YAP regulates neural progenitor cell number via the TEA domain transcription factor

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Tight control of cell proliferation is essential for proper growth during development and for tissue homeostasis in mature animals. The evolutionarily conserved Hippo pathway restrains proliferation through a kinase cascade that culminates in the inhibition of the transcriptional coactivator YAP. Unphosphorylated YAP activates genes involved in cell proliferation and survival by interacting with a DNA-binding factor. Here we show that during vertebrate neural tube development, the TEA domain transcription factor (TEAD) is the cognate DNA-binding partner of YAP. YAP and TEAD gain of function causes marked expansion of the neural progenitor population, partly owing to their ability to promote cell cycle progression by inducing *cyclin D1* and to inhibit differentiation by suppressing *NeuroM*. Their loss of function results in increased apoptosis, whereas repressing their target genes leads to premature neuronal differentiation. Inhibiting the upstream kinases of the Hippo pathway also causes neural progenitor overproliferation. Thus, the Hippo pathway plays critical roles in regulating neural progenitor cell number by affecting proliferation, fate choice, and cell survival.

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In the developing vertebrate neural tube, neural progenitor cells reside along the ventricle and form a pseudostratified epithelium. With their ability to carry out rounds of cell divisions and to produce progeny of different fates, neural progenitor cells ultimately give rise to the vast numbers and diverse types of neurons and glia that constitute the mature nervous system (Merkle and Alvarez-Buylla 2006). Molecular pathways controlling neural progenitor cell number are not only essential for achieving the proper size and composition of the nervous system, but are also likely to have participated in the expansion of brain size during evolution (Rakic 1995). Dysregulation of these pathways can lead to malformations and/or tumorigenesis in the nervous system (Walsh 1999; Dyer 2004).

The number of neural progenitor cells can be influenced by their proliferation properties (length and rounds of cell cycles), cell fate decisions (to remain as a progenitor or to differentiate), and survival. Recently, the Hippo pathway has been shown to control cell proliferation and

survival in *Drosophila* (Saucedo and Edgar 2007). Its core components consist of two serine/threonine kinases, the Ste-20 family kinase Hippo (Hpo) and the nuclear Dbf2-related (NDR) family kinase Warts (Wts), and a transcriptional coactivator named Yorkie (Yki). Hpo phosphorylates and activates Wts, which in turn phosphorylates Yki. Phosphorylated Yki is sequestered in the cytoplasm and is incapable of activating transcription. Inactivation of either kinase or overexpression of Yki leads to the accumulation of unphosphorylated Yki, which activates genes that promote cell proliferation and survival and causes cancerous growth. Because Yki lacks an intrinsic DNA-binding activity, its target gene specificity is dictated by interactions with other factors.

In addition to the core components, adaptor proteins Salvador (Sav) and Mats facilitate the phosphorylation cascade. The cell surface protocadherin Fat and membrane-associated proteins Merlin and Expanded act as upstream activators of the pathway. Most of the identified components of the fly Hippo pathway have conserved vertebrate orthologs, and a number of the Hippo pathway components have been implicated in human cancers (Saucedo and Edgar 2007). Mice lacking *lats1*, the vertebrate ortholog of *wts*, develop soft-tissue sarcomas and ovarian tumors and are sensitized to carcinogenic treatments (St John et al. 1999). Overexpression of *YAP*, the

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vertebrate ortholog of *yki*, in the mouse liver dramatically increases liver size; likewise, its overexpression in the intestine expands the progenitor pool (Camargo et al. 2007; Dong et al. 2007). These findings suggest that individual components of the Hippo pathway have conserved functions in regulating cell proliferation and survival in vertebrates. However, the functional interactions of these components have not been studied in vivo, in particular with the goal of defining developmental signaling pathways.

A missing key component of the Hippo pathway is the DNA-binding transcription factor(s) that interacts with Yki/YAP and guides it to its target genes. YAP has been shown to interact with nine proteins/protein families in cultured mammalian cells, including Yes, Runx, EBP50, p73, p53BP-2, TEAD, 14-3-3, ErbB-4, and hnRNP U (Saucedo and Edgar 2007), at least five of which are transcription factors/cofactors. The lack of a physiological assay system has made it difficult to determine which one is the cognate partner that mediates YAP function in vivo. Here we show that the vertebrate Hippo pathway regulates neural progenitor cell number during neural tube development and the TEA domain transcription factor (TEAD) is the cognate DNA-binding partner of YAP. During the preparation of this manuscript, works in *Drosophila* and a mammalian epithelial cell line have also found that the TEAD proteins link YAP/Yki to its target genes (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008; Zhao et al. 2008).

Results

Overexpressing YAP causes a marked expansion of the neural progenitor pool

In the early vertebrate neural tube, progenitor cells and neurons occupy distinct regions along the medial–lateral axis of the neural tube, whereby progenitor cells reside medially along the ventricle and cells undergoing differentiation migrate laterally to form the mantle zone of post-mitotic neurons. In the mouse neural tube, YAP is expressed by ventricular zone progenitor cells (Milewski et al. 2004). To study the function of the Hippo pathway in vertebrate neural development, we first defined the expression pattern of YAP in the chick neural tube. At Hamburger-Hamilton (HH) stage (Hamburger and Hamilton 1992) 27–28 (equivalent to mouse embryonic day 12), YAP mRNA was detected in the ventricular zone (Fig. 1A). In the human YAP protein, the key site phosphorylated by the Hippo kinase cascade is S127 (Dong et al. 2007; Zhao et al. 2007), which corresponds to S126 in chick YAP. An antibody specific for the phosphorylated YAP (pYAP) revealed that, within the neural tube, the domain of pYAP matched the progenitor zone demarcated by the neural progenitor cell marker Sox2 (Fig. 1B,C; Bylund et al. 2003). The pYAP labeling pattern suggests that the Hippo signaling pathway is active in neural progenitor cells.

