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# Inhibition of YAP/TAZ-driven TEAD activity prevents growth of NF2-null schwannoma and meningioma

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**Running title:** Hippo signalling in NF2-null tumours

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## 1 **Abstract.**

2 Schwannoma tumours typically arise on the 8<sup>th</sup> cranial nerve and are mostly caused by loss of the  
3 tumour suppressor Merlin (*NF2*). There are no approved chemotherapies for these tumours and  
4 the surgical removal of the tumour carries a high risk of damage to the 8<sup>th</sup> or other close cranial  
5 nerve tissue. New treatments for schwannoma and other NF2-null tumours such as meningioma  
6 are urgently required.

7 Using a combination of human primary tumour cells and mouse models of schwannoma, we  
8 have examined the role of the Hippo signalling pathway in driving tumour cell growth. Using  
9 both genetic ablation of the Hippo effectors YAP and TAZ, as well as novel TEAD  
10 palmitoylation inhibitors, we show that Hippo signalling may be successfully targeted in vitro  
11 and in vivo to both block and, remarkably, regress schwannoma tumour growth. In particular,  
12 successful use of TEAD palmitoylation inhibitors in a pre-clinical mouse model of schwannoma  
13 points to their potential future clinical use. We also identify the cancer stem cell marker aldehyde  
14 dehydrogenase 1A1 (*ALDH1A1*) as a Hippo signalling target, driven by the TAZ protein in  
15 human and mouse NF2-null schwannoma cells, as well as in NF2-null meningioma cells, and  
16 examine the potential future role of this new target in halting schwannoma and meningioma  
17 tumour growth.

18

## 19 **Introduction**

20 Schwannomas are benign nervous system tumours that arise either sporadically or as part of the  
21 condition Neurofibromatosis type 2 (NF2) or other schwannomatoses. The annual incidence of  
22 schwannomas is 2.1 per 100,000 individuals<sup>1,2</sup>. In NF2 (incidence 1/25,000), development of  
23 schwannomas is associated with other nervous system tumours such as meningiomas and  
24 ependymomas as well as peripheral neuropathies. Whilst bilateral vestibular schwannomas are a  
25 distinctive feature of NF2, patients may also develop schwannomas on other peripheral nerves<sup>3-6</sup>.  
26 NF2 patients present with hearing loss, tinnitus or balance problems due to vestibular nerve  
27 schwannomas. These tumours may compress the facial nerve, causing additional symptoms and  
28 difficulties with surgical removal of the tumour<sup>7</sup>. While current therapeutic alternatives to  
29 surgery or radiotherapy for schwannomas, such as the anti-VEGF monoclonal antibody  
30 bevacizumab, have shown effect, their use was not without side effects during long-term

1 treatment<sup>8-10</sup>. While in this work we largely focus upon schwannoma as a model for an *NF2-null*  
2 tumour, we also use primary human meningioma tumour cells and cell lines. The ultimate aim  
3 for NF2 patients would be a single treatment for both schwannoma and meningioma tumours,  
4 both potentially seen in the same individual.

5 Loss of the *NF2* tumour suppressor gene product Merlin dysregulates many signalling pathways,  
6 including mitogen activated protein kinase pathways, control of the CRL4<sup>DCAF1</sup> E3 ubiquitin  
7 ligase and increased growth factor receptor expression, leading to loss of contact inhibition, cell  
8 proliferation and tumour development<sup>11-14</sup>. Recently, there has been interest in the Hippo  
9 signalling pathway effectors YAP and TAZ in driving schwannoma development. Merlin has  
10 been shown to suppress YAP/TAZ nuclear translocation via positive regulation of the Hippo  
11 signaling pathway<sup>15</sup>. Inactivation of the Lats1/2 kinases, which phosphorylate YAP and TAZ, led  
12 to widespread development of schwannoma tumours in a mouse model<sup>16</sup>.

13 YAP and TAZ associate with the DNA-binding TEAD proteins (TEADs 1-4) to activate  
14 expression of regulators of the cell cycle and apoptosis to drive tumour growth<sup>17</sup>. Recent findings  
15 that YAP and/or TAZ are essential for tumour growth highlighted an urgent need to block their  
16 activity<sup>18</sup>. Many approaches have been trialled to block interaction between YAP/TAZ and  
17 TEAD proteins, thus blocking tumour growth. The photosensitizer verteporfin has been used to  
18 prevent YAP/TEAD interaction<sup>19,20</sup> and a peptide mimicking the function of the vestigial-like  
19 protein 4, which blocks YAP-TEAD interaction, also suppressed tumour growth in gastric  
20 cancer<sup>21</sup>.

21 A more recent approach has been to target the palmitoylation of TEAD proteins, which is  
22 necessary for protein stability, interaction with YAP or TAZ and TEAD-dependent  
23 transcription<sup>22</sup>. In one study, the use of an auto-palmitoylation inhibitor decreased tumour cell  
24 proliferation in a xenograft mouse model<sup>23</sup>. New inhibitors of TEAD auto-palmitoylation have  
25 now been described that are active at clinically relevant oral doses and block growth of *NF2-null*  
26 mesothelioma tumours in vivo<sup>24</sup>.

27 In this paper, we have also investigated the cancer stem cell marker aldehyde dehydrogenase  
28 1A1 (ALDH1A1) as a potential driver of schwannoma and meningioma tumorigenesis.  
29 ALDH1A1 is a member of the aldehyde dehydrogenase superfamily that detoxify aldehyde  
30 substrates and regulate retinoic acid signalling<sup>25,26</sup>. ALDH1A1 has been proposed as a cancer

1 stem cell marker and high levels of expression correlate with both cytotoxic drug resistance and  
2 poor prognosis<sup>27-30</sup>. Understanding ALDH1A1 function in schwannoma and meningioma  
3 tumours may open up new treatment possibilities.

4 Using a combination of primary human schwannoma cells and the Periostin-CRE NF2<sup>fl/fl</sup> mouse  
5 model<sup>31</sup>, we examine the roles of both YAP and TAZ in schwannoma tumour growth and the use  
6 of novel TEAD auto-palmitoylation inhibitors. In both human *in vitro* and mouse *in vivo* models  
7 of schwannoma, the TEAD inhibitors both block tumour growth and cause tumour shrinkage  
8 without any side effects *in vivo*, pointing to their strong potential as a future therapy for  
9 schwannoma and potentially other NF2-null tumours.

10

## 11 **Materials and methods**

12

### 13 **Clinical material**

14 For ALDH1A1 staining of paraffin sections, 10 cases of schwannoma were included in this  
15 study: Five vestibular schwannomas, four spinal schwannomas and one schwannoma from an  
16 NF2 patient. Histologically, eight cases had features of benign schwannoma (WHO grade 1) and  
17 2 cases were reported as cellular schwannoma (WHO grade 1). The NF2 patient also had  
18 multiple meningiomas and schwannomas at other sites. Normal peripheral (sural) nerve was used  
19 as control.

20

### 21 **Cell culture**

22 Primary schwannoma and meningioma cultures were generated from resected human tumours.  
23 Tumours were cut into small pieces, incubated in DMEM/10% FBS (Gibco), 100 U/mL  
24 penicillin/streptomycin (Gibco), 1.25U/mL dispase grade 1 and 160 U/mL collagenase type 1A  
25 (Worthington Biochemical Corp.) overnight at 37<sup>0</sup>C in 5% CO<sub>2</sub>. Tumour pieces were broken up  
26 by pipetting, then pelleted by centrifugation at 250 g. Cells were resuspended in DMEM/10%  
27 FBS; 100 U/mL penicillin/streptomycin; 1% D-glucose and 2mM L-glutamine (Gibco) at 37<sup>0</sup>C  
28 in 5% CO<sub>2</sub>. Adherent cells were passaged into tissue culture flasks. Schwannomas were cultured  
29 on poly-L-lysine (PLL)/laminin coated plates: 0.1 mg/mL PLL (Merck)/PBS (Gibco; pH 7.2) (30

1 minutes) and then 0.004 mg/mL laminin (Merck)/PBS (120 minutes). Schwannomas were  
2 cultured in growth factor medium (GFM), DMEM/20% FBS; 100 U/mL penicillin/streptomycin;  
3 0.5µM forskolin (Merck); 2.5 µg/mL amphotericin B (Merck); 2.5µg/mL insulin (Merck); 10nM  
4 β1-heregulin (Merck) and 0.5mM 3-Isobutyl-1-methylxanthine (IBMX) (Merck).

5 The NF2-null primary meningioma cells and meningioma cell lines BenMen-1 (Grade 1) and  
6 KT21-MG1 (Grade 3) were cultured in DMEM/10% FBS; 100 U/mL penicillin/streptomycin;  
7 1% D-glucose and 2mM L-glutamine. NF2-status of primary meningioma and schwannoma cell  
8 cultures was confirmed by western blot (data not shown). Human meningeal cells (HMC) were  
9 from ScienCell™ and cultured in manufacturer's recommended medium and supplements at  
10 37°C/5% CO<sub>2</sub>.

