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Nf2-Yap signaling controls the expansion of DRG progenitors and glia during DRG development

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Abstract

Molecular mechanisms governing the maintenance and proliferation of dorsal root ganglia (DRG) progenitors are largely unknown. Here we reveal that the Hippo pathway regulates the expansion of DRG progenitors and glia during mammalian DRG development. The key effectors of this pathway, transcriptional coactivators Yap and Taz, are expressed in DRG progenitors and glia during DRG development but are at least partially inhibited from activating transcription. Aberrant YAP activation leads to overexpansion of DRG progenitor and glial populations. We further show that the Neurofibromatosis 2 (Nf2) tumor suppressor inhibits Yap during DRG development. Loss of *Nf2* leads to similar phenotypes as does YAP hyperactivation, and deleting *Yap* suppresses these phenotypes. Our study demonstrates that Nf2-Yap signaling plays important roles in controlling the expansion of DRG progenitors and glia during DRG development.

Keywords

the peripheral nervous system; neural crest; satellite glia; sensory neurons

INTRODUCTION

The dorsal root ganglia (DRG) are clusters of sensory neurons and glia found at the dorsal root of the spinal nerves. They transmit sensory information from the body to the central nervous system (CNS) (Zigmond et al., 1999). Neurons and glia in the DRG are derived from neural crest (NC) cells, whose precursors reside within the dorsal neural tube (Le

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AUTHOR CONTRIBUTIONS

X.C. conceived the project. Y.S. and X.C. designed the experiments. Y.S. performed the experiments and analyzed the results. J.P. maintained the mouse colony. M.G. generated the *Nf2^{Flox}* mouse line. Y.S. and X.C. wrote the manuscript.

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Douarin and Smith, 1988). Upon delaminating from the neural tube around embryonic day (E) 8.5 in mice, some NC cells migrate ventrally between somites and the neural tube and coalesce to generate the DRG (Frank and Sanes, 1991; Serbedzija et al., 1992). During migration and after condensation into a ganglion, multipotent NC cells (here we refer to the migrating ones as migratory NC cells and those within the DRG as DRG progenitors) become committed to neuronal or glial fates and give rise to sensory neurons and satellite glia in DRG (Frank and Sanes, 1991; Ma et al., 1999; Rifkin et al., 2000; Serbedzija et al., 1992). In mice, starting from around E9.5, a subset of multipotent NC cells give rise to sensory neurons in DRG in two temporally distinct but overlapping waves, which are regulated by basic helix-loop-helix transcription factors Neurogenin (Ngn) 2 and Ngn1 (Ma et al., 1999; Rifkin et al., 2000). The first wave of neurogenesis, dependent on Ngn2 activity, is completed by E11.5 in mice and mostly gives rise to large-diameter neurotrophic tyrosine kinase receptor (Trk) B-positive (TrkB⁺) mechanoreceptive and TrkC⁺ proprioceptive neurons. Ngn1-dependent second wave of neurogenesis occurs between E10.5 and E13.5 and mainly produces small-diameter TrkA⁺ nociceptive neurons (Marmigère and Ernfors, 2007). All sensory neurons express markers Isl1/2 and Tuj1. Satellite glia are first detected around E11 by the early glial marker brain lipid binding protein (Blbp) (Kurtz et al., 1994). Although factors regulating DRG neurogenesis and gliogenesis have been studied extensively (Marmigère and Ernfors, 2007; Woodhoo and Sommer, 2008), those governing the maintenance and proliferation of multipotent DRG progenitors are largely unknown.

The Hippo pathway is an evolutionarily conserved signaling pathway that regulates organ growth and tumorigenesis (Yu and Guan, 2013; Zhao et al., 2011). Central to this pathway are kinases Mst1/2 and Lats1/2, which phosphorylate transcriptional coactivators Yap and Taz (here referred to as Yap/Taz), resulting in their cytoplasmic retention. Inactivation of Mst1/2 or Lats1/2 kinases or overexpression of Yap leads to accumulation of unphosphorylated Yap, which translocates into the nucleus and activates genes that promote proliferation and survival. Nf2/Merlin is an upstream activator of the Hippo pathway (Hamaratoglu et al., 2006; Milton et al., 2010; Zhang et al., 2010; Zhao et al., 2007). Encoded by the tumor suppressor gene *Neurofibromatosis 2*, Nf2 is a close relative of the Ezrin/Radixin/Moesin (ERM) protein family, which links the cytoskeleton to membrane proteins (Bretscher et al., 2002). In addition to its functions in maintaining cell-cell contact and cell polarity, Nf2 mediates contact-dependent inhibition of cell proliferation *in vitro* and controls tissue homeostasis and tumorigenesis *in vivo* (Li et al., 2012).

The Hippo-Yap/Taz pathway controls self-renewal and expansion of mouse and human embryonic stem cells (Lian et al., 2010; Varelas et al., 2008) and tissue-specific stem/progenitor cells (Camargo et al., 2007; Lee et al., 2010; Zhang et al., 2010). We previously showed that Yap regulates neural progenitor cell number during vertebrate CNS development (Cao et al., 2008) and recently found that Nf2 inhibits Yap/Taz to limit the expansion of the neural progenitor pool during mammalian brain development (Lavado et al., 2013). In *Drosophila*, the Yap ortholog Yki promotes the expansion of optic lobe neuroepithelial and glial cells (Reddy and Irvine, 2011; Reddy et al., 2010). Taken together, these studies establish Yap as an important regulator of the sizes of CNS neural progenitor and glial populations.

Here we investigated the function of Yap and Nf2 during mouse DRG development. We found that Yap/Taz are expressed in migratory NC cells, DRG progenitors, and the glial lineage but not in the neuronal lineage. Elevation of YAP expression in DRG progenitors and glial cells expands these cell populations. Furthermore, we found that Nf2 inhibits Yap during DRG development. In the absence of Nf2, the numbers of DRG progenitors and glial cells are increased and that of neurons is reduced, mimicking the phenotypes of YAP gain-of-function mutants. Deletion of *Yap* in the *Nf2* conditional knockout (cKO) background suppresses these phenotypes. We further show that Nf2-Yap signaling regulates progenitor expansion during the development of another NC derivative, the sympathetic ganglia (SG), in a similar fashion. Our findings provide novel insights into the function of Nf2-Yap signaling during NC development.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the guidelines set by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital (SJCRH). The *TetO-YAP* line (Camargo et al., 2007) was provided by Thijn R. Brummelkamp and Fernando D. Camargo (Children's Hospital, Boston, MA). *Sox10-rtTA* line (Ludwig et al., 2004) was a gift from Michael Wegner (Universität Erlangen-Nürnberg, Erlangen, Germany). *Wnt1-Cre* (Stock No: 007807) and *Axin2-rtTA* (Stock No: 016997) lines were obtained from the Jackson Laboratory. The *Yap^{Flox/Flox}* line (Xin et al., 2011) was provided by Eric N. Olson (University of Texas Southwestern Medical Center, Dallas, TX). The *Nf2^{Flox/Flox}* line has been described previously (Giovannini et al., 2000). An *Nf2* allele was generated by breeding *Nf2^{Flox/Flox}* mice with the *Ella-Cre* line (Stock No: 003724, Jackson Laboratory). As both *Nf2* and *Wnt1-Cre* are on Chromosome 11, a recombined allele, *Nf2^{Wnt1-Cre}*, was generated by extensive breeding. For BrdU pulse-labeling, timed pregnant mice were given intraperitoneal injections of BrdU (10 mg/ml in 0.9% saline) at a dose of 100 mg/kg. Doxycycline was delivered in drinking water at 500 mg/L at the time points indicated.

Histology and immunostaining

Embryos younger than E15.5 were fixed with 4% paraformaldehyde in PBS overnight at 4°C. Embryos at E15.5 or older were intracardially perfused with 4% paraformaldehyde and post-fixed overnight at 4°C. Samples were embedded in OCT and sectioned at 16-µm thickness. Immunostainings were performed according to standard protocols. Primary antibodies were as follows: Blbp (Millipore, ABN14; rabbit, 1:500), BrdU (Abcam, ab6326; rat, 1:100), Isl1/2 (Developmental Studies Hybridoma Bank, Clone # 39.4D5; mouse, 1:500), Ki67 (Vector Laboratories, VP-RM04; rabbit, 1:250), Nf2 (Sigma, HPA003097; rabbit, 1:1000 and amplification with Invitrogen TSA kit), p75 (Promega, G3231; rabbit, 1:2000), S100β (Sigma, S2532; mouse, 1:100 and amplification with Invitrogen TSA kit), Sox10 (Santa Cruz, sc-17342; goat, 1:250), TrkA (provided by Louis Reichardt, University of California, San Francisco, CA; rabbit, 1:1000), Tuj1 (Covance, MMS-435P; mouse, 1:1000), Yap/Taz (Cell Signaling, 8418; rabbit, 1:200 and amplification with Invitrogen TSA kit), and pYap (Cell Signaling, 4911; rabbit, 1:200). Secondary antibodies were from

Invitrogen and Jackson ImmunoResearch. Fluorescent images were acquired with a Zeiss LSM 510 or 780 confocal microscope.