To study the function of this pathway, we electroporated a constitutive YAP expression clone into one side of the chick neural tube at HH12–14, at which time the

neural tube is composed almost entirely of progenitor cells. At 22 h post-electroporation (hpe), the transfected side exhibited reduced neuronal differentiation, as monitored by the neuronal marker Tuj1 (Fig. 1D). At 45 hpe, in addition to the reduced differentiation (data not shown), there was a marked increase in the number of Sox2⁺ progenitor cells. In mild cases, ectopic Sox2⁺ cells were found in the mantle zone of the transfected side (Fig. 1E). Most often, the transfected side filled almost entirely with Sox2⁺ cells, these cells expanded into the ventricle, accompanied by the deformation of the neural tube (Fig. 1F). The increase in progenitor cell number became even more evident 3 d post-transfection, with the transfected side being significantly broader than the control side (Fig. 1G). The ectopic Sox2⁺ cells in the mantle zone continued to proliferate and were labeled by 5-bromodeoxyuridine (BrdU) (Fig. 1H). In the neural tube, progenitor cells form a pseudostratified epithelium with their apical ends anchored at the ventricular surface via adherens junctions, and their nuclei oscillate across the width of the ventricular zone, where they undergo mitosis at the ventricular surface (Gotz and Huttner 2005). The presence of ectopic phospho-histone H3⁺ mitotic cells (pH3) (Fig. 1I) that became self-organized around foci of aPKC⁺ adherens junctions (Fig. 1J) indicated that YAP overexpression had induced the formation of rosettes similar to the Homer-Wright rosettes found in many kinds of human neurological tumors (Dyer 2004). These results provide evidence that overactivation of YAP, a critical effector of the Hippo pathway, causes an unchecked expansion of the neural progenitor pool.

A transcriptionally active form of TEAD dramatically increases neural progenitor cell number

Although YAP has been shown to interact with at least five transcription factors/cofactors in cultured cells, including Runx, TEAD, ErbB-4, p73, and hnRNP U, which one mediates YAP function in vivo remains unknown. Five lines of evidence suggest that TEAD may participate in YAP-mediated gene regulation in neural progenitor cells. First, its DNA-binding domain is conserved across all eukaryotes (Anbanandam et al. 2006); thus it potentially could be a component of the evolutionarily conserved Hippo pathway. Second, homozygotes of strong alleles of *scalloped* (*sd*), which encodes the *Drosophila* TEAD protein, die as first instar larvae (Campbell et al. 1992), similar to *yki*-null mutants (Huang et al. 2005). Third, the four mammalian TEAD proteins, TEAD1–4, are all expressed in the ventricular zone of the neural tube (Milewski et al. 2004). Fourth, mouse TEAD has been shown to activate the promoter of Pax3, a transcription factor expressed by neural progenitor cells (Milewski et al. 2004). Finally, TEAD proteins have been shown to lack intrinsic transactivation activity and require an obligate coactivator to turn on transcription (Vassilev et al. 2001).

At least three TEAD proteins—TEAD1, TEAD3, and TEAD4—are encoded in the chick genome (Azakie et al.

Figure 1. Overexpressing YAP in the neural tube results in reduced differentiation and a marked expansion of the progenitor population. (A) In situ hybridization shows YAP expression in the ventricular zone of HH27–28 chick neural tube. (B,C) Phosphorylated YAP (pYAP) was detected in neural progenitor cells marked by Sox2 immunostaining. The boxed area in B is shown at higher magnification in C. (D–G) Transfecting YAP caused a reduction in TuJ1⁺ neurons (D, arrows) and an increase in Sox2⁺ progenitor cells (E–G, arrows). In all cases, the right side of the neural tube was transfected. Images on the left of the dashed lines show the full neural tube cross sections, those on the right only the transfected side. (D–F) Overexpressed YAP was detected by a YAP antibody, which was not sensitive enough to detect endogenous YAP. (G) At 69 hpe, the transfected side was marked by cotransfected GFP. (H–J) Ectopic progenitor cells continued to proliferate at 69 hpe (shown in H). They divided at the mantle zone (I, arrows) and formed tumor-like rosettes with foci of aPKC⁺ adherens junctions (J, arrows; areas in dashed squares are shown at higher magnification in the images to the right of the dashed line).

1996). With an antibody specific for TEAD1 (Supplemental Fig. S1A), we examined its expression pattern in the HH27–28 neural tube. At cervical, brachial, and lumbar levels, TEAD1 was expressed in the ventricular zone but not in the TuJ1⁺ mantle zone (Fig. 2A). Closer examination revealed that the cell domain expressing TEAD1 was slightly broader than the progenitor zone marked by Sox2 and pYAP (Fig. 2B; Supplemental Fig. S1B). Some TEAD1⁺ cells also expressed the proneural basic-helix-loop-helix (bHLH) transcription factor Neurogenin 2 (Ngn2) (Fig. 2B; Ma et al. 1996). At the thoracic level, TEAD1 was also present in a group of cells located at the ventral-lateral region of the neural tube (Supplemental Fig. S1C–E). We also detected *TEAD4* mRNA in the ventricular zone of the chick neural tube (Supplemental Fig. S1F). Unfortunately, several in situ hybridization probes we tested for *TEAD3* did not yield specific signals, and therefore we cannot yet conclude whether *TEAD3* is expressed in the chick neural tube. We confirmed the interaction between YAP and TEAD in neural progenitor cells by performing coimmunoprecipitation in cell lysates of HH27–28 forebrain and midbrain, where both YAP and TEAD1 are expressed in the ventricular progenitor zone (Supplemental Fig. S1G; data not shown).

As a first step in testing the hypothesis that TEAD is the cognate DNA-binding factor that mediates YAP function in the neural tube, we examined whether increasing TEAD levels could mimic the effect of YAP overexpression. We did not detect a change in neural progenitor proliferation or differentiation following TEAD1 overexpression (Fig. 2C,D,M). This result is consistent with TEAD proteins lacking intrinsic transactivation activity. It also implies that the amount/availability of the coactivator is limited in neural progenitor cells.

If TEAD mediates the function of YAP, expressing a transcriptionally active form of TEAD should recapitulate the phenotype of YAP overexpression. We engineered such a TEAD1 construct by replacing the YAP-binding domain (Vassilev et al. 2001) with the activation domain (AD) of VP16 (TEA-VP16) (Fig. 2E), as previous studies in cultured cells have shown that the activity of YAP AD is as potent as that of VP16 AD (Yagi et al. 1999). Structural studies suggest that the guanidino group of R59 (the 59th amino acid of the TEA domain) is likely to contact DNA directly (Anbanandam et al. 2006). We therefore generated a DNA binding-defective control by mutating R59 into lysine (TEA^{R59K}-VP16) (Fig. 2E). An allele of *Drosophila* *sd* harboring the same

