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

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List of abbreviations used

AHA, American Heart Association; ARS, Axenfeld-Rieger syndrome; CFL1, cofilin 1; ChIP, chromatin immunoprecipitation; CKO, conditional knock out; CNC, cranial neural crest; CVH, cerebellar vermis hypoplasia; DAPI, diamidino-2-phenylindole; dUTP, deoxynucleotidyl transferase; DWM, Dandy-Walker malformation; FACS, fluorescence-activated cell sorting; Fox, forkhead box; GFP, green fluorescent protein; H&E, hematoxylin and eosin; NC, neural crest; OPT, optical projection tomography; PBS, phosphate buffered saline; Pdgfb, Platelet-derived growth factor b; PFA, paraformaldehyde; pYAP, phosphorylated Yap; RNA-Seq, RNA-sequencing; SDG, Scientist Development Grant; SFRP, secreted Frizzled-related protein; SMA, smooth muscle actin; TEA, transcriptional enhancer activator; Tead, TEA domain; TUNEL, terminal deoxynucleotidyl transferase nick end labeling.
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, $Wnt1^{Cre2SOR}$ embryos had an open cranial neural tube phenotype that was not evident in $Wnt1^{Cre}$ embryos. In O9-1 CNC cells, the loss of $Yap$ and $Taz$ 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 further 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.
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 cells 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 a critical 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 Lats 1/2 (orthologous to Drosophila Warts). Lats 1/2, in turn, phosphorylate the most downstream Hippo signaling components Yap and Taz, thus promoting their binding to 14-3-3 proteins and inhibiting them from shuttling into the nucleus. In the absence of Hippo signaling repressive activity, Yap and Taz localize in 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 YAP1 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 Yap and Taz function in the CNC. Using two Wnt1 Cre drivers, we uncover important functions Yap and Taz CNC proliferation and subsequent CNC 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 CNC Wnt1Cre and Wnt1Cre2SOR drivers. We collected embryos at multiple developmental stages to analyze the morphogenesis of several CNC-derived structures. Importantly, the Wnt1Cre driver (Chai et al., 2000) has been shown to induce ectopic expression of Wnt1, leading to defects in midbrain development, whereas the Wnt1Cre2SOR driver does not have these issues (Lewis et al., 2013).

We first evaluated embryos in which Yap and Taz were deleted using the Wnt1Cre driver. In control embryos, phosphorylated Yap (pYAP)—a readout for Hippo signaling activity—was present in CNC-derived cells, such as mandibular mesenchymal cells (Fig. 1A,B). However, in Wnt1Cre; 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 Wnt1Cre driver efficiently inactivated Yap and Taz in CNC-derived cells. Among the different mutant genotypes, both Wnt1Cre; Yapff; Tazf/+ and Wnt1Cre; Yap; Taz dCKO were embryonic lethal at embryonic day 10.5 (E10.5) (Table S1). Mutant embryos with the reciprocal genotype of Wnt1Cre; Yapf/++; Tazff displayed lethality over a wide range of developmental time points, from E14.5 to postnatal week 8 (Table S1).

