Schwannoma development is mediated by Hippo pathway dysregulation and modified by RAS/MAPK signaling

Zhiguo Chen, … , Thomas J. Carroll, Lu Q. Le


Graphical abstract

Find the latest version:
https://jci.me/141514/pdf
Schwannoma Development is Mediated by Hippo Pathway Dysregulation and Modified by RAS/MAPK Signaling

Zhiguo Chen1, Stephen Li1,2, Juan Mo1, Eric Hawley3, Yong Wang1, Yongzheng He3, Jean-Philippe Brosseau1,*, Tracey Shipman1, D. Wade Clapp3, Thomas J. Carroll4,6, Lu Q. Le1,5,6

1Department of Dermatology, UT Southwestern Medical Center, Dallas, TX, USA
2Medical Scientist Training Program, UT Southwestern Medical Center, Dallas, TX, USA
3Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN, USA
4Department of Molecular Biology, UT Southwestern Medical Center, Dallas, TX, USA
5Comprehensive Neurofibromatosis Clinic, UT Southwestern Medical Center, Dallas, TX, USA
6Simmons Comprehensive Cancer Center, UT Southwestern Medical Center, Dallas, TX, USA
*Current address: Department of Biochemistry and Functional Genomics, University of Sherbrooke, Sherbrooke, Canada, J1E 4K8

Author for correspondence:
Lu Q. Le, M.D., Ph.D.
Professor
Department of Dermatology
Simmons Comprehensive Cancer Center
Hamon Center for Regenerative Science and Medicine
UT Southwestern Medical Center
Phone: (214) 648-5781
Fax: (214) 648-5553
E-mail: lu.le@utsouthwestern.edu

Conflict of interest:
The authors have declared that no conflict of interest exists.

Running title: Hippo Pathway in Schwannoma Development

Keywords: Hippo Pathway, Neurofibromatosis type 2 (NF2), Schwannoma, Schwannomatosis, Merlin, MAPK pathway, Schwann cell
Abstract

Schwannomas are tumors of the Schwann cells that cause chronic pain, numbness, and potentially life-threatening impairment of vital organs. Despite the identification of causative genes including NF2 (Merlin), INI1/SMARCB1, and LZTR1, the exact molecular mechanism of schwannoma development is still poorly understood. Several studies have identified Merlin as a key regulator of the Hippo, MAPK, and PI3K signaling pathways, however definitive evidence demonstrating the importance of these pathways in schwannoma pathogenesis is absent. Here, we provide direct genetic evidence that dysregulation of the Hippo pathway in the Schwann cell lineage causes development of multiple Schwannomas in mice. We found that canonical Hippo signaling through the effectors YAP/TAZ is required for schwannomagenesis and that MAPK signaling modifies schwannoma formation. Furthermore, co-targeting YAP/TAZ transcriptional activity and MAPK signaling demonstrated a synergistic therapeutic effect on schwannoma. Our new model provides a tractable platform to dissect the molecular mechanisms underpinning schwannoma formation and the role of combinatorial targeted therapy in schwannoma treatment.
Introduction

Schwannomas are tumors of Schwann cell origin found in patients with Neurofibromatosis Type 2 (NF2) and schwannomatosis, a recently characterized third major form of neurofibromatosis. In NF2, schwannomas can develop along peripheral, spinal, and cranial nerves, and often develop along the eighth cranial nerve resulting in bilateral acoustic (vestibular) schwannomas that cause hearing and balance problems. Patients with schwannomatosis lack this hallmark vestibular schwannoma, but develop multiple non-cutaneous schwannomas elsewhere throughout the body. In both NF2 and schwannomatosis, schwannomas can compress nearby nervous tissue resulting in significant neurological impairment resulting in pain, numbness, and weakness of the extremities; in schwannomatosis, the pain can be particularly debilitating, and is often the first presenting symptom (1). Schwannomas most often occur sporadically outside any tumor-predisposition syndromes, however they can undergo rare malignant transformation and cause life-threatening invasion of nearby vital organs (2-4).

Several genes including NF2 (Merlin), INI1/SMARCBI and the newly identified schwannomatosis-predisposing gene LZTR1 are implicated in schwannoma development (5-8), however their molecular roles are poorly defined. Using P0-Cre induced Nf2 gene deletion, Marco Giovannini first demonstrated that loss of NF2 in the Schwann cell lineage was sufficient for schwannoma development and recapitulation of the human phenotype (9). It was subsequently shown that NF2 acts as a regulator of the Hippo pathway, a highly conserved kinase cascade initially discovered in Drosophila that regulates cell proliferation and organ size (10). Merlin activates the Hippo pathway by forming a complex with Hpo and Sav (Orthologs of mammalian Mst1/2 and Sav1, respectively) in Drosophila (11, 12). The Mst1/2-Sav1 complex then phosphorylates and activates LATS1/2. In mammals, Sav1 recruits MST1/2 kinases to the plasma membrane for activation by upstream regulators. In parallel, Merlin recruits LATS1/2 kinases to the plasma membrane for phosphorylation and activation by MST1/2 kinases (13). Merlin can also modulate LATS1/2 activity through CRL4DCAF1 (14). Activated LATS1/2 in turn phosphorylates and induces cytoplasmic retention and degradation of the transcription factors YAP and TAZ (15). In the absence of Hippo pathway signaling, YAP and TAZ
translocate to the nucleus to form a transcriptional complex with TEAD1-4 and other transcription factors including the bromodomain-containing protein 4 (BRD4), a member of the Bromodomain and Extraterminal (BET) family. This complex then initiates expression of target genes that stimulate proliferation and inhibit apoptosis (16, 17) (Supplemental Figure 1).

While it has been hypothesized that NF2 mediates schwannoma development through Hippo pathway signaling, no direct evidence has been shown. Additionally, new evidence suggests that additional pathways may also be important for schwannoma development. In addition to its role in Hippo signaling, NF2 also regulates PI3K/mTOR/Akt, MAPK, RAS/RAF/ERK, RAC/CDC42/p21-activated kinases, and RhoGTPase family signaling pathways (18-24) (Supplemental Figure 1). Moreover, only about 60% of patients with schwannomas carry biallelic loss of NF2 (25). Furthermore, although INI1/SMARCB1 and LZTR1 mutations show strong correlation with schwannomatosis, there is no direct evidence for their role in Hippo pathway signaling.

We reasoned that if Hippo pathway dysregulation was required for schwannomagenesis, then mutation of the downstream kinases (i.e. LATS1/2) should also lead to schwannomagenesis. Previous studies have shown that knockout of Lats1/2 gene with a broad Schwann cell Cre, such as Dhh-Cre, results in direct malignant transformation and bypasses the benign schwannoma stage (26). Therefore, we hypothesized that a more restricted Schwann cell Cre might slow malignant transformation, enabling detection of the benign schwannoma stage. In this study, we generated a new mouse model of schwannomatosis using Hoxb7-Cre driven Lats1/2 gene deletion. We provide direct evidence that dysregulation of the Hippo pathway is necessary for schwannomagenesis and that MAPK signaling acts as a modifier for schwannoma formation. Moreover, pharmacological co-inhibition of YAP/TAZ transcriptional activity and MAPK signaling shows a synergistic size reduction of mouse schwannoma. Our new model provides a framework to further clarify the molecular mechanisms of schwannoma development and identify potential therapeutic targets.
Results

Hippo pathway inactivation in Hoxb7+ lineage cells results in formation of multiple schwannomas

We previously showed that Hoxb7-Cre was a more restricted Schwann cell Cre and that the Hoxb7+ lineage comprises a subset of Schwann cells in peripheral nerves with tumorigenic potential (27). In order to determine whether Hippo pathway inactivation is sufficient for schwannomagenesis, we crossed the Hoxb7-Cre mice with Lats1\textsuperscript{fl/fl};Lats2\textsuperscript{fl/fl} mice to obtain the Hoxb7-Cre;Lats1\textsuperscript{fl/fl};Lats2\textsuperscript{+/+}, Hoxb7-Cre;Lats1\textsuperscript{fl/+};Lats2\textsuperscript{fl/fl}, Hoxb7-Cre;Lats1\textsuperscript{fl/+};Lats2\textsuperscript{+/+}, Hoxb7-Cre;Lats1\textsuperscript{fl/+};Lats2\textsuperscript{fl/fl} (hereafter called H7;Lats1/2mut2), Hoxb7-Cre;Lats1\textsuperscript{fl/fl};Lats2\textsuperscript{fl/+}, Hoxb7-Cre;Lats1\textsuperscript{fl/+};Lats2\textsuperscript{fl/fl} (hereafter called H7;Lats1/2mut3) and Hoxb7-Cre;Lats1\textsuperscript{fl/fl};Lats2\textsuperscript{fl/fl} (hereafter called H7;Lats1/2mut4) respectively. H7;Lats1/2mut2 mice did not develop tumors and H7;Lats1/2mut4 mice were embryonic lethal; only H7;Lats1/2mut3 mice gave rise to multiple masses in skin, soft tissue, and dorsal root ganglions (DRG) (Supplemental Table 1 and 2) (Figure 1A). Further characterization of these well-circumscribed masses indicated a mixture of hypercellular (Antoni A) areas and hypocellular (Antoni B) areas, diffuse/strong expression of Schwann cell marker S100β and GFAP, neural crest lineage marker SOX10 and abundant pericellular collagen type IV (Figure 1A). These results recapitulate the histology of human schwannoma (Figure 1B) and meet the pathologic diagnostic criteria for schwannoma (28). Some of these tumors underwent malignant transformation as indicated by phosphohistone H3 (p-H3, a mitosis marker) staining, consistent with increased mitotic activity, and allograft assay in nude mice (Figure 1C and 1D).

If the Hippo pathway is crucial for schwannoma development, inactivation of Lats1/2 should result in YAP/TAZ dephosphorylation and nuclear localization. As expected, YAP/TAZ was mainly localized to nuclei of cultured mouse tumor cells (Hoxb7-Cre;Lats1\textsuperscript{fl/+};Lats2\textsuperscript{fl/fl}) (Figure 1D). In addition, we also observed nuclear staining of YAP/TAZ in both human and mouse schwannoma tissues (Figure 1E). Altogether, these data suggest that dysregulation of Hippo pathway signaling is sufficient for schwannomagenesis.
Lats1 or Lats2 loss of heterozygosity in H7;Lats1/2mut3 mice is required for schwannoma development

H7;Lats1/2mut3 mice each developed an average of 15.4 tumors. While Hoxb7-lineage cells comprise only a small subset of Schwann cells in peripheral nerves, the total Hoxb7+ cell number is still considerable (27). Therefore, if triallelic loss of Lats1/2 (Lats1f/f;Lats2f/+ or Lats1f/+;Lats2f/f) in the Hoxb7+ cell lineage is sufficient for schwannomagenesis, we would expect to see a greater number of tumors formed. We hypothesized that loss of heterozygosity of the last Lats1 or Lats2 wild-type (WT) allele in H7;Lats1/2mut3 mice is required for schwannomagenesis. To test our hypothesis, we first analyzed LATS1/2 expression in these tumors. Immunohistochemical staining showed complete absence of LATS1/2 expression in H7;Lats1/2mut3 tumors (Figure 2A), suggesting loss of the remaining allele. Next, we designed genotyping PCR primers to amplify WT, flox, and Δfloxed Lats1 and Lats2 alleles (Figure 2B). As a positive control, we used embryonic day 13.5 (E13.5) Lats1f/+;Lats2f/+ DRG/nerve root sphere cells (DNSCs). We previously showed that DNSCs are embryonic Schwann cell precursors with tumorigenic potential that give rise to Schwann cell tumors when cultured and transplanted into mice (29). We infected these DNSCs with Adenovirus-CMV-Cre-EGFP (Ad-Cre) to delete the floxed Lats1 and Lats2 alleles. Using PCR amplification, we were able to detect WT, flox, and Δfloxed Lats1 and Lats2 alleles in infected or uninfected DNSCs (Figure 2C). Next, we genotyped primary tumor cell lines derived from H7;Lats1/2mut3 mouse schwannomas (Figure 2C). We were unable to detect the WT allele of Lats1 or Lats2, further demonstrating loss of heterozygosity.