Figure 2. Overexpressing a transcriptionally active form of TEAD1 increases neural progenitor cell number. (A,B) TEAD1 expression pattern examined by immunostaining. The region examined in B corresponds to that within the dashed square in A. (C,D) Overexpressing TEAD1 did not significantly affect differentiation (C) or progenitor cell number (D). (E) Domain structures of TEAD1 and derivatives. Mammalian and avian TEAD proteins are composed of an N-terminal TEA domain and a C-terminal YAP-binding domain (YAP BD) connected by a linker region. (VP16AD) The activation domain of VP16. (F–K) Reduced neuronal differentiation (F, arrows) and increased progenitor cells (G–I, arrows) upon TEA-VP16 expression. Transfected side showed severe dysplasia with disruptions of ventricular surface and the formation of tumor-like rosettes (K, arrows; areas in dashed squares are shown at higher magnification in the images right of the dashed line). (L) TEA^{R59K}-VP16 did not have a strong effect on progenitor cell number. (C,F–H) TEAD constructs have an N-terminal Myc tag and were detected by a Myc antibody. (D,I,L) At 69 hpe, the transfected side was marked by cotransfected GFP. (M) Quantifications comparing the transfected side with the control side. In this and all following graphs, results are mean $\pm$ SEM. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

mutation is a strong loss-of-function allele (Srivastava et al. 2004). Introducing TEA-VP16 into the neural tube caused phenotypes similar to YAP overexpression: Neuronal differentiation was reduced at 22 hpe (Fig. 2F,M); there was a marked expansion in progenitor cells at 45 hpe, often accompanied by the deformation of the neural tube (Fig. 2G,H,M); and the overproliferation phenotype became even more severe at 69 hpe with the formation of

tumor-like rosettes (Fig. 2I–K,M). In contrast, TEA^{R59K}-VP16 did not significantly alter progenitor cell number (Fig. 2L,M), suggesting that the effect caused by TEA-VP16 is dependent on DNA binding. Despite the structural distortion of YAP- and TEA-VP16-transfected neural tubes, dorsal–ventral patterning, determined by the expression of regional markers Pax3, Pax7, and Pax6, was largely normal (data not shown).

Misexpressing YAP^{Δ60-89} fails to expand the neural progenitor pool

The above results showed that constitutive expression of YAP and the active form of TEAD caused identical phenotypes. To test whether YAP exerts its effect through TEAD, we generated a YAP mutant by removing 30 amino acids within the TEAD-binding domain (YAP^{Δ60-89}) (Fig. 3A; Vassilev et al. 2001). This deletion is unlikely to perturb the interactions between YAP and other known transcription partners, which are mediated by either the WW domain or the N terminus of YAP (Yagi et al. 1999; Strano et al. 2001; Komuro et al. 2003; Howell et al. 2004). A coimmunoprecipitation experiment confirmed that this mutant no longer interacted with TEAD1 (Fig. 3B). In contrast to the severe phenotype caused by YAP overexpression, neural tubes transfected with YAP^{Δ60-89} were largely normal (Fig. 3C,D). This result supports the hypothesis that YAP functions through binding to TEAD, although we cannot rule out that the deletion may abolish the interaction between YAP and other unidentified partners.

The increase in progenitor cell number results from accelerated proliferation and reduced cell cycle exit

We next analyzed the mechanisms responsible for the large expansion of neural progenitors associated with

YAP and TEA-VP16 misexpression. An increase in cell number can result from accelerated proliferation, decreased cell cycle exit, and/or reduced cell death. The level of cell death in the early neural tube is very low (Kuida et al. 1998). Thus, reducing cell death is unlikely to have contributed significantly to the large increase in cell number. The reduction in neuronal differentiation upon YAP and TEA-VP16 overexpression suggests that cell cycle exit was decreased. At early stages of neural tube development, most cell divisions produce two progenitor cells (Morin et al. 2007). Thus, an increase in the rate of proliferative divisions can exponentially increase cell number. We assessed the cell cycle length of transfected progenitor cells by 30-min BrdU pulse-labeling: An increase in the percent of cycling cells that are labeled with BrdU indicates the cell cycle length has shortened. We quantified the fraction of BrdU⁺ cells among the transfected cells that remained cycling at the time of labeling (22 hpe) and compared this fraction with that of the control side to obtain a BrdU-labeling ratio. We used Sox2 as a marker for cycling cells because all Sox2⁺ cells express the proliferating cell marker PCNA at this stage (data not shown). Thus, the BrdU labeling ratio for YAP-transfected neural tubes is $(\text{BrdU}^+\text{YAP}^+\text{Sox2}^+/\text{YAP}^+\text{Sox2}^+)/(\text{BrdU}^+\text{Sox2}^+/\text{Sox2}^+)_{\text{control side}}$ and that for TEA-VP16-transfected ones is $(\text{BrdU}^+\text{Myc}^+\text{Sox2}^+/\text{Myc}^+\text{Sox2}^+)/(\text{BrdU}^+\text{Sox2}^+/\text{Sox2}^+)_{\text{control side}}$. Both YAP- and TEA-VP16-transfected neural tubes had a BrdU-labeling ratio of more than 1 (1.26 ± 0.07 , $n = 18$ for YAP; 1.29 ± 0.06 , $n = 14$ for TEA-VP16) (Fig. 4A), suggesting that the transfected cells have a shortened cell cycle length. In comparison, the cell cycle length of YAP^{Δ60-89}- and TEA^{R59K}-VP16-transfected cells was not significantly different from that of untransfected cells (BrdU labeling ratio was 1.03 ± 0.06 , $n = 15$ for YAP^{Δ60-89} and 0.98 ± 0.07 , $n = 11$ for TEA^{R59K}-VP16). Together, these results suggest that the increase in neural progenitor cell number was caused by accelerated cell cycle progression and reduced cell cycle exit.

Figure 3. YAP^{Δ60-89} cannot interact with TEAD and fails to increase neural progenitor cell number. (A) Domain structures of YAP and the deletion construct. Chick YAP is composed of an N-terminal TEAD-binding domain (TEAD BD), a central WW domain, and a C-terminal activation domain (AD). (B) A coimmunoprecipitation experiment shows that YAP^{Δ60-89} could not interact with TEAD1. 293T cells were transfected with TEAD1 and HA-YAP or HA-YAP^{Δ60-89}. Cell lysates were immunoprecipitated (IP) with an HA antibody and blotted with a TEAD1 antibody. (C) Overexpressing YAP^{Δ60-89} did not significantly increase neural progenitor cells. The transfected side was marked by cotransfected GFP. (D) Quantifications comparing the effects of misexpressing YAP and YAP^{Δ60-89}.

Overexpressing YAP and TEA-VP16 induces Cyclin D1 expression

We next searched for the molecular mechanism underlying the changes in cell cycle parameters. Hyperactivating YAP has been shown to induce *cyclin D1* and *c-Myc* expression in the intestine and liver, respectively (Camargo et al. 2007; Dong et al. 2007). We therefore surveyed a number of genes that have been implicated in cell cycle control, including *cyclin D1*, *cyclin D2*, *c-Myc*, and *N-Myc*. Among them, *cyclin D1* mRNA was strongly up-regulated upon YAP and TEA-VP16 overexpression (Fig. 4B,C; cf. Supplemental Fig. S2).