11

## 12 **shRNA knockdown**

13 Mission® shRNA (Merck) bacterial stocks were used to obtain transfection grade plasmid DNA  
14 to generate viral media for lentiviral-mediated knockdown. Sequences for  
15 ALDH1A1/Scramble/YAP/TAZ knockdown inserted in pLKO.1-puro vector:

16 ALDH1A1 (TRCN0000026415) – 5'-GCCAAATCATTCTTGGGAATT-3';

17 Scramble (TRC1/1.5; SHC002) – 5'- CAACAAGATGAAGAGCACCAA-3';

18 TAZ (TRCN0000307197) – 5'- CGGACTTCATTCAAGAGGAAT-3'

19 and YAP (TRCN0000107266) – 5'- GCCACCAAGCTAGATAAAGAA-3'.

20 Plasmids were packaged using pCMV-VSV-G envelope and pCMV-dr8.2 packaging plasmids  
21 (Addgene). Viral media was produced using 293FT cells transfected using Fugene 6 (Promega)  
22 in optiMEM (Gibco). Primary schwannoma cells were transduced with 1:1 mix of  
23 GFM/lentiviral media/16 µg/mL protamine sulphate (Merck) for 48 h before selection with 4  
24 µg/mL puromycin (Gibco).

25

## 26 **Transgenic Mice**

27 Periostin-CRE mice were provided by S.Conway (Indiana University) and crossed with NF2<sup>fl/fl</sup>  
28 animals (RIKEN Bioresource Research Centre) to make Periostin-CRE;NF2<sup>fl/fl</sup> animals<sup>31</sup>; These

1 mice were either crossed with YAP<sup>fl/fl</sup> <sup>32</sup> or TAZ<sup>fl/fl</sup> <sup>33</sup> mice to generate Periostin-CRE;NF2<sup>fl/fl</sup>  
2 YAP<sup>fl/fl</sup> (NF2<sup>fl/fl</sup>YAP<sup>fl/fl</sup>-CRE+) and Periostin-CRE;NF2<sup>fl/fl</sup> TAZ<sup>fl/fl</sup> (NF2<sup>fl/fl</sup>TAZ<sup>fl/fl</sup>-CRE+) mice  
3 respectively, resulting in additional deletion of YAP or TAZ. Age-matched CRE- littermates  
4 were used in experiments.

5 Schwann cell-specific NF2-null mice generated with the P0-CRE line and the sciatic nerve injury  
6 model have been described <sup>34,35</sup>. For all experiments, male and female animals were used in  
7 approximately equal number. Mice were kept in SPF conditions and fed with standard rodent  
8 diet and water *ad libitum*.

9 In experiments using the Periostin-CRE line, mice were only kept until 9 months as permitted by  
10 our UK Home Office project license, to avoid the substantial mortality observed in the NF2<sup>fl/fl</sup>-  
11 CRE+ animals after this timepoint<sup>31</sup>.

### 13 **Mouse tumour dissection**

14 Mice were killed using carbon dioxide and cervical dislocation. Following fixation in 4%  
15 paraformaldehyde (PFA), dorsal root ganglia (DRGs) were dissected as described<sup>36</sup>. Vestibular  
16 ganglia (VGs) were dissected by cutting the head sagittally, then fixing in 4% PFA. Vestibular  
17 nerves and ganglia were revealed within the internal auditory meatus to expose the  
18 vestibulocochlear apparatus, surrounding bones removed and vestibular ganglia dissected. VG  
19 and DRG volumes were calculated using length and width values as previously described<sup>31</sup>.

### 21 **Western blotting**

22 Protein expression was analysed using western blotting<sup>35</sup>. Cells/sciatic nerves were lysed in  
23 RIPA buffer (Thermo Fisher); sciatic nerves were sonicated into lysis buffer using a Q500  
24 sonicator (Thermo Fisher). Lysates were run on SDS-polyacrylamide gels (Bio-Rad), transferred  
25 onto PVDF membranes (Cytiva), blocked in 5% BSA, incubated with primary antibodies in BSA  
26 overnight at 4<sup>0</sup>C and then HRP-conjugated secondary antibodies in 5% BSA for 1h at room  
27 temperature. Blots were visualised using Pierce<sup>TM</sup> ECL (Thermo Fisher) on a PXi developer  
28 (Syngene), quantified by densitometry and normalised to GAPDH or vinculin loading controls  
29 using ImageJ. In Figures 2, 5E and, 6E, blots shown are representative of independent biological

1 repeats; Figure 5L shows collated blots from the same three paired biological repeats. Figure 7D  
2 shows three paired biological repeats on the same blot. Figure 8C and E show representative  
3 blots of technical repeats.

4

#### 5 **Immunohistochemistry, immunocytochemistry, EdU and TUNEL assays**

6 Immunofluorescence was conducted on 4% PFA fixed cells, frozen tissue sections or tissue  
7 whole mounts. Cells were immunostained on coverslips; frozen tissue sections were made by  
8 cryoprotecting tissue with 30% sucrose/PBS for 48h and freezing in OCT (Agar Scientific) and  
9 cutting 10 $\mu$ m sections using a cryostat (Leica). Wholemounds of VGs were performed as  
10 previously<sup>37</sup>. Cells/tissues were permeabilised with 1% Triton-X100/PBS, blocked with 3%  
11 BSA/PBS for 1h at room temperature, then incubated with primary antibodies overnight at 4<sup>o</sup>C  
12 and the following day with secondary antibodies and Hoechst (Thermo Fisher), diluted in 3%  
13 BSA/PBS, for 1h at room temperature. Formalin fixed paraffin embedded tissue sections (4 $\mu$ m  
14 thick) were stained with either Mayer's Haematoxylin and Eosin (H & E; Thermo Fisher) or in  
15 combination with primary antibodies using a Vectastain Elite ABC kit (Vector Labs) and 3,3'-  
16 diaminobenzidine (DAB)<sup>16,38</sup>. Incorporation of EdU into DNA was used to measure cell  
17 proliferation. For cultured cells, EdU/DMSO was added at final concentration of 10 $\mu$ M in media  
18 4h before fixation in 4% PFA. For mice, 100 mg/kg of EdU was dissolved in DMSO, diluted  
19 1/10 in PBS and given by intraperitoneal injection 24h before killing and fixation of tissue in 4%  
20 PFA. EdU-positive cells were detected using Click-iT™ EdU cell proliferation kit (Thermo  
21 Fisher), according to manufacturer's instructions. Apoptosis was detected using a TUNEL assay  
22 kit (Invitrogen) according to manufacturer's instructions.

23

#### 24 **Drug treatments**

25 Small molecule inhibitors of TEAD auto-palmitoylation (VTs), developed by Vivace  
26 Therapeutics, were used to treat cultured cells and mice. VTs, dissolved in dimethylsulfoxide  
27 (DMSO; Merck), were added to culture medium for in vitro experiments. For adult mice, the  
28 vehicle was an aqueous solution consisting of 5% glucose (w/v, Thermo-Fisher) containing 5%  
29 DMSO and 10% Kolliphor HS-15 (Merck). VT compounds were diluted in DMSO and



1 Kolliphor HS-15 and aqueous 5% glucose (w/v) added to match vehicle solution. VT compounds  
2 were further diluted in vehicle solution to 5 mg/mL (VT1) or 10 mg/mL (VT2). VTs were  
3 administered by gavage each day using feeding tubes (Instech). Randomized groups of mice  
4 were given either vehicle, 10 mg/kg VT1 or 30 mg/kg VT2 daily for 21 days. Details of the VT1  
5 and VT3 inhibitors (referred to as VT104 and VT107 respectively) have been published<sup>24</sup>. VT1  
6 (VT104) and VT2 have different pharmacokinetics in mice and the dosing concentration of each  
7 compound was chosen empirically, based on their minimum efficacy dose and at a dose that  
8 provided maximum efficacy in models of NF2-deficient mesothelioma without adverse effect on  
9 body weight. The ALDH1A1 inhibitor (NCT-505) was a gift from NIH NCATS<sup>39</sup> and used as  
10 stated.

11 For studies with BenMen-1 meningioma cells, cells were treated with ALDH1A1 inhibitors and  
12 cisplatin (Selleckchem), dissolved in DMSO vehicle. For ALDH1A1 inhibitors and cisplatin  
13 individual/combo experiments, cells were plated onto coverslips; after 2 hours, cells were  
14 topped up with media containing either DMSO vehicle or the relevant drug concentration.  
15 Primary schwannoma cells were treated with 10  $\mu$ M MG132 (Merck) or DMSO vehicle for 3  
16 hours to monitor proteasomal-dependent degradation.