Western blot analysis

DRG were dissected from E13.5 embryos. Total DRG lysates were prepared in 20 mM HEPES (pH 7.4), 150 mM NaCl, 2% SDS, and 5% glycerol supplemented with the Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). The following primary antibodies were used: Nf2 (Sigma, HPA003097; rabbit, 1:1,000) and GAPDH (Sigma, G8795, mouse; 1:10,000).

In situ hybridization

In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993). *Ngn1* probe was provided by Andy Groves (Baylor College of Medicine Houston, TX) (Raft et al., 2007). *Ngn2* and *TrkB* probes were provided by Qifu Ma (Harvard Medical School, Boston, MA) (Ma et al., 1999). A probe specific for the TrkC tyrosine kinase domain was designed based on Fagan et al., 1996. Total RNA was extracted from E13.5 wild-type mouse brain using TRIzol reagent (Invitrogen) and reverse transcribed by using the SuperScript III cDNA kit (Invitrogen). A 738 bp-long cDNA fragment encoding a portion of the kinase domain was amplified using forward primer CATCAAGAGGAGAGATATCGTGTGAAGAG and reverse primer GGTCTCTTCTAGACACGGCC, where underlined sequences show the restriction enzyme sites integrated into the fragment by PCR (EcoRV and XbaI, respectively). The fragment was cloned into pBluescript plasmid at EcoRV and XbaI sites. Antisense probe was generated by linearizing the vector with EcoRV and transcribing with T3 RNA polymerase. Images were acquired with a Zeiss Axio Imager M2 microscope equipped with a Zeiss AxioCam MRc camera.

Quantification and statistical analysis

Cell counting was performed using the ImageJ software. Three embryos for each genotype were quantified. Quantifications were performed on sections at similar rostrocaudal levels. Absolute numbers shown in bar graphs are mean number of cells per section. All values shown are mean \pm s.e.m. *P*-values were calculated with two-tailed, unpaired *t*-test.

RESULTS

Yap/Taz expression pattern in the developing DRG

To begin investigating the potential function of the Hippo pathway during DRG development, we first examined the expression pattern of Yap/Taz in mouse embryos using an antibody that recognizes both Yap and Taz. At E9.5, Yap/Taz were detected in Sox10⁺ migrating NC cells (Kuhlbrodt et al., 1998; Paratore et al., 2001) (Fig. 1A). At E10.5, when migrating NC cells have coalesced into the forming DRG, Yap/Taz were expressed in all Sox10⁺ cells (Fig. 1C), a population consisting of p75⁺ DRG progenitors (Fig. 1D, arrowheads) and p75⁻ glial precursors which retain Sox10 expression but have not yet started expression of the glial marker Blbp (Hu et al., 2011; Sonnenberg-Riethmacher et al., 2001) (Fig. 1D, arrows). Cells of the neuronal lineage, marked by Isl1/2, did not express

Yap/Taz (Fig. 1E), whether they were proliferating BrdU⁺ neuronal precursors (Fig. 1E, arrowheads, 2-hour BrdU pulse) or BrdU⁻ postmitotic neurons (Fig. 1E, arrows). At E15.5, Yap/Taz expression persisted in Sox10⁺ cells (Fig. 1G,G'), almost all of which coexpressed the glial marker Blbp at this stage (Fig. 1H,H'). Neurons, marked by Tuj1, did not express Yap/Taz (Fig. 1I,I'). In summary, during mouse DRG development, Yap/Taz are expressed in migratory NC cells, DRG progenitors, glial precursors, and satellite glia, but not in neuronal precursors or post-mitotic neurons.

Throughout the developmental stages examined, Yap/Taz were present in the cytoplasm (Fig. 1A,C,G,G'), suggesting that their transcriptional activity is at least partially inhibited. Indeed, the S112-phosphorylated form of Yap (S127 in human YAP), which is the product of the Hippo kinase cascade and is unable to activate transcription, was readily detectible in Sox10⁺ cells at each stage (Fig. 1B,F,J,J').

YAP overexpression in NC precursors, migratory NC cells, and DRG progenitors leads to overexpansion of DRG progenitors

To understand the functional significance of keeping Yap/Taz suppressed, we decided to examine the consequences of their aberrant activation on DRG development. To this end, we utilized a mouse line that carries a doxycycline (Dox)-dependent allele of human *YAP1* (*TetO-YAP1^{S127A}*, here referred to as *TetO-YAP*) (Camargo et al., 2007). Mating these mice with those expressing the tetracycline-dependent transactivator rtTA under the control of the *Axin2* promoter (*Axin2-rtTA*) (Yu et al., 2007) and providing dams with Dox enable overexpression of the S127A mutant version of YAP, which presumably has enhanced nuclear localization compared to the wild-type protein due to the lack of phosphorylation and therefore inhibition by the Hippo kinase cascade. The *Axin2* promoter is active in NC precursors within the dorsal neural tube starting at E8.5 and in migratory NC cells (Yu et al., 2007). To examine the effect of YAP overexpression at early stages of DRG development, we gave Dox-containing water to pregnant females starting from E5.5. At E10.5, YAP was overexpressed in the DRG of *TetO-YAP;Axin2-rtTA* double transgenic embryos and the overexpression was restricted to Sox10⁺p75⁺ DRG progenitors (Fig. 2A–D', arrowheads) and Sox10⁺p75⁻ glial precursors (Fig. 2A–D', arrows). At E12.5, YAP was no longer overexpressed in *TetO-YAP;Axin2-rtTA* DRG (Fig. 2E,F) despite the presence of Dox, which is likely due to the downregulation of the *Axin2* promoter. Thus, *TetO-YAP;Axin2-rtTA* embryos experienced elevated YAP activity in NC precursors, migratory NC cells, DRG progenitors, and glial precursors before E12.5.

At E10.5, the DRG of *TetO-YAP;Axin2-rtTA* embryos contained significantly more Sox10⁺p75⁺ DRG progenitors (Fig. 2G–I) than did the DRG of control embryos. To determine the cellular basis underlying these phenotypes, we analyzed the proliferation of DRG progenitors. We labeled S-phase cells with BrdU 2 hours before harvesting the embryos and quantified the percentage of BrdU⁺ DRG progenitors (BrdU⁺Sox10⁺p75⁺, Fig. 2J–K', arrowheads) within the progenitor population (Sox10⁺p75⁺). The fraction of proliferating (BrdU⁺) DRG progenitors was markedly higher in *TetO-YAP;Axin2-rtTA* embryos than in controls (Fig. 2L). To measure cell-cycle exit, we labeled S-phase cells with BrdU 24 hours prior to harvesting the embryos and quantified the fraction of BrdU⁺ cells

that no longer expressed the proliferating-cell marker Ki67 (BrdU⁺Ki67⁻/BrdU⁺ cells) (Fig. 2M–N'), which indexes the fraction of cells that have exited the cell-cycle within the past 24-hour period. Cell-cycle exit was significantly reduced in *TetO-YAP;Axin2-rtTA* DRG (Fig. 2O). Taken together, these results demonstrate that YAP augments the proliferation capacity of DRG progenitors during the early stages of DRG development.

To determine whether YAP hyperactivation at early stages of DRG development affects neurogenesis, we examined the neuronal population at two stages of DRG development. At E10.5, *TetO-YAP;Axin2-rtTA* DRG contained fewer Isl1/2⁺ neurons (Fig. 3A,B,G) than did the DRG of control embryos. The number of *TrkC*⁺ neurons was significantly reduced whereas the numbers of *TrkA*⁺ or *TrkB*⁺ neurons were unchanged in *TetO-YAP;Axin2-rtTA* DRG (Fig. 3H–N). At postnatal day 0 (P0), when YAP was no longer overexpressed in *TetO-YAP;Axin2-rtTA* DRG (Fig. 3C,D), both the total number of neurons (Fig. 3E–G) and the numbers of each subtype of neurons (Fig. 3O–U) were restored to control levels. These results suggest that YAP hyperactivation at early stages of DRG development delays the production of *TrkC*⁺ neurons.