D-type cyclins (D1, D2, and D3), in response to mitogenic signals, act during early to mid-G1 phase to drive cell cycle progression (Massague 2004). We asked whether *cyclin D1* up-regulation was responsible for the phenotypes associated with YAP and TEA-VP16 overexpression. In cultured cells, overexpressing cyclin D1 has been shown to shorten cell cycle length (Quelle et al. 1993).

Figure 4. YAP and TEA-VP16 overexpression promotes cell cycle progression. (A) Overexpressing YAP and TEA-VP16 accelerated the cell cycle. (B,C) In situ hybridization (ISH) reveals up-regulation of *cyclin D1* by YAP and TEA-VP16 transfection. YAP and TEA-VP16 cDNAs are followed by IRES-GFP to monitor their expression. The image next to the ISH photograph shows GFP expression in an adjacent section. (D,E) Overexpressing cyclin D1 reduced differentiation (D, arrows) and increased progenitor cell number (E, arrows). Cyclin D1 cDNA is followed by IRES-GFP to monitor its expression. (F) Quantifications comparing the effect of cyclin D1 overexpression with that of YAP and TEA-VP16. Neural tubes transfected with TEA^{R59K}-VP16 serve as the baseline.

Thus, *cyclin D1* up-regulation likely accounts for the accelerated cell cycle rate in YAP- and TEA-VP16-transfected neural tubes. Consistent with a previous report (Lobjois et al. 2004), we found that forced expression of cyclin D1 inhibited neuronal differentiation at 22 hpe (Fig. 4D,F). At 45 hpe, there was a substantial increase in the number of Sox2⁺ cells in the transfected side ($26 \pm 2\%$, $n = 10$) (Fig. 4E,F). The scale of the increase was comparable with that caused by the overexpression of YAP ($27 \pm 3\%$, $n = 10$) and TEA-VP16 ($27 \pm 4\%$, $n = 10$). The morphological effect of cyclin D1 overexpression, however, was quite different from that of YAP and TEA-VP16. Al-

though we always observed expansion in the width of the ventricular zone (Fig. 4E, arrows), we found fewer ectopic Sox2⁺ cells in the mantle zone and never encountered deformations of the neural tube like those caused by YAP and TEA-VP16 overexpression. By 69 hpe, the increase in Sox2⁺ cells, though still present, was much diminished ($12 \pm 2\%$, $n = 6$) and was significantly lower than that caused by YAP and TEA-VP16 transfection (Fig. 4F). Together, these data suggest that *cyclin D1* up-regulation could partially account for the overproliferation phenotype caused by YAP and TEA-VP16 overexpression.

YAP and TEAD loss of function leads to increased cell death

To study the effect of YAP loss of function in the neural tube, we generated two shRNA plasmids. Both shRNAs inhibited the expression of cotransfected YAP cDNA in chick fibroblasts (Supplemental Fig. S3A). Upon introduction into the neural tube, they reduced the level of endogenous YAP mRNA (Fig. 5A,D). The transfected side was much thinner than the control side at 45 hpe, suggestive of increased cell death or/and reduced proliferation. TUNEL assays revealed that both shRNAs caused a marked increase in cell death at 22 hpe (Fig. 5B,E,F). Two observations suggest this phenotype is specific. First, cotransfection of mouse YAP cDNA (mYAP), which is insensitive to the chick YAP shRNAs, completely rescued the cell death phenotype (Fig. 5C,F). Second, a control shRNA consisting of the scrambled sequence of shYAP#1 did not cause significant cell death (Fig. 5F).

To confirm these findings, we employed an independent method to inhibit YAP function by expressing a dominant-negative construct. We first created a truncation mutant (YAP²⁷⁹) in which the activation domain of YAP was removed. We reasoned that, upon misexpression, YAP²⁷⁹ should compete with endogenous YAP for binding to its partners but lack the ability to activate transcription, thereby inhibiting the function of endogenous YAP. We transfected YAP²⁷⁹ into the neural tube but did not detect increased cell death or any other effects (data not shown). When we checked the subcellular localization of YAP²⁷⁹, we found that it was almost exclusively cytoplasmic (data not shown). Thus it is possible that the amount of nuclear YAP²⁷⁹ was insufficient to compete with endogenous YAP. We therefore attached a nuclear localization signal (NLS) to YAP²⁷⁹ (YAP_N²⁷⁹) (Fig. 5G). Consistent with the cell death phenotype triggered by shRNA-mediated knockdown, misexpressing YAP_N²⁷⁹ caused a fivefold increase in cell death (Fig. 5H,J). In addition to the increased TUNEL signals, cleaved caspase 3 was strongly induced by YAP_N²⁷⁹ (Supplemental Figs. S3B). A deletion within YAP_N²⁷⁹ that abolishes the interaction between YAP and TEAD (YAP_N^{279Δ}) (Fig. 5G) greatly reduced the apoptotic effect (Fig. 5I,J), suggesting that the dominant-negative activity of YAP_N²⁷⁹ was probably achieved by competing with endogenous YAP for TEAD binding.

Figure 5. YAP and TEAD loss of function in the neural tube leads to increased cell death. (A) ISH shows reduced *YAP* mRNA level in shYAP#1-transfected neural tube (arrows). The shRNA vector also encodes dsRED to mark transfected cells. The image next to the ISH photograph shows dsRED expression in an adjacent section. Blue signals are DAPI. (B,C) TUNEL assay detected increased cell death in shYAP#1-transfected side (B, arrows). The cell death phenotype was rescued by cotransfecting mYAP (C). (D,E) shYAP#2 reduced *YAP* mRNA level (D, arrows) and caused increased cell death (E, arrows). (F) Quantifications of the cell death phenotype. For each embryo, the sum of TUNEL signals in the transfected side (four to 10 sections per embryo) was divided by that in the control side to obtain a TUNEL ratio. (G) Domain structures of YAP dominant-negative constructs. The pink oval represents NLS. (H–J) YAP²⁷⁹, but not YAP^{279Δ}, caused increased cell death (H, arrows). The transfected side was marked by cotransfected GFP. (K) Domain structures of TEAD dominant-negative constructs. (L–N) TEA, but not TEA^{R59K} or TEA^{R59K}, triggered increased cell death (arrows in L). The transfected side was marked by cotransfected GFP. (O–Q) ISH shows inhibiting YAP and TEAD did not significantly affect *cyclin D1* expression. Transfection efficiency was monitored by the expression of dsRED encoded in the shRNA vector (O) or cotransfected GFP (P,Q) in adjacent sections. (R) Inhibiting YAP and TEAD with YAP²⁷⁹ and TEA, respectively, did not significantly affect BrdU incorporation during a 2-h labeling period. Transfected embryos were harvested at 22 hpe. Neural tubes transfected with d4EGFP_N were used as the control.