In addition, we used the Wnt1Cre2SOR driver (Lewis et al., 2013) to inactivate Yap and Taz in CNC. Similar to Wnt1Cre embryos, lethality was observed at E10.5 in both Wnt1Cre2SOR; Yapff; Tazf/+ and Wnt1Cre2SOR; Yap; Taz dCKO embryos, whereas Wnt1Cre2SOR; Yapf/++; Tazff 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 Wnt1Cre and Wnt1Cre2SOR 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 Wnt1Cre (Fig. S1A-C), nor Wnt1Cre; Yapf/++; Tazff
embryos (Fig. S1D-F) displayed any obvious craniofacial defects. The \textit{Wnt1}^\text{Cre}; \textit{Yap}^{\text{ff}}; \textit{Taz}^{\text{f/+}} (Fig. S1G-I) and \textit{Wnt1}^\text{Cre}; \textit{Yap}; \textit{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 \textit{Wnt1}^\text{Cre}; \textit{Yap}; \textit{Taz} mutant embryos, E10.5, lethality was observed in \textit{Wnt1}^\text{Cre}^{2SOR}; \textit{Yap}; \textit{Taz} dCKO (Fig. 2A-D) and \textit{Wnt1}^\text{Cre}^{2SOR}; \textit{Yap}^{\text{ff}}; \textit{Taz}^{\text{f/+}} (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 \textit{Wnt1}^\text{Cre} compound mutant embryos. These early CNC phenotypes were not observed in \textit{Wnt1}^\text{Cre}^{2SOR}; \textit{Yap}^{\text{ff}}; \textit{Taz}^{\text{ff}} embryos that have one functional copy of \textit{Yap} or controls (Fig. 2E-L). \textit{Wnt1}^\text{Cre}^{2SOR} compound mutant embryos lacked any obvious morphologic defects at E9.5. Notably, both E10.5 \textit{Wnt1}^\text{Cre}^{2SOR}; \textit{Yap}^{\text{ff}}; \textit{Taz}^{\text{f/+}} (Fig. 2P,p) and \textit{Wnt1}^\text{Cre}^{2SOR}; \textit{Yap}; \textit{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) \textit{Wnt1}^\text{Cre}^{2SOR}; \textit{Yap}; \textit{Taz} dCKO embryos displayed open anterior neural tubes (Fig. 2D,R), and most (7/11) \textit{Wnt1}^\text{Cre}^{2SOR}; \textit{Yap}^{\text{ff}}; \textit{Taz}^{\text{f/+}} embryos presented with less severe, but still abnormal, anterior neural tube morphology (Fig. 2P,p). Neural tube defects were not observed in any of the \textit{Yap}; \textit{Taz} compound mutant embryos generated using \textit{Wnt1}^\text{Cre} (Fig. S1).

Histologic analysis indicated that, compared to control embryos (Fig. 3A-D; Fig. S2A-C), both \textit{Wnt1}^\text{Cre}; \textit{Yap}; \textit{Taz} dCKO (Fig. 3E-L; Fig. S2D-F) and \textit{Wnt1}^\text{Cre}; \textit{Yap}^{\text{ff}}; \textit{Taz}^{\text{f/+}} (Fig. 3M-P; Fig. S2 G-I) embryos had enlarged blood vessels. Moreover, the mandibular mesenchyme was disorganized in \textit{Wnt1}^\text{Cre}; \textit{Yap}; \textit{Taz} dCKO (Fig. 3G,H) and \textit{Wnt1}^\text{Cre}; \textit{Yap}^{\text{ff}}; \textit{Taz}^{\text{f/+}} (Fig. 3O,P) embryos. The sparse mesenchyme in the mandible suggests that there are fewer pericytes to be recruited from the surrounding mesenchyme due to lack of in \textit{Yap} and \textit{Taz} in CNC-derived tissues, thus deficient support from the blood vessels surrounding cells likely caused hemorrhage in mutants.

\textbf{\textit{Yap} and \textit{Taz} deletion causes severe neural tube vessel regression}