YAP overexpression in DRG progenitors and glial cells expands these populations

Because the *Axin2* promoter was downregulated in DRG by E12.5 (Fig. 2E,F), we were unable to analyze the effect of aberrant YAP activation on DRG development at later stages. We therefore crossed *TetO-YAP* mice with those expressing rtTA under the control of the *Sox10* promoter (*Sox10-rtTA*) (Ludwig et al., 2004), which is first turned-on in migratory NC cells around E8.5 and stays active in DRG progenitors and the glial lineage during DRG development. Dox administration starting from E7.5 led to increased YAP levels in Sox10⁺ DRG progenitors and glia in the DRG of *TetO-YAP;Sox10-rtTA* embryos at E12.5 (Fig. 4A,B). These DRG contained significantly more Sox10⁺p75⁺ DRG progenitors (Fig. 4C–E, arrowheads) and Sox10⁺p75⁻ glia (Fig. 4C–D',F, arrows) than did control DRG. The number of Isl1/2⁺ neurons, however, was unperturbed at either E12.5 (Fig. 4G–I) or E11.5 (Fig. S1A–E). Furthermore, the numbers of each subtype of neurons were similar between control and *TetO-YAP;Sox10-rtTA* DRG (Fig. S1F–L). Thus, YAP hyperactivation in Sox10⁺ NC cells expands the DRG progenitor and glial populations but does not affect neurogenesis.

To examine the effect of YAP overexpression in the glial lineage specifically, we started Dox delivery at E12.5, when most Sox10⁺ cells in control embryos have differentiated into the glial lineage (Fig. 4E,F; ~14 DRG progenitors vs. ~100 glia in control embryos) (Hu et al., 2011). At E18.5, YAP was overexpressed in Sox10⁺ cells in *TetO-YAP;Sox10-rtTA* DRG (Fig. 4J–K') and almost all Sox10⁺ cells expressed the glial marker S100β (Fig. 4L–M'). We quantified the number of glia by counting Sox10⁺ cells. *TetO-YAP;Sox10-rtTA* DRG contained significantly more glial cells at E18.5 than did controls (Fig. 4N). These results demonstrate that aberrant YAP activation in glial cells causes overexpansion of this population.

Nf2 is expressed in the developing DRG

So far, our data demonstrate that Yap/Taz are expressed in DRG progenitors and glial cells during normal DRG development and that aberrant YAP activation in these cells causes overexpansion of these cell populations. We next sought to identify upstream regulators of Yap/Taz. We hypothesized that Nf2 is an inhibitor of Yap during DRG development. The *Nf2* transcript is widely expressed during mouse embryogenesis (Gutmann et al., 1995; Huynh et al., 1996), and high levels of *Nf2* promoter activity are seen in migrating NC cells (Akhmamyeva et al., 2006). We performed immunostaining to monitor the expression and localization of Nf2 proteins in the developing DRG. At E10.5, Nf2 proteins were detected in the soma of Sox10⁺ DRG progenitors and glial precursors (Fig. 5A,A', arrowheads) and in the soma and processes of Tuj1⁺ sensory neurons (Fig. 5A,A', arrows). At E12.5, Nf2 was present in Blbp⁺ satellite glia (Fig. 5B–D; arrows and arrowheads mark three examples of glial processes) and Tuj1⁺ sensory neurons (Fig. 5E,E'). Thus, Nf2 is expressed in all cell types in the developing DRG and is coexpressed with Yap/Taz in DRG progenitors and the glial lineage (see Fig. 1).

Loss of Nf2 leads to similar defects in DRG development as does YAP hyperactivation

To test our hypothesis that Nf2 is an inhibitor of Yap during DRG development, we conditionally deleted *Nf2* in the NC lineage using *Wnt1-Cre*, which is expressed in NC precursors in the dorsal neural tube starting from E8.5 (Danielian et al., 1998). We confirmed that Nf2 proteins were no longer detected in *Nf2* cKO (*Nf2^{Flox/};Wnt1-Cre*) DRG at E13.5, the earliest stage we could collect sufficient amount of tissues for western blot analysis (Fig. 5F).

Because *YAP* overexpression affected DRG progenitors, we first examined the effect of *Nf2* deletion on this population. At E10.5, the number of Sox10⁺p75⁺ DRG progenitors was significantly increased in *Nf2* cKO embryos (Fig. 6A–C). The percentage of proliferating DRG progenitors within the progenitor population, measured by BrdU incorporation after a 2-hour pulse (BrdU⁺Sox10⁺p75⁺/Sox10⁺p75⁺ cells), was also significantly increased (Fig. 6D–F), whereas cell-cycle exit during the 24-hour period between E9.5 and E10.5 was reduced (BrdU⁺Ki67⁻/BrdU⁺ cells) (Fig. 6G–I). At E12.5, most Sox10⁺ cells in both control and *Nf2* cKO DRG no longer expressed p75 (Fig. 6J–K', arrows), and the numbers of Sox10⁺p75⁺ DRG progenitors in control and *Nf2* cKO DRG were similar (Fig. 6L). These phenotypes closely resemble those of *TetO-YAP;Axin2-rtTA* embryos (see Fig. 2G–O), thus supporting our hypothesis that Nf2 is a Yap inhibitor during DRG development.

We next analyzed gliogenesis and neurogenesis in *Nf2* cKO DRG. Glia are Sox10⁺Blbp⁺ at E12.5 and Sox10⁺S100β⁺ at E18.5 (Fig. 6M–P). We quantified glia by counting Sox10⁺ cells at these stages. This approach gives a close estimate of the quantity of glial cells because, from E12.5 and onward, Sox10⁺p75⁺ DRG progenitors constitute less than 5% of the cells in DRG and their numbers were similar in control and *Nf2* cKO DRG (Fig. 6J–L'). At E12.5 and E18.5, the number of glia was significantly increased in *Nf2* cKO DRG (Fig. 6Q). The number of neurons, marked by *Isl1/2* at E10.5 and Tuj1 at E18.5, was reduced in *Nf2* cKO DRG (Fig. 7A–E). At E10.5, the numbers of *TrkB*⁺ and *TrkC*⁺ neurons were significantly reduced but the number of *TrkA*⁺ neurons was unchanged (Fig. 7F–L). At

E18.5, the numbers of all three subtypes of neurons were reduced in *Nf2* cKO DRG (Fig 7M–S). The expansion in the glial population at E12.5 and E18.5 and reduction in neuron numbers at E10.5 in *Nf2* cKO DRG resembles the effects of *YAP* overexpression (see Fig. 2–4), reinforcing our hypothesis that *Nf2* is a *Yap* inhibitor in the developing DRG.

Yap deletion rescues *Nf2* cKO phenotypes in DRG

To further confirm that *Nf2* functions by inhibiting *Yap*, we generated *Nf2* and *Yap* double cKO animals (*Nf2^{Flox/}; Wnt1-Cre; Yap^{Flox/Flox}*, herein called *Nf2;Yap* cKO). *Yap* cKO (*Yap^{Flox/Flox}; Wnt1-Cre*) and *Nf2;Yap* cKO embryos all died around E10.5 with massive forebrain bleeding (data not shown), probably due to defects in cerebral vessel formation. We therefore focused our analyses at E10.5. *Yap* deletion alone in the NC lineage did not affect DRG progenitor (Fig. 8A,B,E) or neuronal populations (Fig. 8F,G,J). However, deletion of *Yap* in the *Nf2* cKO background suppressed the expansion of DRG progenitors (Fig. 8A,C–E) and rescued the reduction in the neuronal population (Fig. 8F,H–J). These results strongly support that *Nf2* is an inhibitor of *Yap* during DRG development.