We previously demonstrated the value of E13.5 DNSCs for preclinical drug screening and gene editing (27, 29). Therefore, we tested whether 3- or 4-allele ablation of Lats1 and Lats2 in E13.5 DNSCs was sufficient for tumorigenesis in nude mice. We infected E13.5 DNSCs (Lats1/2mut3 and Lats1/2mut4) with Ad-Cre and injected them into the sciatic nerve (SN) and subcutaneous tissue of nude mice. Adenovirus-CMV-EGFP (Ad-GFP) transduction was used as control (Figure 2D). Ad-Cre transduced Lats1/2mut3 DNSCs did not undergo obvious morphological change. However, Ad-Cre transduced
Lats1/2mut4 DNSCs (Supplemental Figure 2) underwent a striking morphological change from small and thin to wide-spreading and flat after Ad-Cre transduction (Figure 2E). When injected into SN and subcutaneous tissue of nude mice, only Lats1/2mut4-Ad-Cre DNSCs gave rise to tumors (Figure 2F). Our results show that loss of heterozygosity of the residual WT Lats1 or Lats2 allele in H7;Lats1/2mut3 mice is required for schwannoma development. However, since NF2 loss in the Schwann cell lineage could also lead to schwannoma development and, although the likelihood is very low, it is still possible that schwannomagenesis may result from NF2 loss/down-regulation as a result of Lats1/2 loss. To exclude this possibility, we performed immunostaining of NF2 in mouse schwannoma. We observed that NF2 was highly expressed in mouse schwannoma (Supplemental Figure 3A). In addition, western blot analysis also showed that NF2 and other Hippo pathway components upstream of LATS1/2, including MST1/2, SAV1, and MOB1 were also expressed (Supplemental Figure 3B), which suggests that NF2 was not lost and was not involved in schwannomagenesis of H7;Lats1/2mut3 mice. These data suggest that both Lats1 and Lats2 loss are required for schwannomagenesis.

**Loss of YAP or TAZ alone does not inhibit schwannoma proliferation**

LATS1/2 are negative regulators of YAP/TAZ in canonical Hippo signaling (Supplemental Figure 1). Given that YAP exerts a stronger influence on multiple cellular processes than TAZ (30), we hypothesized that loss of YAP would impair schwannoma growth. We first tested whether knockdown of YAP transcript would inhibit the proliferation of tumor cells by employing non-inducible small hairpin RNAs (shRNAs). Tumor cells derived from Hoxb7-Cre;Lats1f/+;Lats2f/f mice were transduced with lentivirus harboring either scrambled shRNA (pLKO.1-shCon) or YAP shRNAs (pLKO.1-shYAP-5 and pLKO.1-shYAP-7). Transduction of shYAP-5 and shYAP-7 resulted in complete knockdown of YAP protein (Figure 3A). To study the influence of YAP on the tumorigenic capacity of these cells, we performed subcutaneous injection of shCon, shYAP-5, and shYAP-7 tumor cells into nude mice. Unexpectedly, loss of YAP did not significantly impair schwannoma growth in vivo, with results being highly variable depending on the specific shRNA and experimental replicate (Figure 3B-D). One explanation is that the puromycin-
selected cell populations may have been contaminated with puromycin-resistant shYAP-negative cell clones. Another possibility is functional redundancy of TAZ. In order to rule out contamination, we analyzed the expression of YAP and TAZ in these tumors and tumor-derived cells. We found persistent loss of YAP expression in both shYAP1-5 and shYAP1-7 tumors and tumor-derived cells (Figure 3E and 3F), while TAZ expression remained present. Similarly, we tested whether ablation of YAP alone could inhibit schwannoma proliferation in PostnCre;Nf2<sup>fl/fl</sup>;YAP<sup>fl/fl</sup> mice. Previous studies have shown that PostnCre drives reporter gene expression in Schwann cell progenitors starting at E10 (31, 32). We found that YAP deletion did not affect tumor burden or lifespan (Supplemental Figure 4A-C). These results suggest that YAP alone is not essential for schwannoma development and that TAZ may compensate for YAP loss of function.

Next, we dissected the function of TAZ in schwannomagenesis using the same pLKO.1-shRNA system. Four different pLKO.1-shTAZ lentiviruses were constructed and transduced into tumor cells (Hoxb7-Cre;Lats1<sup>fl/fl</sup>;Lats2<sup>fl/fl</sup>). We chose pLKO.1-shTAZ-6a and pLKO.1-shTAZ-8f for further experiments based on degree of protein knockdown (Figure 3G). After subcutaneous injection of tumor cells into nude mice, we observed similar results to the YAP knockdown. Knockdown of TAZ did not result in significantly reduced tumor growth, with highly varying results dependent on the shRNA used and experimental replicate (Figure 3H-J). Immunohistochemistry and western blot confirmed loss of TAZ protein in transplanted tumors and tumor-derived cells (Figure 3K-L). Taken together, these results suggest that TAZ alone is also not essential for schwannoma development due to potential functional redundancy with YAP.

**YAP/TAZ gene dosage determines the tumor burden and survival**

As key downstream effectors of the Hippo pathway, YAP and TAZ may have both distinct and overlapping functions (30, 33). This potential redundancy may enable schwannomas to tolerate loss of either YAP or TAZ. In order to test whether simultaneous ablation of both YAP and TAZ in Hoxb7<sup>+</sup> lineage cells prevents or rescues the tumor phenotype of H7;Lats1/2mut3 mice, we crossed H7;Lats1/2mut3 mice with YAP<sup>fl/fl</sup>;TAZ<sup>fl/fl</sup> mice to obtain
H7;Lats1/2mut3;YAP/TAZmut4 (YAP\textsuperscript{fl};TAZ\textsuperscript{fl}), H7;Lats1/2mut3;YAP/TAZmut3 (including YAP\textsuperscript{fl};TAZ\textsuperscript{fl/+} and YAP\textsuperscript{fl/+};TAZ\textsuperscript{fl} ) and H7;Lats1/2mut3;YAP/TAZmut2 (including YAP\textsuperscript{fl/+};TAZ\textsuperscript{fl/+}, YAP\textsuperscript{fl/+};TAZ\textsuperscript{fl/+} and YAP\textsuperscript{fl/+};TAZ\textsuperscript{fl}) mice.

All of the H7;Lats1/2mut3;YAP/TAZmut mice were viable, fertile, and normal in size, with no gross behavioral or physical abnormalities except for multiple schwannomas with 100% penetrance. Compared to H7;Lats1/2mut3 mice, H7;Lats1/2mut3;YAP/TAZmut4, H7;Lats1/2mut3;YAP/TAZmut3 and H7;Lats1/2mut3;YAP/TAZmut2 mice exhibited a YAP/TAZ dosage-dependent increase in time to tumor onset and decrease in tumor number (Figure 4A and 4B). We also observed significantly improved survival in H7;Lats1/2mut3;YAP/TAZmut3 and H7;Lats1/2mut3;YAP/TAZmut4 mice compared to H7;lats1/2mut3 (Figure 4A and 4B) (Supplemental Table 1 and 2). Interestingly, while H7;Lats1/2mut4 mice die in utero, several H7;Lats1/2mut4;YAP/TAZmut4 mice survived until post-natal day 10-20, which suggested YAP/TAZ deletion partially rescued not only the tumorigenic phenotype in H7;Lats1/2mut3 mice but also the survival of H7;Lats1/2mut4 mice.

While H7;Lats1/2mut3;YAP/TAZ mut4 mice showed significantly reduced tumor burden, they still developed schwannomas. To confirm whether YAP/TAZ were completely ablated in the tumors of these mice, we performed immunostaining of YAP and TAZ. Surprisingly, strong YAP/TAZ signal was still detectable in these tumors suggesting incomplete ablation of the YAP/TAZ allele (Figure 4C-D). Further genotyping confirmed that at least one intact YAP or TAZ allele was present in all tumor and tumor-derived cell lines, indicating that a subpopulation of tumor cells may have escaped Cre-mediated recombination resulting in delayed tumor formation (Figure 4E). These data suggest that YAP/TAZ gene dosage determines tumor burden and survival, and that a single allele of YAP or TAZ is sufficient for schwannoma formation. Immunohistochemical analysis showed increased phospho-ERK in tumor from Hoxb7;Lats1/2mut3;YAP/TAZmut4 compared to mice Hoxb7;Lats1/2mut3 indicating that YAP/TAZ ablation activated the MAPK pathway (Supplemental Figure 5).
Canonical Hippo signaling through YAP/TAZ is required for schwannomagenesis

To investigate whether YAP/TAZ are critical for schwannomagenesis, we first tested whether or not YAP/TAZ could be completely ablated. We cultured tumor cell lines derived from H7;Lats1/2mut3;YAP/TAZmut4 mice - 459T cells - and transduced them with Ad-Cre to ablate residual YAP and TAZ. We optimized the virus concentration to minimize cytotoxicity and ensure at least 95% gene delivery for each transduction by monitoring GFP expression. We found that a single round of Ad-Cre transduction resulted in only partial recombination of the YAP loxP sites (Figure 5A). We then tested up to six rounds of serial infections. Surprisingly, only YAP was ablated after multiple infections. The TAZ flox allele was present even after six rounds of serial infections with Ad-Cre (Figure 5A). Furthermore, addition of Lentivirus-Cre did not result in recombination of the TAZ allele. We observed similar results in multiple H7;Lats1/2mut3;YAP/TAZmut4 tumor-derived cell lines.

To test whether complete YAP/TAZ ablation inhibits the proliferation of schwannoma in vivo, we chose a H7;Lats1/2mut3;YAP/TAZmut4-derived tumor cell line, 454T, harboring complete YAP loss and two intact TAZ alleles. After six rounds of Ad-Cre transduction and one round of Lenti-Cre infection (Ad-Crex6-Lv-Cre), only a small portion of TAZ alleles remained intact (Figure 5B). Next, we injected these cells into nude mice subcutaneously. Cells infected with six rounds of Adenovirus-GFP (Ad-GFP) and one of Lentivirus-GFP (Lenti-GFP) were injected subcutaneously as a control (Ad-GFX6-Lv-GFP). We found that partial ablation of TAZ resulted in enhanced tumor growth (Figure 5B). Immunostaining and genotyping confirmed that TAZ was still highly expressed in transplanted tumors, suggesting positive clonal selection based on TAZ status (Figure 5B and Supplemental Figure 6A). Given that the genotyping PCR results were based on a mixture of cells, we performed flow cytometry to isolate and seed single cells into each well of 96 well plates to obtain single cell clones from 454T-Ad-Crex6-Lv-Cre cells and their allografted tumors. After 18 days in culture, only 2 of the 96 sorted 454T-Ad-Crex6-Lv-Cre cells exhibited clonal expansion (Figure 5C). However, 42 of the 96 454T-Ad-Crex6-Lv-Cre allograft-derived cells exhibited clonal expansion (Figure 5C). We carefully
searched individual wells of non-expanding clones and observed that single cells isolated from 454T-Ad-Crex6-Lv-Cre cells remained non-proliferative even at 28 days in culture (Figure 5C).