To more closely examine the hemangioma phenotype in the forebrain, branchial arch, and mandibular regions in \textit{Wnt1}^\text{Cre}; \textit{Yap}; \textit{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 \textit{Wnt1}^\text{Cre}; \textit{Yap}; \textit{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 that the vessels were 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 Wnt1Cre; Yap; Taz dCKO embryos. By comparing control embryos (Fig. 4D-F) with Wnt1Cre; Yapf/f; Tazf/f embryos (Fig. 4G-I), we detected several regions where vessel regression and/or disorganization was present in Wnt1Cre; Yapf/f; Tazf/f embryos (regions labeled as 1-4 in Fig. 4G-I), demonstrating that 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 Wnt1Cre; Yap; Taz dCKO and control embryos. RNA-Seq analysis of the mandibular tissue from Wnt1Cre; 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 Wnt1Cre; 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 Wnt1Cre; 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, including the secreted Frizzled-related protein (SFRP) Sfrp2, a Wnt inhibitor that directly binds Wnt ligands (Ladher et al., 2000). Notably, Wnt1Cre; 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 Wnt1Cre 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 Prospero Homeobox Protein 1 (Prox1) expression is upregulated in Wnt1Cre; 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 (Lindahl et al., 1997). Wnt1Cre; Yap; Taz dCKO embryos also had upregulated 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 Wnt1Cre; Yap; Taz dCKO embryos compared to 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 Wnt1Cre; 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 Wnt1Cre; 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 Wnt1Cre; 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 Yap and Taz, as well as Lats1 and Lats2, levels 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 to cells treated with control siRNA (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 control siRNA-treated cells (Fig. 6N).

Although cell proliferation was reduced in the mandible of Wnt1\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} dCKO embryos, these mutant embryos showed no obvious defects in neural tube morphogenesis. We also evaluated cell proliferation in Wnt1\textsuperscript{Cre2SOR} Yap\textsuperscript{-}; Taz\textsuperscript{-} 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\textsuperscript{Cre2SOR} Yap\textsuperscript{-}; Taz\textsuperscript{-} 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\textsuperscript{Cre2SOR} Yap\textsuperscript{-}; Taz\textsuperscript{-} dCKO and control embryos (Fig. S5).

Our RNA-Seq data indicated that the expression levels of negative regulators of apoptosis were lower in Wnt1\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} 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\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} dCKO mutant embryos and control embryos. Our TUNEL data indicated that cell apoptosis was significantly increased in E9.5 Wnt1\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} dCKO embryos compared to 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\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} dCKO embryos when compared with control embryos (Fig. 5C). Moreover, Lightsheet microscopy revealed severe vessel defects in Wnt1\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} 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\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} dCKO and Wnt1\textsuperscript{Cre}; Yap\textsuperscript{-}; Taz\textsuperscript{-} dCKO embryos, we hypothesized that Yap alone plays a critical 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 (the strategy is shown in Fig. 7A and details are provided in the Methods section). Compared to 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 Methods section), 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 is SMC differentiation perhaps because it is more highly expressed in CNC (Fig. 7C-E).

**Hydrocephalus in Yap^{f/+}; Taz^{f/f} CKO embryos**

Although Wnt1^Cre; Yap^{f/+}; Taz^{f/f} and Wnt1^Cre^{2SOR}; Yap^{f/+}; Taz^{f/f} 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. Figure S6E-G shows a representative example of an E12.5 Wnt1^Cre; Yap^{f/+}; Taz^{f/f} 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^{f/+}; Taz^{f/f} embryos (Fig. S6H,h). The hydrocephalus phenotype was detected as early as E11 in Wnt1^Cre; Yap^{f/+}; Taz^{f/f} embryos, but it was not observed in either Wnt1^Cre; Yap^{f/f}; Taz^{f/+} or Wnt1^Cre; Yap; Taz dCKO embryos, most likely because of their lethality at E10.5. Moreover, Wnt1^Cre; Yap^{f/f} embryos at E11 also had severe hydrocephalus (Fig. S8M-P) when compared to controls (Fig. S8I-L). Wnt1^Cre^{2SOR}; Yap^{f/+}; Taz^{f/f} embryos at E12.5 presented more severe hydrocephalus (Fig. S7D-F) than that in Wnt1^Cre; Yap^{f/+}; Taz^{f/f} embryos in the hindbrain region and forebrain region. Notably, our RNA-Seq data (Fig. 5) suggested that genes required for forebrain and hindbrain morphogenesis were regulated by Taz and Yap, such as Foxc1. 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. 8A-b2; coronal view in Fig. S9A-b2), yet Foxc1 expression was missing or reduced in the majority of mesenchymal cells and unchanged in the mandibular epithelial cells in Wnt1Cre; Yap; Taz dCKO embryos (sagittal view in Fig. 8C-d2; coronal view in Fig. S9C-d2). On the basis of cell counting, the number of Foxc1-positive cells was significantly lower in the Wnt1Cre; Yap; Taz dCKO mandible than in the control mandible (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 set (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, \textit{Yap} and \textit{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 \textit{Foxc1} is an important downstream target of Yap and Taz. Significantly, \textit{Foxc1} loss of function in mouse embryos phenocopies several of the morphological defects present in \textit{Yap} and \textit{Taz} mutant embryos.