Nf2-Yap signaling controls the expansion of sympathetic ganglia (SG) progenitors

So far we have shown that *Nf2*-*Yap* signaling controls the expansion of DRG progenitors. Since *Yap/Taz* are expressed in migrating NC cells at E9.5 (Fig. 1A), we asked whether progenitors in other NC derivatives are also regulated by *Nf2*-*Yap* signaling. To this end, we examined the development of SG, which are a part of the autonomic nervous system that regulates the homeostasis of the internal environment of the organism (Rohrer, 2011). In control SG, *Yap/Taz* were expressed in *Sox10⁺* SG progenitors (Fig. 9A) but not in *Isl1/2⁺* sympathetic neuroblasts (data not shown). In the aforementioned *TetO-Yap;Axin2-rtTA* double transgenic embryos (Dox starting from E5.5), *YAP* was overexpressed in *Sox10⁺* SG progenitors at E10.5 (Fig. 9A,B). At this stage, the SG of *TetO-Yap;Axin2-rtTA* embryos contained significantly more *Sox10⁺* SG progenitors (Fig. 9C–E). The percentage of proliferating SG progenitors within the progenitor population, measured by BrdU incorporation after a 2-hour pulse (*BrdU⁺Sox10⁺/Sox10⁺* cells), was also markedly increased (Fig. 9F–H). *Nf2* deletion in NC cells led to similar phenotypes as *YAP* hyperactivation. At E10.5, the SG progenitor population was expanded in *Nf2* cKO embryos (Fig. 9I–K). The fraction of proliferating SG progenitors was markedly higher in *Nf2* cKO embryos than in controls (Fig. 9L–N). Finally, although *Yap* deletion alone did not affect the SG progenitor population (Fig. 9O,P,S), it suppressed the expansion of SG progenitors in the *Nf2* cKO background (Fig. 9O,Q–S). These results demonstrate that *Nf2*-*Yap* signaling controls the expansion of SG progenitors.

DISCUSSION

The Hippo pathway is recently recognized as a crucial regulator of tissue growth and tumorigenesis. Here we show that, during mammalian DRG development, the key effectors of this pathway, transcriptional coactivators *Yap/Taz*, are expressed in DRG progenitors and glia but not in the neuronal lineage. The cytoplasmic localization of *Yap/Taz* proteins and the presence of the inactive, phosphorylated form of *Yap* indicate that their transcriptional activities are at least partially inhibited. We show that aberrant *YAP* activation leads to

overexpansion of the DRG progenitor and glial populations. We further identified that Nf2 is an inhibitor of Yap in the developing DRG; *Nf2* cKO embryos exhibit similar phenotypes as those with elevated YAP activity and these phenotypes are suppressed by *Yap* deletion. Our study demonstrates that Nf2-Yap/Taz signaling is an important regulator of mammalian DRG development.

Nf2-Yap signaling regulates the proliferation of DRG progenitors

Our YAP gain-of-function experiments using the *TetO-YAP* transgenic mouse line demonstrate that aberrant YAP activation in DRG progenitors promotes their proliferation and over-expands the progenitor pool. Loss of Nf2 in the NC lineage leads to nearly identical phenotypes. Furthermore, deleting *Yap* in the *Nf2* cKO background suppresses overexpansion of the DRG progenitor pool. These results strongly suggest that Nf2-Yap signaling controls the size of the progenitor pool during normal DRG development.

It is unclear whether YAP overactivation promotes the maintenance of the multipotent state of DRG progenitors as it does in mouse embryonic stem cells (Lian et al., 2010). Moreover, it remains an open question whether Yap/Taz are required for normal DRG development. The early lethality of *Yap^{Flox/Flox};Wnt1-Cre* embryos prevented us from thoroughly investigating Yap's role during DRG development. Nevertheless, our preliminary analysis of E10.5 *Yap^{Flox/Flox};Wnt1-Cre* DRG did not reveal any obvious defects. It is quite possible that Taz may compensate for the loss of Yap. Future studies using *Yap;Taz Wnt1-Cre* double cKO embryos, *Yap* or *Yap;Taz* cKO with Cre lines that act either at later stages or in more restricted lineages (e.g., *Sox10-Cre* (Matsuoka et al., 2005) and *Plp-CreERT2* (Hari et al., 2012; Leone et al., 2003)), and neural crest stem cell cultures (Morrison et al., 2000; Paratore et al., 2001; Paratore et al., 2002; Taylor et al., 2007) prepared from gain⁻ and loss-of-function mutant embryos will provide more insights into the function of Yap/Taz during DRG development.

One phenotypic difference between *Nf2* cKO and YAP gain-of-function mutants is that, although overexpressing YAP using the *Sox10-rtTA* line is able to over-expand the DRG progenitor population at E12.5, *Nf2* deletion fails to do so (compare Fig. 4E vs. Fig. 6L). We think this difference is likely because the level of Yap activation achieved by *Nf2* deletion is lower than that obtained by YAP overexpression. We were unable to detect, by immunostaining, increased Yap/Taz nuclear localization in *Nf2* cKO DRG (data not shown), which is likely because the change is too small to be quantitatively detected by immunostaining. Previously, we were able to detect increased Yap/Taz nuclear localization in *Nf2* cKO brains by subcellular fractionation and quantitative western blot analysis (Lavado et al., 2013). This approach, however, is not feasible for analyzing DRG samples because the tissue amount is too limited.

The functions of Yap and Nf2 during DRG gliogenesis

By selectively elevating YAP level in the glial lineage of DRG, we show that YAP hyperactivation over-expands the glial population, probably by increasing the proliferation of glial precursors. *Nf2* cKO mice exhibit a similar phenotype. Although the phenotypic similarity suggests that, as in DRG progenitors, Nf2 regulates Yap activity, we cannot yet

prove this genetically due to the early lethality of *Yap^{Flox/Flox};Wnt1-Cre* embryos. It remains possible that Nf2 functions independently of Yap/Taz during glia development. Indeed, previous studies have shown that Nf2 regulates CNS glial cell proliferation by inhibiting ErbB2-dependent Src signaling (Houshmandi et al., 2009) and Schwann cell proliferation and function through a number of signaling pathways (Li et al., 2012; McClatchey, 2005). These possibilities need to be addressed by more molecular and genetic studies in the future.

The functions of Yap and Nf2 during DRG neurogenesis

In addition to the effects on DRG progenitor and glial populations, YAP gain-of-function and Nf2 loss also affect the amount of DRG sensory neurons. The mutant phenotypes are quite complex. *TetO-YAP;Axin2-rtTA* mice show a transient reduction of early born TrkC⁺ neurons at E10.5 but, by P0, neuron numbers are restored to normal levels. *TetO-YAP;Sox10-rtTA* mice, with Dox administration starting from E7.5, do not exhibit neuron number defects at E11.5 or E12.5. *Nf2* cKO mice, however, exhibit reductions of early born TrkB⁺ neurons and TrkC⁺ neurons at E10.5 and reductions of all subtypes of neurons at E18.5. Deleting *Yap* in the *Nf2* cKO background restores the total number of neurons at E10.5. Based on these results, we postulate the following.

The genetic rescue experiment indicates that, at least before E10.5, Nf2 regulates DRG neurogenesis by inhibiting Yap. Only neurons born during the first wave of neurogenesis are affected in *TetO-YAP;Axin2-rtTA* and *Nf2* cKO embryos, suggesting that Nf2-Yap signaling probably selectively regulates the first wave of neurogenesis but not the second wave. We have examined *Ngn2* and *Ngn1* expression in YAP gain-of-function and *Nf2* cKO mutants but did not detect obvious differences in their expression patterns or levels (Fig. S2). It is possible that Nf2-Yap signaling regulates a neuronal differentiation step downstream of *Ngn2* expression, for example the upregulation of NeuroD or Isl1/2.

It is interesting and somewhat puzzling that our two YAP gain-of-function mouse models display different neurogenesis phenotypes: *TetO-YAP;Axin2-rtTA* but not *TetO-YAP;Sox10-rtTA* embryos exhibit a delay in neurogenesis. The key difference between these two models is the timing of rtTA expression: *Axin2-rtTA* expression starts in NC precursors within the neural tube (Yu et al., 2007), whereas *Sox10-rtTA* expression starts in migratory NC cells (Kuhlbrodt et al., 1998). Our results thus suggest that YAP hyperactivation has to occur very early during DRG development to have an impact on neurogenesis. This might explain why only TrkC⁺ neurons are affected in *TetO-YAP;Axin2-rtTA* embryos, as they are the first subtype of neurons to be born during DRG development (Ma et al., 1999). Accumulating evidence suggests that at least some NC precursors within the neural tube are already pre-specified into lineage-restricted subpopulations (George et al., 2007; Lefcort and George, 2007; Nitzan et al., 2013). It is thus possible that elevated Yap activity alters the relative proportion of these subpopulations of multipotent NC cells in the neural tube, for example by converting one subpopulation into another or by preferentially promoting the production of one subpopulation. It is also possible that elevated Yap activity promotes proliferative divisions of multipotent DRG progenitors, which then transition into glial precursors to generate glia, thus reducing neuron production, although we think this is less likely considering that *TetO-YAP;Sox10-rtTA* embryos do not exhibit neurogenesis defects. Lastly,

the difference in the mode of action by *Axin2-rtTA*, which leads to transient overexpression mainly in multipotent NC cells, and *Wnt1-Cre*, which causes permanent gene deletion, might account for the difference between *TetO-YAP;Axin2-rtTA* DRG (reduction of TrkC⁺ neurons only) and *Nf2* cKO DRG (reductions of both TrkB⁺ and TrkC⁺ neurons). In summary, more detailed *in vivo* lineage-tracing experiments are required to address these possibilities.

