the loss of *Yap* impaired smooth muscle cell differentiation. RNA-sequencing data indicated that *Yap* and *Taz* regulate genes encoding Fox transcription factors, specifically *Foxc1*. Proliferation was reduced in the branchial arch mesenchyme of *Yap* and *Taz* CNC conditional knockout (CKO) embryos. Moreover, *Yap* and *Taz* CKO embryos had cerebellar aplasia similar to Dandy-Walker spectrum malformations observed in human patients and mouse embryos with mutations in *Foxc1*. In embryos and O9-1 cells deficient for *Yap* and *Taz*, *Foxc1* expression was significantly reduced. Analysis of *Foxc1* regulatory regions revealed a conserved recognition element for the *Yap* and *Taz* DNA binding co-factor Tead. ChIP-PCR experiments supported the conclusion that *Foxc1* is directly regulated by the *Yap*-Tead complex. Our findings uncover important roles for *Yap* and *Taz* in CNC diversification and development.

KEY WORDS: *Yap* and *Taz*, Cranial neural crest, Craniofacial development

INTRODUCTION

The neural crest (NC) is a migratory, multipotent cell population that originates in the embryonic dorsal neural tube. Developmental defects in NC formation result in numerous human congenital anomalies. Based on the site of origin, NC cells are divided into cranial, cardiac and trunk populations, each of which has its own unique developmental potential. The cranial neural crest (CNC) ultimately diversifies into multiple cell types, including neuronal, glial, cartilage, bone and smooth muscle cells (Santagati and Rijli, 2003). Mutations in the genes required for CNC development are

often associated with the pathophysiology of human congenital malformations (Acloque et al., 2009; Cordero et al., 2011), highlighting the clinical importance of understanding the molecular mechanisms governing CNC development. Much effort has been expended interrogating the gene regulatory networks underlying NC development (Sauka-Spengler and Bronner-Fraser, 2008). Major signaling pathways, including Wnt, Fgf, Bmp and Notch signaling, have been shown to play important roles in regulating NC induction, proliferation and migration (Sela-Donenfeld and Kalcheim, 1999; Garcia-Castro et al., 2002; Coles et al., 2004; Glavic et al., 2004; Carmona-Fontaine et al., 2008). However, the function of Hippo signaling in CNC development remains poorly understood.

Hippo signaling is an essential pathway that regulates organ size through the modulation of cell proliferation (Heallen et al., 2011). The key components of the Hippo signaling pathway are evolutionarily conserved. In mice, Mst1/2 (orthologous to *Drosophila melanogaster* Hpo) and Salv (orthologous to human WW45) form a complex that phosphorylates the kinases Lats1 and Lats2 (orthologous to *Drosophila* Warts). Lats1/2, in turn, phosphorylate the most downstream Hippo signaling components *Yap* (also known as Yap1 – Mouse Genome Informatics) and *Taz*, thus promoting their binding to 14-3-3 proteins and inhibiting them from shuttling into the nucleus. In the absence of the repressive activity from Hippo signaling, *Yap* and *Taz* localize to the nucleus and partner with transcription factors, such as transcriptional enhancer activator (TEA) domain (TEAD) family members, to promote gene programs favoring proliferation. Recently, familial studies have shown that heterozygous nonsense mutations in *YAPI* are associated with variable phenotypes in the affected families, including orofacial clefting and intellectual disability (Williamson et al., 2014). However, the mechanisms underlying these phenotypic alterations remain unclear. In this study, we investigated the function of *Yap* and *Taz* in the CNC. Using two *Wnt1* Cre drivers, we uncover important functions of *Yap* and *Taz* in CNC proliferation and subsequent differentiation.

RESULTS

Yap and *Taz* deletion in CNC-derived cells results in embryonic lethality

To determine the function of *Yap* and *Taz* in the CNC, we generated compound *Yap* and *Taz* conditional mutants by using conditional null alleles and the *Wnt1^{Cre}* and *Wnt1^{Cre2SOR}* drivers. We collected embryos at multiple developmental stages to analyze the morphogenesis of several CNC-derived structures. Importantly, the *Wnt1^{Cre}* driver (Chai et al., 2000) has been shown to induce ectopic expression of *Wnt1*, leading to defects in midbrain development, whereas the *Wnt1^{Cre2SOR}* driver does not have these issues (Lewis et al., 2013).

We first evaluated embryos in which *Yap* and *Taz* were deleted using the *Wnt1^{Cre}* driver. In control embryos, phosphorylated *Yap*

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(pYAP) – a readout for Hippo signaling activity – was present in CNC-derived cells, such as mandibular mesenchymal cells (Fig. 1A, B). However, in *Wnt1^{Cre}; Yap; Taz* double conditional knockout (dCKO) embryos, the level of pYAP was dramatically reduced in CNC-derived cells, but unchanged in non-CNC-derived cells such as endothelium (Fig. 1C,D). These data indicated that the *Wnt1^{Cre}* driver efficiently inactivated *Yap* and *Taz* in CNC-derived cells. Among the different mutant genotypes, both *Wnt1^{Cre}; Yap^{fl/fl}; Taz^{fl/fl}* and *Wnt1^{Cre}; Yap; Taz* dCKO were embryonic lethal at embryonic day (E) 10.5 (Table S1). Mutant embryos with the reciprocal genotype of *Wnt1^{Cre}; Yap^{fl/fl}; Taz^{fl/fl}* displayed lethality over a wide range of developmental time points, from E14.5 to postnatal week 8 (Table S1).

In addition, we used the *Wnt1^{Cre2SOR}* driver (Lewis et al., 2013) to inactivate *Yap* and *Taz* in CNC. Similar to *Wnt1^{Cre}* embryos, lethality was observed at E10.5 in both *Wnt1^{Cre2SOR}; Yap^{fl/fl}; Taz^{fl/fl}* and *Wnt1^{Cre2SOR}; Yap; Taz* dCKO embryos, whereas *Wnt1^{Cre2SOR}; Yap^{fl/fl}; Taz^{fl/fl}* embryos survived until E15.5, the latest developmental stage examined in this study (Table S2). Together, our findings indicate that embryos with *Yap* and *Taz* compound loss of function or *Yap* deletion with *Taz* haploinsufficiency in the CNC exhibit early embryonic lethality, whereas embryos with *Taz* deletion and *Yap* haploinsufficiency in the CNC survive until later developmental stages.

Yap and Taz deletion in *Wnt1^{Cre}* and *Wnt1^{Cre2SOR}* embryos results in similar vascular defects, but distinct neural tube phenotypes

No obvious craniofacial morphologic defects were observed in *Yap* and *Taz* compound mutants at E9.5. At E10.5, neither control

embryos, including *Yap* and *Taz* compound heterozygous embryos and embryos without *Wnt1^{Cre}* (Fig. S1A–C), nor *Wnt1^{Cre}; Yap^{fl/fl}; Taz^{fl/fl}* embryos (Fig. S1D–F) displayed any obvious craniofacial defects. The *Wnt1^{Cre}; Yap^{fl/fl}; Taz^{fl/fl}* (Fig. S1G–I) and *Wnt1^{Cre}; Yap; Taz* dCKO (Fig. S1J–L,P–R) embryos survived until E10.5 and showed disrupted craniofacial structures, including enlarged blood vessels in the branchial arch and hemorrhage in the forebrain and mandible at E10.5.

Similar to *Wnt1^{Cre}; Yap; Taz* mutant embryos, lethality at E10.5 was observed in *Wnt1^{Cre2SOR}; Yap; Taz* dCKO (Fig. 2A–D) and *Wnt1^{Cre2SOR}; Yap^{fl/fl}; Taz^{fl/fl}* (Fig. 2M–P) embryos. The E10.5 mutant embryos exhibited disrupted craniofacial structures, including blood vessel enlargement and hemorrhage, which are phenotypes we observed in *Wnt1^{Cre}* compound mutant embryos. These early CNC phenotypes were not observed in *Wnt1^{Cre2SOR}; Yap^{fl/fl}; Taz^{fl/fl}* embryos that have one functional copy of *Yap* or in controls (Fig. 2E–L). *Wnt1^{Cre2SOR}* compound mutant embryos lacked any obvious morphologic defects at E9.5. Notably, both E10.5 *Wnt1^{Cre2SOR}; Yap^{fl/fl}; Taz^{fl/fl}* (Fig. 2Pa) and *Wnt1^{Cre2SOR}; Yap; Taz* dCKO (Fig. 2D,R) embryos had neural tube defects, which is one of the most common human birth defects. At E10.5, all (14/14) *Wnt1^{Cre2SOR}; Yap; Taz* dCKO embryos displayed open anterior neural tubes (Fig. 2D,R), and most (7/11) *Wnt1^{Cre2SOR}; Yap^{fl/fl}; Taz^{fl/fl}* embryos presented with less severe, but still abnormal, anterior neural tube morphology (Fig. 2Pa). Neural tube defects were not observed in any of the *Yap; Taz* compound mutant embryos generated using *Wnt1^{Cre}* (Fig. S1).

Histological analysis indicated that, compared with control embryos (Fig. 3A–D, Fig. S2A–C), both *Wnt1^{Cre}; Yap; Taz* dCKO

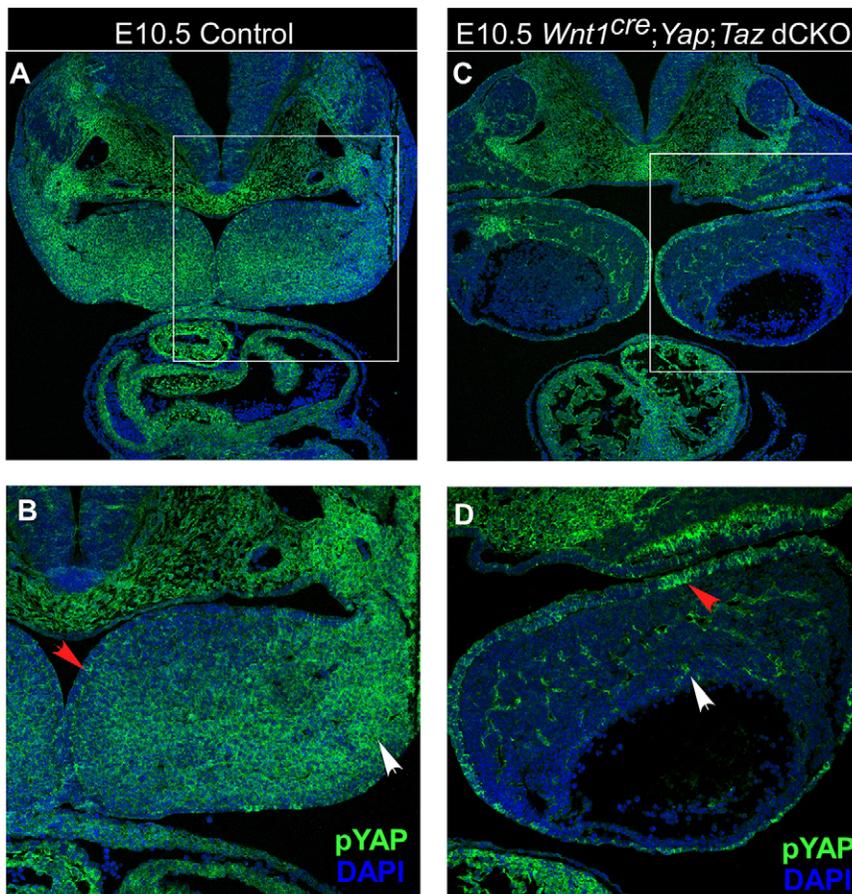


Fig. 1. Efficient deletion of *Yap* and *Taz* in CNC-derived cells. Hippo signaling activity, indicated by the level of phosphorylated Yap (pYAP) in control embryos (A,B) and in *Wnt1^{Cre}; Yap^{fl/fl}; Taz^{fl/fl}* embryos (C,D). pYAP levels were reduced in CNC-derived cells (white arrows) but not in non-CNC derived cells (red arrows). Hippo-active cells (green) were stained with pYAP; nuclei (blue) were stained with DAPI.