Overlapping functions for \textit{Yap} and \textit{Taz} during craniofacial development
Using either a \textit{Wnt1\textsuperscript{Cre}} or \textit{Wnt1\textsuperscript{Cre2SOR}} driver, \textit{Yap} and \textit{Taz} conditional CNC mutants presented with disrupted craniofacial vascular development and hemorrhage. We also observed hydrocephalus in \textit{Yap\textsuperscript{f/+}; Taz\textsuperscript{f/f}} mutants established by using both Cre drivers, at later embryonic stages. It is most likely that \textit{Yap} and \textit{Taz} have redundant functions when they are co-expressed, but at earlier developmental stages, \textit{Taz} expression is lower than that of \textit{Yap}. Interestingly, we did observe a phenotypic difference in embryos when we deleted \textit{Yap} and \textit{Taz} using the two Cre drivers. Deleting \textit{Yap} and \textit{Taz} with the \textit{Wnt1\textsuperscript{Cre2SOR}} driver resulted in anterior neural tube closure defects at E10.5, whereas \textit{Wnt1\textsuperscript{Cre}; Yap\textsuperscript{f/f}; Taz\textsuperscript{f/+}} and \textit{Wnt1\textsuperscript{Cre}; Yap; Taz} dCKO embryos showed no neural tube closure defects. \textit{Wnt1\textsuperscript{Cre}} directs ectopic Wnt1 activity in the midbrain, whereas \textit{Wnt1\textsuperscript{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 \textit{Wnt1\textsuperscript{Cre}} and \textit{Wnt1\textsuperscript{Cre2SOR}} may be caused by elevated Wnt signaling from the \textit{Wnt1\textsuperscript{Cre}} transgene (Chai et al., 2000).

\textit{Yap} and \textit{Taz} regulate vascular development
The diminished expression of CD31 in E10.5 \textit{Yap} and \textit{Taz} mutants indicated that \textit{Yap} and \textit{Taz} have an essential role in craniofacial vascular development. We found that the multipotent O9-1 NC cells treated with siRNAs targeting \textit{Yap} and \textit{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 \textit{Yap} and \textit{Taz} mutants, suggesting that other developmental events may 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\textsuperscript{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 \textit{in vivo} setting.

\textit{Proxl}, a regulator of lymphatic development, transcripts were also elevated in \textit{Yap} and \textit{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 Proxl levels may conceivably disrupt normal vascular specification. It will be interesting to determine in future experiments whether increased \textit{Proxl} expression leads to fate switching to a predominantly lymphatic endothelial cell phenotype.

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

\textbf{The Hippo pathway regulates CNC proliferation and apoptosis}

We showed that proliferation was significantly reduced in the mandibular mesenchyme in \textit{Yap} and \textit{Taz} mutant embryos. This diminished proliferation is consistent with our observation of reduced cell density surrounding the enlarged vessels in \textit{Yap} and \textit{Taz} mutant embryos. Furthermore, a recent study showed that NC migration and fate specification were spared in Wnt1\textsuperscript{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\textsuperscript{cre2SOR}, Yap; Taz embryos, we also found that proliferation was intact in the neural tube, despite the open neural tube phenotype, indicating that other mechanisms likely account for the failure of neural tube closure.