Due to the presence of multiple TEAD proteins in the neural tube and the difficulty we encountered when trying to knock them down simultaneously with shRNAs, we resorted to the dominant-negative approach to study the

loss-of-function effect of TEAD proteins. The dominant inhibitor, TEA, contains only the TEA domain and surrounding sequences of TEAD1 but lacks the YAP-binding domain (Fig. 5K). It should compete with endogenous

TEAD proteins for DNA binding but lack the ability to activate transcription. For the negative control, we introduced the R59K mutation that abolishes/weakens the DNA-binding ability of TEA domain (TEA^{R59K}) (Fig. 5K). Upon examining the subcellular localization of these proteins in transfected neural tubes, we found that, while TEA was enriched in the nucleus, TEA^{R59K} was present in both the nucleus and the cytoplasm (data not shown). Therefore, we attached a NLS to its N terminus (TEA_N^{R59K}) (Fig. 5K). Overexpression of TEA, but not TEA^{R59K} or TEA_N^{R59K}, caused a marked increase in apoptosis detected by TUNEL assays (Fig. 5L–N) and immunostaining for cleaved caspase 3 (Supplemental Fig. S3C).

We next investigated whether YAP and TEAD loss of function affected proliferation. Since YAP and TEA-VP16 overexpression strongly elevated *cyclin D1* mRNA levels, we examined whether their loss of function diminished *cyclin D1* expression. To our surprise, we did not detect a decrease in *cyclin D1* mRNA in neural tubes transfected with YAP shRNAs, YAP inhibitor YAP_N²⁷⁹, or TEAD inhibitor TEA at 8, 12, 16, and 22 hpe (Fig. 5O–Q; Supplemental Fig. S3F–I; data not shown). We also measured proliferation by BrdU labeling. The fraction of YAP_N²⁷⁹- and TEA-transfected cells that incorporated BrdU was the same as that of cells transfected with the control plasmid d4EGFP_N (nuclear-localized destabilized EGFP) (Fig. 5R; Supplemental Fig. S3D,E). We also evaluated neuronal differentiation with markers including Tuj1, neurofilament-associated protein (NF), and Lim1/2 and did not observe obvious phenotypes in neural tubes transfected with YAP shRNAs, YAP_N²⁷⁹, or TEA except for a small decrease in the number of cells expressing these markers (data not shown), which was probably due to the increased cell death. Dorsal–ventral patterning of transfected neural tubes also appeared normal (data not shown). Together, these findings suggest that cell cycle progression and cell fate specification can operate in the absence of YAP and TEAD function.

Repressing YAP and TEAD target genes induces cell cycle exit and neuronal differentiation

Next, we investigated the effect of repressing YAP and TEAD downstream genes. We replaced the activation domain of YAP and the YAP-binding domain of TEAD1 with the transcription repression domain of *Drosophila* Engrailed (YAP²⁷⁹-EnR and TEA-EnR; Fig. 6A). Consistent with YAP and TEAD loss-of-function phenotypes, introducing each construct into the neural tube induced cell death at 22 hpe (data not shown). Strikingly, in contrast to YAP loss of function, which had no obvious effect on proliferation, most cells transfected with YAP²⁷⁹-EnR exited the cell cycle by 22 hpe, as shown by the lack of BrdU incorporation (Fig. 6B,H). Most transfected cells had migrated to the mantle zone and were expressing neuronal markers NF and Lim1/2 (Fig. 6B–D,H). There was an increase in the number of neurons in the transfected side compared with the control side (Fig. 6H, right

graph), suggesting that YAP²⁷⁹-EnR promoted neuronal differentiation. These phenotypes were absent in neural tubes transfected with the deleted version, YAP^{279Δ}-EnR (Fig. 6H; Supplemental Fig. S4A–D). Misexpressing TEA-EnR, but not TEA^{R59K}-EnR, also triggered cell cycle exit and neuronal differentiation, and the differentiation phenotype was even stronger (Fig. 6E–H; cf. Supplemental Fig. S4E–G).

Next we examined the earlier events that led to neuronal differentiation. As early as 8 hpe, both YAP²⁷⁹-EnR and TEA-EnR had repressed *cyclin D1* expression (Fig. 6I,L). Although we did not observe a reduction in the mRNA levels of *Hes1* and *Hes5*—bHLH factors that inhibit neuronal differentiation and maintain the progenitor population (Hatakeyama et al. 2004)—or an up-regulation of the proneural gene *Ngn2* (data not shown), the expression of the neurogenic bHLH factor *NeuroM/NeuroD4/Math3* (Roztocil et al. 1997; Lee and Pfaff 2003) was strongly induced by TEA-EnR at 8 hpe (Fig. 6M). Induction of *NeuroM* by YAP²⁷⁹-EnR was weak at 8 hpe (data not shown) but became much stronger at 12 hpe (Fig. 6J). The linking of neuronal differentiation to *NeuroM* regulation prompted us to check whether *NeuroM* expression was affected in the opposite manner in YAP- and TEA-VP16-transfected neural tubes, in which differentiation was inhibited. Indeed, both YAP and TEA-VP16 overexpression reduced *NeuroM* mRNA levels (Fig. 6K,N) without inducing *Hes1* or *Hes5* expression (data not shown). Together, these data demonstrate that repressing YAP and TEAD target genes leads to cell cycle exit and neuronal differentiation. These effects are, at least partially, due to the down-regulation of *cyclin D1* and the induction of *NeuroM*.

Epistasis analyses of YAP and TEAD

Our manipulations of YAP and TEAD functions, either through gain-of-function approaches, loss-of-function approaches, or by repressing their target genes, yielded similar results for these two proteins, strongly suggesting that YAP and TEAD act in the same pathway. The deletion mutants of YAP (Δ60–89) that cannot interact with TEAD were inactive in all three settings, indicating that YAP operates through binding to TEAD. To further confirm that TEAD is the DNA-binding partner of YAP in the neural tube, we performed double-transfection experiments to test their epistatic relationship.