We then designed nested PCR primers to detect TAZ status on a single-cell level. Consistent with our clonal analysis, we found that 95.6% (43 of 45) of single 454T-Ad-Crex6-Lv-Cre cells had complete recombination of the remaining TAZ allele, while the remaining 4.4% (2 of 45) carried both a flox and Δfloxed TAZ. For sorted 454T-Ad-Crex6-Lv-Cre allograft cells, only 6.8% (3 of 44) of single cell clones exhibited recombination of the remaining TAZ allele, while 79.5% (35 of 44) carried both a flox and Δfloxed TAZ allele and 13.6% (6 of 44) carried only floxed TAZ alleles (Figure 5D). Taken together, our data show that complete ablation of YAP/TAZ inhibits the proliferation of tumor cells, providing definitive evidence that canonical Hippo signaling is necessary and sufficient for schwannoma development. In addition, our studies demonstrate a need to develop inhibitors that simultaneously target both YAP and TAZ or their interaction for transcriptional activity.

The MAPK pathway is a modifier of schwannomagenesis

We previously demonstrated that YAP/TAZ were activated in both human and mouse NF1-null cutaneous neurofibromas, a tumor driven by dysregulated RAS/MAPK signaling (27). Several studies have demonstrated that RAS/MAPK signaling may regulate the Hippo pathway through a WTIP-LATS interaction or Raf1-MST2 interaction (34, 35). Therefore, we hypothesized that the RAS/MAPK pathway may modify and accelerate schwannoma development. Since NF1 (neurofibromin) is a negative regulator of RAS and loss of NF1 activates RAS/MAPK signaling, to test our hypothesis we crossed H7;Lats1/2mut3 mice with Nf1\textsuperscript{lf} or Nf1\textsuperscript{lf-} (hereafter called Nf1mut) to obtain H7;Lats1/2mut;Nf1mut mice (Supplemental Figure 7A). As hypothesized, loss of Nf1 significantly increased the tumor burden of H7;Lats1/2mut3;Nf1mut mice (Figure 6A). H7;Lats1f/+;Lats2f/f and H7;Lats1ff;Lats2f/+ mice developed about 9.2 and 21.2 tumors in each mouse, respectively (Figure 6B). However, H7;Lats1f/+;Lats2ff;Nf1mut and
H7;Lats1f/f;Lats2f/+;Nf1mut mice developed about 21.1 and 33.4 tumors in each mouse, respectively (Figure 6B).

Next, we investigated the impact of RAS/MAPK pathway activation via Nf1 deletion on the overall survival of H7;Lats1/2mut3 mice. At 100 days, approximately 93% of H7;Lats1f/+;Lats2f/f;Nf1mut and 100% of H7;Lats1f/f;Lats2f/+;Nf1mut had been sacrificed after reaching tumor size limits, whereas none of H7;Lats1f/+;Lats2f/f;Nf1wt mice and only 24% of H7;Lats1f/f;Lats2f/+;Nf1wt mice needed to be sacrificed (Figure 6C). Consistent with these findings, immunostaining confirmed that the loss of Nf1 in H7;Lats1/2mut3 tumor enhanced the expression of p-ERK, a RAS/MAPK pathway activation marker (Figure 6D and Supplemental Figure 7B), and these tumors were well circumscribed with hypercellular (Antoni A) areas and hypocellular (Antoni B) areas of predominantly organized Schwann cells, consistent with schwannomas histologically and molecularly (Supplemental Figure 8).

Taken together, these data suggest that the RAS/MAPK pathway may serve as a modifier of schwannomagenesis and that inhibition of RAS/MAPK signaling may serve as an alternative therapeutic strategy.

**MAPK pathway inhibition sensitizes schwannoma to JQ1 treatment**

There are currently no targeted pharmacologic therapies for schwannomas. We sought to investigate the therapeutic effect of YAP/TAZ inhibition on Schwannoma proliferation. Verteporfin was the first identified small molecule inhibitor of YAP and functions by disrupting the interaction between YAP and TEAD (36). It has been shown to have some degree of therapeutic effect on high YAP-expressing tumor mouse models (37, 38). Unfortunately, verteporfin does not disrupt the interaction between TAZ and TEAD, and an optimal inhibitory effect requires very high concentrations that make it unsuitable for in vivo use. Consistent with this, a previous report demonstrated that although high-dose verteporfin alone suppressed Lats1/2-deficient tumor cell growth in vivo relative to vehicle, it increased risk of mortality due to toxicity (26). Additionally, a lower dose of verteporfin only showed a gradual and modest inhibition of tumor growth (26), indicating a need for
alternative YAP/TAZ inhibitors. Recent studies suggest that pharmacologic inhibition of BRD4, a required cofactor for YAP/TAZ transcriptional activity, blunts growth of YAP/TAZ-addicted breast tumors. Interestingly, we also observed high BRD4 expression in YAP/TAZ-activated mouse schwannoma (Figure 7), indicating the therapeutic potential of BET inhibitors in the treatment of schwannoma (17). However, single inhibitor therapy of tumors often results in compensatory signaling upregulation and blunted therapeutic effects. For example, YAP/TAZ inactivation may cause compensatory lysosome-mediated activation of MAPK signaling in NF2 tumor growth (39). Additionally, YAP may also mediate resistance to MEK1/2 inhibition in neuroblastomas with hyperactivated RAS signaling (40). These studies suggest that targeting of both YAP/TAZ and MAPK signaling may provide additive or synergistic therapeutic benefit. Therefore, we tested the combined effect of a BRD4 inhibitor (JQ1) and MAPK pathway inhibitor (PD0325901, hereafter called 901) on schwannoma in vitro and in vivo. In mouse tumor cells, 901 significantly inhibited the phosphorylation of ERK1/2 and slightly inhibited the mRNA expression of YAP/TAZ but not their downstream target genes CTGF and Cyr61 (Figure 7A). JQ1 treatment alone downregulated mRNA expression of YAP and Cyr61, indicating partial inhibition of YAP/TAZ transcriptional activity (Figure 7B). Consistent with previous findings, we observed compensatory activation of the MAPK pathway upon JQ1 treatment (Figure 7B) (39). When we combined 901 and JQ1, we found dramatic inhibition of ERK1/2 phosphorylation and downregulation of YAP/TAZ transcriptional targets (Figure 7C). These data suggest a synergistic inhibitory effect of MAPK and Hippo pathway targeting that enables circumvention of established compensatory signaling mechanisms.

Next, we assessed whether tumor cells were sensitive to combined treatment of 901 and JQ1 in vitro. 901 treatment alone slightly suppressed the proliferation of tumor cells while JQ1 alone demonstrated a greater effect. Combined 901 and JQ1 treatment significantly slowed schwannoma cell growth, outweighing the effect of either drug alone (Figure 7D). In order to validate our findings in vivo, we administered 901 and JQ1 in H7;Lats1/2mut3 mice. These mice developed tumors around 2-3 months of age. Once the largest tumor diameter reached 5-10 mm, mice were randomized into 4 groups and treated with vehicle, 901, JQ1, or a combination of 901 and JQ1. Consistent with our in vitro data, H7;Lats1/2mut3 mice treated with either 901 or JQ1 showed slowed tumor growth.
However, there was no significant reduction in tumor size from baseline, with only 3 of 33 (9.1%) JQ1-treated tumors seeing size reduction. When mice were treated with combined 901 and JQ1, we found a marked reduction in tumor growth (Figure 7E). Furthermore, nearly half of the tumors (44.2%, 19 of 43) atrophied after one month (Figure 7F). Immunohistochemistry of 901 and JQ1 treated tumors confirmed reduced BRD4 expression and ERK1/2 phosphorylation (Figure 7G-H). Altogether, our data demonstrate that co-targeting YAP/TAZ transcriptional activity and the MAPK signaling pathway may be a promising therapeutic strategy for Schwannoma.

Discussion

*Dysregulated Hippo-Lats1/2-YAP/TAZ pathway leads to schwannoma development*

The significance of canonical Hippo signaling in schwannoma pathogenesis has long been hypothesized, despite a lack of definitive evidence. In the present study, we provide multiple lines of evidence supporting the requirement of canonical Hippo signaling in schwannomagenesis. Firstly, complete loss of both *Lats1* and *Lats2* in the Hoxb7+ lineage resulted in multiple schwannoma formation. While the key effectors of the Hippo pathway, YAP/TAZ, are also regulated by alternative pathways such as Wnt and G-protein-coupled receptor signaling (41, 42), these alternative modes of regulation still function through LATS1/2. In addition, activation of the Hippo pathway by reconstituting *NF2* expression in *NF2*-null breast cancer cell lines results in a robust LATS1/2-dependent inhibition of YAP/TAZ activity (43). Secondly, ablation of YAP/TAZ resulted in a gene dosage-dependent reduction in tumor burden and extension of life span. Although genetic ablation of YAP/TAZ only partially rescued the tumor phenotype of *H7;Lats1/2mut3* mice, YAP/TAZ expression were still detectable in these tumors suggesting incomplete Cre recombination resulting in positive selection of YAP or TAZ-retaining tumor cells. This is supported by our single cell clonal analysis, which demonstrated retention of wild-type TAZ alleles in expanding clones only. This natural limitation of the Cre-LoxP system may be due to impaired chromatin accessibility (44). Lastly, although the functions of INI1/SMARCB1 and LZTR1 in schwannomagenesis are still unclear, recent research
shows that subsequent biallelic loss of \textit{Nf2} is necessary to induce schwannoma formation (45). Collectively, our data demonstrate that the dysregulation of Hippo-Lats1/2-YAP/TAZ signaling drives schwannomagenesis.

\textit{Hippo pathway dysregulation in schwannoma and cancer}

Loss of function of the core Hippo pathway kinases have been widely implicated in murine tumor models (9, 26, 46-48). However, with the exception of \textit{NF2} mutations in human schwannoma (49-66%), very few Hippo pathway mutations have been found in human tumors. Only 2% and 1% of human schwannomas carry \textit{Lats1} and \textit{Lats2} mutations, respectively (49), and no \textit{Mst1/2} mutation has been reported in human cancer. One possibility for this discrepancy is the functional redundancy of MST1 and MST2 or LATS1 and LATS2. Accordingly, we and others must ablate four alleles of \textit{Lats1/2} in order to completely block the Hippo pathway for schwannoma induction, making human sporadic loss of function mutations unlikely. Another possibility is epigenetic silencing of these genes. Studies show that promoter methylation of \textit{Lats1} and \textit{Lats2} is common in schwannomas (17% and 30%, respectively) (49) while promoter methylation of \textit{Mst1} and \textit{Mst2} has been detected in 37% and 17% of sarcomas, respectively (50). These findings suggest that non-mutational mechanisms may also inactivate the Hippo pathway and play important roles in tumorigenesis.