Consistent with our \textit{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 supporting that Yap and Taz are critical for NC cell proliferation. We also showed that the knockdown of \textit{Lats1} and \textit{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.

The 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; Kidson et al., 1999; Kume et al., 2001; Tuteja and Kaestner, 2007b; Tuteja and Kaestner, 2007a; 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 Foxcl was downregulated at the mRNA and protein level in Yap and Taz mutant embryos. Foxcl 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 Foxcl in different diseases, it also has critical roles in the development of multiple organs and tissues including cerebellar, skull, ocular, and cardiovascular development (Kume, 2009). Notably, compound Foxcl 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 Foxcl display a variant of the Dandy Walker malformation, including cerebellar vermis hypoplasia (CVH). Notably, Foxcl mutant mice also have an enlarged fourth ventricle roof plate. Similar to Foxcl mutant mice, Yap; Taz dCKO mice also display cerebellar hypoplasia with an enlarged fourth ventricle roof plate. In addition, Foxcl 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 Foxcl 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 Wnt1Cre, Wnt1Cre2SOR, Yaplox/+ and Tazlox/+ mouse lines and alleles used in this study have been described previously (Chai et al., 2000; Xin et al., 2011; Lewis et al., 2013; Xin et al., 2013).

Histology and hematoxylin and eosin (H&E) 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, Minneapolis, MN, 233-FB) and 1000 U leukemia inhibitory factor (EMD Millipore, Danvers, MA, 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, Inc. (Lafayette, CO), and the transfections were performed by following a typical RNAiMAX transfection procedure (Thermo Fisher Scientific, Waltham, MA).

Yap exon 3 deletion by 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'-CACCgtggaattacgtgggtatgtt-3' (sgRNA1-forward), 5'-AAACaaacatacccacgtaatccac-3' (sgRNA1-reverse), 5'-CACCGagatggtctaatgtagtga-3' (sgRNA2-forward), and 5'-AAACtcactacattagaccatctC-3' (sgRNA2-reverse). The paired oligonucleotides were annealed and ligated into a pSpCas9(bb)-2a-GFP vector (Addgene, Cambridge, MA, 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. The 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’-GGCCATCATAGATCCTGGACG-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 (OPT) 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, St. Louis, MO) for 2 hours 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 Scientifics) and 2 parts benzyl benzoate (Acros Organics, Waltham, MA). Samples were then imaged on a home-build OPT system (BCM-OPT) (Singh et. al submitted) 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 1.38x 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 degrees, with each view taken with a 0.3-degree step size. Acquired images were reconstructed by using NRecon Reconstruction software, and 3D rendering was performed by using CT Vox 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 (Fisher Scientific, Pittsburgh, PA). The sections were rehydrated using xylene and a graded ethanol series to a final concentration of 70% and then processed for antigen retrieval.

For the immunofluorescence analysis, the antigens were retrieved by incubating the slides in citrate buffer (10 mM) for 2 minutes in a microwave oven. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) at a 1:500 dilution. Primary antibodies against phospho-histone H3 (Cell Signaling Technology, Inc., Danvers, MA) and FoxC1 (Abcam, Cambridge, MA) 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, Boston, MA) and a Zeiss LSM 510 confocal microscope.

For the immunohistochemistry analysis, the antigens were retrieved by boiling the sections in ethylenediaminetetraacetic acid for 15 minutes, and then the tissues were permeabilized by incubating the sections with 0.5% triton X-100 for 15 minutes. Afterwards, the sections were treated with 0.3% H₂O₂ for 15 minutes and blocked with 10% donkey serum for 1 hour. The sections were incubated with primary antibody against FoxC1 (Novus Biologicals, Littleton, CO) 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 hour and then developed with DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 2 minutes and 30 seconds. Sections were subsequently washed with H₂O, stained with hematoxylin for 3 minutes, and then washed with H₂O again for 5 minutes. Then, they were dehydrated with an ethanol series to a final concentration of 100% and 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% H₂O₂ / 95% methanol overnight, and rehydrated to PBS-Tween (0.1%). The embryos were blocked
for 2 hours (PBS / 5% goat serum / 0.5% Triton X-100), then incubated with an anti-CD31 antibody (MEC13.3, BD Biosciences, San Jose, CA, 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, Inc., 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 (Hama H et al., 2011, Nature Neuroscience) 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 5X 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, Irvine, CA), and 3D rendering was performed with Imaris software (Bitplane USA, Concord, MA).