## 18 **Antibodies**

19 Primary antibodies used for immunostaining were: Neurofilament (1:1000; ab4680; Abcam),  
20 ALDH1A1 (1:200; ab52492; Abcam), YAP (1:100; #14074; CST), TAZ (1:100; sc-48805; Santa  
21 Cruz), Ki67 (1:100; ab15580; Abcam), S100 (pre-diluted; GA504; Dako). Species-specific  
22 AlexaFluor<sup>TM</sup> secondary antibodies (Thermo Fisher) were used at 1:200.

23 Primary antibodies used for western blotting: YAP (1:1000; #14074; CST), TAZ (1:500; sc-  
24 48805; Santa Cruz), Pan-TEAD (1:1000; #13295; CST), GAPDH (1:5000; AB2302; Merck),  
25 ALDH1A1 (1:1000, ab52492; Abcam) and CTGF (1:500, ab6992, Abcam). For detection of  
26 primary antibodies, HRP-conjugated goat anti-rabbit (1:5000; #1706515; Bio-Rad) and HRP-  
27 goat anti-mouse (1:5000; #1721011; Bio-Rad) were used.

28

29

## 1 **Statistical Analysis**

2 Statistical analysis was performed using GraphPad Prism 8. Statistical tests performed for are  
3 stated in Figure legends; in all cases \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; and \*\*\*  $P \leq 0.001$ . Because of  
4 small sample sizes ( $n < 5$  for most comparisons), assumptions of normality and equal variances  
5 for the data couldn't be assessed. Sample size was not predetermined by statistical methods and  
6 randomization was not applied. In gavage experiments the investigators were not blinded  
7 because the  $NF2^{fl/fl}$ -CRE+ mice were frequently smaller than  $NF2^{fl/fl}$ -CRE- littermates and  
8 vehicle solution looked visibly different to the drug suspension. No samples were excluded from  
9 the analyses. Biological repeats were used in all experiments and data presented as mean  $\pm$  SEM  
10 with the  $n$  number reported in each Figure legend.

11

## 12 **Study Approval**

13 For schwannoma and meningioma tumour tissue, anonymised MN samples from the 'Identifying  
14 and validating molecular targets in low grade brain tumours' (MOT) project (REC No:  
15 14/SW/0119; IRAS project ID: 153351) and Plymouth Brain Tumour Biobank (REC No:  
16 19/SC/0267; IRAS No: 246667) were collected under ethical approval from University Hospitals  
17 Plymouth NHS trust and North Bristol NHS trust. All animal experiments conformed to UK  
18 Home Office regulations under the Animals (Scientific Procedures) Act 1986, followed ARRIVE  
19 guidelines and were approved by the Plymouth University Animal Welfare and Ethical Review  
20 Board.

21

## 22 **Data Availability**

23 The authors confirm that the data supporting the findings in this paper are available within the  
24 article and/or its supplementary material.

25

26

## 1 Results

### 3 YAP/TAZ are required for schwannoma development in the DRGs and VGs of Periostin- 4 CRE NF2<sup>fl/fl</sup> mice

5 The Periostin (Postn)-CRE NF2<sup>fl/fl</sup> mouse model has been widely used as a model of spontaneous  
6 schwannoma formation *in vivo*. Mice with Postn-CRE-driven loss of Merlin (*NF2*) develop  
7 tumours in dorsal root ganglia (DRG), vestibular ganglia (VG) and vestibular nerves<sup>31,40,41</sup>. Use  
8 of a Rosa TdTomato line, which expresses Tomato RFP in cells following recombination showed  
9 high Postn-CRE-driven recombination in glial cells of the DRG (Supplementary Figure 1N, O).

10 For analysis of schwannoma tissue in DRG, we analysed mice at 3, 5 and 9 months.  
11 Haematoxylin and eosin (H & E) staining of DRG sections showed progressive and clear  
12 hyperplasia in DRG of Postn-CRE+ NF2<sup>fl/fl</sup> (NF2<sup>fl/fl</sup>-CRE+) animals compared to controls  
13 (NF2<sup>fl/fl</sup>-CRE-) (Supplementary Figure 1A-M). A 24 hour EdU pulse showed EdU-positive cells  
14 in DRG and VG tissue of NF2<sup>fl/fl</sup>-CRE+ mice (Figure 1 B, F), allowing quantification of effects  
15 of loss of YAP or TAZ in such tumours or efficacy of TEAD auto-palmitoylation inhibition of  
16 proliferation *in vivo*.

17 We next studied effects of either YAP or TAZ loss in *NF2-null* cells upon schwannoma  
18 proliferation in both DRG and VG tissue<sup>31</sup>. Counts of total non-neuronal cells per area within the  
19 DRG showed increased numbers in *NF2* single null animals, which further increased with age  
20 (Supplementary Figure 1M). For EdU quantification, neurofilament antibody stain revealed  
21 neuronal cell bodies within DRG and VG and numbers of EdU positive cells per tissue area  
22 around these neuronal cell bodies were used for quantification of proliferating cells. At both  
23 DRG and VG tumour sites, loss of either YAP or TAZ significantly reduced cell proliferation;  
24 although loss of either YAP or TAZ seemingly had a greater effect on proliferation in the VG  
25 than the DRG (Figure 1A-J).

26 Staining of DRG paraffin sections from control (NF2<sup>fl/fl</sup>-CRE-), NF2 single null (NF2<sup>fl/fl</sup>-CRE+),  
27 NF2/YAP double knockout (NF2<sup>fl/fl</sup>YAP<sup>fl/fl</sup>-CRE+) and NF2/TAZ double knockout  
28 (NF2<sup>fl/fl</sup>TAZ<sup>fl/fl</sup>-CRE+), showed an elevation of both YAP and TAZ in NF2 single null tissue  
29 compared to control tissue. NF2/YAP double null and NF2/TAZ double null tissue showed

1 reduced stain for YAP and TAZ respectively compared to NF2 single null, confirming their loss  
2 in the double knockout tissue (Figure 1 K-R).

3

#### 4 **Use of pan-TEAD auto-palmitoylation inhibitors reduces VG and DRG schwannoma** 5 **tumour cell growth rates in vivo**

6 We next used NF2 single null animals to trial two novel pan-TEAD auto-palmitoylation  
7 inhibitors, VT1 and VT2, and effects upon proliferation in vivo. The use of VT1 has previously  
8 been described<sup>24</sup> (designated VT104), but details of VT2 have not yet been published. Both  
9 inhibitors are orally available and were administered by gavage for 21 days. There were no  
10 apparent side effects or weight loss in animals, as in their previous use<sup>24</sup>; Figure 2 shows the  
11 results of our experiments in VG tissue in 3-month old animals. Both VT1 and VT2 showed  
12 significant decreases in tumour cell proliferation (73% and 52% respectively) within the VG  
13 (Figure 2, A-F, quantification in G, H). A similar inhibition of proliferation by VT1 and VT2  
14 was also seen in the VG from 5-month-old animals (Supplementary Figure 2).

15 As blocking TEAD auto-palmitoylation has been shown to regulate both TEAD protein stability  
16 and block TEAD target gene transcription, we measured levels of TEAD proteins and the TEAD  
17 target connective tissue growth factor (CTGF) in sciatic nerve of animals treated with vehicle,  
18 VT1 or VT2. Sciatic nerve was used as it is Schwann cell-rich (>70% of total cell number<sup>42</sup>).  
19 CTGF was elevated in NF2 single null sciatic nerve compared to control and significantly  
20 decreased by VT1 or VT2 treatment (Figure 2I, J, L, M). Thus, both inhibitors are engaging with  
21 their target and blocking TEAD-dependent transcription in NF2-null Schwann cells. For TEAD  
22 protein expression in VT1 or VT2 treated animals, a pan-TEAD antibody showed significant  
23 changes in TEAD proteins expression only with VT2 drug in vivo (compare Figure 2K for VT1;  
24 2N for VT2).

25 We next studied effects of VT1 and VT2 upon schwannoma proliferation in the DRG at 3 and 5  
26 months. Both VT1 and VT2 significantly reduced cell proliferation at both timepoints  
27 (Supplementary Figure 3, 3-month and Supplementary Figure 4 for 5-month DRG). For those  
28 experiments shown in Supplementary Figure 4 A-F, the Postn-CRE NF2<sup>fl/fl</sup> animals were crossed  
29 with PLP-GFP expressing mice, expressing GFP in Schwann cells and satellite glial cells<sup>43,44</sup>;  
30 thus confirming that EdU positive cells within the DRG were glial cells. Similar to the earlier

1 timepoint at 3 months (Figure 2), both VT1 and VT2 blocked expression of CTGF and VT2, but  
2 not VT1, reduced levels of total TEAD protein (Supplementary Figure 4I-N).