It is worth noting that \textit{YAP/TAZ} may be dispensable for tumorigenesis in certain cancers such as malignant hematopoiesis (51). However, our present work and other studies suggest that \textit{YAP/TAZ} are likely required for tumors that form as a consequence of Hippo pathway mutations. If true, then the mutational status or promoter methylation of Hippo pathway components may be used to predict the therapeutic potential of \textit{YAP/TAZ} inhibitors.

\textit{YAP/TAZ and MAPK pathway act as the co-therapeutic target of schwannoma}
In addition to their essential roles in regulating tumor proliferation and stemness (52), YAP/TAZ also enable resistance to chemotherapy and targeted therapy (53). YAP/TAZ are also dispensable for tissue homeostasis, (including of the Hoxb7+ Schwann cell lineage) making them ideal therapeutic targets (51, 52). Unfortunately, our single cell analyses clearly indicate that complete inhibition of both YAP and TAZ is required to block schwannoma cell proliferation. Therefore, adequate pharmacologic therapy necessitates targeting of both YAP and TAZ. However, there is currently no TAZ-specific inhibitor.

Alternatively, researchers had looked to transcriptional coactivators of YAP/TAZ for pharmacologic targets. BRD4 is one of the required transcriptional coactivators of YAP/TAZ. It has been shown that YAP/TAZ target genes have significant vulnerability to BRD4 inhibitors and that a relevant fraction of BRD4’s oncogenic functions are associated with YAP/TAZ (17). In line with these notions, a majority of the cell population in our mouse schwannoma tumor highly expressed BRD4 and YAP/TAZ. In addition, BRD4 inhibition by JQ1 partially blocked transcriptional activity of YAP/TAZ and reduced mouse schwannoma growth. However, when compared to the tumor size before JQ1 treatment, only 3 of 33 tumors atrophied while the majority of tumors continued growing. These data suggest that YAP/TAZ inhibition by JQ1 results in cytostasis rather than cell death.

Because our single cell clone forming assay also demonstrated that complete YAP/TAZ loss may only induce cytostasis, we looked for alternative pathways that may serve as adjunct targets. Many studies, including the present, indicate that BET inhibition activates compensatory MAPK signaling to provide a cell-protective effect (54). As we expected, combined pharmacologic targeting of BRD4 and the MAPK pathway achieved promising therapeutic results on mouse schwannoma. We found nearly half of the tumors atrophied, indicating that co-treatment may trigger tumor apoptosis or cell death. Furthermore, combined treatment decreased the phosphorylation of ERK and protein levels of BRD4 and YAP/TAZ, suggesting a synergistic effect. Interestingly, a previously published study demonstrated that co-targeting of BRD4 and MAPK signaling promoted cell death in a NF1 loss-of-function mouse model of malignant peripheral nerve sheath tumor (55). The authors demonstrated that targeting of both pathways resulted in PRC2/SUZ12-mediated epigenetic remodeling that repressed RAS transcriptional output. Given the crosstalk between the Hippo and MAPK pathways, it is interesting to postulate that JQ1 sensitivity
in MPNST may be due to a YAP/TAZ-dependent mechanism. It is also known that phosphorylation of ERK induces cyclin D expression while YAP/TAZ activation upregulates cyclin dependent kinase 6 (CDK6) expression (56, 57). Cyclin D forms a complex with and activates CDK4/6, an important regulator of DUB3-mediated de-ubiquitination and stability of BRD4 (58, 59). This may represent another mechanism through which co-targeting BRD4 and phosphorylation of ERK results in decreased protein level of BRD4 and impairment of schwannoma proliferation.

Finally, our studies using Nf1 deletion to activate the MAPK pathway suggested that this signaling pathway might modify schwannoma development. However, neurofibromin also regulates a number of MAPK-independent pathways, and thus it is possible that one or more of these other pathways may also affects schwannoma development.

In conclusion, we provide genetic evidence that dysregulation of Hippo-Lats1/2-YAP/TAZ signaling mediates schwannomagenesis. Furthermore, the MAPK pathway acts as a modifier of schwannoma development and co-targeting of YAP/TAZ and the MAPK pathway is a promising therapeutic strategy for schwannomas. Although the herein described mouse model produces schwannomas with matching histology, the spectrum of this mouse tumor syndrome is beyond the scope of human schwannomas or schwannomatosis. Nonetheless, our new model provides a framework to begin to dissect the molecular mechanisms of schwannomagenesis and identify novel therapeutic targets.

Methods

Mice

The Nf1 knockout (60), Nf1 flox (61), Hoxb7-Cre (62), Lats1 flox (63), Lats2 flox (63) Yap flox (64), Taz flox (65) and athymic nude mice (Foxn1/−) are available from Jackson Laboratory (Bar Harbor, ME). Genotyping was performed by PCR as previously reported (60-65). All mice were housed in the Animal Care Facility at the University of Texas Southwestern Medical Center (Dallas, TX).
Animal studies

Mice were monitored and sacrificed when the largest tumor diameter reached 1 cm. The hair of mice was removed and total palpable tumor number was counted. The dates of sacrifice were documented for survival analysis. Tumors arising near the spine could occasionally infiltrate and compress the spinal cord, leading to paralysis of mice. These mice were euthanized before the largest tumor diameter reached 1 cm. In this case, the dates of sacrifice were still documented; however the total tumor number was excluded for quality purposes.

To analyze the therapeutic effect of JQ1, PD0325901 (901), or combined JQ1 and 901 treatment on tumor burdened H7;Lats1/2mut3 mice, we closely monitored the tumor size of each mouse. When the biggest tumor diameter reached 0.5 to 1 cm, the tumor burdened H7;Lats1/2mut3 mice were randomly assigned into 4 groups (5 mice/group). Group 1 received vehicle daily; Group 2 received JQ1 (45 mg/kg intraperitoneally) daily in a 10% (2-hydroxypropyl)-β-cyclodextrin solution. Group 3 received 901 by oral gavage daily at 1.5 mg/kg (vehicle (0.5% (w/v) methylcellulose solution with 0.2% (v/v) Tween 80)). Group 4 was administered a combination of JQ1 (45 mg/kg intraperitoneally) and 901 (1.5 mg/kg, oral gavage) sequentially (55). All groups were treated for 1 month. Tumor size was measured weekly and tumor volume was calculated by measuring length and width of the lesion with the formula (length) × (width)^2 × (0.52) (66).

E13.5 DRG/nerve roots and tumorsphere cell culture

E13.5 DRGs/nerve roots were isolated and neurospheres generated as previously described (29). Briefly, mouse embryos were removed from anesthetized 13.5-day-old pregnant female mice. Embryos were sacrificed and the spinal cord of each embryo was removed. DRGs/nerve roots were dissected from the vertebral column with the aid of a stereomicroscope. The DRGs/nerve roots were digested with 1 mg/ml collagenase at 37°C for 30 min, and then washed twice with Dulbecco’s modified Eagle’s medium (DMEM)/F12. The cells were counted and plated on uncoated, ultra-low attachment 6-well plates (Corning) to allow the sphere formation in proliferation media: DMEM/F12
containing penicillin/streptomycin (0.1%); fungizone (40 mg/ml); B27 (without vitamin A), epidermal growth factor (20 ng/ml), and basic fibroblast growth factor (40 ng/ml; Sigma-Aldrich). The sphere cells were fed every 3–4 days and passaged every 7 days. Sphere cells were then seeded on fibronectin-coated cell culture plates for monolayer culture with aforementioned proliferation media.

For tumorsphere cell culture, the tumors were removed from the mice. Tumors were minced with fine scissors and tumorsphere cell culture was performed using the same procedure mentioned above. The schwannoma cell line 1162 was generated in this way from a mouse schwannoma tumor harvested from a Hoxb7-Cre;Lats1/2mut3 mouse.

**In Vitro Growth Assays**

ATP CellTiter Glo assay (Promega) was performed per manufacturer’s instructions. Luminescence was quantified via Synergy|HT 96-well plate reader (BioTek).

**Cytoplasmic and nuclear fractionation**

Cytoplasm and nuclear extracts were isolated as previously described (67) with some modifications. Cells were harvested by trypsin-EDTA, collected by centrifugation and washed twice in ice-cold PBS. The cell pellets were resuspended by one volume PBS and two volume hypotonic lysis buffer (HLB; 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and protease inhibitors) and incubated for 10 min on ice. NP-40 was added at final concentration of 0.1% total volume. The lysed cell solution was placed on ice for 10 min. Nuclei were then pelleted by centrifugation at 5000 g for 5 min at 4°C and supernatant containing cytoplasmic proteins was collected and stored at −80°C. The nuclear pellet was washed twice with ice cold 0.1% NP40-HLB buffer. The nuclear pellet was resuspended in 1 volume of 0.1% NP40-HLB buffer with protease inhibitors, then 0.2 volume of 5x nuclei lysis buffer (0.5% NP40, 500 mM NaCl and 50 mM Tris) was added. The lysed nuclei solution was incubated on ice for 10 min and then centrifuged at 16,000
RPM for 10 min. The supernatant containing nucleus proteins was collected and stored at −80°C. Both cytoplasmic and nuclear fractions were analyzed by Western blotting.

RNA Isolation, cDNA Synthesis, qRT-PCR

RNEasy mini kit (Qiagen) was used to isolate total RNA from cells, followed by cDNA synthesis with iScript Select cDNA synthesis kit (Bio-Rad), and then qRT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad). The following primers were used: YAP-F: 5’-ACCAATAGTTCCGATCCCTTTTC-3’; YAP-R: 5’-TGTCTCCTGTATCCATTTCATCC-3’; TAZ-F: 5’-CGTCCATCACTCCACCTC-3’; TAZ-R: 5’-GTTGGTTCTGAGTCCGGGTG-3’; β-actin-F: 5’-TTCTACAATGAGCTGCGTG-3’; β-actin-R: 5’-GGGGTGTTGAAGGTCTCAAA-3’; CTGF-F: 5’-CTGCCTACCGACTGGAAGAC-3’; CTGF-R: 5’-CATTGGTAACTCGGGTGGAG-3’; Cyr61-F: 5’-GCTCAGTCAGAAGGCAGGACAGG-3’; Cyr61-R: 5’-GCTCTTGGGGACACAGAGGA-3’;

Immunohistochemistry

For immunohistochemistry, paraffin sections were deparaffinized, rehydrated, and subjected to antigen retrieval prior to incubation with the primary antibodies as described below. The primary antibodies were visualized by treating the sections with biotinylated secondary antibody followed by amplification with peroxidase-conjugated avidin and 3, 30-diaminobenzidine substrate per manufacturer’s protocol (Vector Labs). The primary antibodies used in this study were as follows: rabbit anti-S100 β (Agilent Cat# Z0311, RRID:AB_10013383), rabbit anti-GFAP (Agilent Cat# Z0334, RRID:AB_10013382), rabbit anti-SOX10 (Abcam Cat# ab180862, RRID:AB_2721184), rabbit anti-Collagen IV (Abcam Cat# ab6586, RRID:AB_305584), rabbit anti-YAP (Cell Signaling Technology Cat# 14074, RRID:AB_2650491), rabbit anti-TAZ (ABCclonal Cat# A8202, RRID:AB_2721146), rabbit anti- Phospho-Histone H3 (Cell Signaling Technology Cat# 9701, RRID:AB_331535), rabbit anti-LATS1 (Proteintech Cat# 17049-1-AP, RRID:AB_2281011), rabbit anti-LATS2 (Proteintech Cat# 20276-1-AP,
RRID:AB_10697657), rabbit anti-total ERK1/2 (Cell Signaling Technology Cat# 4695, RRID:AB_390779), rabbit anti-phospho ERK1/2 (Cell Signaling Technology Cat# 4370, RRID:AB_2315112), and rabbit anti-NF2 (Thermo Fisher Scientific Cat# PA5-82060, RRID:AB_2789221).