**RNA-Seq and data analysis**

Mandibular tissues from *Wnt1Cre*, *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=ejajwkiyvenvjuj&acc=GSE69311.

**Chromatin immunoprecipitation (ChIP)**

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). Normal rabbit 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′- TAGTCCTATCCAGTGAGCATC -3′ (antisense).

**Real-time PCR**

For the 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 SEM.

**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 3 embryos for each genotype. For O9-1 cell counting, each experiment was performed in duplicate and all experiments were repeated 3 times or more. Image J software (National Institutes of Health, Bethesda, MD) was used to perform the cell counting and over 200 cells was counted for each experiment. The percentage of proliferating cells was determined by counting the pH3-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 ± SEM. The 2-tailed t-test was used to determine statistical significance, and $P<0.05$ was considered statistically significant.
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Competing interests
No competing interests declared.

Author contributions

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Fig. 1. Efficient deletion of *Yap* and *Taz* in CNC-derived cells. Hippo signaling activity, indicated by the level of phosphorylated Yap (pYAP), is shown in control embryos (A-B) and in *Wnt1Cre; Yap<sup>fl/fl</sup>;Taz<sup>fl/fl</sup>* 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 Wnt1Cre2SOR Cre driver. Both Wnt1Cre2SOR; Yap; Taz dCKO (A-D) and Wnt1Cre2SOR; Yap^f/f; Taz^{f/+} (M-P) embryos showed lethality at E10.5 with severe vascular
defects, hemorrhage, and defects in neural tube closure, whereas control embryos (I-L) and $Wnt1^{Cre2SOR}; Yap^{f/+}; Taz^{dCKO}$ embryos (E-H) showed no obvious defects. Zoomed-in images of branchial arch vessel defects, hemorrhage, and neural tube defects of $Wnt1^{Cre2SOR}; Yap^{f/+}; Taz^{dCKO}$ embryos are shown (m-p). Three dimensional imaging using optical projection tomography microscopy indicated that the neural tube of control embryos closed normally (Q), whereas $Wnt1^{Cre2SOR}; Yap; Taz$ dCKO embryos had a defect in neural tube closure (R).
Fig. 3. Histologic 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^f/f; Taz^f/+* (M–P) embryos had 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.
Fig. 4. Severe vessel defects caused by Yap and Taz deletion. Whole mount CD31 immunofluorescence staining in a control (A) and a Wnt1Cre; Yap; Taz dCKO mutant (B) revealed vessel defects and endothelial-lined hemangiomas in the forebrain and mandible in Wnt1Cre; Yap; Taz dCKO mutants (B). The boxed area in B is shown at a higher magnification in C, focusing in on the endothelial-lined hemangiomas in the branchial arch in the Yap; Taz dCKO embryo (C). Whereas control embryos had normal vessel development in brain (D-F), Wnt1Cre; Yap; Taz dCKO mutants had vessel regression and disorganization (different regions are labeled as 1-4). (G-I). Endothelial cells (green) are stained with CD31 antibody; smooth muscle cells (red) are stained with SMA antibody; and auto fluorescence is shown in blue.