An important phenotypic difference between *Nf2* cKO mutants and YAP gain-of-functions mutants is that, at the end of embryogenesis, the numbers of sensory neurons are reduced in *Nf2* cKO mutants but are normal in YAP gain-of-function mutants. These results indicate that, during the later stages of DRG development, Nf2's function in sensory neurons is independent of Yap/Taz (which are not expressed in sensory neurons). Nf2 loss might impair the proliferation of neuronal precursors or the survival of DRG neurons. The exact function of Nf2 in DRG neurons needs to be determined by future studies. Interestingly, a recent work found that, in neurons of both the CNS and the peripheral nervous system (PNS), including in DRG neurons, Nf2 promotes neurofilament phosphorylation by activating the GTPase RhoA and thereby is essential for maintaining axonal integrity (Schulz et al., 2013).

Conclusions

By using YAP gain-of-function and Nf2 loss-of-function mouse models, we demonstrate that Nf2-Yap signaling regulates the development of two NC derivatives, the DRG and the SG. The observation that significant amount of Yap/Taz proteins remain localized in the cytoplasm in *Nf2* cKO DRG (see above, data not shown) suggests that other factors are involved in sequestering Yap/Taz in the cytoplasm. Consistent with this idea, although the *TetO-YAP* transgenic animals used here express the S127A mutant form of YAP (Camargo et al., 2007), which is thought to have increased nuclear localization than the wild-type protein because it cannot be inhibited by the Hippo kinase cascade (Yu and Guan, 2013), a large fraction of YAP-S127A proteins reside in the cytoplasm (see Fig. 2B,D and Fig. 4B,K,K'), indicating that factors besides the Hippo kinase cascade are involved in Yap/Taz suppression during DRG development. A rapidly growing list of Yap/Taz regulators are being discovered (Yu and Guan, 2013). An important task for future studies is to understand how these regulators operate in intact animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. The function of the Hippo pathway during mammalian DRG development is investigated.
2. YAP hyperactivation expands the DRG progenitor and glial populations.
3. Deletion of *Nf2* recapitulates Yap hyperactivation phenotypes in DRG.
4. *Nf2* inhibits Yap during DRG development.
5. *Nf2*-Yap regulates the expansion of DRG progenitors and glia during DRG development.

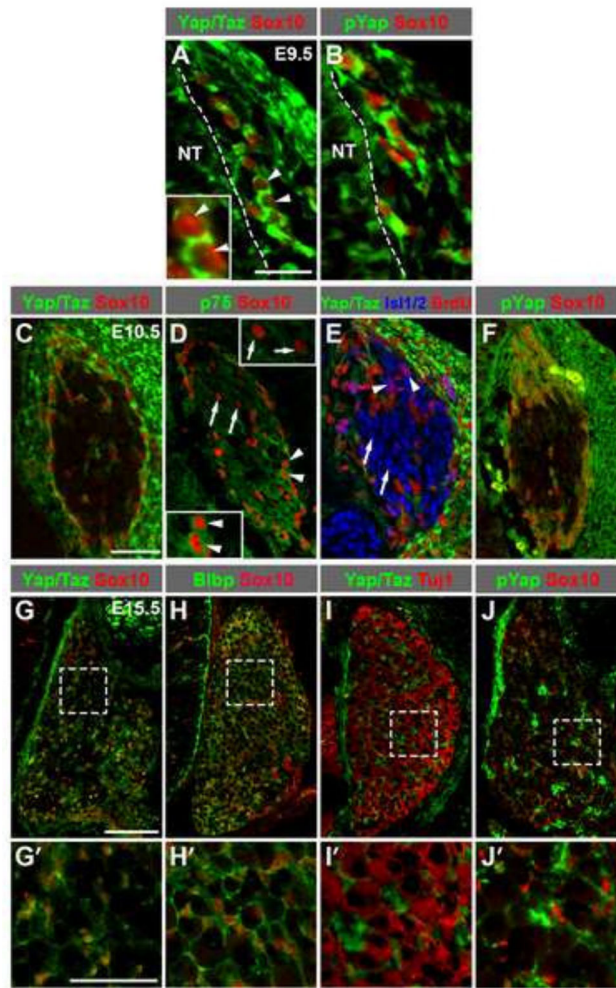


Fig. 1. Yap/Taz are expressed in migratory NC cells, DRG progenitors, glial precursors, and satellite glia during DRG development

(A) At E9.5, Yap/Taz are expressed in Sox10⁺ migratory NC cells. Two cells marked by arrowheads are magnified in the inset. (B) At E9.5, pYap is expressed in Sox10⁺ cells. Dashed lines in A,B mark the neural tube (NT) border. (C–E) At E10.5, Yap/Taz are expressed in Sox10⁺ cells (C) consisting of p75⁺ DRG progenitors (D, arrowheads, lower left corner inset) and p75⁻ glial precursors (D, arrows, upper right corner inset). Neuronal precursors (E, arrowheads, Isl1/2⁺BrdU⁺, 2-hour BrdU pulse) or postmitotic neurons (E, arrows, Isl1/2⁺BrdU⁻) do not express Yap/Taz. (G–I) At E15.5, Yap/Taz are expressed in Sox10⁺ cells (G,G'), almost all of which coexpress the glial marker Blbp (H,H'). Tuj1⁺ neurons do not express Yap/Taz (I,I'). (J,J') pYap is detected in Sox10⁺ cells at each stage. Panels labeled with primes are the magnified view of the boxed areas in corresponding panels. Scale bars: 25 μm in A,G'; 50 μm in C; 100 μm in G.

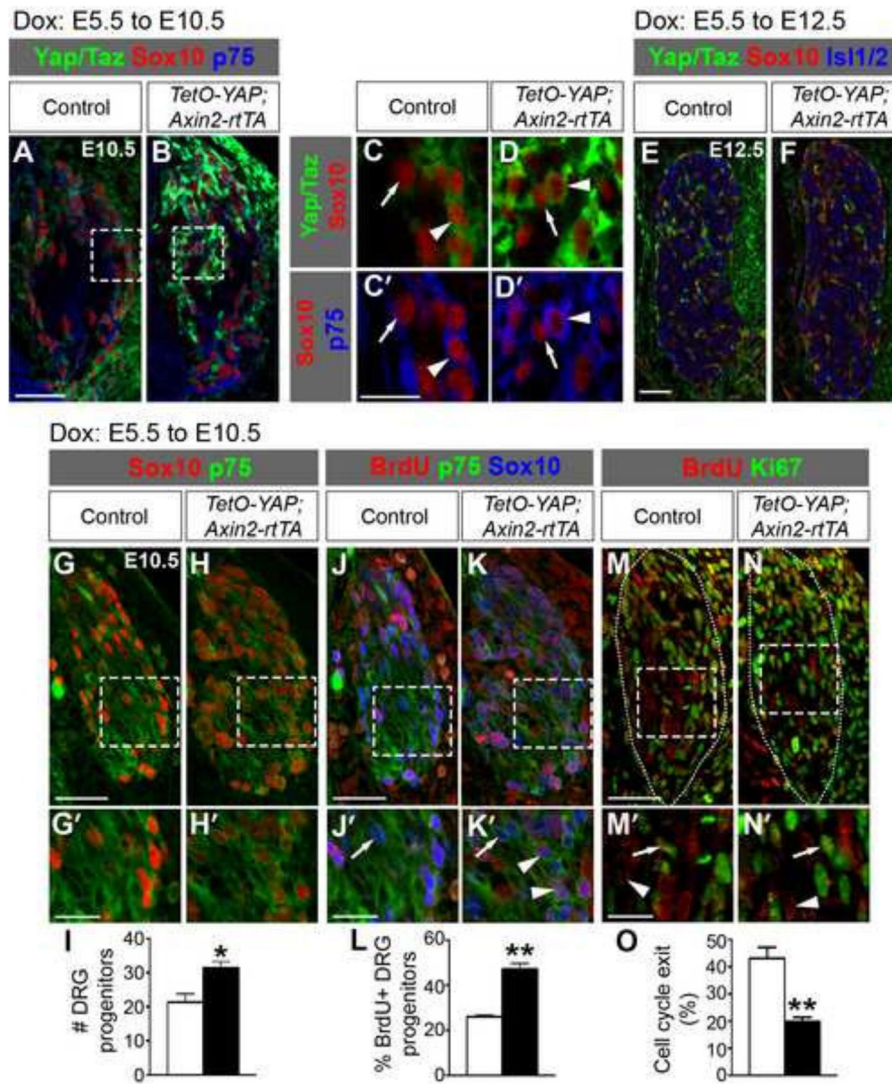


Fig. 2. YAP overexpression in NC precursors, migratory NC cells, and DRG progenitors expands the DRG progenitor pool