Mouse whole spinal cord dissection

Whole spinal cord dissection was performed as previously reported (68). To perform mouse anesthesia, a mixture of ketamine (10 mg/ml) and xylazine (1 mg/ml) solution (100 μL per 25g of mouse weight) was administered intraperitoneally. After 15-20 min, mice were placed face-up in a surgical field and the chest area was sprayed with 70% ethanol. The left thoracic cage was removed, a catheter was installed in the heart left ventricle and mice were perfused intracardially with 4% para-formaldehyde. Then, the mouse was prepared for microscopic dissection by removing gross tissue (cervical decapitation, whole skin removal and all internal organs). Next, muscle and other tissue were carefully removed and bones from the vertebrate column were broken one by one under dissection microscope to end up with intact spinal cord and peripheral nerves. Finally, whole spinal cord and peripheral nerves were rinsed with PBS 1X and immersed in 10% formalin-buffered solution.

Dorsal root ganglion volume measurement

DRG and spinal nerves were dissected out under a microscope after being fixed and decalcified in 5% formic acid. The tumor volume was calculated according to a specific formula as volume = length X width² X 0.52, which approximates the volume of a spheroid.

Transplantation experiments

Sciatic nerve implantation of E13.5 DNSCs was performed as previously reported (69). Briefly, mice were anesthetized by intraperitoneal injection of 120 μl of a mixture of ketamine (10 mg/ml) and xylazine (1 mg/ml) solution. A skin incision was made above the femur. Using iris scissors, a pocket was created within the quadriceps muscles to expose the SN. 40 μl of L15 medium containing 1 ×10⁶ viable DNSCs was then deposited into
this pocket so that DNSCs can be in contact with the SN. The quadriiceps muscles were then closed with 4-0 Vicryl suture, and the skin was closed with 5-0 prolene suture.

For subcutaneous implantation, the nude mouse was manually restrained by hand and then placed on a clean towel. $1 \times 10^6$ DNSCs or tumor cells were injected under the skin of the shoulder area.

**Genotyping PCR and single cell nested PCR**

Genotyping PCR primers are as follows: \textit{Lats1}-F: 5'-CCTTTATGCTGATGCCCTAAGA-3', \textit{Lats1}-R: 5'-ATGAATGAACCTGAGGCTGC-3'. \textit{Lats2}-F: 5'-AAAGCACAGGGCTTTTACA-3'. \textit{Lats2}-R: 5'-ACACATTCCCCCTCCACTGAC-3'. \textit{β-actin} F: 5'-CCTAGGCACCAGGGTGTGAT-3'. \textit{β-actin} R: 5'-TCACGGTTGGCCTTAGGGTT-3'.

For single cell nested PCR: Single cells were isolated by flow cytometry into each well of a 96-well PCR plate. Single cell nested PCR was performed as previously reported (70). Briefly, 10 μl of single cell lysis buffer was added to well containing one single cell/well. 90 μl of reaction mixture including first PCR primers was added. PCRs were performed in 100 μl of reaction buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM Mg$^{2+}$, 200 μM of each dNTP, and 1.25 μ Taq polymerase. Twenty-five picomoles of each primer was used. The primers were designed as follows: the outer forward primer (1F) and reverse primer (1R) sequences were located upstream and downstream of the two \textit{loxP} sites of \textit{TAZ} respectively; The inner forward primer (2F) and reverse primer (2R) sequences were located inside the 1F and 1R sequence but outside the two \textit{loxP} sites of \textit{TAZ} such that the \textit{floxed} and \textit{Δfloxed} alleles of \textit{TAZ} could be detected (Figure 5). Two reverse primers with recognition sequences within the \textit{loxP}-flanked exon were also generated (R1.5 and R2.5). The primer sequences are as follows: \textit{TAZ} F1: ATCTTGCTCTGGACACT; \textit{TAZ} R1: GGCAAAGCACAGGGTAAGAA; \textit{TAZ} R1.5: TCCTTTCTGGAAAAGTTGCATT; \textit{TAZ} F2: GGCCACTGCATTGGACATTC; \textit{TAZ} R2: CATCAGAAAACACAGCAGCA; \textit{TAZ} R2.5: AATGCTTCTCCCAAGACTG.
Two consecutive PCRs with nested primers were performed. For the first PCR with outer primers (F1+R1+R1.5): following 5 min of incubation at 94°C, 30 cycles were carried out with denaturing for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 2 min at 72°C, with a final extension step of 10 min at 72°C. For the second PCR with inner primers (F2+R2+R2.5): 20 μl of the reaction buffer described above and 1 μl of the first PCR product were mixed. Thirty cycles of 35 s at 94°C, 30 s at 70°C, and 40 s at 72°C were followed by a final extension step of 10 min at 72°C.

Western blot analysis

Protein isolation, and subsequent Western blot analysis was performed as described previously (66). The following antibodies were used: rabbit anti-YAP/TAZ (Cell Signaling Technology Cat# 8418, RRID:AB_10950494), rabbit anti-Histone H3 (Cell Signaling Technology Cat# 9717, RRID:AB_331222), mouse anti-GAPDH (Santa Cruz Biotechnology Cat# sc-47724, RRID:AB_627678), rabbit anti-BRD4 (Bethyl Cat# A700-004, RRID:AB_2631885), rabbit anti-ERK1/2 (Cell Signaling Technology Cat# 4695, RRID:AB_390779), rabbit anti-p-ERK1/2 (Cell Signaling Technology Cat# 4370, RRID:AB_2315112), rabbit anti-LATS1 (Proteintech Cat# 17049-1-AP, RRID:AB_2281011), rabbit anti-LATS2 (Proteintech Cat# 20276-1-AP, RRID:AB_10697657), rabbit anti-MST1 (Cell Signaling Technology Cat# 3682, RRID:AB_2144632), rabbit anti-MST2 (Cell Signaling Technology Cat# 3952, RRID:AB_2196471), rabbit anti-NF2 (Thermo Fisher Scientific Cat# PA5-82060, RRID:AB_2789221), rabbit anti-SAV1 (Cell Signaling Technology Cat# 13301, RRID:AB_2798176) and rabbit anti-MOB1 (Cell Signaling Technology Cat# 13730, RRID:AB_2783010).

shRNA and lentiviral constructs

pLKO.1-TRC cloning vector was a gift from David Root (Addgene plasmid # 10878; http://n2t.net/addgene:10878; RRID:Addgene_10878)(71). pLKO-shRNA plasmids were
generated as described on the website: https://www.addgene.org/tools/protocols/plko/.
The following constructs were used: pLKO-shYAP-5: 5’-TGAGAACAAATGACAACCAATA -3’(TRCN0000238436), pLKO-shYAP-6: 5’-GCAGACAGATTTCTTTGTTAA -3’(TRCN0000095864), pLKO-shYAP-7: 5’-GAAGCGCTGAGTTCCGAAATC -3’(TRCN000238432), pLKO-shYAP-8: 5’-TCCAACCAGCAGCAGCAAATA -3’(TRCN0000095853), pLKO-shYAP-9: 5’-CCATGAGCACAGATAGATAGAT -3’(TRCN0000095952), pLKO-shTAZ-6: 5’-CAGCCGAATCTCGCAATGAAT-3’(TRCN0000095951), pLKO-shTAZ-7: 5’-CCATGAGCACAGATAGATAGAT -3’(TRCN0000095953), pLKO-shTAZ-8: 5’-GTGATGAATCAGCCTCTGAAT-3’(TRCN0000095952), pLKO-shTAZ-9: 5’-CCTTCTTTAAGGAGCCGATT-3’(TRCN0000095950). Packaging vectors psPAX2 and pMD2.g were gifts from Didier Trono (Addgene plasmid # 12260; http://n2t.net/addgene:12260; RRID:Addgene_12260; Addgene plasmid # 12259 ; http://n2t.net/addgene:12259; RRID:Addgene_12259). psPAX2 and pMD2.g packaging vectors were used for lentivirus production.

Quantification and statistical analysis

All data are displayed as the mean ± SEM unless specified otherwise. For mouse survival analysis, Kaplan-Meier estimator with log-rank statistical test was employed. One-way analysis of variance and Student's t test and one-way ANOVA with Tukey's test for multiple comparisons were applied to evaluate statistical significance (* = p < 0.05; ** = p < 0.01; *** = p < 0.001).

Study approval

All mouse procedures were approved by Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center and conformed to NIH guidelines.

Authors’ Contributions

Acknowledgments
We thank all members of the Le laboratory for helpful suggestions and discussions, Renee McKay for critical review and editing of the manuscript, and Megan Cermak for generating the initial mouse crosses and phenotype observation. S.L. is supported through the Medical Scientist Training Program and an F30 fellowship from the National Institute of Aging of the National Institute of Health (F30AG056075). J.M. and J.P.B. are Early Investigator Research Awardees from the US Department of Defense. L.Q.L. holds the Thomas L. Shields, M.D. Professorship in Dermatology. T.C. was supported by funding from CPRIT RP160340 and the NIH R01DK095057. Mouse strains were provided by the UT Southwestern George M. O’Brien Kidney Research Core Center P30DK079328. D.W.C. was supported by funding from U.S. Department of Defense grant # NF170058. This work was supported by funding from the National Cancer Institute of the National Institutes of Health (grant number R01 CA166593) and the U.S. Department of Defense (grant number W81XWH -17-1-0148) to L.Q.L.

References


Figure 1. Hippo pathway inactivation in Hoxb7+ lineage cells results in multiple schwannoma formation.

A) Dissection and histological characterization of mouse schwannoma: H&E and immunohistochemistry of Schwann cell markers (S100β and GFAP), a neural crest marker (Sox10), and collagen IV. (B) H&E and immunohistochemistry of S100β, GFAP, SOX10 and Collagen IV on human schwannoma tissue sections. (C) Immunohistochemistry of phospho-Histone H3 on mice and human schwannoma tissue sections. (D) Mouse schwannomas were harvested, and tumorsphere cell culture was performed. Tumorspheres were then seeded to fibronectin-coated plates for monolayer culture. Both cytoplasmic and nuclear fractions isolated from these monolayer cultured cells were analyzed by Western blotting. The tumor cells were injected into nude mice subcutaneously (n = 6). (E) Immunohistochemistry of YAP and TAZ on mouse and human schwannoma tissue sections. Scale bars, 50 μm.
Figure 2. *Lats1* or *Lats2* loss of heterozygosity in *H7;Lats1/2mut3* mice is required for schwannoma development.