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 Wnt1Cre Taz; Yap dKO mutants. (A) RNA-Seq analysis indicates that Yap and Taz expression levels (indicated by reads) were decreased by 40% to 50% in Wnt1Cre Taz; Yap dKO embryos compared to control embryos. (B) Gene ontology analysis shows genes that are upregulated in Wnt1Cre Taz; Yap dKO embryos compared to control embryos, which includes genes that regulate adherens junctions, vasoconstriction, and the cytoskeleton. (C) Gene ontology analysis shows genes that are downregulated in Wnt1Cre Taz; Yap dKO embryos compared to control embryos, and that negatively regulate canonical Wnt. (D) Heat map of RNA-Seq data shows that, compared to controls, Wnt1Cre Taz; Yap dKO mutants had downregulated expression of Foxc1 and upregulated expression of Foxe1, Prox1, Pdgfb, and Jak-Stat genes including Jak3, Ptk2b, Stat3, Stat5a, and Stat5b.
Fig. 6. The regulation of proliferation and apoptosis in CNC cells by the Hippo pathway. Compared to control embryos, \( Wnt^{Cre}; Yap; Taz \) dCKO embryos had a significantly diminished percentage of pHH3-positive proliferating cells in the mandible at E9.5 and E10.5 (A-J). Proliferating cells (green) were stained with pHH3 antibody; smooth muscle cells (red) were stained with SMA antibody; and nuclei (blue) were stained with DAPI. *p<0.05. (K-N) Compared to O9-1 cells transfected with control (con) siRNA, O9-1 cells transfected with \( Yap \) and \( Taz \) siRNA had significantly reduced proliferation, and O9-1 cells transfected with \( Lats1 \) and \( Lats2 \) siRNA had significantly increased proliferation (*p<0.05). Proliferating cells (green) were stained with pHH3 antibody; nuclei (red) were stained with DAPI. (O-S) Compared to control embryos, \( Wnt^{Cre}; Yap; Taz \) dCKO embryos had a significantly increased percentage of apoptotic cells in the mandible at E9.5 (*p<0.05). Apoptotic cells (green) were stained with TUNEL; nuclei (blue) were stained with DAPI. All error bars represent SEM.
Fig. 7. The requirement of *Yap* for smooth muscle differentiation. The strategy for establishing a *Yap* knockout (KO) O9-1 cell line by using CRISPR/Cas9 system (A). Specifically, exon 3 was deleted from *Yap*. Western blot data shows diminished SMA and pYap expression in *Yap* KO O9-1 cells compared to wild type (wt) O9-1 cells (B). Under differentiation conditions, most wild type O9-1 cells give rise to smooth muscle cells (C), whereas *Yap* KO O9-1 cells did not give rise to smooth muscle cells (D) Arrows designate SMA positive cells. Cell counting data show that the percentage of SMA-positive cells was significantly reduced in *Yap* KO O9-1 cells compared to wild type O9-1 cells (*p<0.01). Error bars represent SEM. (E). Smooth muscle cells (red) were stained with SMA antibody; nuclei (blue) were stained with DAPI.
Fig. 8. The 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-B, b1-b2), whereas the expression of Foxc1 was diminished in Wnt1Cre; Yap; Taz dCKO embryos (C-D, d1-d2). Boxed areas are shown at higher magnification in panels to the right, as labeled. Cell counting data show that the percentage of cells positive for Foxc1 expression in the mandible was significantly reduced in Wnt1Cre; Yap; Taz dCKO embryos compared to control embryos (E) (*p<0.01). Immunohistochemical staining of Foxc1 in O9-1 cells shows significantly decreased Foxc1 expression in Yap knockout (KO); Taz knockdown (KD) O9-1 cells compared to wild-type O9-1 cells (*p<0.05) (F-H). Arrows designate Foxc1 positive cells. Western blot analysis of Foxc1 in O9-1 cells shows decreased Foxc1 expression in response to decreased Yap and Taz expression level (I). (J) Conserved Tead binding site located in the upstream region of FOXC1. Peaks in ATAC-seq data (accession no. GSE70751) indicated chromatin accessibility (Prescott et al., 2015). (K) In vivo real-time PCR using ChIP DNA indicates that Foxc1 was bound by the Yap-Tead complex in embryonic facial tissue. p<0.05. All error bars represent SEM.