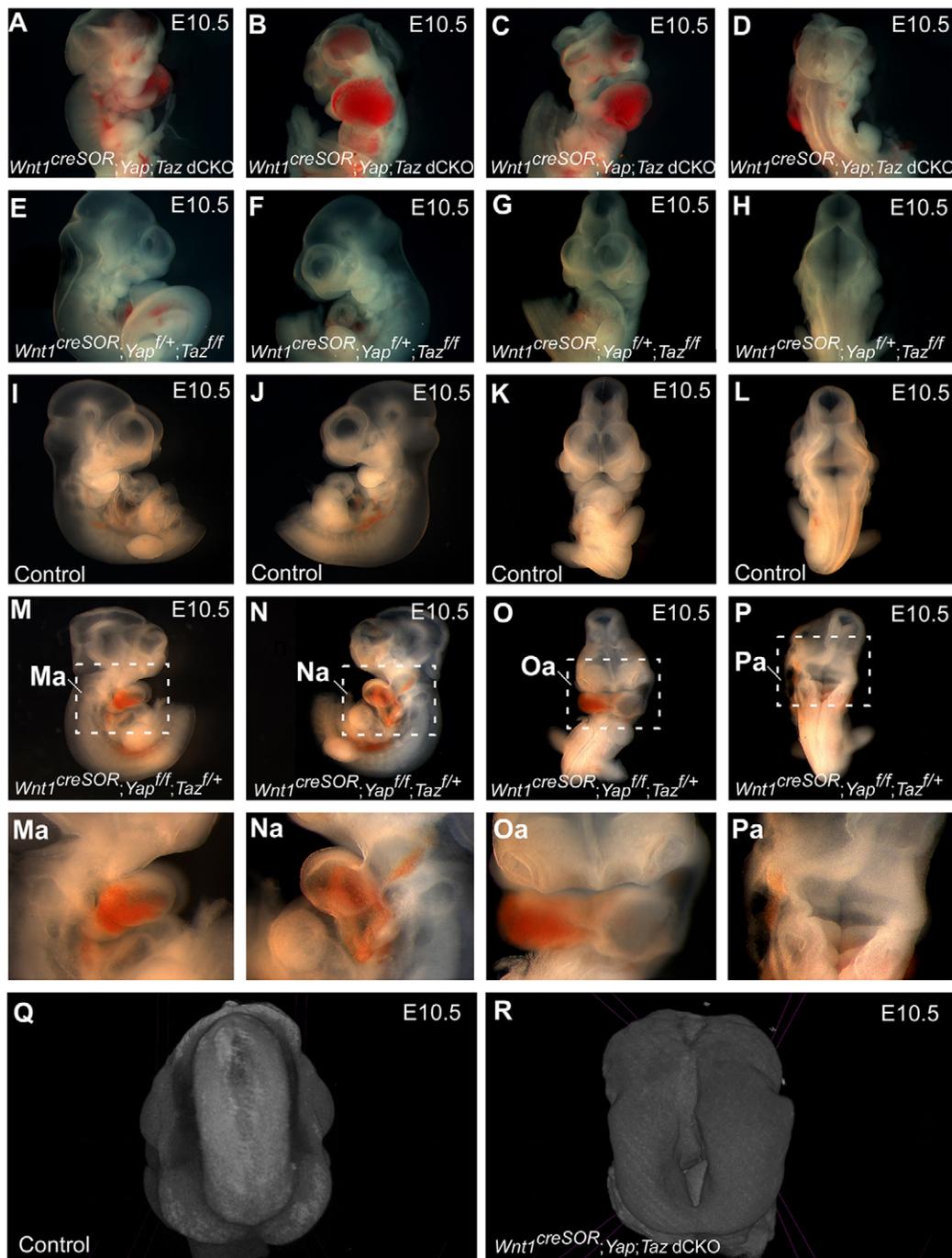


Fig. 2. Vascular defects, hemorrhage and neural tube defects following *Yap* and *Taz* inactivation using the *Wnt1*^{Cre2SOR} Cre driver. Both *Wnt1*^{Cre2SOR}; *Yap*; *Taz* dCKO (A-D) and *Wnt1*^{Cre2SOR}; *Yap*^{f/f}; *Taz*^{f/+} (M-P) embryos show lethality at E10.5 with severe vascular defects, hemorrhage and defects in neural tube closure, whereas control embryos (I-L) and *Wnt1*^{Cre2SOR}; *Yap*^{f/+}; *Taz*^{f/f} embryos (E-H) show no obvious defects. (Ma-Pa) Zoom images of branchial arch vessel defects, hemorrhage and neural tube defects of *Wnt1*^{Cre2SOR}; *Yap*^{f/f}; *Taz*^{f/+} embryos. Three-dimensional imaging using optical projection tomography microscopy indicates that the neural tube of control embryos closed normally (Q), whereas *Wnt1*^{Cre2SOR}; *Yap*; *Taz* dCKO embryos have a defect in neural tube closure (R).

(Fig. 3E-L, Fig. S2D-F) and *Wnt1*^{Cre}; *Yap*^{f/f}; *Taz*^{f/+} (Fig. 3M-P, Fig. S2G-I) embryos had enlarged blood vessels. Moreover, the mandibular mesenchyme was disorganized in *Wnt1*^{Cre}; *Yap*; *Taz* dCKO (Fig. 3G,H) and *Wnt1*^{Cre}; *Yap*^{f/f}; *Taz*^{f/+} (Fig. 3O,P) embryos. The sparse mesenchyme in the mandible suggests that there are fewer pericytes to be recruited from the surrounding mesenchyme because of the lack of *Yap* and *Taz* in CNC-derived

tissues; thus, deficient support from the blood vessels surrounding cells probably caused hemorrhage in mutants.

***Yap* and *Taz* deletion causes severe neural tube vessel regression**

To more closely examine the hemangioma phenotype in the forebrain, branchial arch and mandibular regions in

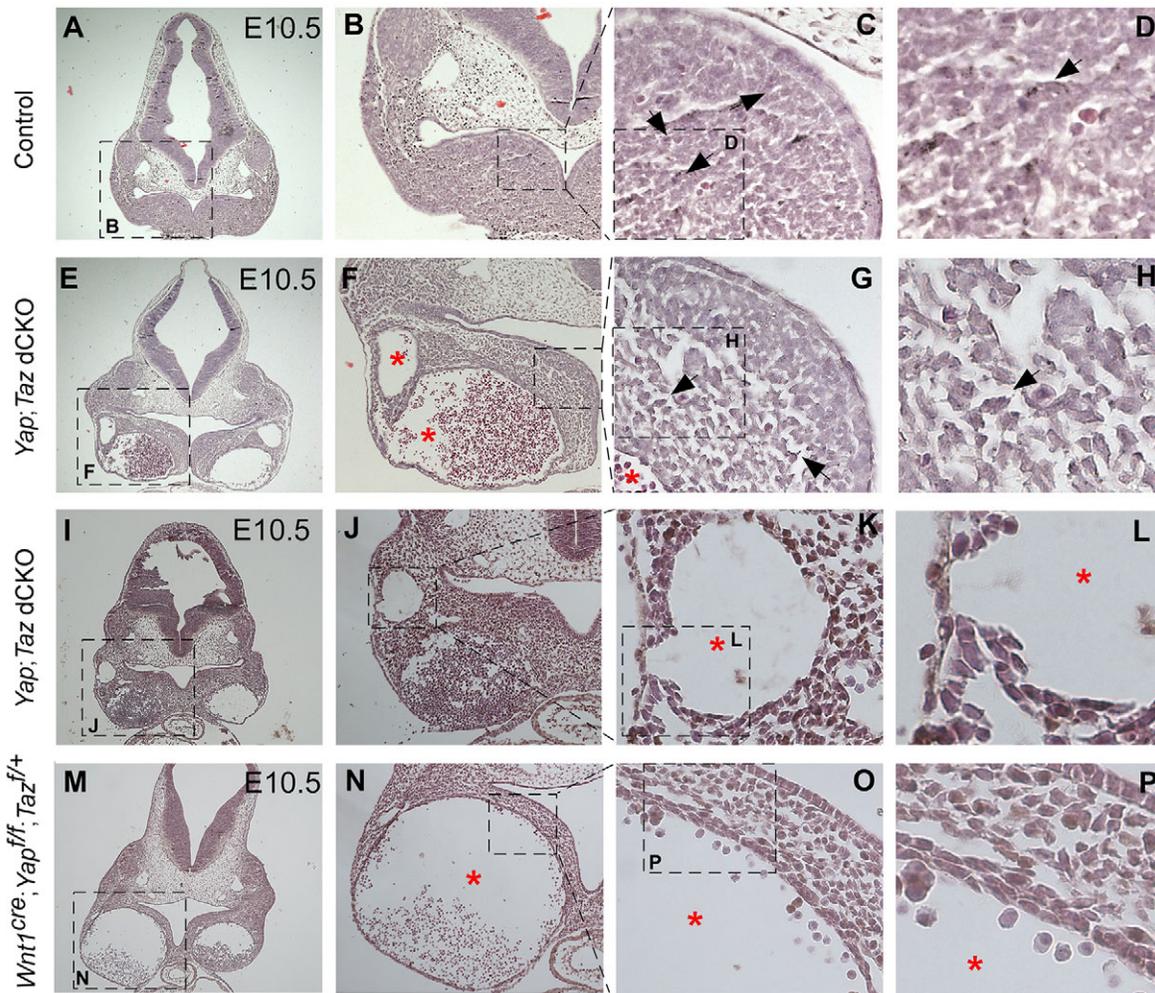


Fig. 3. Histological analysis showing the disruption of mandibular structure in *Yap* and *Taz*-deficient embryos. Coronal sections stained with hematoxylin and eosin showing that, in contrast to control embryos (A-D), both *Wnt1^{Cre}; Yap; Taz* dCKO (E-L) and *Wnt1^{Cre}; Yap^{fl}; Taz^{fl/+}* (M-P) embryos have disorganized, sparse mesenchyme (black arrows) and enlarged vessels (red stars) in the mandible. Boxed areas are shown at higher magnification in panels to the right, as labeled.

Wnt1^{Cre}; Yap; Taz dCKO embryos, we performed whole-mount immunofluorescence staining for CD31 (Pecam1, endothelial cell marker) and smooth muscle actin (SMA, a smooth muscle cell marker), followed by imaging with Lightsheet microscopy. In contrast to control embryos (Fig. 4A), the hemangiomas present in the branchial arch of *Wnt1^{Cre}; Yap; Taz* dCKO embryos are encapsulated by CD31-positive endothelium (Fig. 4B). In addition, the results of three-dimensional rendering and maximum intensity projection of the branchial arch in a *Yap; Taz* dCKO embryo further supported the finding that the vessels are wrapped around the hemangioma in the branchial arch of the *Yap; Taz* dCKO embryo (Fig. 4C). Similar endothelial-lined hemangiomas were also identified in the forebrain and mandibular regions in other dCKO embryos. Images of CD31 immunostaining also revealed abnormal vessel regression throughout the brain of *Wnt1^{Cre}; Yap; Taz* dCKO embryos. By comparing control embryos (Fig. 4D-F) with *Wnt1^{Cre}; Yap^{fl}; Taz^{fl}* embryos (Fig. 4G-I), we detected several regions where vessel regression and/or disorganization was present in *Wnt1^{Cre}; Yap^{fl}; Taz^{fl}* embryos (regions labeled 1-4 in Fig. 4G-I), demonstrating that expression of both *Yap* and *Taz* within the CNC is required for normal vascularization of the early brain and mandibular region.

RNA sequencing reveals genes regulated by the Hippo pathway in the CNC

Because the mandibular phenotype in *Yap; Taz* dCKO embryos appeared to be consistent in both CNC-specific Cre lines, we focused our attention on the molecular mechanism underlying the enlarged cranial and facial vessels. We performed RNA-sequencing (RNA-Seq) analysis by using mandibular RNA isolated from E10.5 *Wnt1^{Cre}; Yap; Taz* dCKO and control embryos. RNA-Seq analysis of the mandibular tissue from *Wnt1^{Cre}; Yap; Taz* dCKO embryos revealed efficient ablation of *Yap* and *Taz* (Fig. 5A). Among the differentially expressed genes, 355 were upregulated and 77 were downregulated (Fig. S3A). We noted that a large percentage of the differentially expressed genes (11%) were DNA binding factors (Fig. S3B).

Gene ontology analysis indicated that genes upregulated in *Wnt1^{Cre}; Yap; Taz* dCKO embryos are involved in regulating adherens junction formation, vasoconstriction, cytoskeleton and positive regulation of endothelial cell migration (Fig. 5B). Genes downregulated in *Wnt1^{Cre}; Yap; Taz* dCKO embryos primarily function in cell proliferation, extracellular matrix organization and vasculogenesis (Fig. 5C).