3

#### 4 **Reduction of tumour size and apoptosis in vivo with VT1 and VT2 inhibitors**

5 While our data shows that both VT1 and VT2 inhibitors significantly reduce proliferation rates  
6 of schwannoma tumours in vivo, we next tested whether there was any shrinkage of the tumours  
7 in vivo by VT1 or VT2. We used 9 month old control and NF2-null animals and examined  
8 vestibular ganglion sizes in animals treated for 21 days with VT1 or VT2. We observed  
9 significant reductions in tumour volume in VT1- or VT2-treated animals (Figure A-D;  
10 quantification in E). A similar decrease in size was observed in the DRG (Figure 3F). In  
11 correlation with this finding of tumour shrinkage, we found increased apoptosis of tumour cells  
12 in both VG and DRG with VT2 by TUNEL assay after 10 days of treatment (Figure 3G-R;  
13 quantification in S, T).

14

#### 15 **Increased macrophage numbers within NF2-null mouse schwannoma tissue are YAP and** 16 **TAZ-dependent**

17 Proliferation of human schwannomas has been shown to positively correlate with macrophage  
18 numbers within the tumour<sup>45,46</sup>. Furthermore, mouse models of schwannoma show high  
19 macrophage numbers within tumours<sup>34,35</sup>. Using the pan-macrophage marker Iba1, we  
20 determined percentages of Iba1 positive cells in control and NF2 single null DRG tissue (Figure  
21 4 A, E, I and B, F, J respectively) and VG tissue (Figure 4, M-P). While we see macrophages  
22 within the DRG and VG in controls, loss of NF2 significantly increased macrophage numbers  
23 within tumours in both locations (Figure 4Q, R). For DRG tissue in NF2 null animals, a stepwise  
24 increase was seen in percentages of macrophages between 3, 5 and 9 months (compare Figure  
25 4A, E, I and B, F, J). We tested whether loss of YAP or TAZ in NF2 single null animals would  
26 alter macrophage numbers. Loss of either YAP (Figure 4C, G, K) or TAZ (Figure 4D, H, L)  
27 significantly decreased macrophage numbers in DRG at all ages (Figure 4Q), correlating with  
28 reduced schwannoma cell proliferation in the NF2/YAP and NF2/TAZ double nulls (Figure 1 I,  
29 J).

## 1 **Knockdown of YAP/TAZ or TEAD inhibition blocks human schwannoma and meningioma** 2 **cell proliferation**

3 To complement the in vivo mouse data, we next tested primary human schwannoma cells for the  
4 roles of YAP and TAZ in proliferation. Knockdown of either YAP or TAZ significantly reduced  
5 schwannoma cell proliferation (Figure 5A-D); successful knockdown of YAP or TAZ was  
6 confirmed by western blot (Figure 5E-G). In these experiments, however, while knockdown of  
7 TAZ did not affect YAP expression, knockdown of YAP did reduce TAZ in primary  
8 schwannoma cells, so while both knockdown of either YAP or TAZ reduces cell proliferation, an  
9 additional effect upon TAZ expression may mediate some of the effects of YAP knockdown  
10 (Figure 5 E-G).

11 It is unknown which TEAD proteins are expressed in human *NF2*-null schwannoma cells, so we  
12 next performed western blotting on 3 primary schwannoma tumours (S<sub>1</sub>-S<sub>3</sub>) using TEAD 1-4  
13 specific antibodies. TEAD expression was remarkably variable between tumours (Figure 5L, left  
14 panel). For this reason, as in the mouse model, we tested a pan-TEAD auto-palmitoylation  
15 inhibitor (VT3) for effects upon human schwannoma proliferation, TEAD expression and  
16 inhibition of CTGF. VT3 blocked human schwannoma proliferation with an IC<sub>50</sub> of 39nM  
17 (Figure 5H-K, quantification in M) and reduced CTGF expression. Use of VT1 or VT2 pan-  
18 TEAD inhibitors, as used for in vivo use in the Postn-CRE *NF2*<sup>fl/fl</sup> animals, also significantly  
19 reduced CTGF levels and proliferation of human *NF2*-null schwannoma (HEI193) cells  
20 (Supplementary Figure 5).

21 We also tested the effects of VT1, VT2 and VT3 pan-TEAD inhibitors on proliferation of human  
22 *NF2*-null meningioma cells. All three TEAD inhibitors significantly inhibited the proliferation of  
23 human meningioma cells (Supplementary Figure 6), demonstrating the potential to extend their  
24 future use to other *NF2*-null tumour types.

## 25 26 **The cancer stem cell marker ALDH1A1 is regulated by TAZ in *NF2*-null Schwann and** 27 **schwannoma cells**

28 Having shown roles for TEAD activity in schwannoma cell proliferation, we wished to identify  
29 new YAP or TAZ targets driving cell proliferation in schwannoma and other *NF2*-null tumours.

1 Recent work showed expression of ALDH1 in human schwannoma tissue, but the mechanism of  
2 ALDH1 upregulation and its potential function is unclear<sup>47</sup>.

3 To further define the subtype of ALDH1 expressed, given its roles in cancer stem cell biology,  
4 we examined ALDH1A1 expression in both Postn-CRE NF2<sup>fl/fl</sup> mice and in human  
5 schwannoma. In the Postn-CRE NF2<sup>fl/fl</sup> animals, we examined adult sciatic nerve and DRG in  
6 control and NF2-null animals. In sciatic nerve, we observed weak ALDH1A1 expression in non-  
7 myelinating Schwann cells (Figure 6A). This finding corresponds to the recent published data  
8 from the Sciatic Nerve Atlas (<https://snat.ethz.ch/search.html?q=aldh1a1>), showing *aldh1a1*  
9 mRNA expression in the non-myelinating cells of adult sciatic nerve<sup>48</sup>. Compared to control  
10 nerves, ALDH1A1 protein expression was elevated in the sciatic nerves of NF2-null mice, again  
11 only in the non-myelinating cell population (Figure 6C). In the DRG, levels of ALDH1A1 were  
12 much higher in the glial cells surrounding the neuronal cell bodies in NF2-null animals (Figure  
13 6B, D). To test whether YAP or TAZ drive ALDH1A1 expression in NF2-null Schwann cells,  
14 we examined sciatic nerves of control, NF2 single null, NF2/YAP and NF2/TAZ double null  
15 mice. By western blot and immunolabelling we showed that it was TAZ, not YAP, driving  
16 ALDH1A1 expression in NF2-null sciatic nerve (Figure 6E-I) and DRG (Figure 6J-M). Analysis  
17 of TAZ single null sciatic nerve showed that ALDH1A1 expression seen in the non-myelinating  
18 Schwann cells (Figure 6A) was TAZ-dependent (data not shown). The increase in ALDH1A1  
19 levels appeared to be transcriptional, as we observed increased *aldh1a1* mRNA in both sciatic  
20 nerve and DRG of NF2-null animals, along with other Hippo pathway responsive genes.  
21 Correspondingly, treatment of NF2-null animals with VT2 for 7 days significantly reduced  
22 *aldh1a1* mRNA levels in both tissues (Supplementary Figure 10). The effects upon *aldh1a1* and  
23 other Hippo targets were more marked in sciatic nerve than DRG, probably reflecting the higher  
24 Schwann cell content of sciatic nerve<sup>49,42</sup>. Western blotting of ALDH1A1 *in vivo* also showed a  
25 decrease with VT2 treatment (Figure 6N, O).

26 We have previously shown that peripheral nerve injury leads to schwannoma tumour  
27 development using the P0-CRE+/NF2<sup>fl/fl</sup> mouse model, which also has a Schwann cell-specific  
28 knockout of NF2<sup>34,35,50</sup>. Prior to injury in the P0-CRE+/NF2<sup>fl/fl</sup> animals, we once again saw  
29 elevated levels of ALDH1A1 in the non-myelinating Schwann cells of the sciatic nerve  
30 (Supplementary Figure 7C, E, F). In line with the tumour formation in this model, staining of

1 distal nerve following injury showed an increase ALDH1A1 expression at 7 days post-nerve  
2 crush injury, confirmed by western blot (Supplementary Figure 7D-F).

3 Next, we measured levels of ALDH1A1 protein in human schwannoma tumour tissues and cells.  
4 Analysis of human schwannoma showed strong ALDH1A1 expression in all tumours (n=10),  
5 with no expression in control (sural) nerve (n=3) (Figure 7A-C and not shown). As in the Postn-  
6 CRE/NF2<sup>fl/fl</sup> mouse model, loss of TAZ (by shRNA knockdown) in human primary schwannoma  
7 cells reduced ALDH1A1 expression (Figure 7D, E). Experiments using the proteasome inhibitor  
8 MG132 to determine whether changes in ALDH1A1 protein levels by TAZ may be mediated by  
9 proteasomal degradation showed no changes in cells with TAZ knockdown treated with MG132  
10 (Figure 7F, G).