If YAP functions by acting as the coactivator of TEAD, then the active form of TEAD, TEA-VP16, should be able to rescue YAP loss of function. Indeed, cotransfection of TEA-VP16 with YAP shRNA largely blocked the cell death phenotype (Fig. 7A,B). Conversely, when YAP was cotransfected with TEA, the dominant inhibitor of TEAD proteins, it was unable to significantly reduce the apoptosis triggered by TEAD loss of function (Fig. 7C,D). Moreover, whereas YAP overexpression inhibited differentiation, cotransfection of TEA-EnR completely reversed this effect, resulting in most transfected cells expressing neuronal markers (Fig. 7E,G), a phenocopy of the TEA-EnR single transfection (Fig. 6F–H). Likewise, cotrans-

Figure 6. Repressing YAP and TEAD downstream genes leads to cell cycle exit and neuronal differentiation. (A) Domain structures of YAP²⁷⁹-EnR and TEA-EnR. EnR: the repression domain of Engrailed. (B–H) Repressing YAP and TEAD target genes through YAP²⁷⁹-EnR and TEA-EnR, respectively, promoted cell cycle exit (B,E,H) and neuronal differentiation (C,D,F,G, arrows, H). YAP²⁷⁹-EnR was detected by a YAP antibody. TEA-EnR has a Myc tag and was detected by a Myc antibody. (I–N) ISH shows that YAP²⁷⁹-EnR and TEA-EnR repressed *cyclin D1* (I,L, arrows) and induced *NeuroM* (J,M, arrows), whereas overexpressing YAP and TEA-VP16 reduced *NeuroM* mRNA levels (K,N, arrows). Transfection efficiency was monitored by GFP expression—either from the cotransfected GFP-expressing plasmids (I,J,L,M) or the downstream IRES-GFP (K,N)—in adjacent sections.

fection of YAP²⁷⁹-EnR and TEA-VP16 led to reduced differentiation (Fig. 7F,G), similar to the effect of TEA-VP16 overexpression and opposite to that of YAP²⁷⁹-EnR. These experiments establish that TEAD is epistatic to YAP and YAP functions through TEAD.

The upstream kinases of the Hippo pathway regulate neural progenitor proliferation and survival

Finally, we investigated whether the upstream kinases of the Hippo pathway regulate neural progenitor cell number. The vertebrate orthologs of *hpo*, *Mst1* and *Mst2*, and those of *wts*, *Lats1* and *Lats2*, are all widely expressed (Creasy and Chernoff 1995a,b; Tao et al. 1999; McPherson et al. 2004). Studies using cultured cells or recombi-

nant proteins have confirmed that the Hpo–Wts–Yki phosphorylation cascade is conserved for the vertebrate orthologs (Chan et al. 2005; Dong et al. 2007; Zhao et al. 2007). However, the functions of Mst1/2 and Lats1/2 in neural development are largely unexplored.

To inhibit Mst1/2 function, we generated a kinase-dead version of Mst2 (Mst2^{KD}), which has been shown to have a dominant-negative effect on the endogenous kinase (Wu et al. 2003). Transfecting Mst2^{KD} into the neural tube induced *cyclin D1* expression at 22 hpe (Fig. 8A). At 69 hpe, the ventricular zone of the transfected side became noticeably wider than the control side, with clusters of ectopic Sox2⁺ cells in the mantle zone (Fig. 8B). The number of Sox2⁺ cells increased $14 \pm 1\%$ ($n = 15$) compared with the untransfected side (Fig. 8E).

Figure 7. Epistasis analyses of YAP and TEAD. (A,B) TUNEL assays show that cotransfecting TEA-VP16 with YAP shRNAs rescued the cell death phenotype. Transfected cells were marked by dsRED expressed from the shRNA vector. TUNEL ratios are shYAP#1: 3.8 ± 0.4 , $n = 12$; shYAP#1 + TEA-VP16: 1.6 ± 0.3 , $n = 4$; shYAP#2: 4.7 ± 0.8 , $n = 9$; shYAP#2 + TEA-VP16: 1.7 ± 0.2 , $n = 6$. (C,D) Cotransfecting YAP with TEA could not rescue the cell death phenotype (arrows in C). YAP cDNA is followed by IRES-GFP to mark transfected cells. TUNEL ratios are TEA: 4.4 ± 0.4 , $n = 6$; TEA + YAP: 3.1 ± 0.4 , $n = 11$. (E–G) Cotransfecting TEA-EnR with YAP increased neuronal differentiation (E, arrows), whereas cotransfecting TEA-VP16 with YAP²⁷⁹-EnR reduced it (F, arrows). In both cases, the phenotype resembled that of the single transfection of the TEAD variant and opposite to that of the YAP variant. A YAP antibody was used to label YAP- and YAP²⁷⁹-EnR-transfected cells.

We also engineered a kinase-dead mutant of Lats2 (Lats2^{KD}; Chan et al. 2005) as well as an shRNA targeting *Lats1* (shLats1) (Supplemental Fig. S5). Cotransfection of shLats1 and Lats2^{KD}, similar to Mst2^{KD} misexpression, increased *cyclin D1* mRNA levels and the number of Sox2⁺ cells (Fig. 8C–E). These results are consistent with the model that endogenous Mst1/2 and Lats1/2 inhibit YAP activity in the neural tube. Two possibilities may account for the lesser severity of the

overproliferation phenotype as compared with that caused by YAP overexpression. First, our loss-of-function approaches might have only partially reduced the activities of endogenous kinases. Second, the amount of active YAP resulted from Mst1/2 and Lats1/2 loss of function, which relieves the inhibition on endogenous YAP, may be much less than that obtained by YAP overexpression.

Conversely, in neural tubes overexpressing Mst2, but not Mst2^{KD}, cell death was significantly increased (Fig. 8F,J), whereas proliferation and differentiation appeared largely normal (data not shown). This phenotype closely resembles YAP and TEAD loss of function, in agreement with the model that Mst1/2 inhibit YAP activity through a phosphorylation-dependent event. However, one caveat is that, in addition to Lats1/2 (and hence the Hippo pathway), Mst1/2 have been shown to phosphorylate a number of substrates and lead to apoptosis (Matallanas et al. 2008). It is thus possible that the apoptosis phenotype in Mst2-transfected neural tubes was not due to YAP inhibition but rather to other Mst1/2 substrates. We therefore tested whether YAP and TEAD could rescue Mst2-induced cell death. Cotransfection of TEA-VP16 and the constitutively active form of YAP (YAP^{S126A}), but not wild-type YAP, with Mst2 greatly reduced the amount of cell death (Fig. 8G–J). These experiments suggest that Mst2 overexpression triggers cell death by inhibiting YAP function.

In summary, both loss-of-function and gain-of-function studies demonstrate that Mst1/2 and Lats1/2, the upstream components of the Hippo pathway, regulate neural progenitor proliferation and survival by inhibiting the activity of YAP.

Discussion

The Hippo pathway plays important roles in the control of cell proliferation and survival. Although extensively studied in fly imaginal discs, the function of this pathway during vertebrate development is largely unknown. The most critical effector of the Hippo pathway is the transcriptional coactivator Yki/YAP, which activates downstream genes via a DNA-binding transcription factor. We found that during vertebrate neural tube development, TEAD is the cognate DNA-binding factor that bridges YAP and its target genes (Fig. 8K). Furthermore, we showed that the Hippo pathway regulates the number of neural progenitor cells by affecting their proliferation, fate choice, and survival.