Among the downregulated genes, we identified those that negatively regulate the canonical Wnt signaling pathway,

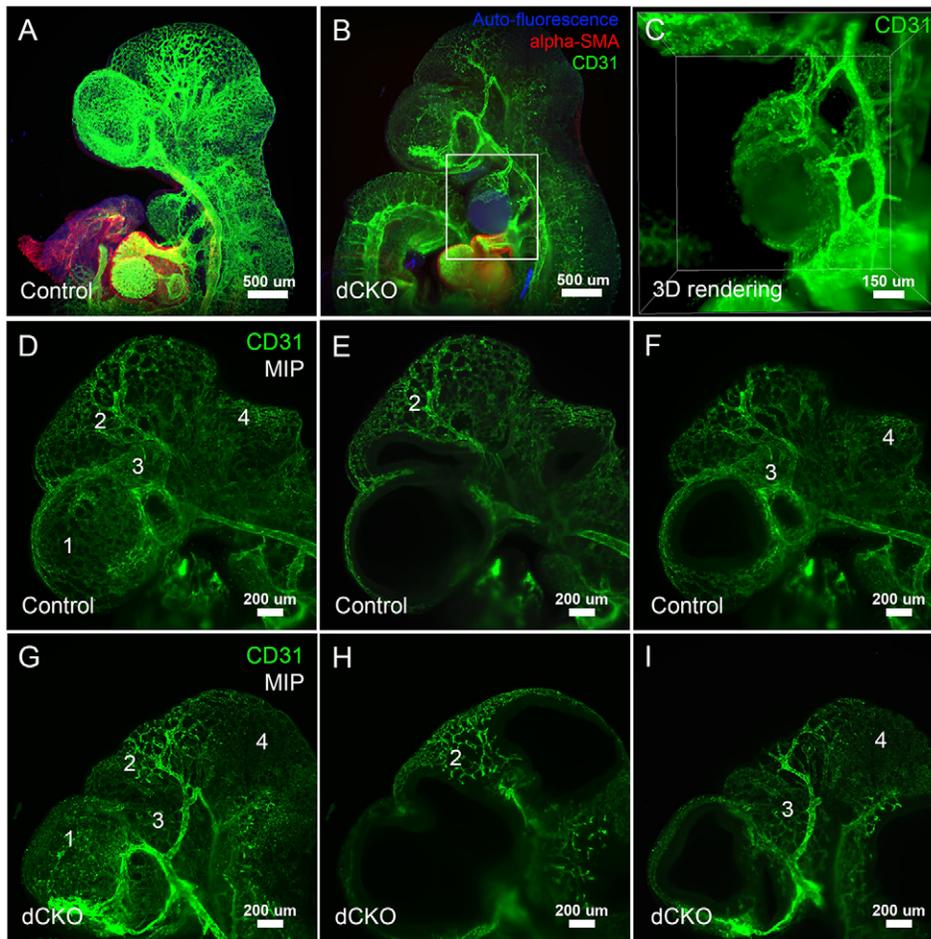


Fig. 4. Severe vessel defects caused by deletion of *Yap* and *Taz*. Whole-mount CD31 immunofluorescence staining in a control (A) and a *Wnt1^{Cre}; Yap; Taz* dCKO mutant (B) reveals vessel defects and endothelial-lined hemangiomas in the forebrain and mandible in *Wnt1^{Cre}; Yap; Taz* dCKO mutants (B). (C) The boxed area in B at a higher magnification, focusing on the endothelial-lined hemangiomas in the branchial arch in the *Yap; Taz* dCKO embryo. Whereas control embryos have normal vessel development in brain (D-F), *Wnt1^{Cre}; Yap; Taz* dCKO mutants have vessel regression and disorganization (different regions are labeled 1-4) (G-I). Endothelial cells are stained with CD31 antibody (green), smooth muscle cells are stained with SMA antibody (red) and auto fluorescence is blue.

including the secreted Frizzled-related protein (SFRP) *Sfrp2*, a Wnt inhibitor that directly binds Wnt ligands (Ladher et al., 2000). Notably, *Wnt1^{Cre}; Yap; Taz* dCKO embryos had reduced *Foxc1* expression (Fig. 5D). *Foxc1* has been implicated in ocular and cerebellar malformations in human patients, as well as in vascular malformations in mice (Kume et al., 2001; Kume, 2009; Delahaye et al., 2012; Haldipur et al., 2014).

Among the transcripts upregulated in *Wnt1^{Cre}; Yap; Taz* dCKO embryos, we identified genes encoding components of the Jak-Stat cascade, including *Jak3*, *Ptk2b*, *Stat3*, *Stat5a* and *Stat5b* (Fig. 5D). Interestingly, Jak-Stat signaling controls organ size in *Drosophila*, much like the Hippo pathway. In vertebrates, the Jak-Stat pathway is involved in growth hormone signaling, although it is less clear what the predicted consequence of increased Jak-Stat levels would be during development. We also found that mRNA expression of prospero homeobox protein 1 (*Prox1*) is upregulated in *Wnt1^{Cre}; Yap; Taz* dCKO embryos. *Prox1* encodes a transcription factor that is a master regulator of lymphatic endothelial cell specification and identity, and is essential for normal vascular development (Wigle and Oliver, 1999; Wigle et al., 2002; Johnson et al., 2008). Another upregulated vascular mitogen was platelet-derived growth factor b (*Pdgfb*), which promotes the recruitment and proliferation of vascular cells (Yancopoulos et al., 2000). *Pdgfb* deficiency leads to reduced numbers of microvascular-associated pericytes in mouse embryos (Lindhahl et al., 1997). *Wnt1^{Cre}; Yap; Taz* dCKO embryos also had upregulated mRNA expression of forkhead box E1 (*Foxe1*), which has been implicated as a causative gene in human orofacial clefting (Moreno et al., 2009).

***Yap* and *Taz* regulate proliferation and apoptosis in the CNC**

As indicated by our RNA-Seq data analysis (Fig. 5C), cell proliferation genes were downregulated in *Wnt1^{Cre}; Yap; Taz* dCKO embryos compared with control embryos. To evaluate differences in proliferation *in vivo*, we performed phospho-histone H3 (pHH3) immunofluorescence staining on sections from both E9.5 and E10.5 *Yap; Taz* dCKO and control embryos. The percentage of proliferating, pHH3-positive cells was lower in the mandible of *Wnt1^{Cre}; Yap; Taz* dCKO embryos (E9.5, Fig. 6C,D; E10.5, Fig. 6H,I) than in that of control embryos (E9.5, Fig. 6A,B; E10.5, Fig. 6F,G). Furthermore, quantification of pHH3-positive cells indicated that the rate of proliferation was significantly lower in *Wnt1^{Cre}; Yap; Taz* dCKO embryos than in control embryos at both E9.5 and E10.5 (Fig. 6E,J).

To further evaluate the role of the Hippo pathway in CNC cells, we used the O9-1 cell line for *in vitro* analyses. The O9-1 cell line is a stable, multipotent, mesenchymal CNC cell line, originally derived from *Wnt1^{Cre}*; R26R-green fluorescent protein (GFP)-expressing cells and can differentiate into multiple CNC derivatives, including osteoblasts, chondrocytes, smooth muscle cells and glial cells (Ishii et al., 2012). To examine cell proliferation in response to altered Hippo signaling, we used siRNA-mediated knockdown to reduce expression of *Yap* and *Taz*, as well as *Lats1* and *Lats2*, in O9-1 cells, and performed pHH3 immunofluorescence staining to assess cell proliferation. The percentage of pHH3-positive cells was decreased in cells treated with siRNA against *Yap* and *Taz* (Fig. 6L), but was increased in cells treated with siRNAs targeting *Lats1* and *Lats2* (Fig. 6M) compared with cells treated with control siRNA

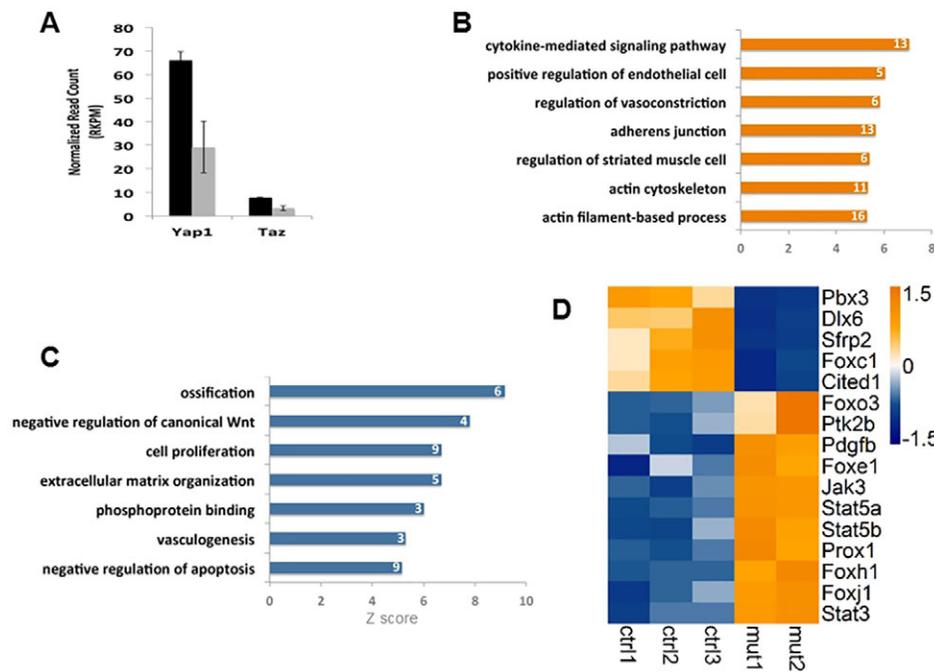


Fig. 5. The regulation of multiple signals by the Hippo pathway in CNC-derived cells.

RNA-Seq analysis was performed by using mandibular tissues from E10.5 control embryos and *Wnt1^{Cre} Taz*; *Yap* dCKO mutants. (A) RNA-Seq analysis indicates that *Yap* and *Taz* expression levels (indicated by reads) decreased in *Wnt1^{Cre} Taz*; *Yap* dCKO embryos compared with control embryos. (B) Gene ontology analysis shows genes that are upregulated in *Wnt1^{Cre} Taz*; *Yap* dCKO embryos compared with control embryos, which includes genes that regulate adherens junctions, vasoconstriction and the cytoskeleton. (C) Genes that are downregulated in *Wnt1^{Cre} Taz*; *Yap* dCKO embryos compared with control embryos, which includes genes that regulate cell proliferation and vasculogenesis and that negatively regulate canonical Wnt. Numbers in bars in B and C indicate gene number of each gene ontology term. (D) Heat map of RNA-Seq data shows that, compared with controls, *Wnt1^{Cre} Taz*; *Yap* dCKO mutants downregulate expression of *Foxc1* and upregulate expression of *Foxe1*, *Prox1*, *Pdgfb* and *Jak-Stat* genes, including *Jak3*, *Ptk2b*, *Stat3*, *Stat5a* and *Stat5b*.

(Fig. 6K). Based on cell counting, the proliferation rate was significantly reduced in *Yap* and *Taz*-knockdown cells but significantly increased in *Lats1* and *Lats2*-knockdown cells when compared with the control (Fig. 6N).

Although cell proliferation was reduced in the mandible of *Wnt1^{Cre} Yap*; *Taz* dCKO embryos, these mutant embryos showed no obvious defects in neural tube morphogenesis. We also evaluated cell proliferation in *Wnt1^{Cre2SOR} Yap*; *Taz* dCKO embryos, which did display neural tube closure defects (Fig. 2R). However, no obvious difference was detected in cell proliferation within the neural tube between *Wnt1^{Cre2SOR} Yap*; *Taz* dCKO and control embryos (Fig. S4). Moreover, when we performed immunofluorescence studies to evaluate the expression of the actin-severing protein cofilin 1 (CFL1), an important factor for neural tube closure, we found no obvious difference between *Wnt1^{Cre2SOR} Yap*; *Taz* dCKO and control embryos (Fig. S5).

Our RNA-Seq data indicated that the expression levels of negative regulators of apoptosis were lower in *Wnt1^{Cre} Yap*; *Taz* dCKO embryos than in control embryos (Fig. 5C). To evaluate cell apoptosis *in vivo*, we performed terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) analysis in both E9.5 *Wnt1^{Cre} Yap*; *Taz* dCKO mutant embryos and control embryos. Our TUNEL data indicated that cell apoptosis was significantly increased in E9.5 *Wnt1^{Cre} Yap*; *Taz* dCKO embryos compared with control embryos (Fig. 6O-S).

Yap and Taz promote smooth muscle differentiation

Our RNA-Seq data indicated that genes involved in vasculogenesis are downregulated in *Wnt1^{Cre} Yap*; *Taz* dCKO embryos when compared with control embryos (Fig. 5C). Moreover, Lightsheet microscopy revealed severe vessel defects in *Wnt1^{Cre} Yap*; *Taz* dCKO embryos than in control embryos (Fig. 4). A recent study indicated that *Yap* and *Taz* deficiency gives rise to smooth muscle differentiation defects partially derived from the CNC (Manderfield et al., 2015). Given the similar vessel defects in *Wnt1^{Cre} Yap^{fl/fl} Taz^{fl/fl}* embryos and *Wnt1^{Cre} Yap*; *Taz* dCKO embryos, we hypothesized that *Yap* alone plays a crucial role in the

regulation of smooth muscle differentiation. Accordingly, we created a *Yap*-null O9-1 cell line (*Yap* KO O9-1) by removing exon 3 of *Yap* using CRISPR/Cas9-mediated genome editing (strategy is shown in Fig. 7A; details in Materials and Methods). Compared with wild-type O9-1 cells, *Yap* KO O9-1 cells had diminished SMA protein expression and *Yap* activity (Fig. 7B). SMA immunofluorescence indicated that under differentiation conditions (see Materials and Methods), wild-type O9-1 cells gave rise to SMA-positive smooth muscle cells, but the ability of *Yap* KO O9-1 cells to generate SMA-positive cells was significantly diminished, supporting the idea that *Yap* plays the predominant function in SMC differentiation, perhaps because it is more highly expressed in CNC (Fig. 7C-E).