Dox-containing water was given to pregnant females starting from E5.5 and embryos were collected at E10.5 and E12.5. (A–D') At E10.5, YAP is overexpressed in Sox10⁺ cells in *TetO-YAP;Axin2-rtTA* DRG. C,C' and D,D' are magnified views of the boxed regions in A and B, respectively. Arrowheads: Sox10⁺p75⁺ DRG progenitors; arrows: Sox10⁺p75⁻ glial precursors. (E,F) At E12.5, YAP is no longer overexpressed in *TetO-YAP;Axin2-rtTA* DRG. (G–I) The number of Sox10⁺p75⁺ DRG progenitors is increased in *TetO-YAP;Axin2-rtTA* DRG compared to controls. Panels labeled with primes are the magnified view of the boxed areas in corresponding panels. (J–L) Quantification of BrdU⁺ cells among Sox10⁺p75⁺ DRG progenitors after a 2-hour BrdU pulse shows increased proliferation of DRG progenitors in *TetO-YAP;Axin2-rtTA* embryos. Arrowheads: BrdU⁺ DRG progenitors; arrows: BrdU⁻ DRG progenitors. (M–O) Quantification of Ki67⁻ cells among BrdU⁺ cells after a 24-hour BrdU pulse shows decreased cell-cycle exit in *TetO-YAP;Axin2-rtTA* DRG.

Arrowheads: BrdU⁺Ki67⁻ cells; arrows: BrdU⁺Ki67⁺ cells. * $P < 0.05$, ** $P < 0.01$. Scale bars: 50 μm in A,E,G,J,M; 25 μm in C',G',J',M'.

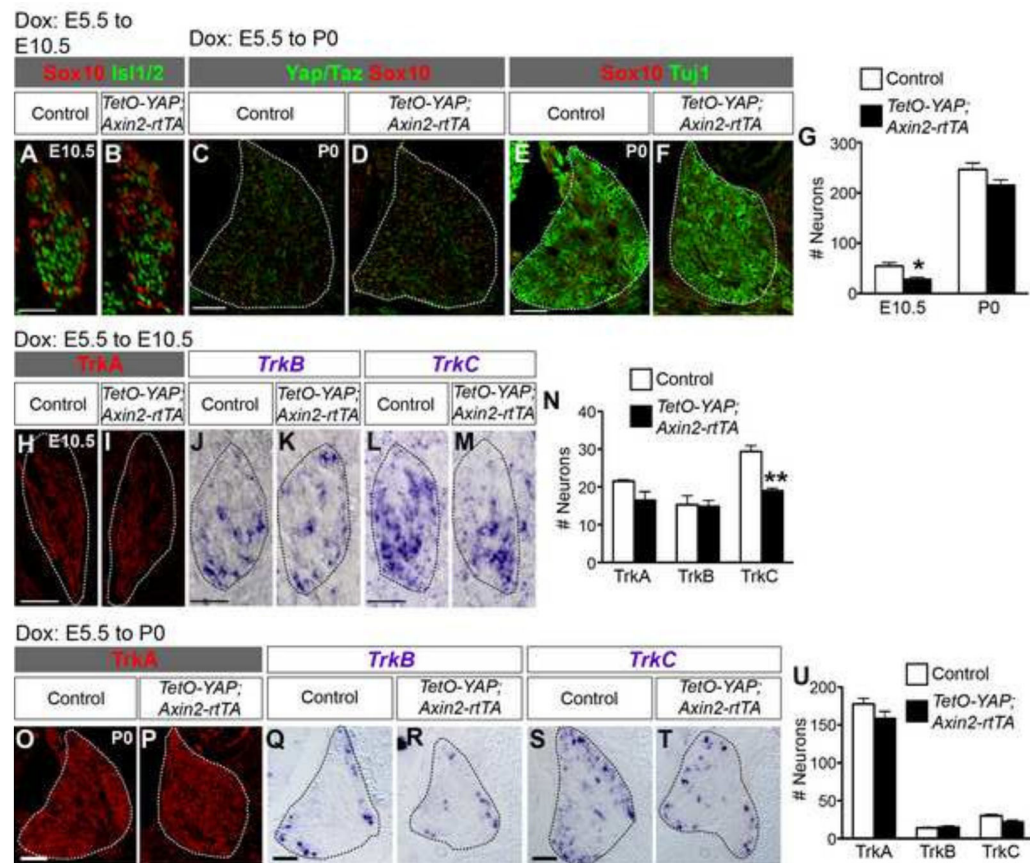


Fig 3. YAP overexpression in NC precursors, migratory NC cells, and DRG progenitors leads to a transient reduction in neuron number

(A–G) The number of neurons is reduced in *TetO-YAP;Axin2-rtTA* DRG at E10.5 but not changed at P0. Neurons are labeled by *Isl1/2* at E10.5 and *Tuj1* at P0. At P0, YAP is no longer overexpressed in *TetO-YAP;Axin2-rtTA* DRG (C,D). (H–N) At E10.5, the number of *TrkC*⁺ neurons is reduced but the numbers of *TrkA*⁺ and *TrkB*⁺ neurons are unchanged in *TetO-YAP;Axin2-rtTA* DRG. (O–U) At P0, the numbers of each subtype of neurons are unchanged in *TetO-YAP;Axin2-rtTA* DRG. * $P < 0.05$, ** $P < 0.01$. Scale bars: 50 μ m in A,H,J,L; 100 μ m in C,E,O,Q,S.