(A) Immunohistochemistry of LATS1 and LATS2 on schwannoma and sciatic nerve sections from *H7;Lats1/2mut3* mice. (B) Diagram of PCR primer design for detecting the WT, Flox or ΔFloxed alleles of *Lats1* or *Lats2*. (C) Gel electrophoresis of DNSC and tumor PCR products. β-actin was used as an internal control (n = 3-4). Y= Ad-Cre infected. N= No Ad-Cre infected. T= Tumors. M= DNA marker. (D) Diagram of experimental design for testing tumor formation potential of E13.5 DNSCs *in vivo*. (E) Morphology of E13.5 DNSCs (*Lats1/2mut*) infected with Ad-GFP or Ad-Cre. (F) Left panel: Gross picture of nude mice injected with E13.5 DNSCs (*Lats1/2mut4-Ad-GFP*) (left side) or E13.5 DNSCs (*Lats1/2mut4-Ad-Cre*) (right side, red circles) (n = 4). Right panels: H&E histology (top) and immunohistochemistry (bottom) for LATS1 and LATS2 in *Lats1/2mut4-Ad-Cre* tumor. *L1/2mut4*: *Lats1/2mut4*. +Con: positive control. *H7*: *Hoxb7-Cre*. *L1*: *Lats1*. *L2*: *Lats2*. Scale bars, 50 μm.
Figure 3. Loss of YAP or TAZ alone does not inhibit schwannoma proliferation.

(A) Western blot analysis of YAP and TAZ protein level in shControl, shYAP-5 and shYAP-7 transduced tumor cells (Hoxb7-Cre;Lats1ff;Lats2ff). shCon=shControl. (B) Tumor volume of shControl, shYAP-5 and shYAP-7 transduced tumor cells after transplantation into nude mice (n = 5/group). (C) Gross picture of tumors from the experimental endpoint in Figure 3B (n = 5/group). (D) Average weight of excised tumors from Figure 3C (n = 5/group). (E) Representative images of allografted tumor sections stained for YAP and TAZ. (F) Western blot analysis of YAP and TAZ protein level in cultured tumor-derived cells. (G) Western blot analysis of YAP and TAZ protein level in shControl, shTAZ-6, shTAZ-7, shTAZ-8 and shTAZ-9 tumor cells. (H) Tumor volume of shControl, shTAZ-6, and shTAZ-8 schwannomas in nude mice (n = 7/group). (I) Gross picture of tumors from the experimental endpoint in Figure 3H (n = 7/group). (J) Average weight of excised tumors from Figure 3I (n = 7/group). (K) Representative images of tumor sections stained for YAP and TAZ. (L) Western blot analysis of YAP and TAZ protein levels in cultured tumor cells derived from tumors in Figure 3I. Scale bars, 50 μm. One-way analysis of variance was applied to evaluate statistical significance in B, D, H and J. All statistics are represented as the mean ± SEM (*p<0.05, **p<0.01, ***p < 0.001).
Figure 4

A. Hoxb7-Cre;Lats1/+;Lats2/± YAP/TAZ WT vs YAP/TAZ mut4

B. Hoxb7-Cre;Lats1/+;Lats2/± YAP/TAZ WT vs YAP/TAZ mut4

C. Hoxb7-Cre;Lats1/2mut3

D. YAP and TAZ nuclear positive cells (%)

E. Hoxb7;Lats1/2mut3; YAP/TAZ

Tissues:
- YAP
- TAZ

Cells:
- YAP
- TAZ

Legend:
- Flox
- WT
- ΔFloxed

Significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- p < 0.0001
Figure 4. YAP/TAZ gene dosage determines the tumor burden and survival.

(A, B) Representative pictures of tumor-burdened mice (left panel); scatter plot of the total number of palpable tumors in each mouse (middle panel). Kaplan-Meier plot illustrating the survival curve among the groups with corresponding genotypes (right panel) for $\text{Hoxb7-Cre;Lats1}^{+/+};\text{Lats2}^{f/f}$ (A) and $\text{Hoxb7-Cre;Lats1}^{f/f};\text{Lats2}^{+/+}$ (B) mice. $\text{H7;Lats1}^{+/+};\text{Lats2}^{f/f};\text{YAP/TAZmut4}$: $n = 12$. $\text{H7;Lats1}^{f/f};\text{Lats2}^{f/f};\text{YAP/TAZmut3}$: $n = 44$. $\text{H7;Lats1}^{f/f};\text{Lats2}^{f/f};\text{YAP/TAZmut2}$: $n = 60$. $\text{H7;Lats1}^{f/f};\text{Lats2}^{f/f};\text{YAP/TAZWT}$: $n = 24$. $\text{H7;Lats1}^{f/f};\text{Lats2}^{f/f};\text{YAP/TAZmut4}$: $n = 15$. $\text{H7;Lats1}^{f/f};\text{Lats2}^{f/f};\text{YAP/TAZmut3}$: $n = 29$. $\text{H7;Lats1}^{f/f};\text{Lats2}^{f/f};\text{YAP/TAZmut2}$: $n = 39$. $\text{H7;Lats1}^{f/f};\text{Lats2}^{f/f};\text{YAP/TAZWT}$: $n = 25$. (C) Immunohistochemistry of YAP and TAZ on tumor sections. (D) Quantification of immunohistochemistry in (C) ($n = 3$/group). (E) Genotyping of tumor tissues (upper panel) and tumor-derived cell lines (lower panel). Red star, intact Flox allele. Green dot, non-specific amplification. Scale bars, 50 μm. One-way ANOVA with Tukey’s test for multiple comparisons were applied to evaluate statistical significance in A, B and D. Log-rank statistical test was employed in A and B. Statistics are represented as the mean ± SEM (*p<0.05, **p<0.01, ***p < 0.001).
Figure 5. Canonical Hippo signaling through YAP/TAZ is required for schwannomagenesis.

(A) Genotyping (left) and western blot (right) of YAP and TAZ in 459T cells serially infected with Ad-GFP/Lv-GFP or Ad-Cre/Lv-Cre. (B) Genotyping (left top panel) and western blot (right top panel) of YAP and TAZ in 454T cells serially infected with Ad-GFP or Ad-Cre/Lv-Cre; tumor volume of 454T-Ad-GFPx6-Lv-GFP and 454T-Ad-CreX6-Lv-Cre schwannoma tumor in nude mice (middle left panel) (n = 10/group); gross picture of tumors from experimental endpoint in Figure 5B (lower left panel) (n = 10/group); Immunohistochemistry (lower right panel) and genotyping analysis of TAZ (lower left panel) in transplanted nude mice tumor tissue and its derived tumor cell lines. (C) Single cell clonal analysis of 454T-Ad-Crex6-Lv-Cre cells (upper panel) and their transplanted tumor-derived cells (lower panel); Yellow colored cell culture media indicates the proliferation of single cell clone; pictures were taken at 28 days (454T-Ad-Crex6-Lv-Cre cells) and 8 days (transplanted tumor-derived cells). (D) Single cell nested PCR for TAZ. Diagram of PCR primer design for detecting the Flox or ΔFloxed allele of TAZ in single cell level. F1, R1, and R1.5 primers were used for the first PCR. F2, R2 and R2.5 primers were used for the second PCR (upper panel). Representative pictures of gel electrophoresis for single cell nested PCR products (middle panel). Quantification of Flox and ΔFloxed alleles of TAZ from 454T-Ad-Crex6-Lv-Cre cells and their allograft-derived cells (Lower panel). 454T-Ad-Crex6-Lv-Cre cells: n = 45. 454T-Ad-Crex6-Lv-Cre allograft cell: n = 44. First lane: DNA marker; empty lane: failure to detect the signal. Each lane represents a single cell. Scale bars, 50 μm. Student's t test was applied to evaluate statistical significance in B. Statistics are represented as the mean ± SEM (*p<0.05, **p<0.01).
Figure 6. The MAPK pathway is a modifier of schwannomagenesis.

(A) Representative pictures of $H7;Lats1/2mut3;Nf1wt$ (upper panel) and $H7;Lats1/2mut3;Nf1mut$ (lower panel) mice. (B) Scatter plot of the total palpable tumor number in each mouse. $H7;Lats1^{f/+};Lats2^{f/f};Nf1wt$: n=24. $H7;Lats1^{f/+};Lats2^{f/f};Nf1mut$: n = 14. $H7;Lats1^{f/f};Lats2^{f/+};Nf1wt$: n = 24. $H7;Lats1^{f/f};Lats2^{f/+};Nf1mut$: n = 29. (C) Survival comparison between the $H7;Lats1^{f/+};Lats2^{f/f};Nf1wt$ and $H7;Lats1^{f/+};Lats2^{f/f};Nf1mut$ groups (left panel); Survival comparison between the $H7;Lats1^{f/f};Lats2^{f/+};Nf1wt$ and $H7;Lats1^{f/f};Lats2^{f/+};Nf1mut$ groups (middle panel). Legend for Figure 6B and 6C (right panel). $H7;Lats1^{f/+};Lats2^{f/f};Nf1wt$: n = 24. $H7;Lats1^{f/+};Lats2^{f/f};Nf1mut$: n = 14. $H7;Lats1^{f/f};Lats2^{f/+};Nf1wt$: n = 24. $H7;Lats1^{f/f};Lats2^{f/+};Nf1mut$: n = 29. (D) Immunohistochemistry of phospho-ERK1/2 and total ERK1/2 in $H7;Lats1/2mut3;Nf1wt$ and $H7;Lats1/2mut3;Nf1mut$ tumor sections. Scale bars, 50 μm. Student’s t test was applied to evaluate statistical significance in B. Log-rank statistical test was employed in C. Statistics are represented as the mean ± SEM **p<0.01).
Figure 7. MAPK pathway inhibition sensitizes schwannoma to JQ1 treatment.

(A-C) Western blot (top) and qPCR (bottom) analysis of a mouse schwannoma cell line (1162, generated from a schwannoma of a H7;Lats1/2mut3 mouse) treated with 901 (A), JQ1 (B) or both (C). (D) Effect of treatment of 901, JQ1, or both on mouse schwannoma cell growth using ATP CellTiter Glo assay. n = 3/group. (E) Fold change of tumor volume with treatment of 901, JQ1 or both on tumor-burdened H7;Lats1/2mut3 mice. Vehicle: n = 34. 901: n = 48. JQ1: n = 33. JQ1+901: n = 43. (F) Waterfall plot depicting fold change of tumor volume from baseline (Day 0) after treatment with 901, JQ1, or both. Vehicle: n = 34. 901: n = 48. JQ1: n = 33. JQ1+901: n = 43. (G) Immunohistochemistry of BRD4 and phospho-ERK1/2 in mouse schwannomas treated with vehicle, 901, JQ1, or both. (H) Quantification of immunohistochemistry in (G). Scale bars, 50 μm. All statistical comparisons made to JQ1+901. n = 3/group. One-way analysis of variance was applied to evaluate statistical significance in D and E. Box plots show median (line) and 25th to 75th percentile (box); The end of the whiskers represents the minimum and the maximum of all of the data in A, B and C. One-way ANOVA with Tukey’s test for multiple comparisons were applied to evaluate statistical significance in H. All statistics are represented as the mean ± SEM (*p<0.05, ***p < 0.001).
Supplemental Figure 1

Hippo pathway
Up stream signal

Cytoplasm

Nucleus

Downstream targets

RAC CDC42

PAK1

RAS

RAF

MEK

ERK

YAP/TAZ

Mst1/2

Lats1/2

YAP/TAZ

PI3K

TSC1/2

AKT

Rheb

mTORC1

Downstream targets

BRDd4 YAP/TAZ

TEAD

Target genes
Supplemental Figure 1. Schematic diagram of Merlin and Hippo pathway signaling.