TEAD is the DNA-binding factor of the Hippo pathway during vertebrate neural tube development

We presented five lines of evidence demonstrating that, during neural tube development, TEAD is the DNA-binding partner of YAP. First, overexpressing YAP and a transcriptionally active form of TEAD in the neural tube both led to reduced neuronal differentiation and a marked increase in progenitor cell number. Up-regulation of *cyclin D1*, repression of *NeuroM*, accelerated pro-

Figure 8. Mst1/2 and Lats1/2 regulate neural progenitor proliferation and survival. (A–E) Inhibiting Mst1/2 with Mst2^{KD} and Lats1/2 with shLats1 and Lats2^{KD} caused an up-regulation of *cyclin D1* expression at 22 hpe (A,C, arrows) as well as a widening of the Sox2⁺ progenitor zone and the appearance of ectopic Sox2⁺ cells in the mantle zone at 69 hpe (B,D, arrows). Blue signals are DAPI. (E) “Sox2 ratio” was obtained by dividing Sox2 area in the transfected side by that in the control side. Neural tubes transfected with dsRED was used as the baseline for statistics analysis. (F–I) Overexpressing Mst2 induced cell death (arrows in F). Cotransfection of TEA-VP16 and YAP^{S126A}, but not YAP, reduced Mst2-induced cell death. Transfected side was marked by cotransfected GFP. TUNEL ratios are Mst2: 3.5 ± 0.3 , $n = 5$; Mst2^{KD}: 0.98 ± 0.1 , $n = 4$; Mst2 + TEA-VP16: 1.39 ± 0.08 , $n = 4$; Mst2 + YAP: 4.9 ± 0.4 , $n = 5$; Mst2 + YAP^{S126A}: 1.7 ± 0.2 , $n = 5$. (K) A simplified model illustrating that the Hippo pathway regulates neural progenitor cell number through TEAD. Mst kinases phosphorylate and activate Lats kinases, which phosphorylate YAP and inhibit its nuclear translocation. Unphosphorylated YAP enters the nucleus and interacts with the DNA-binding factor TEAD. Together, they activate genes that promote proliferation, survival, and the progenitor fate.

liferation, and disruption of the neuroepithelial structure were observed in both cases. Second, YAP and TEAD loss of function also resulted in the same phenotype: increased cell death without severe defects in proliferation and differentiation. Third, repressing the downstream genes of YAP and TEAD both led to down-regulation of *cyclin D1* and induction of *NeuroM*, resulting in cell cycle exit and neuronal differentiation. These three lines of evidence suggest that YAP and TEAD converge on the same set of target genes. The fourth line of evidence is based on a deletion within YAP that abolishes its interaction with TEAD. This mutant abrogated all of the above phenotypes, suggesting that TEAD binding is important for YAP function. Fifth, the active form of TEAD was able to rescue YAP loss of function, whereas YAP could not res-

cue TEAD loss of function, indicating that TEAD is epistatic to YAP. Our finding is consistent with recent works in *Drosophila* and a mammalian epithelial cell line that also identified the TEAD proteins as the bridge between YAP/Yki and its target genes (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008; Zhao et al. 2008).

The vertebrate Hippo pathway regulates neural progenitor proliferation and fate choice

We found that activating YAP and TEAD and inhibiting Mst1/2 and Lats1/2 all induced *cyclin D1* expression in the neural tube, thereby promoting neural progenitor cell cycle progression. On the other hand, our data as well as others' show that constitutive expression of cy-

clin D1 can only transiently increase neural progenitor cell number (Lobjois et al. 2008). Cells forced to express cyclin D1 undergo a few extra rounds of mitosis and, probably due to the up-regulation of cell cycle inhibitors associated with neuronal differentiation, eventually exit the cell cycle. Thus, to achieve the dramatic overproliferation phenotype, YAP and TEA-VP16 overexpression likely has induced factors that inhibit differentiation. In support of the notion that YAP and TEAD affect cell fate, we found that activating YAP/TEAD inhibits the expression of the neurogenic bHLH factor *NeuroM*. Conversely, repressing YAP/TEAD target genes up-regulates *NeuroM* and leads to premature neuronal differentiation.

Recent studies in mice also implicate the Hippo pathway in the fate choices of several progenitor cell types. Overexpressing YAP in the intestine increases the number of progenitor cells, which are maintained in an undifferentiated state until YAP expression is repressed (Camargo et al. 2007). In mice lacking *WW45*, the mammalian ortholog of *sav*, epithelial progenitors in many organs are defective in terminal differentiation (Lee et al. 2008). How the Hippo pathway governs neural progenitor fate choice remains to be determined. A number of observations suggest that the Notch and Wnt pathways may be involved. In the mouse intestine, both Notch and Wnt signalings seem to be stimulated shortly after YAP activation (Camargo et al. 2007). In *Drosophila*, *fat* and *wts* mutant clones as well as *yki* overexpression clones have increased levels of the Notch ligand Serrate in leg imaginal discs and of Wingless in wing discs (Cho et al. 2006). It is well established that the Notch pathway plays a critical role in maintaining the progenitor fate in the early neural tube. Wnt signaling has also been shown to inhibit neuronal differentiation. Excessive activation of each pathway leads to a large expansion of the neural progenitor population (Lardelli et al. 1996; Chenn and Walsh 2002; Megason and McMahon 2002), whereas blocking their signaling results in premature differentiation (Zechner et al. 2003; Hatakeyama et al. 2004).

We found that repressing YAP/TEAD target genes leads to precocious neuronal differentiation. It appears that active repression is necessary, as overexpressing the TEA domain alone—which binds to DNA but does not activate or repress transcription—was unable to do so. A similar finding was obtained with the HMG-box factors Sox1–3, which act as transcriptional activators and maintain the progenitor fate: Expression of HMG-EnR but not HMG domain alone induces differentiation (Bylund et al. 2003). Upon the initiation of neuronal differentiation, the target genes of Sox1–3 are repressed by Sox21, which shares the conserved HMG domains but acts as a transcriptional repressor (Sandberg et al. 2005). It is tempting to speculate that such an activator–repressor switch also operates on TEAD target genes during neurogenesis. In support of this hypothesis, we observed that some cells lateral to the Sox2⁺ progenitor zone appear to express TEAD1 but not YAP (Fig. 2B). These cells, judged from their location, are likely to be *NeuroM*⁺ cells that have just commenced neuronal differentiation (Bylund et al. 2003). TEAD1 may interact with a

transcriptional repressor in these cells to facilitate differentiation.