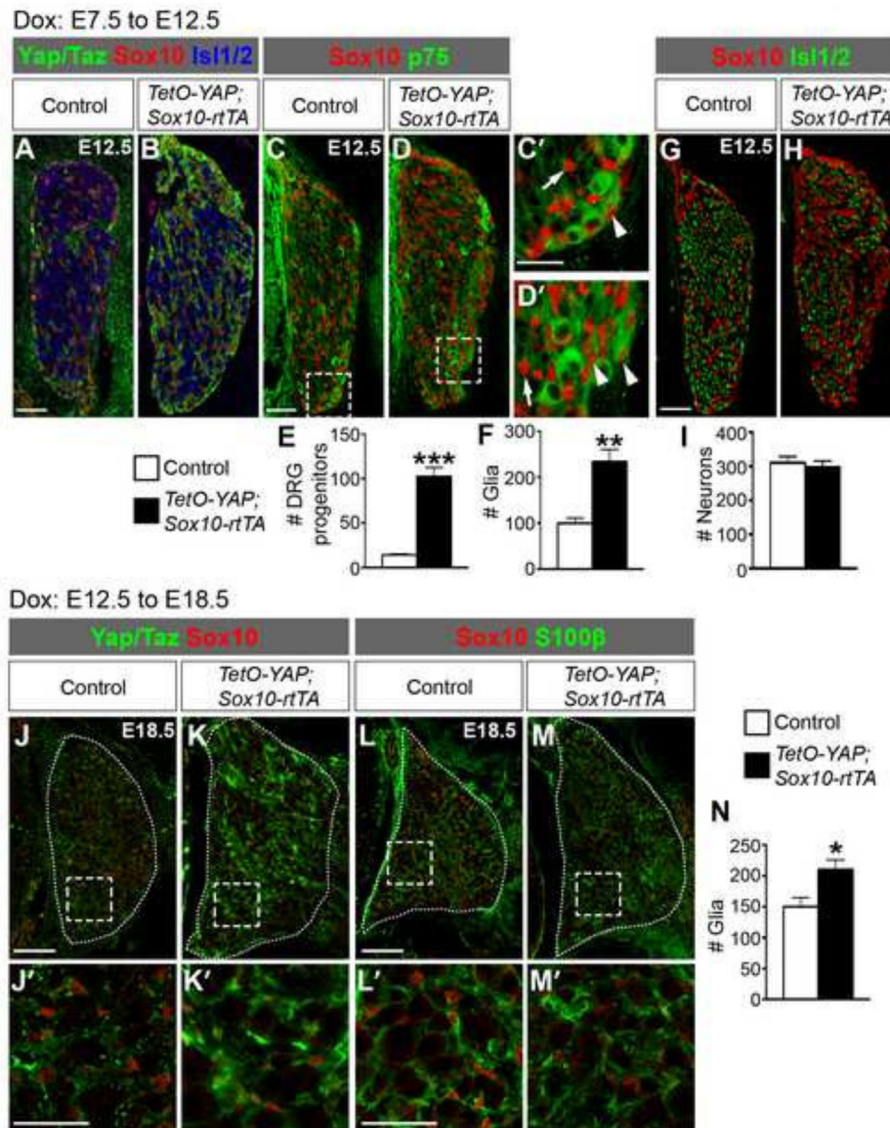


Fig. 4. YAP overexpression in DRG progenitors and glial cells expands these populations (A–I) Dox-containing water was given to pregnant females starting from E7.5 and embryos were collected at E12.5. (A,B) YAP is overexpressed in Sox10⁺ cells in E12.5 *TetO-YAP;Sox10-rtTA* DRG. (C–F) At E12.5, *TetO-YAP;Sox10-rtTA* DRG have more Sox10⁺p75⁺ DRG progenitors (arrowheads in C',D') and Sox10⁺p75⁻ glia (arrows in C',D') compared to controls. Panels labeled with primes are the magnified view of the boxed areas in corresponding panels. (G–I) The number of Isl1/2⁺ neurons is not changed in *TetO-YAP;Sox10-rtTA* DRG at E12.5. (J–N) Dox-containing water was given to pregnant females starting from E12.5 and embryos were collected at E18.5. (J–K') YAP is overexpressed in Sox10⁺ cells in E18.5 *TetO-YAP;Sox10-rtTA* DRG. (L–M') Almost all Sox10⁺ cells express the glial marker S100β. (N) The number of glial cells, quantified by counting Sox10⁺ cells, is increased in *TetO-YAP;Sox10-rtTA* DRG compared to controls. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars: 50 μm in A,C,G,J',L'; 25 μm in C'; 100 μm in J,L.

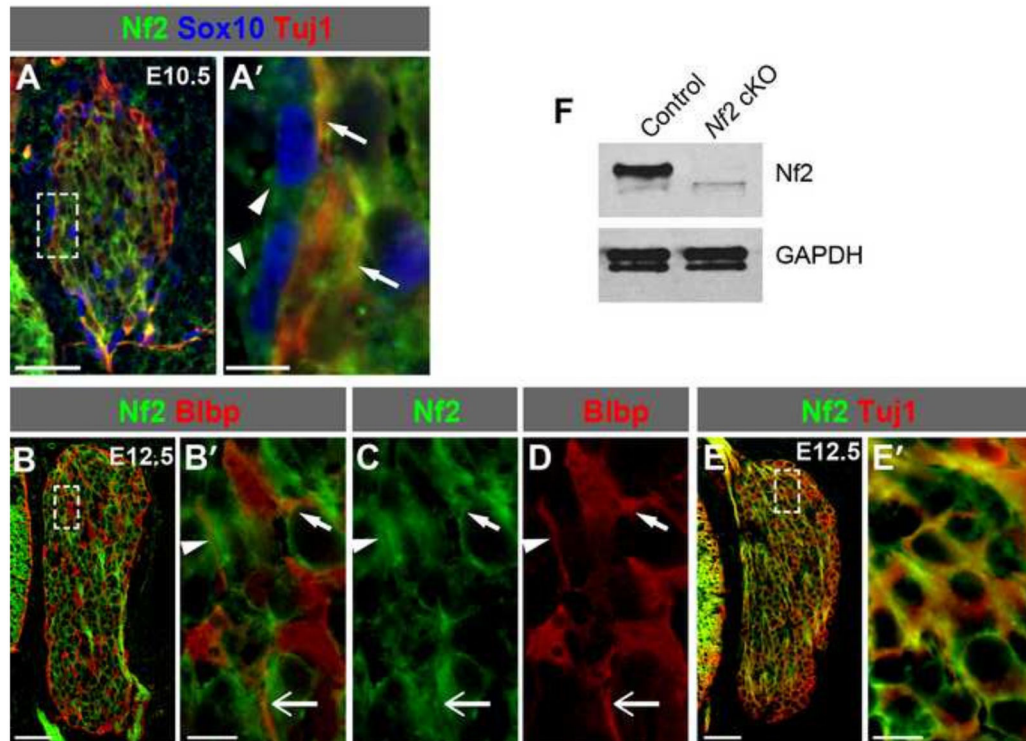


Fig. 5. Nf2 is expressed in the developing DRG

(A,A') At E10.5, Nf2 is detected in the soma of Sox10⁺ DRG progenitors and glia (arrowheads) and in the soma and processes of Tuj1⁺ neurons (arrows). Panels labeled with primes are the magnified view of the boxed areas in corresponding panels. (B–D) At E12.5, Nf2 is detected in Blbp⁺ satellite glia. Arrows and arrowheads point to three examples of glial processes. (E,E') At E12.5, Nf2 is expressed in Tuj1⁺ neurons. (F) Western blot analysis shows that Nf2 is no longer detected in E13.5 Nf2 cKO DRG. Scale bars: 50 μ m in A,B,E; 10 μ m in A',B',E'.

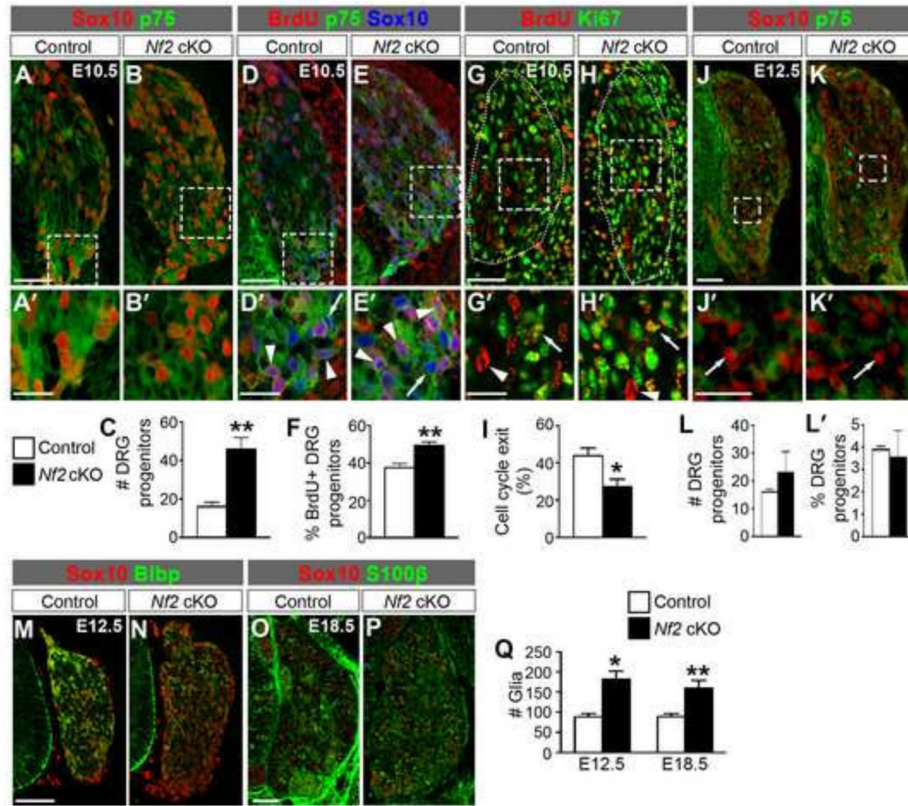


Fig. 6. Loss of *Nf2* leads to similar defects in DRG development as does YAP hyperactivation (A–C) At E10.5, *Nf2* cKO DRG have more Sox10⁺p75⁺ DRG progenitors than controls. Panels labeled with primes are the magnified view of the boxed areas in corresponding panels. (D–F) Quantification of BrdU⁺ cells among Sox10⁺p75⁺ DRG progenitors after a 2-hour BrdU pulse shows increased proliferation in *Nf2* cKO. Arrowheads: BrdU⁺ DRG progenitors; arrows: BrdU⁻ DRG progenitors. (G–I) Quantification of Ki67⁻ cells among BrdU⁺ cells after a 24-hour BrdU pulse shows decreased cell-cycle exit in *Nf2* cKO. Arrowheads: BrdU⁺Ki67⁻ cells; arrows: BrdU⁺Ki67⁺ cells. (J–L') At E12.5, the number of Sox10⁺p75⁺ DRG progenitors and the percentage of DRG progenitors among all DRG DAPI⁺ cells (DAPI staining not shown) in *Nf2* cKO are similar to that in controls. Arrows: Sox10⁺p75⁻ glial cells. (M–Q) The number of glial cells is increased in *Nf2* cKO DRG at E12.5 and E18.5. Glia are Sox10⁺Blbp⁺ at E12.5 and Sox10⁺S100β⁺ at E18.5. **P*<0.05, ***P*<0.01. Scale bars: 50 μm in A,D,G,J,M; 25 μm in A',D',G',J'; 100 μm in O.