11 Interest in ALDH1A1 as a driver of the cancer stem cell phenotype has led to development of  
12 novel ALDH1A1-specific inhibitors, for use either alone, or, as ALDH1A1 can detoxify some  
13 chemotherapy agents, in combination with such agents to potentiate effects. Yang et al reported  
14 the development of novel orally available ALDH1A1 inhibitors<sup>39,51,52</sup>. One such ALDH1A1-  
15 specific inhibitor (NCT-505) reduced proliferation of human NF2-null schwannoma cells in vitro  
16 (Figure 7H-J).

17

### 18 **Increased expression and function of ALDH1A1 in human NF2-null meningioma cells**

19 As NF2 loss is seen in approximately 60% of sporadic human meningioma tumours<sup>53-55</sup>, we also  
20 examined ALDH1A1 expression in human meningioma tissue and cell lines. We observed  
21 increases in ALDH1A1 protein in NF2-null compared to NF2-positive human meningioma tissue  
22 (Figure 8A, B). Analysis of BenMen-1 (Grade 1) and KT21-MG1 (Grade 3) meningioma cell  
23 lines, both NF2-null, also showed raised ALDH1A1 levels compared to control human  
24 meningeal cells (Figure 8C, D and Supplementary Figure 8).

25 Knockdown of YAP or TAZ in BenMen-1 meningioma cells, as for schwannoma cells,  
26 confirmed a dependence upon TAZ for ALDH1A1 expression (Figure 8E, F). As for human  
27 schwannoma cells, use of an ALDH1A1-specific inhibitor slowed the proliferation of BenMen-1  
28 meningioma cells (Figure 8G-J).



1 Finally, we compared the effects upon proliferation between knockdown of ALDH1A1 and  
2 knockdown of TAZ in BenMen-1 cells. Loss of either ALDH1A1 or TAZ both significantly  
3 reduced cell proliferation, but knockdown of TAZ was more effective, perhaps indicating  
4 additional TAZ targets in driving meningioma cell growth (Figure 8K-N).

5 As ALDH1A1 may detoxify platinum-based chemotherapy drugs, use of either ALDH1A1  
6 inhibitors or ALDH1A1 knockdown may sensitise tumour cells to agents such as cisplatin and  
7 paclitaxel in ovarian and lung tumour cells<sup>39,56,57</sup>. Cisplatin exhibits anti-tumour activity in  
8 meningioma cells<sup>58,59</sup> with resistance to cisplatin highest within the cancer stem cell population  
9 of meningioma cells<sup>60</sup>. We performed similar experiments with BenMen1 meningioma cells and  
10 a combination of ALDH1A1 inhibitor and cisplatin. Either reagent alone reduced BenMen-1 cell  
11 proliferation, but the combination was strongly synergistic in reducing cell proliferation  
12 (Supplementary Figure 9).

13

## 14 Discussion

15 We have reported three key findings in the biology of schwannoma tumours. Firstly, the  
16 requirement for Hippo signalling through YAP and TAZ to drive growth of human and mouse  
17 schwannoma tumours *in vitro* and *in vivo* respectively. Secondly, we have shown efficacy for  
18 TEAD auto-palmitoylation inhibitors in blocking schwannoma and meningioma growth and  
19 raised the prospect of these being used clinically. Thirdly, we have characterised the expression  
20 and function of the cancer stem cell marker ALDH1A1, and its regulation by TAZ, in both NF2-  
21 null schwannomas and meningiomas. These findings open up new avenues of treatment for these  
22 two tumour types in patients.

23 Dysregulation of Hippo signalling in *NF2*-null tumours has been widely studied and *NF2* loss  
24 causes reduced phosphorylation of YAP/TAZ by the LATS1/2 kinases, leading to increased  
25 nuclear localisation and raised activity of YAP and TAZ<sup>15,61</sup>. Target genes of YAP and TAZ  
26 include those involved in cell proliferation, cell death and cytoskeletal function<sup>62,63</sup>. We found  
27 that loss of either YAP or TAZ reduced schwannoma tumour growth in both DRG and VG tissue  
28 (Figure 1); however loss of YAP or TAZ alone did not completely halt tumour growth.  
29 Moreover, our data suggests that YAP and TAZ have overlapping but distinct functions in  
30 driving proliferation in schwannoma tumours, for instance the regulation of ALDH1A1 appears

1 only TAZ-dependent in our experiments. However, the relationship between YAP and TAZ  
2 expression is complex<sup>64</sup> with for example YAP reported to inversely regulate levels of TAZ  
3 protein in mammalian cells<sup>65</sup>, although we did not observe such effects in our knockdown  
4 experiments (Figure 5) so such effects may be cell type-specific. Mice with loss of both YAP  
5 and TAZ *in vivo* are not viable to adulthood, either on wild-type or *NF2*-null background, so we  
6 cannot test their combined loss.

7 While removal of the Hippo pathway kinases Lats1/2 in all Schwann cells leads to the malignant  
8 peripheral nerve sheath tumours (MPNSTs)<sup>66</sup>, a more recent paper<sup>16</sup> used the Hoxb7-CRE line to  
9 reduce Lats1/2 activity in a sub-population of Schwann cells leading to widespread schwannoma  
10 tumours in skin, soft tissue and DRGs. Experiments using this Lats1/2 model also showed YAP  
11 and TAZ were required for schwannoma development<sup>16</sup>, in agreement with our findings.

12 It should, however, be noted that the tumours with the Lats1/2 model appear much more  
13 aggressive and more numerous than in our model and are subcutaneous, rather than modelling  
14 the tumour sites seen in *NF2* patients. Recent data has shown that in *NF2*-null cells, Motin  
15 family members control YAP/TAZ activity and mediate the benign nature of most *NF2*-null  
16 tumour types<sup>67</sup>. Additionally, in one study of human schwannomas, Lats1 and Lats2 mutations  
17 were seen in only 2% and 1% of cases respectively, compared to 55% showing mutations in the  
18 *NF2* gene<sup>68</sup>; it is therefore arguable that a schwannoma model with *NF2* loss is more clinically  
19 relevant.

20 A number of new TEAD auto-palmitoylation inhibitors, with differential TEAD selectivity have  
21 now been identified<sup>24</sup>. The pharmacokinetics of these compounds are favourable, they are also  
22 orally available and have no discernible side effects in mice<sup>24</sup>.

23 We trialled two pan-TEAD inhibitors in the Postn-CRE *NF2*<sup>fl/fl</sup> mouse model, which closely  
24 mimics the sites of tumour formation in human patients. We chose to study tumour cell division  
25 in mice at 3, 5 and 9 months, where tumour formation is clearly seen in this model. Both TEAD  
26 inhibitors showed good target engagement, downregulating the TEAD target CTGF, as well as  
27 other Hippo targets and both significantly blocked cell proliferation in DRG and VG tumour sites  
28 (Figure 2; Supplementary Figures 2, 3, 4, 10). Thus, these compounds would seem ideal for  
29 potential translation into clinical trials for patients with schwannoma tumours, although a

1 limitation of our study is that we have not carried out auditory brainstem response (ABR)  
2 measurements in control and treated animals<sup>31,41</sup>.

3 Data using these compounds showed an apparent shrinkage of schwannoma tumours in the VG  
4 and DRG of 9-month-old mice treated with either VT1 or VT2 for 21 days. We have also seen a  
5 clear and significant increase in schwannoma cell apoptosis in the VG and DRG at 10 days of  
6 treatment with VT2 (Figure 3). It has been shown that YAP/TAZ function up-regulates pro-  
7 survival members of the Bcl-2 family, can overcome anoikis-driven apoptosis and prevent the  
8 alternative apoptotic cascade regulated by tumour necrosis factor alpha and FAS ligand<sup>69-71</sup>.  
9 Indeed, we observed raised mRNA levels of *birc5* (survivin), a pro-survival TEAD target gene<sup>69</sup>,  
10 in NF2-null mouse sciatic nerve (Supplementary Figure 10). A schematic (Supplementary Figure  
11 11) illustrates the effects of the auto-palmitoylation inhibitors in the mouse schwannoma model.

12 Macrophages form part of the schwannoma tumour microenvironment<sup>34,35</sup> and numbers of  
13 macrophages within the tumour tissue correlate with tumour growth<sup>45,46</sup>. Similarly, we found that  
14 in both NF2/YAP and NF2/TAZ double null animals, with decreased proliferation compared to  
15 NF2 single null mice, reduced macrophages were observed (Figure 4). Screens for cytokines  
16 produced by NF2-null Schwann cells in a model of injury-induced schwannoma tumour  
17 formation identified a number of cytokines with links to chronic inflammation, such as IL-6 and  
18 SDF-1/CXCL12<sup>34</sup>, but it is unclear if these may be YAP- or TAZ-dependent. Studies in human  
19 meningioma tumours have also shown that NF2-null tumours have higher macrophage numbers  
20 than tumours with other driving mutations (AKT1 E17K)<sup>72</sup>. While roles for macrophages in  
21 driving meningioma tumour growth are unknown, larger numbers of macrophages are seen in  
22 higher grade meningioma tumours<sup>73</sup>.