Core Hippo pathway components are shown. Merlin (MER) activates the Hippo pathway by forming a complex with STE20-like protein kinase 1/2 (MST1/2; mammalian ortholog of Hippo). When phosphorylated and activated by upstream signals, Mst1/2 directly phosphorylates and activates large tumor suppressor homolog 1/2 (LATS1/2). In parallel, Merlin can recruits LATS1/2 kinases to the plasma membrane for phosphorylation and activation by MST1/2 kinases. Merlin can also modulate LATS1/2 activity through the cullin4 (CUL4)-RING E3 ubiquitin ligase complex (CRL4-DCAF1). Activated LATS1/2 in turn phosphorylates and induces cytoplasmic retention and degradation of the transcription factors Yes-associated protein or transcriptional coactivator with PDZ-binding motif (YAP/TAZ). When Hippo signaling is in the “off” state, YAP/TAZ are devoid of serine phosphorylation and so escape from degradation and cytoplasmic retention. They translocate into the nucleus, where they interact with transcriptional enhancer associated domain (TEAD) and other transcription factors such as bromodomain-containing protein 4 (BRD4) to co-activate the expression of target genes. Merlin also interacts with other signaling pathways such as the MAP kinase pathway and the PI3 kinase pathway to activate gene expression.
Supplemental Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>Lats1</th>
<th></th>
<th>Lats2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E956</td>
<td>GFP Cre</td>
<td>E956</td>
<td>Cre</td>
</tr>
<tr>
<td>E954</td>
<td>GFP Cre</td>
<td>E956</td>
<td>Cre</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td>Lats2</td>
<td>Flox</td>
</tr>
</tbody>
</table>

Lats1 Floxed

Lats2 Floxed

β-actin

B

E956

E954

L107;L267

GFP Cre

GFP Cre

LATS1

LATS2

GAPDH
Supplemental Figure 2. \textit{Lats1} or \textit{Lats2} loss of heterozygosity in \textit{H7;Lats1/2mut3} mice is required for schwannoma development.

(A) Genotyping analysis of \textit{Lats1/2mut3} and \textit{Lats1/2mut4} DNSCs with Ad-GFP or Ad-Cre. (B) Western blot analysis of LATS1 and LATS2 expression in indicated cells. E956 genotype: \textit{Lats1}^{f/f};\textit{Lats2}^{f/+}; E954 genotype: \textit{Lats1}^{f/f};\textit{Lats2}^{f/f}. GFP: Ad-GFP. Cre: Ad-Cre. M: molecular weight marker.
Supplemental Figure 3

A

Brain

Tumor

NF2

NF2

B

<table>
<thead>
<tr>
<th></th>
<th>Tumor 1</th>
<th>Tumor 2</th>
<th>Tumor 3</th>
<th>Tumor 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MST1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAV1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Figure 3. NF2 and other Hippo pathway component expression in mouse schwannoma.

(A) Immunohistochemistry of NF2 on Brain (positive control) and tumor sections. (B) Western blot analysis of Hippo pathway components upstream of LATS1/2 on tumor tissues. Scale bars, 50 μm.
Supplemental Figure 4

A

PostnCre-; Nf2+/++; Yap+/+

PostnCre+; Nf2+/++; YAP+/+

PostnCre+; Nf2+/++; YAP+/+

PostnCre+; Nf2+/++; Yap+/+

B

C

DRG volume (mm²)

Percent survival

Age (month)
Supplemental Figure 4. YAP deletion did not affect tumor burden or lifespan.

(A) Representative pictures of spinal cord and peripheral nerve (upper panel) of depicted genotype; Histological evaluation of DRGs (Lower panel). (B) Average DRG volume of mice with different genotypes. (C) Kaplan-Meier plot illustrating the survival curve of mice with indicated genotypes. Scale bars, 50 μm.
Supplemental Figure 5

A

<table>
<thead>
<tr>
<th>p-ERK1/2</th>
<th>ERK1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H7;Lats1/2mut3</td>
<td>H7;Lats1/2mut3;YAP/TAZmut4</td>
</tr>
</tbody>
</table>

[Image of tissue sections for p-ERK1/2 and ERK1/2 staining in different conditions]
Supplemental Figure 5. Deletion of YAP/TAZ activates MAPK signaling.

(A) Immunohistochemistry of phospho-ERK1/2 and total ERK1/2 in $H7;Lats1/2mut3$ and $H7;Lats1/2mut3;YAP/TAZ mut4$ tumor sections. Scale bars, 50 μm.
Supplemental Figure 6

A

![Bar chart showing nuclear positive cells for YAP and TAZ with different treatments like Ad-GFPX6-Lv-GFP and Ad-CreX6-Lv-Cre.]

B

459T Cell line

![Line graph showing tumor volume (mm^3) over days after injection with different treatments like Ad-GFPX6-LV-GFP and Ad-CreX6-LV-Cre.]

C

459T Cell line

![Image showing different tissue samples labeled Ad-GFPx6 +Lv-GFP and Ad-CreX6 +Lv-Cre.]

Supplemental Figure 6. Canonical Hippo signaling through YAP/TAZ is required for schwannomagenesis.

(A) Quantification of YAP and TAZ immunohistochemistry in Figure 5B. n = 5/group. (B) Tumor volume of 459T-Ad-GFPX6-Lv-GFP and 459T-Ad-CreX6-Lv-Cre schwannoma tumor in nude mice. N = 10/group. (C) Gross picture of tumors from experimental endpoint in (B). n = 10/group.
Supplemental Figure 7

A  M  
\[ H7;Lats1/2mut3;Nf1wt \]  \[ H7;Lats1/2mut3;Nf1mut \]  
\[ \text{T1 T2 T3 T4 T5 T6} \]  
\[ \text{T1 T2 T3 T4 T5 T6} \]  
\[ \text{Nf1 Flox} \]  
\[ \text{Nf1 WT} \]  
\[ \text{Nf1Δ Floxed} \]  

B  
\[ H7;Lats1/2mut3;Nf1wt \]  \[ H7;Lats1/2mut3;Nf1mut \]  
\[ \text{T1 T2 T3 T4 T5 T6} \]  
\[ \text{T1 T2 T3 T4 T5 T6} \]  
\[ \text{p-ERK1/2} \]  
\[ \text{ERK1/2} \]
Supplemental Figure 7. MAPK pathway activation following NF1 loss.

(A) Genotyping of Nf1 and (B) Western blot analysis of phospho-ERK1/2 and ERK1/2 in tumors from Hoxb7-Cre;Lats1/2mut3;Nf1wt and Hoxb7-Cre;Lats1/2mut3;Nf1mut mice.
Supplemental Figure 8. *Hoxb7-Cre;Lats1/2mut3;Nf1mut* mice developed schwannoma and not neurofibroma.

Histological and molecular analysis by H&E staining, Toluidine blue staining (Mast cell marker, arrow), Sirius red staining (collagen marker) and SOX10 immunohistochemistry (neural crest lineage marker) of cutaneous neurofibroma from *Hoxb7-Cre;Nf1mut* mice (left column); schwannoma from *Hoxb7-Cre;Lats1/2mut3* mice (middle column) and schwannoma from *Hoxb7-Cre;Lats1/2mut3;Nf1mut* mice (right column). Scale bars, 50 μm.
Supplemental Table 1. Genotype and phenotype of H7;Lats1<sup>f/+</sup>;<Lats2<sup>f/f</sup>; YAP/TAZ(mut/WT) mice.