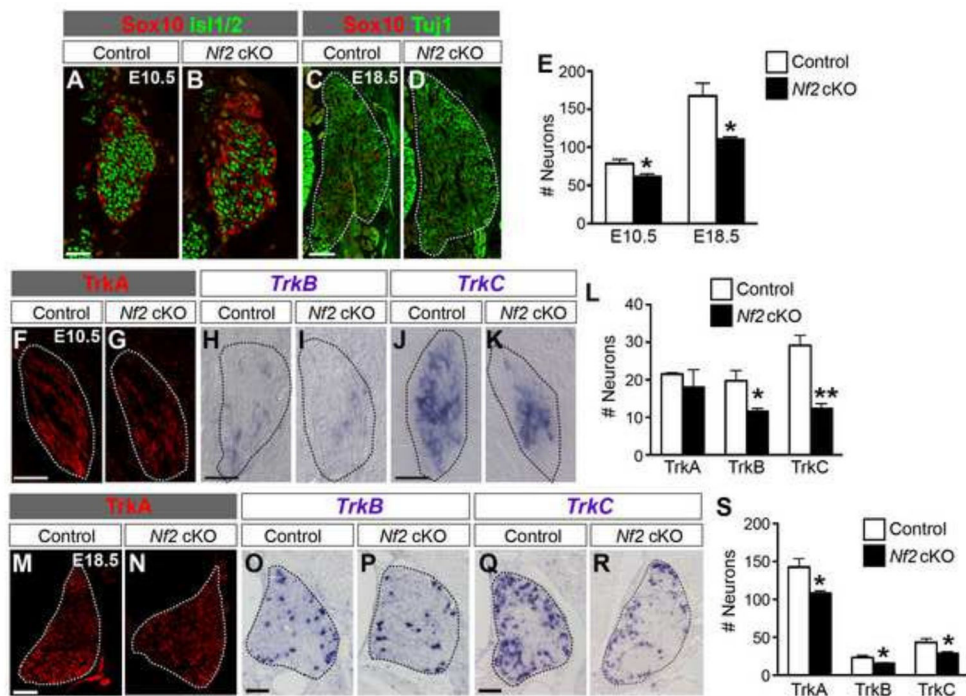


Fig. 7. Deletion of *Nf2* leads to a reduction in neurons

(A–E) The number of neurons, marked by *Is1/2* at E10.5 and by *Tuj1* at E18.5, is reduced in *Nf2* cKO DRG. (F–L) The numbers of *TrkB*⁺ and *TrkC*⁺ neurons are reduced but the number of *TrkA*⁺ neurons is not changed in *Nf2* cKO DRG at E10.5. (M–S) The numbers of each subtype of neurons are reduced in *Nf2* cKO DRG at E18.5. * $P < 0.05$, ** $P < 0.01$. Scale bars: 50 μ m in A,F,H,J; 100 μ m in C,M,O,Q.

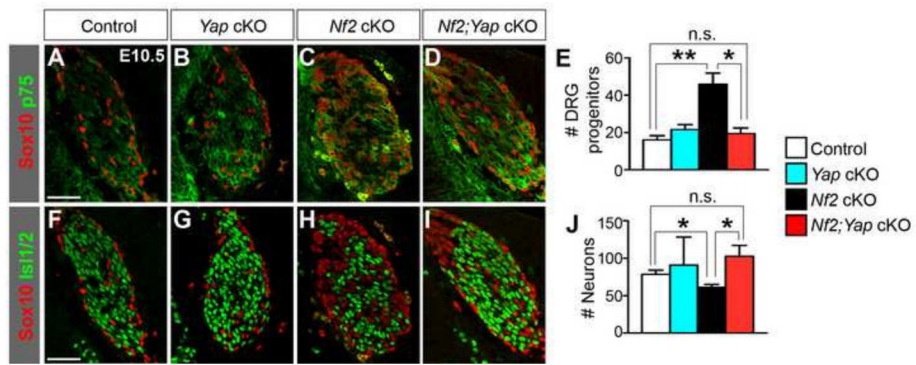


Fig. 8. *Yap* deletion rescues the early DRG defects of *Nf2* cKO embryos

Yap deletion alone does not affect the numbers of Sox10⁺p75⁺ DRG progenitors (A,B,E) and Isl1/2⁺ neurons (F,G,J). However, it suppresses the expansion of DRG progenitors (A–E) and reduction in neurons (F–J) in *Nf2* cKO DRG. **P*<0.05, ***P*<0.01, n.s., not significant. Scale bars: 50 μm.

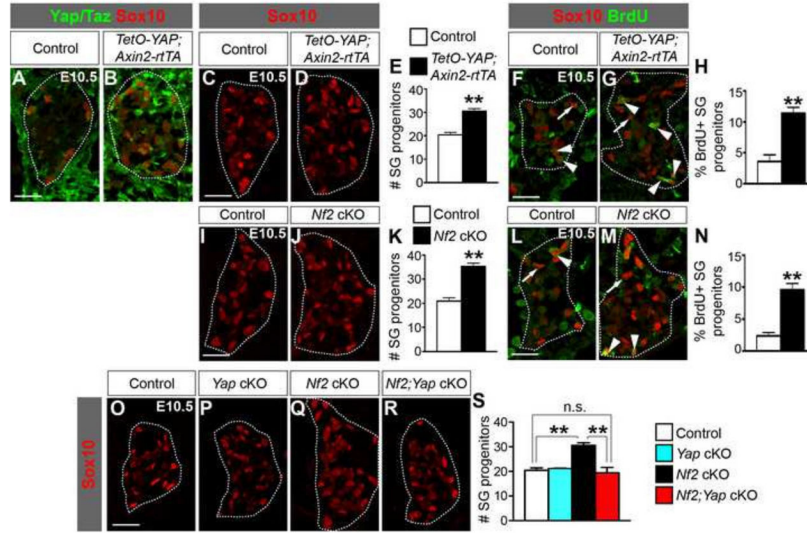


Fig. 9. Nf2-Yap signaling controls the expansion of SG progenitors

(A–H) Dox-containing water was given to pregnant females starting from E5.5 and embryos were collected at E10.5. (A–B) At E10.5, YAP is overexpressed in Sox10⁺ cells in *TetO-YAP;Axin2-rtTA* SG. (C–E) The number of Sox10⁺ SG progenitors is increased in *TetO-YAP;Axin2-rtTA* SG compared to controls. (F–H) Quantification of BrdU⁺ cells among Sox10⁺ SG progenitors after a 2-hour BrdU pulse shows increased proliferation of SG progenitors in *TetO-YAP;Axin2-rtTA* embryos. Arrowheads: BrdU⁺ SG progenitors; arrows: BrdU⁻ SG progenitors. (I–K) At E10.5, *Nf2* cKO SG have more Sox10⁺ SG progenitors than controls. (L–N) Quantification of BrdU⁺ cells among Sox10⁺ SG progenitors after a 2-hour BrdU pulse shows increased proliferation in *Nf2* cKO SG. Arrowheads: BrdU⁺ SG progenitors; arrows: BrdU⁻ SG progenitors. (O–S) *Yap* deletion alone does not affect the number of Sox10⁺ SG progenitors. However, it suppresses the expansion of SG progenitors in *Nf2* cKO SG. ***P*<0.01, n.s., not significant. Scale bars: 50 μm.