23 We found that in human schwannoma tumours, there is remarkable heterogeneity in TEAD  
24 isoform expression, thus decided to use pan-TEAD inhibitors. Using pan-TEAD inhibitors in  
25 experiments with either primary human schwannoma or meningioma cells, they blocked cell  
26 proliferation in the nanomolar concentration range, while slightly less efficacious in the  
27 schwannoma cell line HEI193, possibly highlighting differences in primary cells versus cell lines  
28 (Compare Supplementary Figures 5 and 6).

29 Experiments performed using three TEAD auto-palmitoylation inhibitors in both NF2-null  
30 meningioma cell lines and primary human meningioma cells show they are also highly effective

1 in this tumour type (Supplementary Figure 6). Not only are meningiomas the most common  
2 primary intracranial tumour type<sup>74</sup>, but in individuals with NF2, patients are predisposed to  
3 develop both bilateral vestibular schwannomas as well as meningiomas<sup>75</sup>. Thus, these TEAD  
4 inhibitor compounds hold promise in both treatment of sporadic schwannomas and  
5 meningiomas, but also for NF2 patients with multiple tumours of both types. The pan-TEAD  
6 inhibitor VT2 (also known as VT3989; Tang et al, unpublished) is currently in a phase 1 clinical  
7 trial that includes patients with NF2-deficient mesothelioma (NCT04665206). VT2 and other  
8 TEAD palmitoylation inhibitors have shown efficacy in blocking resistance development when  
9 used in combination with osimertinib in mouse models of non-small cell lung cancer (Tang et al,  
10 AACR, 2022; Haderk et al, Biorxiv <https://doi.org/10.1101/2021.10.23.465573>)

11 While the TEAD auto-palmitoylation inhibitors we have used have high potential, another part of  
12 the data presented in this paper was to identify the cancer stem cell marker ALDH1A1 as a TAZ  
13 target in both NF2-null schwannoma and meningioma tumour cells and characterise its function  
14 (Figures 6-8). ALDH1A1 expression has been seen in a number of different tumour types,  
15 restricted to the cancer stem cell population and has been previously proposed as a TAZ  
16 target<sup>25,26,76</sup>. In lung cancer cells, TAZ was previously shown to activate *Aldh1a1* promoter  
17 activity<sup>76</sup>. Whilst we show that both TEAD inhibition and TAZ regulate ALDH1A1 expression  
18 (Figure 6), the precise mechanism of YAP/TAZ-mediated ALDH1A1 regulation remains to be  
19 investigated. For NF2-null schwannoma, we found strong ALDH1A1 expression in all cells of  
20 mouse and human tumours. Similarly in meningioma, ALDH1A1 was expressed in all cells of  
21 NF2-null tumour tissue. Knockdown or chemical inhibition of ALDH1A1 alone in either  
22 schwannoma or meningioma tumour cells reduced proliferation (Figures 7, 8).

23 Another facet of ALDH1A1 function in cancer stem cells is to mediate drug resistance to  
24 chemotherapy agents such as paclitaxel and cisplatin; knockdown of ALDH1A1 reverses  
25 cisplatin resistance in lung adenocarcinoma cells<sup>57</sup>. Our experiments with the NF2-null BenMen-  
26 1 meningioma cell line showed strong synergistic effects of cisplatin and an ALDH1A1 inhibitor  
27 upon proliferation (Supplementary Figure 9). Whether this kind of approach may be useful  
28 clinically in this and higher grades of meningioma tumour, or indeed even schwannoma tumours,  
29 remains to be seen.

1 In summary, this study has highlighted the therapeutic potential of disrupting YAP/TAZ-driven,  
2 TEAD transcriptional activity in NF2-null schwannoma and meningioma, both *in vitro* and *in*  
3 *vivo*. In addition, the efficacy of TEAD auto-palmitoylation inhibitors in the most clinically  
4 relevant schwannoma mouse model provides a strong mandate for early-phase clinical trials of  
5 these inhibitors.

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## 18 **Competing interests**

19 Tracy T. Tang and Leonard Post are employees of Vivace Therapeutics and have equity interest  
20 in Vivace Therapeutics.

## 22 **Supplementary material**

23 Supplementary material is available at *Brain* online.

24

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- 28

1

2 **Figure Legends:**

3

4 **Figure 1 Proliferation of schwannoma cells in the dorsal root ganglia (DRG) and vestibular**  
5 **ganglia (VG) is dependent upon both YAP and TAZ proteins.** A-H. Images of DRG (A-D)  
6 and VG (E-H) from 5-month-old mice stained for EdU and neurofilament (NF); sections were  
7 counterstained with Hoechst to reveal nuclei (Ho). Arrows indicate EdU positive nuclei in the  
8 areas of the ganglia in close proximity to the neuronal cell bodies; note fewer proliferating cells  
9 in NF2/YAP (C, G) and NF2/TAZ (D, H) than in NF2 single null (B, F) ganglia. I, J.  
10 Quantification of EdU positive cells per area of ganglion tissue of DRG (I) and VG (J). Note  
11 significant decreases in proliferation in both NF2/YAP and NF2/TAZ ganglia. K-R. Staining of  
12 DRG sections from 9-month-old control (NF2<sup>fl/fl</sup>CRE-; K, O), NF2 single null (NF2<sup>fl/fl</sup>CRE+; L,  
13 P), NF2/YAP double null (NF2<sup>fl/fl</sup>/YAP<sup>fl/fl</sup> CRE+; M, Q) and NF2/TAZ double null (NF2<sup>fl/fl</sup>/  
14 TAZ<sup>fl/fl</sup> CRE+; N, R) animals. Panels K-N show staining with YAP antibody; panels O-R  
15 with TAZ antibody. Note raised nuclear expression of YAP in NF2 single (L; arrows) and  
16 NF2/TAZ double (N; arrows) null tissue, which is lost in NF2/YAP double null tissue (M;  
17 arrows). For TAZ staining, note raised nuclear TAZ expression in NF2 single (P; arrows) and  
18 NF2/YAP double (Q; arrows) null tissue, which is not present in NF2/TAZ double null DRG  
19 tissue (R; arrows). A-D and I, n=4; E-H and J, n=3; K-R, n=3 for each age and genotype  
20 examined. Data presented in graphs are means  $\pm$ SEM using one way ANOVA with Bonferroni's  
21 multiple comparison tests. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001; ns not significant. Scale bars: A-H  
22 75 $\mu$ m, K-R 50 $\mu$ m.

23

24 **Figure 2 Treatment of mice with VT1 or VT2 TEAD auto-palmitoylation inhibitors**  
25 **significantly inhibits proliferation of vestibular schwannoma tumours in vivo.** Data from 3-  
26 month-old NF2<sup>fl/fl</sup>-CRE- (CRE-) and NF2<sup>fl/fl</sup>-CRE+ (CRE+) animals treated with vehicle (Veh),  
27 10mg/kg/day VT1 or 30mg/kg/day VT2 by oral gavage for 21 consecutive days. A-H. Both VT1  
28 and VT2 significantly block tumour cell growth as measured by EdU incorporation in vestibular  
29 ganglion tissue. A-F. Representative images of vestibular ganglion from CRE- and CRE+ 3-  
30 month-old animals treated with either vehicle (A, B, D and E), VT1 (C) or VT2 (F). Note no

1 EdU positive cells are seen in CRE- animals with vehicle and that for CRE+ animals, numbers of  
 2 EdU positive cells (indicated by arrows) are reduced upon treatment with either VT1 (C) or VT2  
 3 (F). G, H. Quantification of numbers of EdU positive cells per area of ganglion tissue from  
 4 animals treated with vehicle (Veh.) or VT1 (Drug, G) or VT2 (Drug, H) compounds. Note  
 5 significant decreases in proliferation in CRE+ animals treated with either VT1 or VT2. I-N.  
 6 Representative western blots and quantification to show target engagement of VT1 and VT2 in  
 7 regulating connective tissue growth factor (CTGF) and TEAD protein expression in sciatic nerve  
 8 tissue of CRE- and CRE+ mice. I. Western blot of sciatic nerve of 3-month-old mice treated for  
 9 21d with either Vehicle (Veh.), VT1 (Drug) (I) or Vehicle or VT2 (Drug) (L). J, K.  
 10 Quantification of western blot in I. M, N. Quantification of western blot in L. Note significant  
 11 decrease in TEAD target CTGF by both VT1 and VT2 in sciatic nerve tissue in vivo (J, M) and  
 12 reduction in TEAD protein expression by VT2 (N) but not by VT1 (K). For data in A-H, n=4  
 13 mice for each genotype and drug treatment. For data in J, K, M and N, n=3 mice for each  
 14 genotype and drug treatment. Data presented in graphs are means  $\pm$ SEM using one way ANOVA  
 15 with Bonferroni's multiple comparison tests. \* P<0.05; \*\* P<0.01; \*\*\*, P<0.001. A-F. Scale bar:  
 16 50  $\mu$ m.