Neural tubes with hyperactivated YAP/TEAD manifest disorganized neuroepithelia and tumor-like rosettes, probably resulting from disruptions in adherens junctions and loss of neuroepithelial cell polarity that are observed before the onset of severe tissue dysplasia (data not shown). Similar perturbations in epithelial structure and cell polarity are found in YAP transgenic mice, *WW45* mutant mice, and fly Hippo mutants (Camargo et al. 2007; Meignin et al. 2007; Polesello and Tapon 2007; Lee et al. 2008). These structural changes are reminiscent of a process known as epithelial–mesenchymal transition (EMT), in which epithelial cells lose their apical–basal polarity and adherens junctions and adopt properties that are typical of mesenchymal cells. Interestingly, overexpressing YAP in epithelial cell lines affects the expression of known EMT markers and induces EMT (Zhao et al. 2008). This activity may, at least partially, underlie the structural defects in YAP-transfected neural tubes.

YAP and TEAD are required for the survival of neural progenitor cells

Loss-of-function studies in mice have not been able to determine the roles of YAP and TEAD during neural development. YAP mutant mice are arrested around E8.5 with widespread defects (Morin-Kensicki et al. 2006), preventing an assessment of its function in neural development. TEAD proteins, with their overlapping expression in the neural tube, appear to, at least partially, compensate for each other (Chen et al. 1994; Kaneko et al. 2007; Yagi et al. 2007). We show here that inhibiting YAP and TEAD in the neural tube triggers apoptosis. Recent studies find that overexpressing YAP in the mouse liver and intestine induces several anti-apoptotic factors, including *cIAP1*, *survivin*, *MCL1*, and *Bcl-X_L* (Camargo et al. 2007; Dong et al. 2007). Moreover, *diap1*, the fly ortholog of *cIAP1*, has been shown to be a direct target of Yki/SD-mediated transcription (Wu et al. 2008; Zhang et al. 2008). Thus, YAP and TEAD may directly promote neural progenitor survival. However, it is also possible that the apoptosis phenotype is secondary to other cellular defects. Identifying direct YAP/TEAD target genes in neural progenitor cells is required to address these possibilities.

Mechanism of transcriptional regulation by the Hippo pathway

We identified *cyclin D1* as a potential target gene of the Hippo pathway during neural tube development. Although *cyclin D1* is up-regulated by YAP/TEAD gain of function, its expression is not diminished when YAP/TEAD function is inhibited, suggesting YAP/TEAD is not required for its basal transcription. A similar finding is made with the fly Hippo target gene *expanded*, whose basal expression is *yki* independent. However, the basal

expression of *diap1* does require *yki* (Wu et al. 2008; Zhang et al. 2008). Two distinct Hippo-regulated regions have been isolated from the *diap1* locus. One region is responsive to *yki* overexpression but is largely unresponsive to the loss of *yki* (Wu et al. 2008), whereas the other region, although bound by Yki/SD and required for *diap1* expression, does not respond to *yki* overexpression (Zhang et al. 2008). These observations suggest that the output of the Hippo pathway may be exquisitely sensitive to the efficacy of the signal transduction cascade: Different target genes probably respond to different thresholds of signaling activity, and, even within a target gene, different promoter elements may be tuned to respond differently.

During neural tube development, the Wnt and Shh pathways have mitogenic functions. We show here that the Hippo pathway has an opposing activity and restricts neural progenitor cells from overproliferation. It is very likely that the Hippo, Wnt, and Shh pathways interact with each other and together orchestrate the growth of the neural tube. Moreover, organ growth has to be tightly coupled to patterning. Dissecting the logic of the interplay between the growth pathways and the patterning pathways, such as Shh, BMP, retinoic acid, and Fgf pathways, will offer important insights into the mechanisms controlling nervous system development.

Materials and methods

DNA constructs

A modified pMIW vector (Muramatsu et al. 1997) was used as the backbone for all overexpression constructs. The coding sequence of every gene was PCR amplified (see Supplemental Table S1 for primers) from HH24–25 chick cDNAs. Point mutations were introduced using the QuikChange mutagenesis kit (Stratagene). shRNA constructs were generated using a chick RNAi system (Das et al. 2006). The target sequences are shYAP#1, GCGGACTTAGCATGAGCAGTTA; shYAP#2, CTGAGGACTATGACTACAAATA; shYAP#1scrambled, GGATGCAAGCGAGCCGTTTATA; shLats1, TGATCCAGTTGATCCAGATAAA.

In ovo electroporation, immunostaining, BrdU labeling, in situ hybridization, and TUNEL assay

In ovo electroporation (see Supplemental Table S2 for plasmid concentrations) and immunostaining (see Supplemental Table S3 for antibodies) were performed as described (Megason and McMahon 2002). For BrdU labeling of HH27–28 embryos, 150 μ L of BrdU (0.05 mg/mL) were added at 67 hpe onto the embryos, which were further incubated for 2 h at 38°C. For cell cycle length analysis (Chenn and Walsh 2002), embryos were labeled with 50 μ L of BrdU for 30 min at 38°C. Histological analyses were performed on brachial and thoracic levels of embryos harvested at 22 hpe or earlier stages and on thoracic levels of later stage embryos. For *in situ* hybridization, full-length cDNAs were cloned into pBluescript SK. *NeuroM* probe has been described (Lee and Pfaff 2003). Probes were prepared using the Riboprobe System-T3/T7 (Promega). Hybridization was carried out according to a method described previously (Ma et al. 1996). TUNEL assay was performed with the ApopTag kit (Chemicon).

Cell culture, transfection, and coimmunoprecipitation

293T cells and chick embryonic fibroblasts (American Type Culture Collection) were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum. Cells were transfected with Lipofectamin 2000 (Invitrogen). Coimmunoprecipitation was performed as described (Cao and Sudhof 2004).

Image acquisition and analysis

Fluorescent images were acquired with a Nikon Eclipse TE300 microscope and a Bio-Rad Radiance 2100 laser scanning system. Bright field images were acquired with a Nikon Eclipse E800 microscope and a RT Slider Spot camera (Diagnostic Instruments). The ImagePro software was used for image analysis. Sections with high transfection efficiencies were used for quantifications. For Tuj1 quantification, the sum of the fluorescence intensity in the transfected region (except the lower quarter, where Tuj1 signals are very strong and tend to mask the changes in upper regions) was divided by that in the corresponding region of the control side. NF quantification was also obtained by comparing the sum of the fluorescence intensity in the transfected region with that in the corresponding untransfected region. Sox2 and BrdU were quantified by measuring the area of immunofluorescence signals. pH3, Lim1/2, and TUNEL signals were counted manually.

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