Hydrocephalus in *Yap^{fl/fl} Taz^{fl/fl}* CKO embryos

Although *Wnt1^{Cre} Yap^{fl/fl} Taz^{fl/fl}* and *Wnt1^{Cre2SOR} Yap^{fl/fl} Taz^{fl/fl}* embryos did not show any obvious defects before E10.5 (Fig. 2, Fig. S1), they developed hydrocephalus at later stages (Fig. S6E-G, Fig. S7D-F). Hydrocephalus is mainly characterized by the abnormal widening of brain spaces caused by the excessive accumulation of cerebrospinal fluid, which places harmful pressure on the surrounding tissues of the brain. Fig. S6E-G shows a representative example of an E12.5 *Wnt1^{Cre} Yap^{fl/fl} Taz^{fl/fl}* embryo that had severe hydrocephalus in the hindbrain. In addition, in the sagittal sections of control embryos (Fig. S6D,d), the cerebellum structure is clearly visible, whereas it is missing in the *Wnt1^{Cre} Yap^{fl/fl} Taz^{fl/fl}* embryos (Fig. S6H,h). The hydrocephalus phenotype was detected as early as E11 in *Wnt1^{Cre} Yap^{fl/fl} Taz^{fl/fl}* embryos, but it was not observed in either *Wnt1^{Cre} Yap^{fl/fl} Taz^{fl/fl}* or *Wnt1^{Cre} Yap*; *Taz* dCKO embryos, most likely because of their lethality at E10.5. Moreover, *Wnt1^{Cre} Yap^{fl/fl}* embryos at E11 also had severe hydrocephalus (Fig. S8M-P) when compared with controls (Fig. S8I-L). *Wnt1^{Cre2SOR} Yap^{fl/fl} Taz^{fl/fl}* embryos at E12.5 presented more severe hydrocephalus (Fig. S7D-F) than that in *Wnt1^{Cre} Yap^{fl/fl} Taz^{fl/fl}* embryos in the hindbrain region and forebrain region. Notably, our RNA-Seq data (Fig. 5) suggested that genes required for forebrain and hindbrain morphogenesis, such

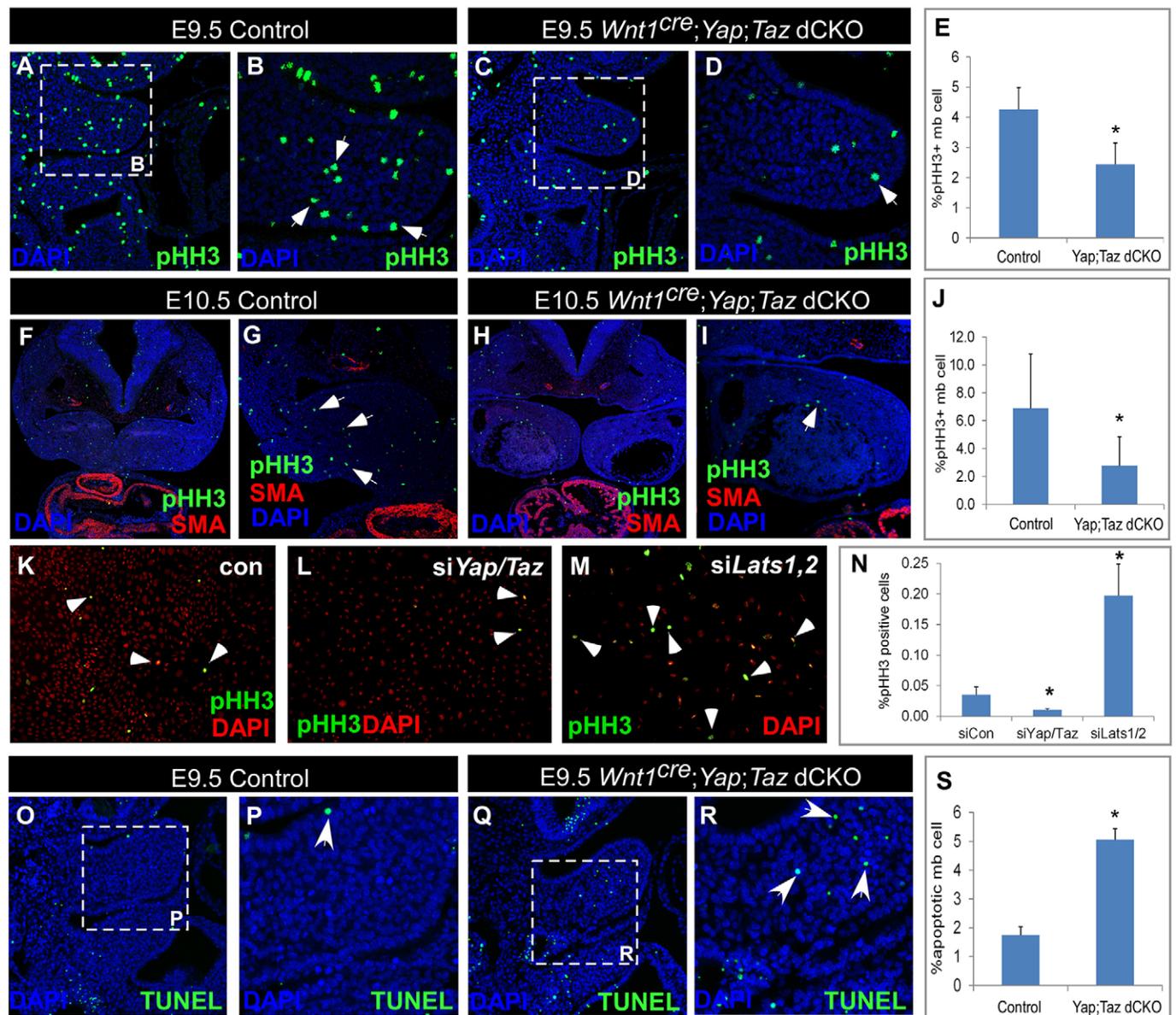


Fig. 6. Regulation of proliferation and apoptosis in CNC cells by the Hippo pathway. (A–J) Compared with control embryos, *Wnt1^{Cre}; Yap; Taz* dCKO embryos have a significantly diminished percentage of pHH3-positive proliferating cells in the mandible at E9.5 and E10.5. Proliferating cells are stained with pHH3 antibody (green), smooth muscle cells with SMA antibody (red) and nuclei with DAPI (blue). * $P < 0.05$. (K–N) Compared with O9-1 cells transfected with control (con) siRNA, O9-1 cells transfected with *Yap* and *Taz* siRNA have significantly reduced proliferation, and O9-1 cells transfected with *Lats1* and *Lats2* siRNA have significantly increased proliferation (* $P < 0.05$). Proliferating cells are stained with pHH3 antibody (green), nuclei with DAPI (red). Arrows indicate proliferating cells. (O–S) Compared with control embryos, *Wnt1^{Cre}; Yap; Taz* dCKO embryos have a significantly increased percentage of apoptotic cells in the mandible at E9.5 (* $P < 0.05$). Apoptotic cells are stained with TUNEL (green), nuclei with DAPI (blue). All error bars represent s.e.m. Arrows indicate apoptotic cells.

as *Foxc1*, were regulated by *Taz* and *Yap*. *Foxc1* loss of function also leads to hydrocephalus in mice (Kume et al., 1998).

Yap and Taz regulate Fox genes

Our RNA-Seq data indicated that *Yap* and *Taz* modulated the expression of multiple members of the winged-helix/forkhead box (*Fox*) transcription factor family, including the upregulation of *Foxe1*, *Foxh1*, *Foxj1* and *Foxo3*, as well as the downregulation of *Foxc1* (Fig. 5D). We focused on *Foxc1* in part because its expression was reduced in *Wnt1^{Cre}; Yap; Taz* dCKO embryos and *Yap* and *Taz* are thought to be transcriptional activators. Indeed, *Yap* ChIP-Seq data showed that genome-wide *Yap* peaks are enriched in active chromatin regions, as defined by H3K27Ac

chromatin marks (Morikawa et al., 2015). We reasoned that downregulated genes were more likely to be direct *Yap/Taz* target genes. Moreover, *Foxc1* is the gene mutated in the congenital hydrocephalus mouse mutant (Kume et al., 1998).

Immunohistochemistry data indicated that *Foxc1* is normally expressed in the majority of the epithelial and mesenchymal cells in the mandible (sagittal view in Fig. 8Bb; coronal view in Fig. S9Bb), yet *Foxc1* expression was missing or reduced in the majority of mesenchymal cells and unchanged in the mandibular epithelial cells in *Wnt1^{Cre}; Yap; Taz* dCKO embryos (sagittal view in Fig. 8Db; coronal view in Fig. S9Db). On the basis of cell counting, the number of *Foxc1*-positive cells was significantly lower in the *Wnt1^{Cre}; Yap; Taz* dCKO mandible than in the control mandible

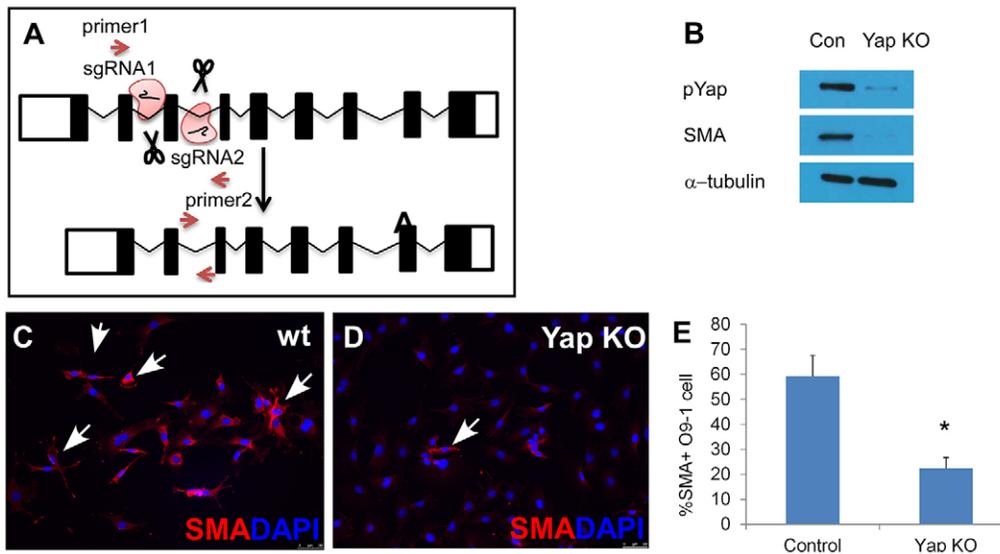


Fig. 7. Yap is required for smooth muscle differentiation. (A) Strategy for establishing a *Yap*-knockout (KO) O9-1 cell line by using CRISPR/Cas9 system. Specifically, exon 3 was deleted from *Yap*. (B) Western blot data shows diminished SMA and pYap expression in *Yap* KO O9-1 cells compared with wild-type (wt) O9-1 cells. (C) Under differentiation conditions, most wild-type O9-1 cells give rise to smooth muscle cells. (D) *Yap* KO O9-1 cells did not give rise to smooth muscle cells. Arrows indicate SMA-positive cells. Smooth muscle cells are stained with SMA antibody (red), nuclei with DAPI (blue). (E) Cell counting data show that the percentage of SMA-positive cells is significantly reduced in *Yap* KO O9-1 cells compared with wild-type O9-1 cells (* $P < 0.01$). Error bars represent s.e.m.

($P < 0.01$, Fig. 8E). Furthermore, *Foxc1* immunofluorescence staining indicated that *Foxc1* expression was significantly lower in *Yap* knockout; *Taz* knockdown O9-1 cells (Fig. 8G) than in wild-type O9-1 cells (Fig. 8F) ($P < 0.05$, Fig. 8H). Western blot analysis of *Foxc1* further indicated that *Foxc1* expression was decreased in response to the decrease in *Yap* and *Taz* expression levels (Fig. 8I).

Importantly, we identified a conserved binding site for Tead, the *Yap* and *Taz* cofactor, in *Foxc1* (Fig. 8J). Craniofacial cis-regulatory landscapes were recently studied by deep-sequencing of transposase-accessible chromatin (ATAC-seq) in human and chimpanzee cranial neural crest cells (Prescott et al., 2015). Bioinformatic analysis of these data sets (Gene Expression Omnibus, accession no. GSE70751) revealed increased chromatin accessibility in a region located upstream of the 5' UTR of *FOXC1*. Significantly, this putative enhancer region contained a potential TEAD DNA-binding element (Fig. 8J). The TEAD binding element was confirmed by chromatin immunoprecipitation (ChIP) PCR in murine embryonic facial tissue using an anti-*Yap* antibody, indicating that a *Yap*-Tead complex directly binds to *Foxc1* chromatin during embryonic facial morphogenesis (Fig. 8K).

DISCUSSION

During normal development, regeneration and cancer progression, Hippo signaling inhibits proliferation while promoting apoptosis. Here, we show that, during craniofacial development, *Yap* and *Taz*, the final downstream effectors of Hippo signaling, regulate multiple events in CNC diversification including vasculogenesis, smooth muscle differentiation, cerebellar development, and neural tube closure. Furthermore, our data indicate that *Foxc1* is an important downstream target of *Yap* and *Taz*. Significantly, *Foxc1* loss of function in mouse embryos phenocopies several of the morphological defects present in *Yap* and *Taz* mutant embryos.