17  
 18 **Figure 3 Treatment of mice with VT auto-palmitoylation inhibitors significantly increases**  
 19 **apoptosis and reduces tumour volumes in vestibular ganglia (VGs) and dorsal root ganglia**  
 20 **(DRGs) in 9-month-old NF2<sup>fl/fl</sup>-CRE+ (NF2<sup>fl/fl</sup>-CRE+) mice.** NF2<sup>fl/fl</sup>-CRE- (NF2<sup>fl/fl</sup>-CRE-)  
 21 and NF2<sup>fl/fl</sup>-CRE+ animals were treated with vehicle (Veh.), 10mg/kg/day VT1 or 30mg/kg/day  
 22 VT2 by oral gavage for 10 or 21 consecutive days. A-D. Representative brightfield micrographs  
 23 of three different unilateral VG from: NF2<sup>fl/fl</sup>-CRE- Veh. treated mice (A), NF2<sup>fl/fl</sup>-CRE+ Veh.  
 24 treated mice (B), NF2<sup>fl/fl</sup>-CRE+ VT1-treated mice (C) and NF2<sup>fl/fl</sup>-CRE+ VT2-treated mice (D),  
 25 all mice were treated for 21 consecutive days. Scale bar = 20  $\mu$ m. E. Quantification of average  
 26 bilateral VG volume in A-D, for NF2<sup>fl/fl</sup>-CRE- Veh. treated mice (n=6 ganglia, n=3 mice),  
 27 NF2<sup>fl/fl</sup>-CRE+ Veh. treated mice (n=4 ganglia, n=3 mice), NF2<sup>fl/fl</sup>-CRE+ VT1 treated mice (n=3  
 28 ganglia, n=3 mice) and NF2<sup>fl/fl</sup>-CRE+ VT2 treated mice (n=3 ganglia, n=3 mice). F.  
 29 Quantification of average bilateral lumbar 4 DRG volume, for NF2<sup>fl/fl</sup>-CRE- Veh. and NF2<sup>fl/fl</sup>-  
 30 CRE+ Veh. treated mice (n=8 ganglia, n=3 mice), for NF2<sup>fl/fl</sup>-CRE+ VT1 and NF2<sup>fl/fl</sup>-CRE+

1 VT2 treated mice (n=3 ganglia, n=3 mice). G-R. Representative immunofluorescence of n=3  
 2 different VGs with *in situ* apoptosis detected by Terminal deoxynucleotidyl transferase dUTP  
 3 Nick-End Labelling (TUNEL) assay, following oral gavage with either vehicle or 30mg/kg/day  
 4 VT2 for 10 days. TUNEL<sup>+</sup> nuclei (green; arrows; J, L, P and R) were significantly increased in  
 5 NF2<sup>fl/fl</sup>-CRE<sup>+</sup> VT2-treated mice compared to NF2<sup>fl/fl</sup>-CRE<sup>+</sup> Veh. treated mice. Neurofilament  
 6 (NF; H and K) counterstain (red and merged with Hoechst counterstain (blue); I, L) reveals  
 7 increased apoptosis in cells surrounding neuronal cell bodies of the VG in NF2<sup>fl/fl</sup>-CRE<sup>+</sup> VT2  
 8 treated mice. S100 counterstain (N, Q (red) and merged with Hoechst counterstain (blue); O, R)  
 9 reveals apoptosis is increased in S100+ schwannoma cells of NF2<sup>fl/fl</sup>-CRE<sup>+</sup> VT2 treated mice.  
 10 Scale bars = 20  $\mu$ m. S, T. Quantification of TUNEL<sup>+</sup> cells/ 100  $\mu$ m<sup>2</sup> in VG (S) and DRG (T). In  
 11 E and F, data presented as mean  $\pm$ SEM using one way ANOVA with Tukey's multiple  
 12 comparisons tests In S and T, data presented as mean  $\pm$ SEM using Brown-Forsythe and Welch  
 13 ANOVA with Dunnett's T3 multiple comparisons test. \*\* P<0.01; \*\*\*, P<0.001; ns= non-  
 14 significant.

15  
 16 **Figure 4 Increased numbers of macrophages in NF2-null DRG tissue is dependent upon**  
 17 **YAP and TAZ.** A-L Staining of DRG sections from NF2<sup>fl/fl</sup>-CRE<sup>-</sup> (A, E, I), NF2<sup>fl/fl</sup>-CRE<sup>+</sup> (B, F,  
 18 J), NF2<sup>fl/fl</sup>YAP<sup>fl/fl</sup>-CRE<sup>+</sup> (C, G, K) and NF2<sup>fl/fl</sup>TAZ<sup>fl/fl</sup>-CRE<sup>+</sup> (D, H, L) animals at 3, 5 and 9  
 19 months of age with pan-macrophage marker Iba1 antibody. Note time-dependent increase in  
 20 numbers of Iba1-positive macrophages in NF2<sup>fl/fl</sup>-CRE<sup>+</sup> DRG between 3 (B), 5 (F) and 9 (J)  
 21 months. M-P. Staining of sections of VG tissue from NF2<sup>fl/fl</sup>-CRE<sup>-</sup> (M, O) and NF2<sup>fl/fl</sup>-CRE<sup>+</sup> (N,  
 22 P) at 3 and 5 months of age with Iba1 antibody. Q. Quantification of % macrophages of total cell  
 23 number in DRG tissues. R. Quantification of percentage of macrophages at 3 and 5 months in  
 24 NF2<sup>fl/fl</sup>-CRE<sup>-</sup> and NF2<sup>fl/fl</sup>-CRE<sup>+</sup> VG tissue. For data presented, n=3 mice for each genotype and  
 25 age. Data presented in graphs are means  $\pm$ SEM; in Q and R, two way ANOVA was used with  
 26 Tukey's multiple comparison test. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001. Scale bars: 25  $\mu$ m.

27  
 28 **Figure 5 Knockdown of either YAP or TAZ or use of TEAD auto-palmitoylation inhibitors**  
 29 **inhibits human schwannoma cell proliferation.** A-G. Lentiviral knockdown of either YAP or  
 30 TAZ significantly reduces proliferation of human NF2-null schwannoma cells. A-C.

1 Representative images of EdU positive cells (red), counterstained with Hoechst (Ho; blue) from  
 2 scrambled control (Scr, A), YAP knockdown (shYAP, B) and TAZ knockdown (shTAZ, C). D.  
 3 Quantification of percentage positive EdU cells for each condition. E-G. Western blot (E) and  
 4 quantification (F and G) confirming YAP or TAZ knockdown in cells; note that knockdown of  
 5 TAZ does not significantly affect levels of YAP (F), but knockdown of YAP does significantly  
 6 lower levels of TAZ (G). H-K. TEAD auto-palmitoylation inhibitor (VT3) decreases human  
 7 schwannoma cell proliferation in a dose-dependent manner. L. Expression of four TEAD  
 8 isoforms (TEAD1-4) in cells from three human schwannoma tumours S<sub>1</sub>, S<sub>2</sub> and S<sub>3</sub> treated with  
 9 either vehicle (left) or 2µm VT3 for 7d (VT, right). M. Quantification of schwannoma cell  
 10 proliferation with increasing concentrations of auto-palmitoylation inhibitor VT3. N=3 for all  
 11 data shown. Data presented in graphs are means ±SEM. Data analysis in (D) was one-way  
 12 ANOVA with Bonferroni's correction; in (F) and (G) were matched one-way ANOVAs with the  
 13 Geisser-Greenhouse correction and Tukey's multiple comparisons test; in (M) Student's t-test  
 14 and in (N) one-way ANOVA with Bonferroni's correction. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001.  
 15 Scale bars: 25µm.