<table>
<thead>
<tr>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>2</td>
<td>193</td>
<td>F</td>
<td>4</td>
<td>175</td>
<td>F</td>
<td>15</td>
<td>116</td>
<td>F</td>
<td>4</td>
<td>158</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>147</td>
<td>F</td>
<td>2</td>
<td>230</td>
<td>M</td>
<td>14</td>
<td>134</td>
<td>F</td>
<td>4</td>
<td>161</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>155</td>
<td>F</td>
<td>2</td>
<td>143</td>
<td>F</td>
<td>6</td>
<td>98</td>
<td>M</td>
<td>3</td>
<td>157</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>188</td>
<td>F</td>
<td>1</td>
<td>193</td>
<td>M</td>
<td>6</td>
<td>109</td>
<td>M</td>
<td>5</td>
<td>177</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>188</td>
<td>M</td>
<td>2</td>
<td>172</td>
<td>F</td>
<td>10</td>
<td>166</td>
<td>F</td>
<td>1</td>
<td>189</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>237</td>
<td>M</td>
<td>4</td>
<td>125</td>
<td>M</td>
<td>6</td>
<td>127</td>
<td>M</td>
<td>3</td>
<td>201</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>326</td>
<td>M</td>
<td>1</td>
<td>205</td>
<td>F</td>
<td>7</td>
<td>136</td>
<td>M</td>
<td>2</td>
<td>213</td>
</tr>
<tr>
<td>M</td>
<td>N.A.</td>
<td>200</td>
<td>F</td>
<td>2</td>
<td>140</td>
<td>M</td>
<td>4</td>
<td>147</td>
<td>M</td>
<td>2</td>
<td>245</td>
</tr>
<tr>
<td>M</td>
<td>1</td>
<td>216</td>
<td>F</td>
<td>2</td>
<td>189</td>
<td>F</td>
<td>2</td>
<td>73</td>
<td>F</td>
<td>2</td>
<td>152</td>
</tr>
<tr>
<td>M</td>
<td>1</td>
<td>207</td>
<td>F</td>
<td>3</td>
<td>165</td>
<td>M</td>
<td>7</td>
<td>161</td>
<td>F</td>
<td>5</td>
<td>160</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>168</td>
<td>M</td>
<td>5</td>
<td>252</td>
<td>F</td>
<td>3</td>
<td>153</td>
<td>M</td>
<td>N.A.</td>
<td>147</td>
</tr>
<tr>
<td>M</td>
<td>5</td>
<td>325</td>
<td>F</td>
<td>4</td>
<td>223</td>
<td>M</td>
<td>4</td>
<td>189</td>
<td>F</td>
<td>8</td>
<td>127</td>
</tr>
<tr>
<td>H7; L&lt;sup&gt;f/+&lt;/sup&gt;; L&lt;sup&gt;f/f&lt;/sup&gt;; Y&lt;sup&gt;f/+&lt;/sup&gt;; T&lt;sup&gt;i/-&lt;/sup&gt;</td>
<td>F</td>
<td>1</td>
<td>90</td>
<td>F</td>
<td>4</td>
<td>131</td>
<td>M</td>
<td>7</td>
<td>172</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>165</td>
<td>M</td>
<td>2</td>
<td>224</td>
<td>F</td>
<td>6</td>
<td>137</td>
<td>M</td>
<td>9</td>
<td>156</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>143</td>
<td>M</td>
<td>5</td>
<td>250</td>
<td>M</td>
<td>3</td>
<td>124</td>
<td>M</td>
<td>10</td>
<td>107</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>143</td>
<td>F</td>
<td>2</td>
<td>198</td>
<td>M</td>
<td>4</td>
<td>137</td>
<td>F</td>
<td>5</td>
<td>107</td>
</tr>
<tr>
<td>F</td>
<td>4</td>
<td>108</td>
<td>F</td>
<td>2</td>
<td>165</td>
<td>F</td>
<td>7</td>
<td>127</td>
<td>F</td>
<td>7</td>
<td>101</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>115</td>
<td>F</td>
<td>1</td>
<td>170</td>
<td>F</td>
<td>4</td>
<td>145</td>
<td>F</td>
<td>10</td>
<td>134</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>145</td>
<td>F</td>
<td>2</td>
<td>266</td>
<td>M</td>
<td>7</td>
<td>145</td>
<td>F</td>
<td>7</td>
<td>112</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>162</td>
<td>F</td>
<td>9</td>
<td>251</td>
<td>F</td>
<td>1</td>
<td>115</td>
<td>F</td>
<td>2</td>
<td>112</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>159</td>
<td>H7; L&lt;sup&gt;f/+&lt;/sup&gt;; L&lt;sup&gt;f/f&lt;/sup&gt;; Y&lt;sup&gt;f/+&lt;/sup&gt;; T&lt;sup&gt;i/-&lt;/sup&gt;</td>
<td>F</td>
<td>10</td>
<td>106</td>
<td>M</td>
<td>5</td>
<td>112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>162</td>
<td>F</td>
<td>6</td>
<td>118</td>
<td>H7; L&lt;sup&gt;f/+&lt;/sup&gt;; L&lt;sup&gt;f/f&lt;/sup&gt;; Y&lt;sup&gt;f/+&lt;/sup&gt;; T&lt;sup&gt;i/-&lt;/sup&gt;</td>
<td>F</td>
<td>8</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>162</td>
<td>F</td>
<td>3</td>
<td>118</td>
<td>M</td>
<td>4</td>
<td>150</td>
<td>F</td>
<td>12</td>
<td>166</td>
</tr>
<tr>
<td>H7; L&lt;sup&gt;f/+&lt;/sup&gt;; L&lt;sup&gt;f/f&lt;/sup&gt;; Y&lt;sup&gt;f/+&lt;/sup&gt;; T&lt;sup&gt;i/-&lt;/sup&gt;</td>
<td>M</td>
<td>1</td>
<td>86</td>
<td>M</td>
<td>4</td>
<td>145</td>
<td>M</td>
<td>5</td>
<td>147</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>142</td>
<td>M</td>
<td>1</td>
<td>174</td>
<td>F</td>
<td>12</td>
<td>154</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>126</td>
<td>F</td>
<td>5</td>
<td>145</td>
<td>F</td>
<td>1</td>
<td>134</td>
<td>F</td>
<td>18</td>
<td>154</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>148</td>
<td>F</td>
<td>5</td>
<td>100</td>
<td>F</td>
<td>4</td>
<td>119</td>
<td>M</td>
<td>11</td>
<td>150</td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>188</td>
<td>M</td>
<td>4</td>
<td>125</td>
<td>M</td>
<td>1</td>
<td>121</td>
<td>F</td>
<td>9</td>
<td>169</td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>126</td>
<td>M</td>
<td>3</td>
<td>123</td>
<td>M</td>
<td>2</td>
<td>170</td>
<td>F</td>
<td>8</td>
<td>120</td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>188</td>
<td>M</td>
<td>N.A.</td>
<td>157</td>
<td>M</td>
<td>1</td>
<td>121</td>
<td>M</td>
<td>16</td>
<td>154</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>256</td>
<td>F</td>
<td>5</td>
<td>173</td>
<td>F</td>
<td>1</td>
<td>134</td>
<td>M</td>
<td>15</td>
<td>148</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>200</td>
<td>M</td>
<td>3</td>
<td>129</td>
<td>M</td>
<td>9</td>
<td>134</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>93</td>
<td>F</td>
<td>8</td>
<td>135</td>
<td>F</td>
<td>2</td>
<td>120</td>
<td>F</td>
<td>10</td>
<td>161</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>156</td>
<td>F</td>
<td>4</td>
<td>135</td>
<td>F</td>
<td>1</td>
<td>147</td>
<td>F</td>
<td>8</td>
<td>154</td>
</tr>
<tr>
<td>M</td>
<td>4</td>
<td>295</td>
<td>M</td>
<td>6</td>
<td>150</td>
<td>M</td>
<td>1</td>
<td>173</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>170</td>
<td>M</td>
<td>15</td>
<td>183</td>
<td>M</td>
<td>1</td>
<td>199</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Supplemental Table 2. Genotype and phenotype of *H7;Lats1<sup>−/−</sup>;Lats2<sup>−/−</sup>;YAP/TAZ(mut/WT)* mice.

<table>
<thead>
<tr>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
<th>Sex</th>
<th>TTN</th>
<th>Life span (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>4</td>
<td>178</td>
<td>M</td>
<td>5</td>
<td>108</td>
<td>F</td>
<td>8</td>
<td>103</td>
<td>M</td>
<td>4</td>
<td>122</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>147</td>
<td>M</td>
<td>8</td>
<td>135</td>
<td>F</td>
<td>5</td>
<td>103</td>
<td>M</td>
<td>N.A.</td>
<td>76</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>233</td>
<td>M</td>
<td>6</td>
<td>127</td>
<td>M</td>
<td>13</td>
<td>145</td>
<td>F</td>
<td>15</td>
<td>131</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>106</td>
<td>M</td>
<td>12</td>
<td>139</td>
<td>M</td>
<td>14</td>
<td>95</td>
<td>M</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>142</td>
<td>F</td>
<td>6</td>
<td>111</td>
<td>F</td>
<td>10</td>
<td>134</td>
<td>M</td>
<td>27</td>
<td>95</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>139</td>
<td>F</td>
<td>3</td>
<td>103</td>
<td>F</td>
<td>11</td>
<td>123</td>
<td>F</td>
<td>6</td>
<td>72</td>
</tr>
<tr>
<td>M</td>
<td>6</td>
<td>151</td>
<td>M</td>
<td>8</td>
<td>236</td>
<td>M</td>
<td>4</td>
<td>85</td>
<td>M</td>
<td>44</td>
<td>122</td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>126</td>
<td>M</td>
<td>3</td>
<td>135</td>
<td>M</td>
<td>13</td>
<td>93</td>
<td>M</td>
<td>14</td>
<td>130</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>159</td>
<td>F</td>
<td>10</td>
<td>58</td>
<td>F</td>
<td>3</td>
<td>74</td>
<td>F</td>
<td>N.A.</td>
<td>122</td>
</tr>
<tr>
<td>M</td>
<td>4</td>
<td>187</td>
<td>F</td>
<td>2</td>
<td>159</td>
<td>F</td>
<td>14</td>
<td>126</td>
<td>F</td>
<td>13</td>
<td>111</td>
</tr>
<tr>
<td>M</td>
<td>2</td>
<td>185</td>
<td>M</td>
<td>1</td>
<td>100</td>
<td>F</td>
<td>19</td>
<td>126</td>
<td>F</td>
<td>11</td>
<td>111</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>154</td>
<td>F</td>
<td>3</td>
<td>107</td>
<td>M</td>
<td>15</td>
<td>143</td>
<td>M</td>
<td>25</td>
<td>139</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>154</td>
<td>M</td>
<td>2</td>
<td>218</td>
<td>F</td>
<td>9</td>
<td>126</td>
<td>F</td>
<td>31</td>
<td>81</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>118</td>
<td>F</td>
<td>3</td>
<td>146</td>
<td>F</td>
<td>27</td>
<td>92</td>
<td>M</td>
<td>70</td>
<td>131</td>
</tr>
<tr>
<td>H7;L1f/f;L2f/+;Yf/f;Tf/f</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>7</td>
<td>109</td>
<td>M</td>
<td>5</td>
<td>104</td>
<td>M</td>
<td>11</td>
<td>97</td>
<td>M</td>
<td>30</td>
<td>123</td>
</tr>
<tr>
<td>M</td>
<td>6</td>
<td>128</td>
<td>M</td>
<td>12</td>
<td>122</td>
<td>F</td>
<td>11</td>
<td>115</td>
<td>F</td>
<td>24</td>
<td>125</td>
</tr>
<tr>
<td>F</td>
<td>4</td>
<td>140</td>
<td>F</td>
<td>12</td>
<td>122</td>
<td>M</td>
<td>15</td>
<td>145</td>
<td>F</td>
<td>27</td>
<td>116</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>126</td>
<td>M</td>
<td>11</td>
<td>122</td>
<td>H7;L1f/f;L2f/+;Yf/+;Tf/+</td>
<td></td>
<td></td>
<td>F</td>
<td>13</td>
<td>159</td>
</tr>
<tr>
<td>M</td>
<td>5</td>
<td>107</td>
<td>F</td>
<td>5</td>
<td>93</td>
<td>F</td>
<td>12</td>
<td>227</td>
<td>M</td>
<td>25</td>
<td>120</td>
</tr>
<tr>
<td>M</td>
<td>4</td>
<td>120</td>
<td>F</td>
<td>6</td>
<td>147</td>
<td>F</td>
<td>7</td>
<td>138</td>
<td>M</td>
<td>18</td>
<td>152</td>
</tr>
<tr>
<td>M</td>
<td>4</td>
<td>127</td>
<td>M</td>
<td>28</td>
<td>138</td>
<td>M</td>
<td>3</td>
<td>157</td>
<td>F</td>
<td>12</td>
<td>105</td>
</tr>
<tr>
<td>M</td>
<td>3</td>
<td>133</td>
<td>M</td>
<td>8</td>
<td>145</td>
<td>F</td>
<td>3</td>
<td>99</td>
<td>F</td>
<td>11</td>
<td>88</td>
</tr>
<tr>
<td>M</td>
<td>8</td>
<td>155</td>
<td>M</td>
<td>8</td>
<td>109</td>
<td>M</td>
<td>2</td>
<td>146</td>
<td>M</td>
<td>7</td>
<td>104</td>
</tr>
<tr>
<td>F</td>
<td>6</td>
<td>156</td>
<td>H7;L1f/f;L2f/+;Yf/+;Tf/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>16</td>
<td>147</td>
<td>M</td>
<td>18</td>
<td>80</td>
<td>M</td>
<td>4</td>
<td>146</td>
<td>M</td>
<td>6</td>
<td>175</td>
</tr>
<tr>
<td>F</td>
<td>8</td>
<td>117</td>
<td>M</td>
<td>14</td>
<td>129</td>
<td>M</td>
<td>11</td>
<td>144</td>
<td>F</td>
<td>7</td>
<td>104</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>118</td>
<td>F</td>
<td>19</td>
<td>107</td>
<td>M</td>
<td>7</td>
<td>143</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>