Overlapping functions for *Yap* and *Taz* during craniofacial development

Using either a *Wnt1^{Cre}* or *Wnt1^{Cre2SOR}* driver, *Yap* and *Taz* conditional CNC mutants presented with disrupted craniofacial

vascular development and hemorrhage. We also observed hydrocephalus in *Yap^{f/f}*; *Taz^{f/f}* mutants established by using both Cre drivers, at later embryonic stages. It is most likely that *Yap* and *Taz* have redundant functions when they are co-expressed, but at earlier developmental stages, *Taz* expression is lower than that of *Yap*. Interestingly, we did observe a phenotypic difference in embryos when we deleted *Yap* and *Taz* using the two Cre drivers. Deleting *Yap* and *Taz* with the *Wnt1^{Cre2SOR}* driver resulted in anterior neural tube closure defects at E10.5, whereas *Wnt1^{Cre}*; *Yap^{f/f}*; *Taz^{f/f}* and *Wnt1^{Cre}*; *Yap*; *Taz* dCKO embryos showed no neural tube closure defects. *Wnt1^{Cre}* directs ectopic *Wnt1* activity in the midbrain, whereas *Wnt1^{Cre2SOR}* has normal *Wnt1* activity in the midbrain (Lewis et al., 2013). Because *Wnt* signaling is known to stabilize *Yap* and *Taz* (Azzolin et al., 2014), our data suggest that the phenotypic differences observed between mutants obtained by using *Wnt1^{Cre}* and *Wnt1^{Cre2SOR}* might be caused by elevated *Wnt* signaling from the *Wnt1^{Cre}* transgene (Chai et al., 2000).

Yap and *Taz* regulate vascular development

The diminished expression of CD31 in E10.5 *Yap* and *Taz* mutants indicated that *Yap* and *Taz* have an essential role in craniofacial vascular development. We found that the multipotent O9-1 NC cells treated with siRNAs targeting *Yap* and *Taz* were defective in their ability to differentiate into smooth muscle cells. Moreover, our RNA-Seq data revealed that genes functioning in adherens junction formation, endothelial cell migration, vasoconstriction and the cytoskeleton were differentially expressed in *Yap* and *Taz* mutants, suggesting that other developmental events also contribute to defective vascular development.

We noted that genes encoding the *Jak-Stat* cascade components, including *Jak3*, *Ptk2b*, *Stat3*, *Stat5a* and *Stat5b*, were upregulated in *Wnt1^{Cre}*; *Yap*; *Taz* dCKO mutants. A previous study reported that the activation of *Stat3* promotes the apoptosis of vascular smooth muscle cells by triggering mitochondria-mediated cell death receptors and cell death pathways (Bai et al., 2008). Further work is required to investigate the role of increased *Jak-Stat* pathway components during craniofacial development in an *in vivo* setting.

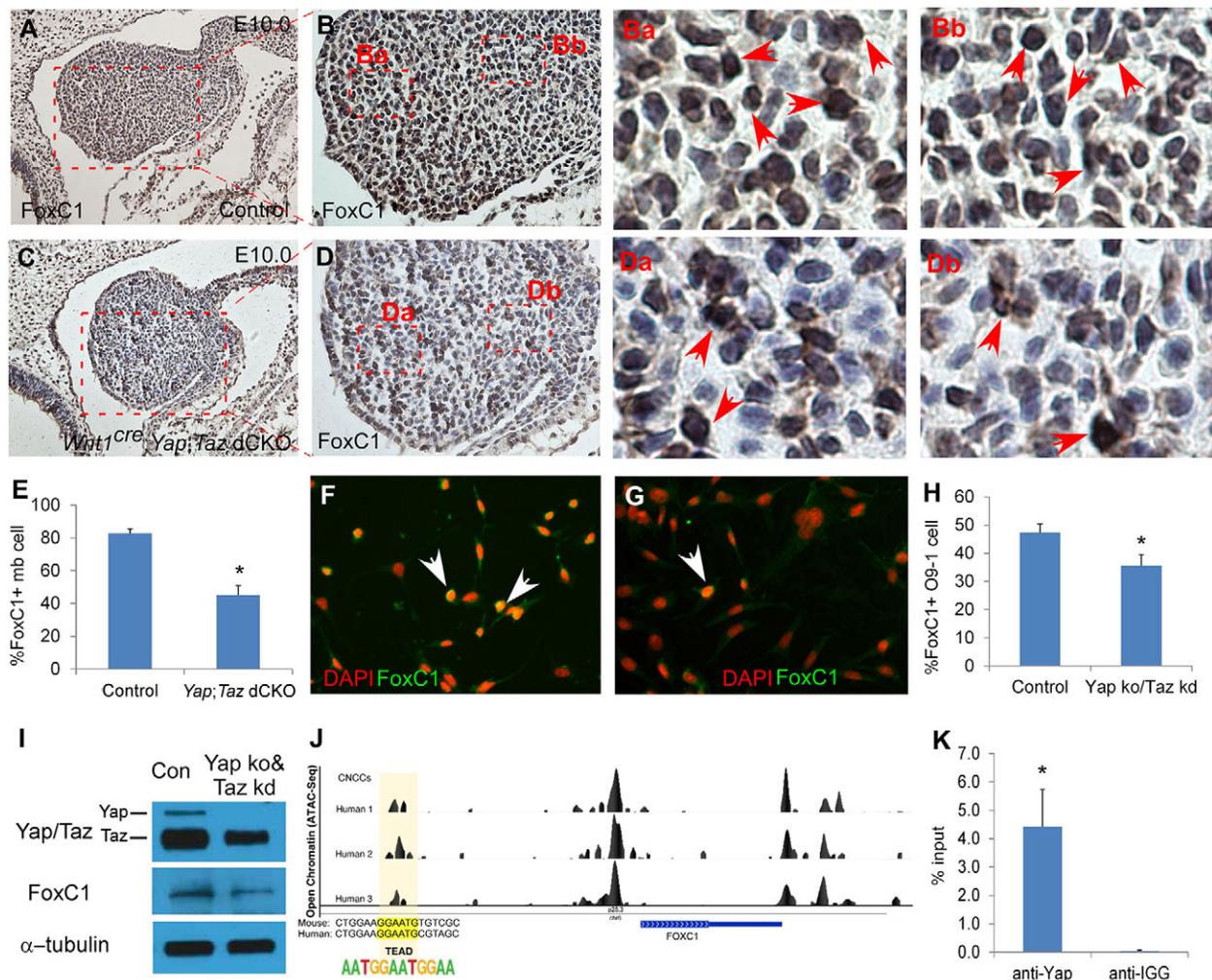


Fig. 8. Regulation of *Foxc1* by Yap and Taz. Immunohistochemical staining of *Foxc1* in sagittal sections of the mandible show that the majority of cells in control embryos express *Foxc1* (A-Bb), whereas the expression of *Foxc1* is diminished in *Wnt1^{Cre}; Yap; Taz* dCKO embryos (C-Db). Boxed areas are shown at higher magnification in panels to the right, as labeled. (E) Cell counting data show that the percentage of cells positive for *Foxc1* expression in the mandible is significantly reduced in *Wnt1^{Cre}; Yap; Taz* dCKO embryos compared with control embryos (* $P < 0.01$). (F-H) Immunohistochemical staining of *Foxc1* in O9-1 cells shows significantly decreased *Foxc1* expression in *Yap* knockout (KO); *Taz* knockdown (KD) O9-1 cells compared with wild-type O9-1 cells (* $P < 0.05$). Arrows indicate *Foxc1*-positive cells. (I) Western blot analysis of *Foxc1* in O9-1 cells shows decreased *Foxc1* expression in response to decreased Yap and Taz expression level. (J) Conserved Tead binding site located in the upstream region of *FOXC1*. Peaks in ATAC-seq data (accession no. GSE70751) indicate chromatin accessibility (Prescott et al., 2015). (K) *In vivo* real-time PCR using ChIP DNA indicates that *Foxc1* is bound by the Yap-Tead complex in embryonic facial tissue. $P < 0.05$. All error bars represent s.e.m.

Transcripts of *Prox1*, a regulator of lymphatic development, were also elevated in *Yap* and *Taz* mutant embryos. Specification of lymphatic endothelial cells is an essential event in vascular development (Wigle and Oliver, 1999; Wigle et al., 2002; Johnson et al., 2008); thus, elevated *Prox1* levels may conceivably disrupt normal vascular specification. It will be interesting to determine in future experiments whether increased *Prox1* expression leads to fate switching to a predominantly lymphatic endothelial cell phenotype.

We found that *Pdgfb* is also upregulated in *Yap* and *Taz* mutant embryos. PDGF signaling has been implicated both directly and indirectly in the regulation of vasculogenesis and angiogenesis. Given the crucial role of *Pdgfb* in regulating the recruitment and proliferation of vascular cells (Yancopoulos et al., 2000), the defective vascular development observed in *Yap* and *Taz* mutant embryos might be related to increased *Pdgfb* expression.

The Hippo pathway regulates CNC proliferation and apoptosis

We showed that proliferation was significantly reduced in the mandibular mesenchyme in *Yap* and *Taz* mutant embryos. This diminished proliferation is consistent with our observation of reduced cell density surrounding the enlarged vessels in *Yap* and *Taz* mutant embryos. Furthermore, a recent study showed that NC migration and fate specification were spared in *Wnt1^{Cre}; Yap; Taz* embryos at E10.5, suggesting that Yap and Taz regulate proliferation in the post migratory CNC (Manderfield et al., 2014). In *Wnt1^{Cre2S0R}; Yap; Taz* embryos, we also found that proliferation was intact in the neural tube, despite the open neural tube phenotype, indicating that other mechanisms might account for the failure of neural tube closure.

Consistent with our *in vivo* observations, we found that the siRNA-mediated knockdown of *Yap* and *Taz* significantly reduced the proliferation of multipotent NC O9-1 cells, further suggesting

that *Yap* and *Taz* are essential for NC cell proliferation. We also showed that the knockdown of *Lats1* and *Lats2* significantly increased the proliferation of O9-1 cells, which is consistent with the idea that *Yap* and *Taz* function as Hippo pathway effectors to regulate NC proliferation.

In addition, apoptosis was significantly increased in the mandibular mesenchyme in *Yap* and *Taz* mutant embryos. Both decreased proliferation and increased apoptosis were observed in E9.5 *Yap* and *Taz* mutant embryos in the absence of any detectable hemorrhage, indicating that the reduced cell proliferation and sparse cell density observed in E10.5 *Yap* and *Taz* mutant embryos were caused by a direct, primary effect of *Yap* and *Taz*, rather than a secondary effect due to the hemorrhaging.

Regulation of Fox genes by Yap and Taz and their potential roles in human disease

Fox transcription factors are key regulators of embryogenesis and control fundamental biological processes, including cell proliferation, fate determination, differentiation and growth (Kaufmann and Knochel, 1996; Kume et al., 1998, 2001; Kidson et al., 1999; Tuteja and Kaestner, 2007a,b; Benayoun et al., 2011; Haldipur et al., 2014). Notably, Fox genes are evolutionarily and functionally conserved across multiple species, including mice and humans, making animal models invaluable tools for understanding the mechanisms underlying human diseases caused by Fox genes.

Expression of *Foxc1* was downregulated at the mRNA and protein level in *Yap* and *Taz* mutant embryos. *Foxc1* has been implicated in several different human disorders including the most common cerebellar malformation, Dandy-Walker malformation (DWM), Axenfeld-Rieger syndrome (ARS), 6p25 deletion syndrome and iridogoniodygenesis (Mears et al., 1998; Nishimura et al., 1998; Maclean et al., 2005; Aldinger et al., 2009; Delahaye et al., 2012). In addition to the function of *Foxc1* in different diseases, it also has crucial roles in the development of multiple organs and tissues, including cerebellar, skull, ocular and cardiovascular development (Kume, 2009). Notably, compound *Foxc1* and *Foxc2*-knockout mouse embryo mutants have craniofacial abnormalities similar to those of *Yap*; *Taz* mutants, such as enlarged blood vessels, sparse mesenchyme and an open neural tube (Kume et al., 2001).

Patients with mutations in *Foxc1* display a variant of the Dandy-Walker malformation, including cerebellar vermis hypoplasia (CVH). Notably, *Foxc1* mutant mice also have an enlarged fourth ventricle roof plate. Similar to *Foxc1* mutant mice, *Yap*; *Taz* dCKO mice also display cerebellar hypoplasia with an enlarged fourth ventricle roof plate. In addition, *Foxc1*-knockout mice are reported to have hydrocephalus (Kume et al., 1998), a severe and common lethal human birth defect that was also observed in mice with *Yap* inactivation and *Taz* heterozygosity in NC cells. Collectively, these findings further support the hypothesis that *Foxc1* is a downstream effector of *Yap* and *Taz*.

MATERIALS AND METHODS

Mouse alleles and transgenic lines

All animal experiments in this study were approved by the Baylor College of Medicine Institutional Review Board. The *Wnt1^{Cre}*, *Wnt1^{Cre2SOS}*, *Yap^{fllox/+}* and *Taz^{fllox/+}* mouse lines and alleles used in this study have been described previously (Chai et al., 2000; Xin et al., 2011, 2013; Lewis et al., 2013).

Histology and hematoxylin and eosin staining

All embryos were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. For H&E staining, the fixed embryos were dehydrated with an ethanol series (70% to 100%) and xylene and then embedded in paraffin. These tissues were subsequently cut

into 7 µm sections and stained with H&E, as previously described (Lu et al., 1999).

O9-1 cell culture and siRNA knockdown

The O9-1 cells were cultured under undifferentiating conditions by following a previously published protocol (Ladher et al., 2000). The culture medium used consisted of Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 55 µM β-mercaptoethanol, 100 units/ml penicillin/streptomycin and 2 mM L-glutamine. Importantly, the medium was conditioned with growth-inhibited STO (mouse embryonic fibroblast cell line) feeder cells overnight, filtered (0.22 µm pore size) and further supplemented with 25 ng/ml basic fibroblast growth factor (R&D Systems, 233-FB) and 1000 U leukemia inhibitory factor (Millipore, ESG1106). The conditions used for smooth muscle differentiation were described previously (Ishii et al., 2012). For the siRNA knockdown experiments in O9-1 cells, siRNA SMARTpools targeting *Yap*, *Taz* and Hippo kinases *Lats1* and *Lats2* were purchased from Dharmacon and the transfections were performed by following a typical RNAiMAX transfection procedure (Thermo Fisher Scientific).

Yap exon 3 deletion using CRISPR/Cas9

To make a *Yap*-null O9-1 cell line, exon 3 of *Yap* was deleted using CRISPR/Cas9 genome editing. Two sgRNAs flanking exon 3 were identified using the sgRNA design tool (<http://www.dna20.com>). Four sgRNA oligonucleotides with overhanging *BbsI* restriction sites were synthesized by IDT (Coralville, IA): 5'-CACCGTggattacgtgggatgtt-3' (sgRNA1-forward), 5'-AAACaataccacgtaatccac-3' (sgRNA1-reverse), 5'-CACCGagatgttctaatgtagtga-3' (sgRNA2-forward) and 5'-AAACtaccattagaccatctC-3' (sgRNA2-reverse). The paired oligonucleotides were annealed and ligated into a pSpCas9(bb)-2a-GFP vector (Addgene, plasmid #48138). To create cell lines with a deletion of *Yap* exon 3, we transfected pSpCas9-GFP-YAP-sgRNA1 and pSpCas9-GFP-YAP-sgRNA2 into O9-1 cells using Lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours after the transfection, the cells underwent fluorescence-activated cell sorting (FACS) with GFP/7-AAD. Viable, single cells (7-AAD⁻;GFP⁺) were seeded into 96-well plates with culture medium. After 4–5 days, single colonies could be observed in some of the wells; wells that had more than one colony were excluded from further analyses. Single colonies were passaged into 12-well plates and incubated for 2–4 days to expand the clones. When the cells were more than 80% confluent, they were dissociated with trypsin and half of the sample was processed for DNA extraction while the other half was used to make a freezer stock culture. To detect the *Yap* exon 3 deletion, we designed PCR primers flanking the sgRNA recognition sites: 5'-AAAACAGTCTCCACTACCCCTT-3' (forward) and 5'-GGCCATCAT-AGATCCTGGACG-3' (reverse). Clones harboring a homozygous deletion for *Yap* exon 3 were retained and used for experimental analyses.

3D embryo imaging with optical projection tomography microscopy

Optical projection tomography microscopy was used to determine embryonic craniofacial morphology. Specimens were embedded in 1% agarose (Amresco LLC, Solon, OH) and dehydrated in 25%, 50%, 75% and 100% ethanol (Sigma-Aldrich) for 2 h each. Samples were then stored in fresh 100% ethanol overnight before clearing. The dehydrated samples were then completely cleared in BABB solution, which contained one part benzyl alcohol (Thermo Fisher Scientific) and two parts benzyl benzoate (Acros Organics, Waltham, MA). Samples were then imaged on a custom-built OPT system (BCM-OPT) (Singh et al., 2015) modified from a previous version (Wong et al., 2013). The BCM-OPT microscope consisted of an Optem Zoom 125C lens (Qioptiq, Waltham, MA) and an auxiliary objective lens attached to a Retiga 4000DC FAST 1394 CCD camera (QImaging, Surrey, BC) with a 1.38× TV tube (Qioptiq). Autofluorescence images of the embryos were acquired with an X-Cite illumination light source (Exfo, Quebec, QE) and both a 425/26 nm BrightLine bandpass excitation filter and a 520/20 nm emission filter (Semrock, Rochester, NY). The embryos were rotated 360°

with each view taken with a 0.3° step size. Acquired images were reconstructed using NRecon Reconstruction software and 3D rendering was performed by using CTVox software (Bruker Corporation, Camarillo, CA).

Immunofluorescence and immunohistochemistry

After the embryos were fixed overnight in 4% PFA and dehydrated, they were embedded in paraffin, cut into 7 µm sections, and collected on Superfrost Plus slides (Thermo Fisher Scientific). The sections were rehydrated using xylene and a graded ethanol series to a final concentration of 70% and then processed for antigen retrieval. For immunofluorescence analysis, the antigens were retrieved by incubating the slides in citrate buffer (10 mM) for 2 min in a microwave oven. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) at a 1:500 dilution. Primary antibodies against phospho-Yap (Ser127) (Cell Signaling, 4911), phospho-histone H3 (Cell Signaling, 9701) and Foxc1 (Abcam, ab5079) were used at a 1:200 dilution. A broad HRP-conjugated secondary antibody (Invitrogen) was used according to the manufacturer's guidelines. An anti-SMA-Cy3 antibody (Sigma-Aldrich, Clone 1A4) was used at a 1:200 dilution. Staining was then visualized by using a TSA Plus Fluorescence System (PerkinElmer) and a Zeiss LSM 510 confocal microscope.

For immunohistochemistry analysis, antigens were retrieved by boiling the sections in ethylenediaminetetraacetic acid for 15 min and then tissues were permeabilized by incubating sections with 0.5% Triton X-100 for 15 min. Next, the sections were treated with 0.3% hydrogen peroxide for 15 min and blocked with 10% donkey serum for 1 h. The sections were incubated with primary antibody against Foxc1 (Abcam, ab5079) at a 1:200 dilution overnight at 4°C. The next day, the sections were incubated with HRP-conjugated anti-goat IgG antibody (1:200 dilution) for 1 h and then developed with DAB substrate (Vector Laboratories) for 2 min and 30 s. Sections were subsequently washed with water, stained with hematoxylin for 3 min and washed with water again for 5 min. Then, they were dehydrated with an ethanol series to a final concentration of 100% ethanol, fixed in xylene and mounted.

Whole-mount immunohistochemistry

E10.5 embryos were processed as described (Wythe et al., 2013). Briefly, the embryos were fixed in 4% PFA overnight, serially dehydrated to absolute methanol, bleached in 5% hydrogen peroxide/95% methanol overnight, and rehydrated to PBS-Tween (0.1%). The embryos were blocked for 2 h (PBS/5% goat serum/0.5% Triton X-100), then incubated with an anti-CD31 antibody (BD Biosciences, MEC13.3, 1:200 dilution) in blocking solution for 3 days. The embryos were then washed and incubated with biotin-conjugated goat anti-rat IgG secondary antibody (Vector Laboratories, 1:250 dilution) for 2 days. The signal was amplified with Alexa Fluor 488 Tyramide (Thermo Fisher Scientific, T20948). The embryos were then washed in blocking solution and incubated with anti-SMA-Cy3 antibody (Sigma-Aldrich, Clone 1A4, 1:200 dilution) for 3 days at 4°C. After washing, the embryos were cleared in ScaleA2 for 2 days, ScaleB4 for 2 days, then embedded in 1% agarose in ddH₂O and cleared for another 2 days in ScaleA2. The embryos were then imaged on a Lightsheet Z.1 (Zeiss) using a 5× air lens (NA=0.16) in ScaleA2. All images were obtained using the same laser power and exposure time. Serial images were aligned using Zen software (Zeiss) and 3D rendering was performed with Imaris software (Bitplane).

RNA-Seq and data analysis

Mandibular tissues from *Wnt1^{cre}*, *Yap* and *Taz* dCKO mutant and control E10.5 embryos were dissected in diethylpyrocarbonate-treated PBS. Total RNA extracted from these tissues was treated with RNase-free DNase I (Ambion) for 30 min at 37°C. Then, the poly(A) RNA was purified with the MicroPoly(A) Purist Kit according to the manufacturer's instructions (Thermo Fisher Scientific). The purified RNA samples were processed for cDNA synthesis and library preparation. cDNA sequencing using the Illumina platform and read mapping were performed as previously described (Nagalakshmi et al., 2008). Datasets were processed for gene ontology analysis, which provided different terms that represent gene properties including cellular components, molecular function and biological processes. The data were deposited in the Gene Expression Omnibus (GEO) database

(accession no. GSE69311). More information is available at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ejajwkiynvevuj&acc=GSE69311>.

Chromatin immunoprecipitation

Embryonic orofacial tissues from wild-type mice at stages E10.5-E11.5 were dissected for Yap ChIP analysis, as previously described (Wang et al., 2013; Morikawa et al., 2015). Normal mouse IgG was used as a replacement control for the anti-Yap ChIP assay to show nonspecific immunoprecipitation of the chromatin. The primers used to amplify the TEAD regulatory element in the 5' upstream region of the *Foxc1* genomic sequence were 5'-CCTTGGCATCTCTCAGAAAGTC-3' (sense) and 5'-TAGTCCTATCCCAGTGAGC-ATC-3' (antisense).

Real-time PCR

For real-time RT-PCR analysis, total RNA was isolated from the mandibles of E10.5 embryos or O9-1 cells by using the RNeasy Micro Kit (Qiagen) and processed for cDNA synthesis using Super Script II Reverse Transcriptase (Invitrogen). For the real-time ChIP-PCR analysis, immunoprecipitated DNA and input DNA were used as templates. All real-time thermal cycling was performed with the StepOne Real-time PCR System (Thermo Fisher Scientific). SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) was used for real-time thermal cycling. All error bars represent s.e.m.

Cell counts and data analyses

For embryonic cell counting, all embryos used were at the same embryonic stage. The embryos were embedded carefully to maintain the same orientation and were sectioned at identical thickness (5 µm). Sections were carefully chosen to represent the matching regions between control and mutant embryos. Our analyses included at least three embryos for each genotype. For O9-1 cell counting, each experiment was performed in duplicate and all experiments were repeated three times or more. ImageJ software (National Institutes of Health, Bethesda, MD) was used to perform the cell counting and over 200 cells were counted for each experiment. The percentage of proliferating cells was determined by counting pHH3-positive cells and dividing that number by the number of DAPI-positive cells. The percentage of apoptotic cells was determined by counting the number of TUNEL-positive cells. All counting data are represented in graphs as the mean±s.e.m. The two-tailed Student's *t*-test was used to determine statistical significance and *P*<0.05 was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.W. and J.F.M. designed research; J.W., Y.X., C.-W.H., I.M.M.-T., Y.B. and J.D.W. performed research; J.W., Y.X., C.-W.H., I.M.M.-T., M.Z., J.D.W., M.E.D. and J.F.M. analyzed data; M.I., R.E.M. and E.N.O. provided reagents; J.W., C.-W.H., J.D.W. and J.F.M. wrote the manuscript.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.126920/-/DC1>

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Table S1. Survival rates of *Yap* and *Taz* mutants at different developmental stages. *Wnt1^{Cre}; Yap^{f/+}; Taz^{f/+}* males were crossed with *Yap^{ff}; Taz^{ff}* females to generate mutant genotypes with inactivated *Yap* and *Taz*. The *Wnt1^{Cre}; Yap^{ff}; Taz^{f/+}* and *Wnt1^{Cre}; Yap^{ff}; Taz^{ff}* genotypes were embryonically lethal by stage E10.5 and resulted in severe craniofacial defects. *Wnt1^{Cre}; Yap^{f/+}; Taz^{ff}* mutants had a range of survival times (from E14.5 to postnatal week 8), and although they showed no obvious defects before stage E11.5, an array of calvarial bone defects were seen at later stages.

Stage	Total embryo number	<i>Wnt1^{Cre}; Yap^{f/+}; Taz^{ff}</i>		<i>Wnt1^{Cre}; Yap^{ff}; Taz^{f/+}</i>		<i>Wnt1^{Cre}; Yap^{ff}; Taz^{ff}</i>	
		Expected (12.5%)	Actual	Expected (12.5%)	Actual	Expected (12.5%)	Actual
E9.5-10.5	124	16	18 (14.5%)	16	16 (12.9%)	16	17 (13.7%)
E11.0-11.5	31*	4	3 (10.1%)	4	0 (2 dead [#])	4	0 (3 dead [#])
E12.0-12.5	23*	3	1 (3.7%)	3	0 (2 dead [#])	3	0 (1 dead [#])
E13.0-13.5	25*	3	5 (11.8%)	3	0 (3 dead [#])	3	0 (1 dead [#])
E14.0-14.5	14	2	3 (15.8%)	2	0 (2 dead [#])	2	0 (0.00%)
E15.0-15.5	18	2	2 (11.1%)	1	0 (1 dead [#])	1	0 (0.00%)
E16.0-16.5	9	1	2 (16.7%)	1	0 (0.00%)	1	0 (1 dead [#])

*There were dead embryos that could not be genotyped.

[#]The embryos were dead but could be genotyped.

Table S2. Survival rates of *Yap* and *Taz* dCKO embryos using *Wnt1*^{Cre2SOR}. Mutants with inactivated *Yap* and *Taz* were derived by crossing *Wnt1*^{Cre2SOR}; *Yap*^{f/+}; *Taz*^{f/+} males with *Yap*^{f/f}; *Taz*^{f/f} females. *Wnt1*^{Cre2SOR}; *Yap*^{f/f}; *Taz*^{f/+} and *Wnt1*^{Cre2SOR}; *Yap*^{f/f}; *Taz*^{f/f} mutants survived to stage E10.5 but had severe craniofacial defects. *Wnt1*^{Cre2SOR}; *Yap*^{f/+}; *Taz*^{f/f} mutants did not show obvious defects before stage E11.5 but had hydrocephalus at later stages.

Stage	Total embryo number	<i>Wnt1</i> ^{Cre2SOR} ; <i>Yap</i> ^{f/+} ; <i>Taz</i> ^{f/f}		<i>Wnt1</i> ^{Cre2SOR} ; <i>Yap</i> ^{f/f} ; <i>Taz</i> ^{f/+}		<i>Wnt1</i> ^{Cre2SOR} ; <i>Yap</i> ^{f/f} ; <i>Taz</i> ^{f/f}	
		Expected (12.5%)	Actual	Expected (12.5%)	Actual	Expected (12.5%)	Actual
E9.5	26	3	5 (19.2%)	3	4 (15.4%)	3	2 (7.7%)
E10.5	79	10	8 (10.1%)	10	11 (13.9%)	10	14 (17.7%)
E11.5	24	3	5 (20.8%)	3	0 (2 dead [#])	3	0 (3 dead [#])
E12.5	25	3	4 (16.0%)	3	0 (4 dead [#])	3	0 (0.00%)
E13.5	21*	3	1 (5.8%)	3	0 (1 dead [#])	3	0 (0.00%)
E14.5	17	2	2 (12.5%)	2	0 (1 dead [#])	2	0 (1 dead [#])

*There were dead embryos that could not be genotyped.

[#]The embryos were dead but could be genotyped.

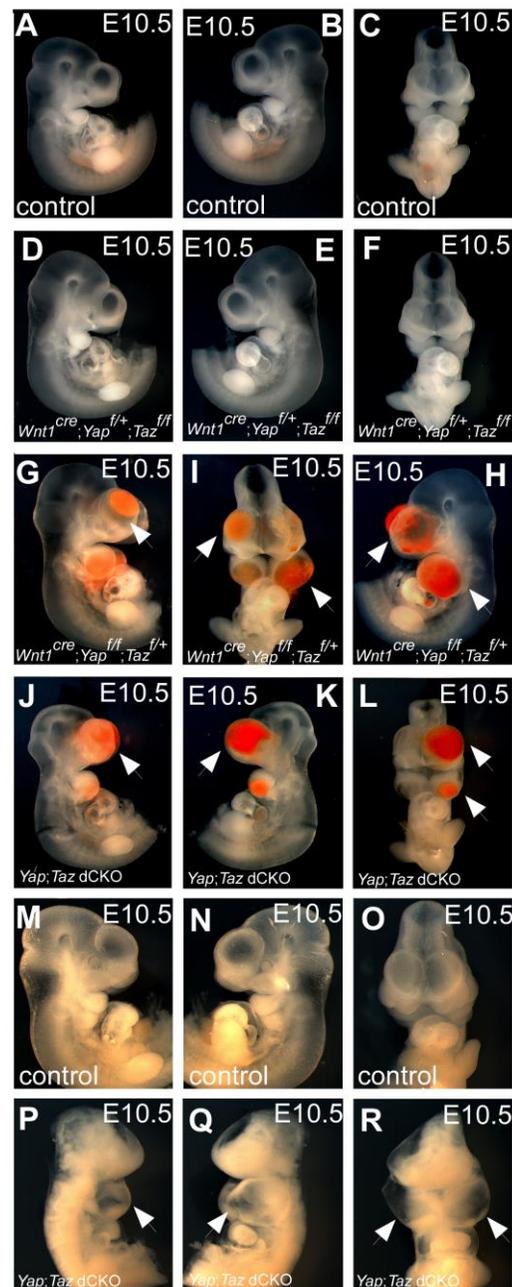


Fig. S1. *Yap* and *Taz* deletions by the *Wnt1^{Cre}* driver affected the vascular phenotype.

Controls (A-C) and *Wnt1^{Cre}; Yap^{f/+}; Taz^{ff}* embryos (D-F) showed no obvious vascular defects. Both the *Wnt1^{Cre}; Taz^{f/+}; Yap^{ff}* (G-H) and *Wnt1^{Cre}; Yap; Taz* dCKO embryos (J-L) were early embryonic lethal at stage E10.5 and had severe vascular defects and hemorrhaging. . A littermate control embryo (M-O) and a dead *Wnt1^{Cre}; Yap; Taz* dCKO embryo (P-R) with severe branchial arch vessel defects are shown at stage E10.5.

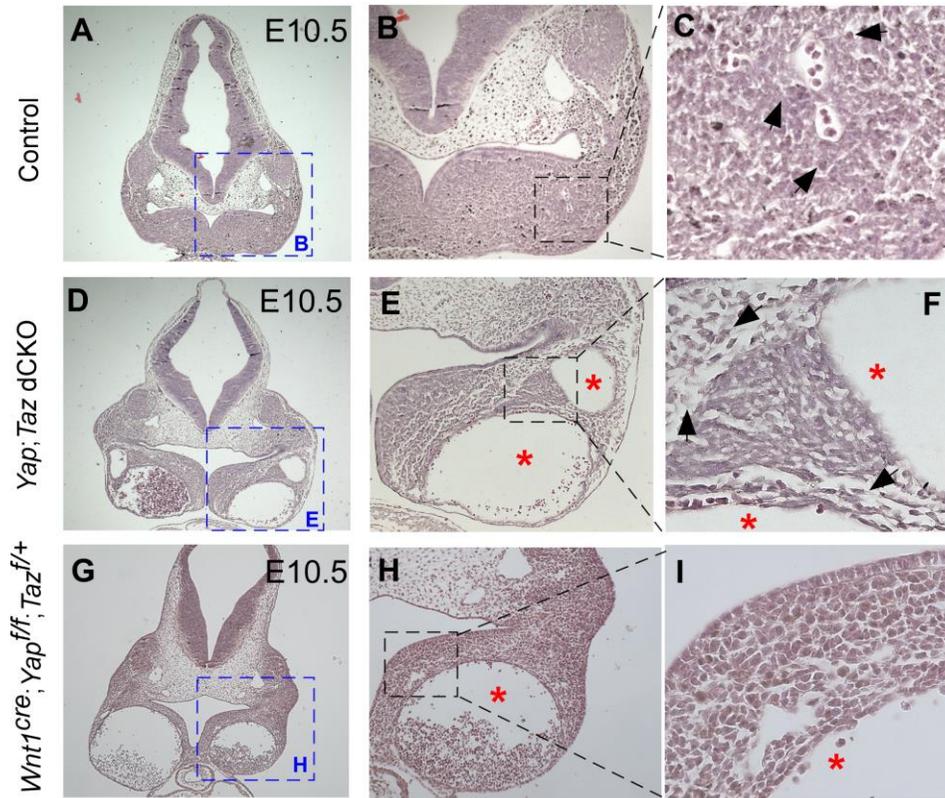


Fig. S2. Coronal sections stained with hematoxylin and eosin showing disrupted mandibular structure in *Yap* and *Taz* deficient mutants. As compared to controls (A-C), both *Wnt1^{Cre}; Yap; Taz* dCKO (D-F) and *Wnt1^{Cre}; Taz^{f/+}; Yap^{ff}* (G-I) embryos had enlarged vessels (red stars) and sparse mesenchyme (black arrows) in the mandible.

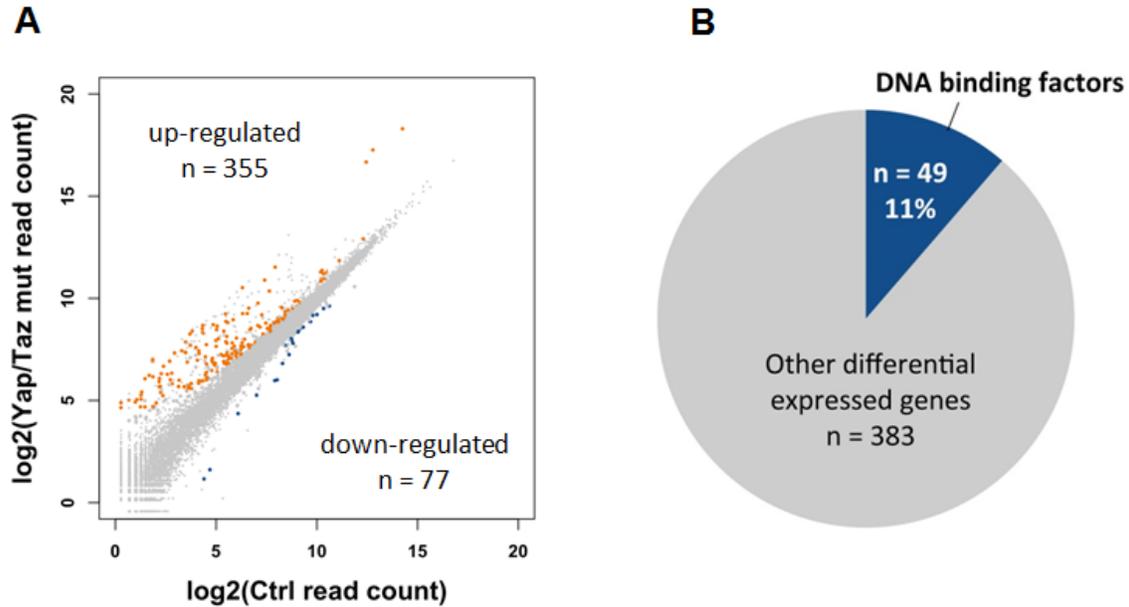


Fig. S3. RNA-Seq analysis of mandibular tissues from controls and *Taz* and *Yap* dCKO embryos at stage E10.5. (A) Given a 10% false discovery rate, 432 genes showed a significant difference in expression level; 355 were upregulated, and 77 were downregulated. (B) Of the 432 genes showing a significant difference, 49 (11%) were DNA-binding factors.

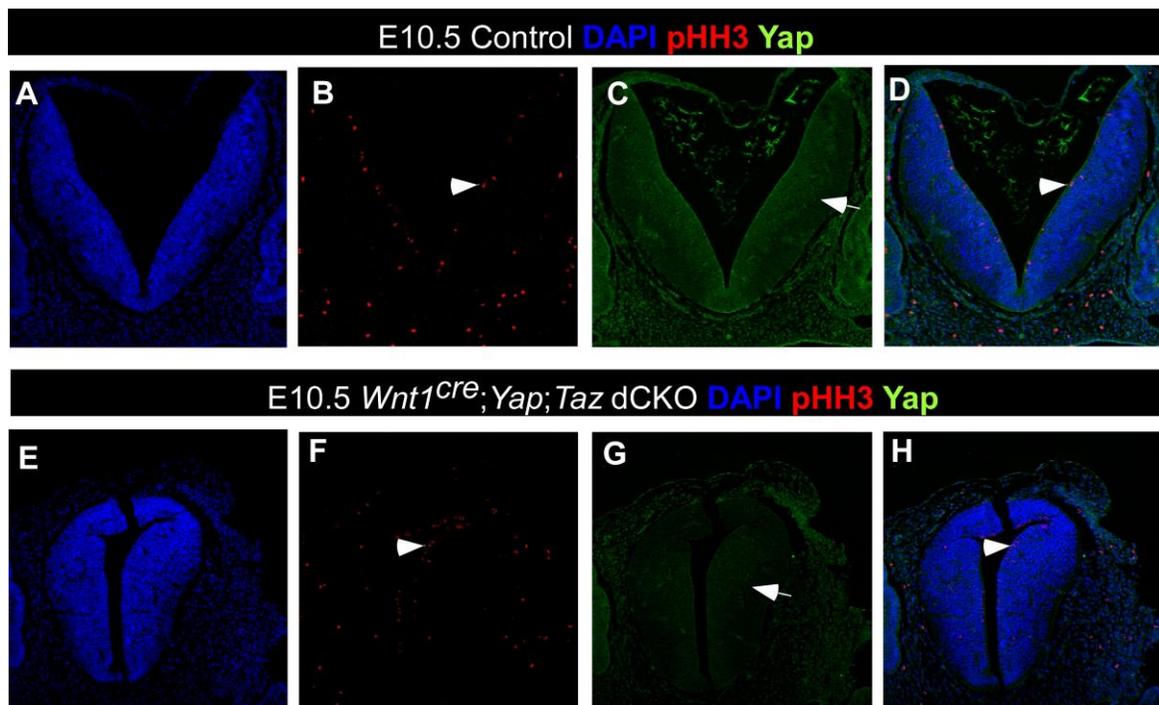


Fig. S4. There was no obvious change in cellular proliferation in the neural tube. Compared to controls (A-D), *Wnt1^{CreSOR}; Yap; Taz* dCKO embryos (E-H) showed no obvious changes in cellular proliferation or Hippo activity in the neural tube. Hippo activity is shown by pYAP staining (green), cellular proliferation is shown by pHH3 staining (red), and nuclei are shown with DAPI staining (blue).

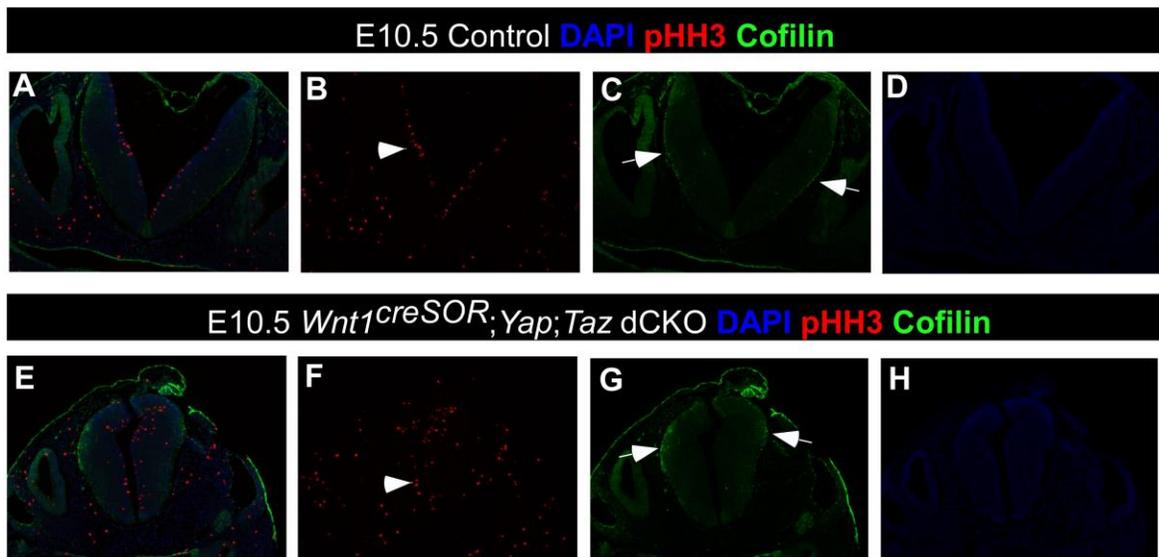


Fig. S5. There was no obvious change in cofilin 1 expression in the neural tube. Compared to control embryos (A-D), *Wnt1^{CreSOR} Taz* and *Yap* dCKO embryos (E-H) showed no obvious change in the expression level of the critical neural tube closure factor cofilin 1. Cofilin staining is shown in green.

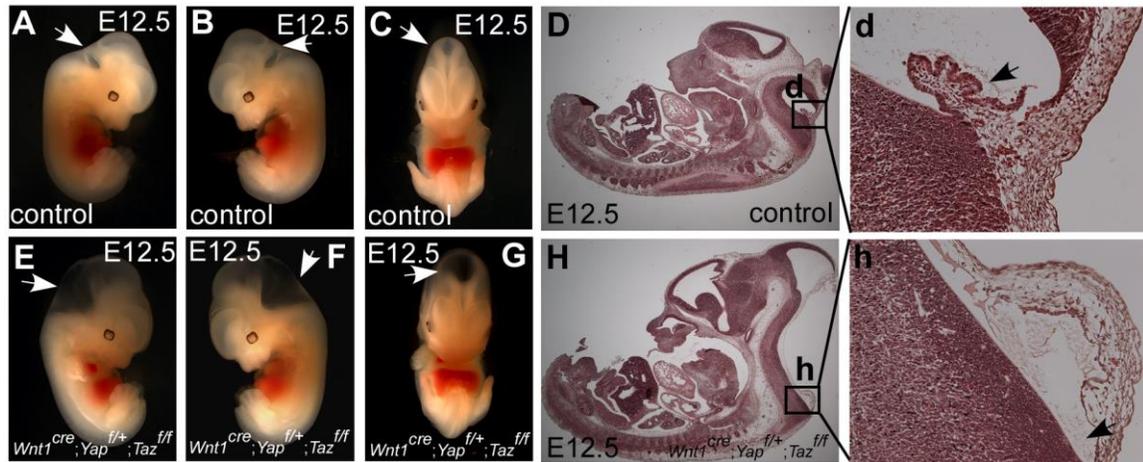


Fig. S6. Hydrocephalus in $Wnt1^{Cre}; Yap^{f/+}; Taz^{ff}$ CKO embryos. Compared to the controls (A-C), $Wnt1^{Cre}; Yap^{f/+}; Taz^{ff}$ embryos (E-G) showed an abnormal widening of brain spaces, indicating that they had hydrocephalus. Moreover, $Wnt1^{Cre}; Yap^{f/+}; Taz^{ff}$ embryos had no cerebellum structure in the sagittal sections (H and h); however, this structure could clearly be seen in the controls (D and d).

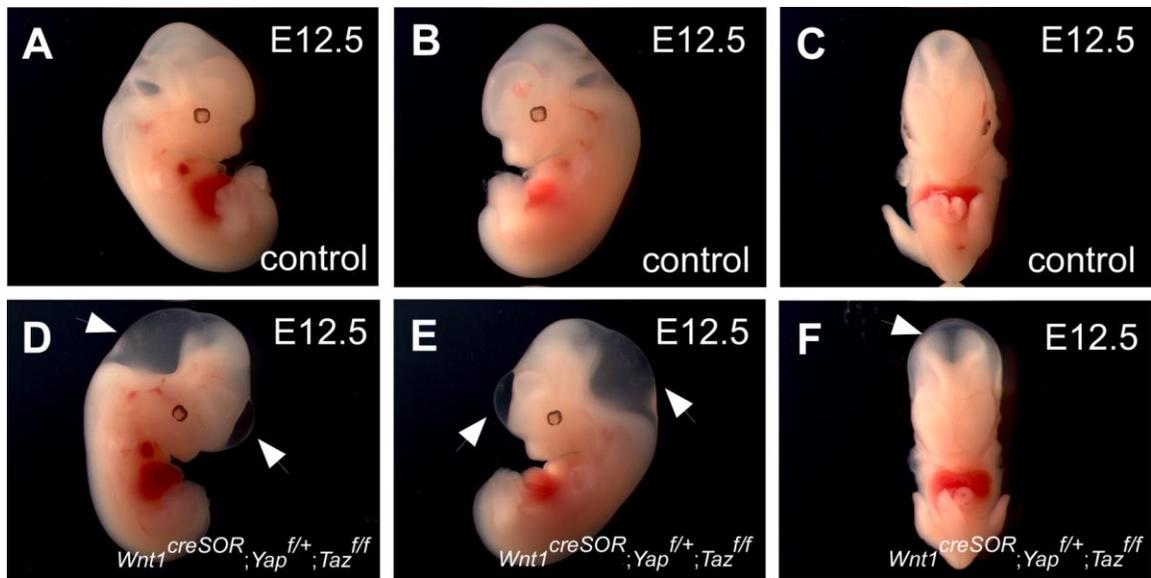


Fig. S7. Hydrocephalus in $Wnt1^{Cre2SOR}; Yap^{f/+}; Taz^{ff}$ CKO embryos. At stage E12.5, hydrocephalus was not detected in any of the controls (A-C), whereas the $Wnt1^{Cre2SOR}; Yap^{f/+}; Taz^{ff}$ mutants (D-F) showed severe hydrocephalus (arrows).

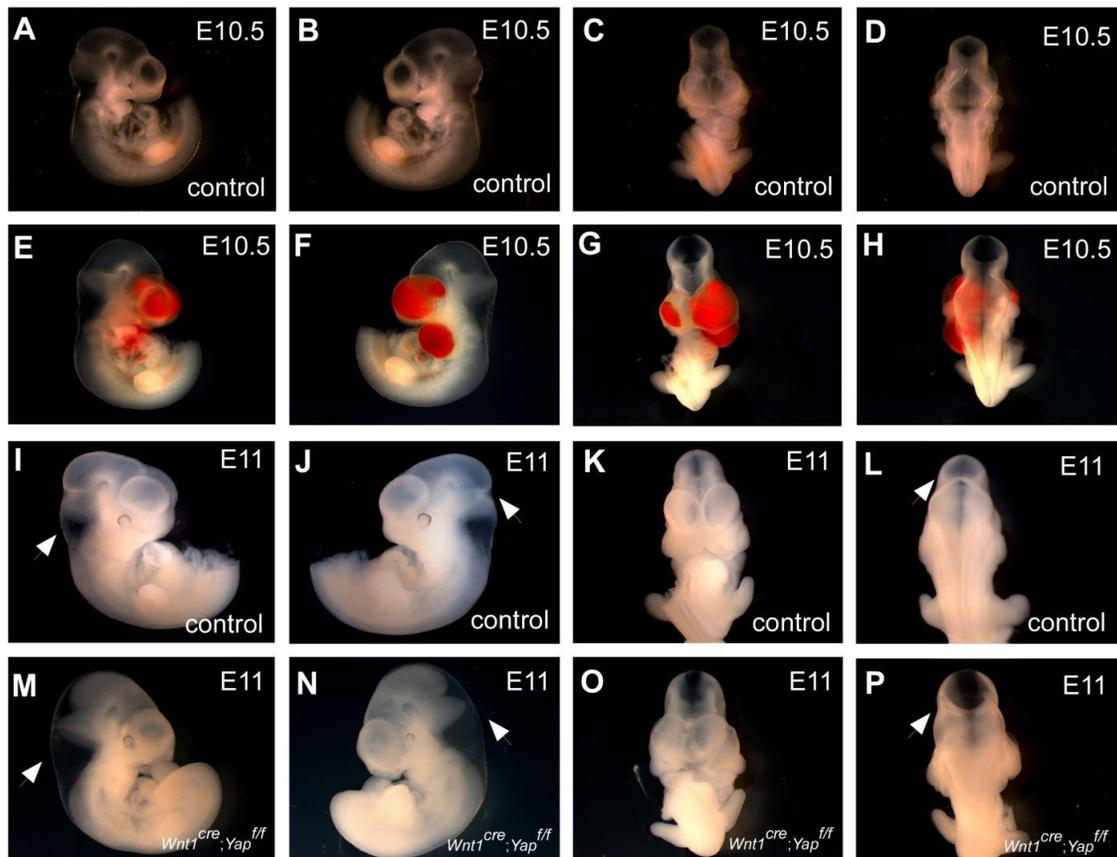


Fig. S8. *Yap* deletion caused embryonic lethality and severe craniofacial defects. The Controls at E10.5 (A-D) and E11 (I-L) showed no obvious craniofacial defects. *Wnt1^{Cre}; Yap^{ff}* genotype was lethal during stages E10.5 to E11.0 and resulted in enlarged vessels and hemorrhaging (E-H). At stage E11.0, hydrocephalus (arrows) was also detected in *Wnt1^{Cre}; Yap^{ff}* mutants (M-P).

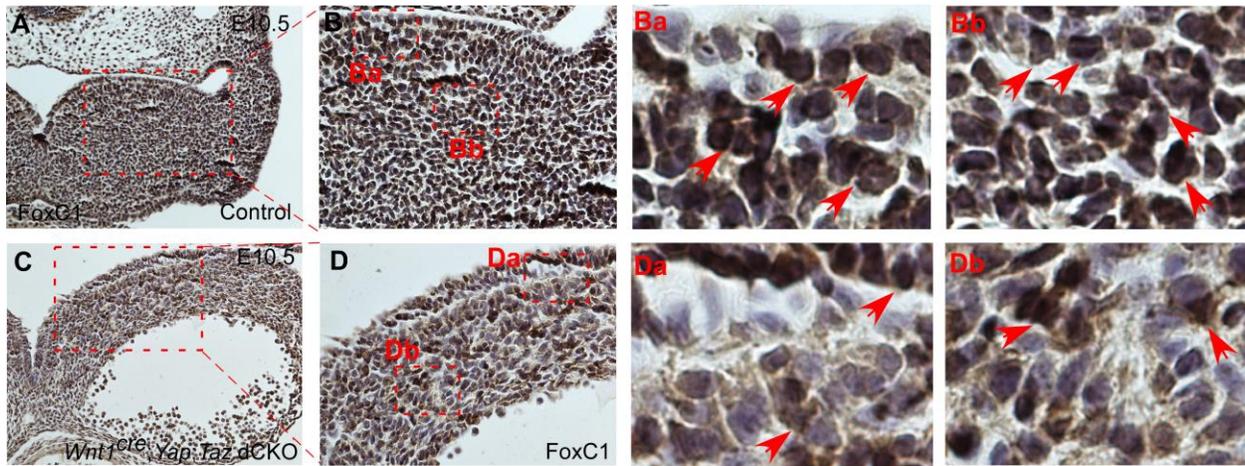


Fig. S9. Yap and Taz regulate *Foxc1*. Immunohistochemistry images of coronal sections showing decreased *Foxc1* expression in the mandibles of *Wnt1^{Cre}; Yap; Taz* dCKO embryos (C-Db), as compared to that in control mandibles (A-Bb). Boxed areas are shown at higher magnification in panels to the right, as labeled.