16  
 17 **Figure 6 Expression of ALDH1A1 is TAZ-dependent in mouse Schwann cells and**  
 18 **schwannoma tissue.** A-D. Immunolabelling of NF2<sup>fl/fl</sup>-CRE- (A, B) and NF2<sup>fl/fl</sup> CRE+ (C, D)  
 19 sciatic nerve (A and C) and dorsal root ganglion (DRG; B and D) tissue showing elevated  
 20 expression of ALDH1A1 (green) in NF2-null mouse tissue. In sciatic nerve, ALDH1A1 staining  
 21 appears associated with the non-myelinating Schwann cells. In DRG, high ALDH1A1  
 22 expression was seen in the cells surrounding the neuronal cell bodies. E, F. Western blot showing  
 23 raised ALDH1A1 expression in NF2<sup>fl/fl</sup> CRE+ sciatic nerve, which was reduced in NF2<sup>fl/fl</sup> TAZ<sup>fl/fl</sup>  
 24 -CRE+ but not NF2<sup>fl/fl</sup> YAP<sup>fl/fl</sup> -CRE+ animals. F. Quantification of western blot in E. G-I.  
 25 Immunolabelling of transverse sections of sciatic nerve, showing elevated ALDH1A1 (red) in  
 26 NF2<sup>fl/fl</sup> -CRE+ nerve (H), but reduced in NF2<sup>fl/fl</sup> TAZ<sup>fl/fl</sup> -CRE+ double null nerves (I). J-M.  
 27 ALDH1A1 staining of DRG tissue paraffin sections from control NF2<sup>fl/fl</sup>-CRE- (J), NF2<sup>fl/fl</sup>-  
 28 CRE+ (K), NF2<sup>fl/fl</sup> YAP<sup>fl/fl</sup> -CRE+ (L) and NF2<sup>fl/fl</sup> TAZ<sup>fl/fl</sup> -CRE+ (M) double-null mice. N, O.  
 29 Representative western blot (N) and quantification (O) of ALDH1A1 expression in sciatic nerve  
 30 from NF2<sup>fl/fl</sup>-CRE- and CRE+ vehicle or VT2 treated mice. For data presented, n=3 mice for



1 each genotype and age. Data presented in graphs are means  $\pm$ SEM. In F, for analysis, a one-way  
 2 ANOVA with Bonferroni's correction was used. In O, for analysis, a one-way ANOVA with  
 3 Tukey's multiple comparisons correction used. \*  $P < 0.05$ , \*\*  $P < 0.01$ ; ns not significant. Scale  
 4 bars: A-D, G-I 50  $\mu$ m, J-M 25  $\mu$ m.

5  
 6 **Figure 7 ALDH1A1 is upregulated in human schwannoma and is a TAZ target.** A-C.  
 7 Staining of control human nerve (A) and two human schwannoma samples (B, C) showing  
 8 strong expression of ALDH1A1 in all tumour cells. D, E. Knockdown experiments in human  
 9 schwannoma cells. Lentiviral-mediated shRNA knockdown of TAZ (shTAZ) reduces ALDH1A1  
 10 protein levels in cells; knockdown of ALDH1A1 was used as a positive control. E.  
 11 Quantification of western blot data in D. F, G. Representative western blot for ALDH1A1  
 12 expression in primary human schwannoma cells with either knockdown of TAZ (shTAZ) or  
 13 scramble control (shSCR), treated with 10 $\mu$ M MG132 (+MG132) or DMSO vehicle control (-  
 14 MG132) (F). Note suppression of ALDH1A1 protein levels is not reversed by proteasome  
 15 inhibition by MG132. G. quantification of F. H-J. Use of ALDH1A1-specific inhibitor reduces  
 16 proliferation of primary human schwannoma cells. Ki67 stain of vehicle control (H) or 10  $\mu$ M  
 17 ALDH1A1 inhibitor 1 (I). J. Quantification of percentage Ki-67 positive schwannoma cells with  
 18 increasing concentrations of ALDH1A1 inhibitor 1. For data presented, n=3. Data presented in  
 19 graphs are means  $\pm$ SEM. Statistical analysis shown in E and J is one way ANOVA with  
 20 Bonferroni's correction; in G a one-way ANOVA with Tukey's multiple comparisons test. \*  
 21  $P < 0.05$ ; \*\*\*  $P < 0.001$ ; ns not significant. Scale bars: A-C, F, G 25  $\mu$ m.

22  
 23 **Figure 8 ALDH1A1 is upregulated in NF2-null human meningioma tissue and cells.** A, B.  
 24 Staining of sections of NF2-positive (NF2<sup>+/+</sup>, A) and NF2-null (NF2<sup>-/-</sup>, B) meningioma tissue  
 25 shows higher ALDH1A1 in NF2-null tumours. Boxes show enlarged section of the tumour with  
 26 strong cytoplasmic stain of ALDH1A1 protein. C. Western blot of control human meningeal  
 27 cells (HMC), BenMen-1 (BM1) and KT21-MG1 (KT21) meningioma cells showing elevated  
 28 ALDH1A1 expression in both human NF2-null cell lines. D. Quantification of western blot in C.  
 29 E. Lentiviral shRNA knockdown of YAP or TAZ in BenMen-1 cells showing that ALDH1A1  
 30 expression is dependent upon TAZ. F. Quantification of western blot in E. G-I. ALDH1A1

1 inhibitor 1 reduces proliferation of BenMen-1 cells. J. Quantification of percentage Ki-67  
2 positive cells with increasing ALDH1A1 inhibitor concentrations. K-N. Comparison of the  
3 effects of shRNA knockdown of ALDH1A1 (L) and TAZ (M) upon cell proliferation in  
4 BenMen-1 cells. N. Quantification of percentage EdU positive cells. For data presented, n=3.  
5 Data presented in graphs are means  $\pm$ SEM. Statistical analysis shown is a one-way ANOVA  
6 with Bonferroni's correction. \* P<0.05; \*\* P<0.01; \*\*\* P<0.001; ns not significant. Scale bars:  
7 25  $\mu$ m.  
8  
9

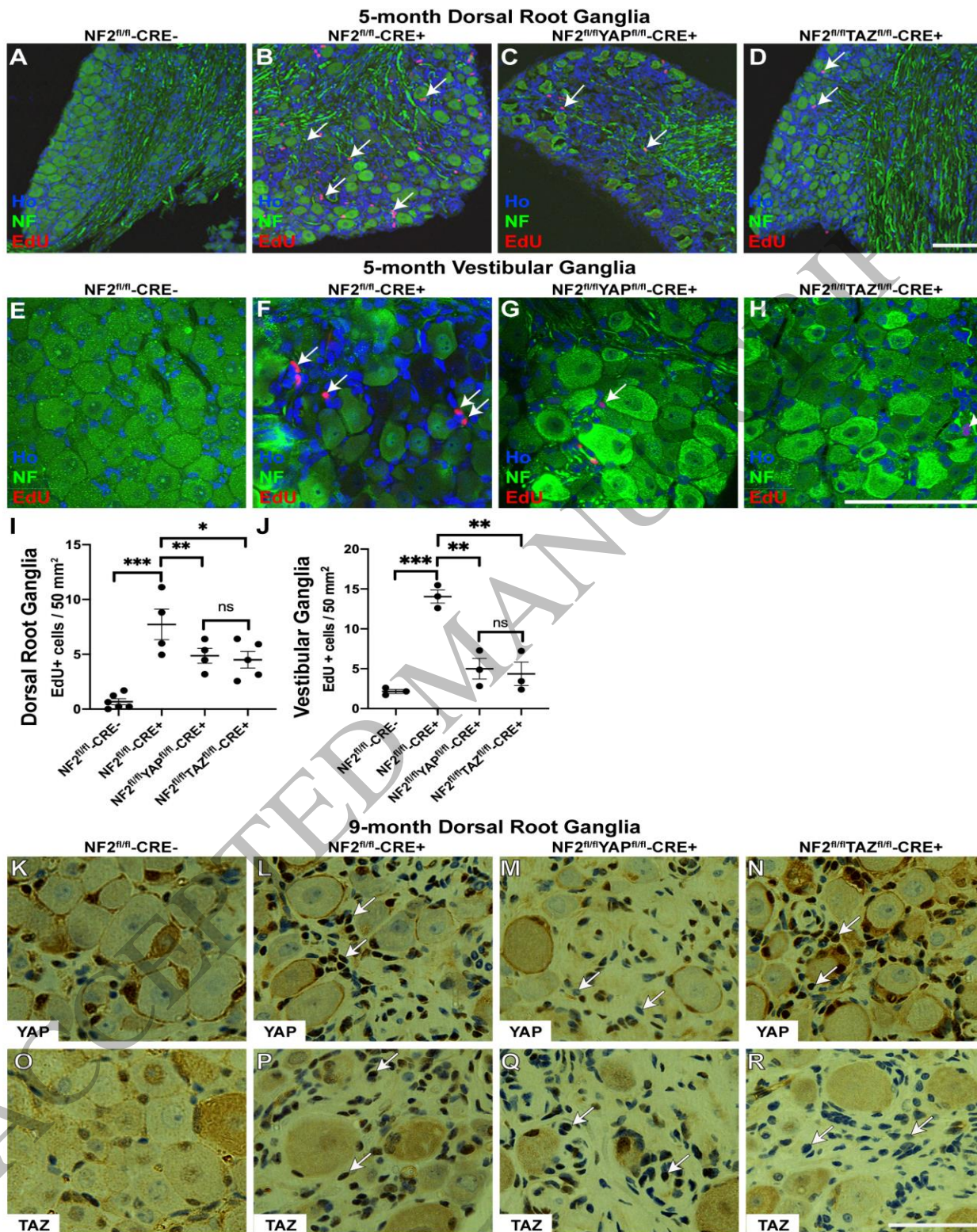


Figure 1  
 167x236 mm ( x DPI)

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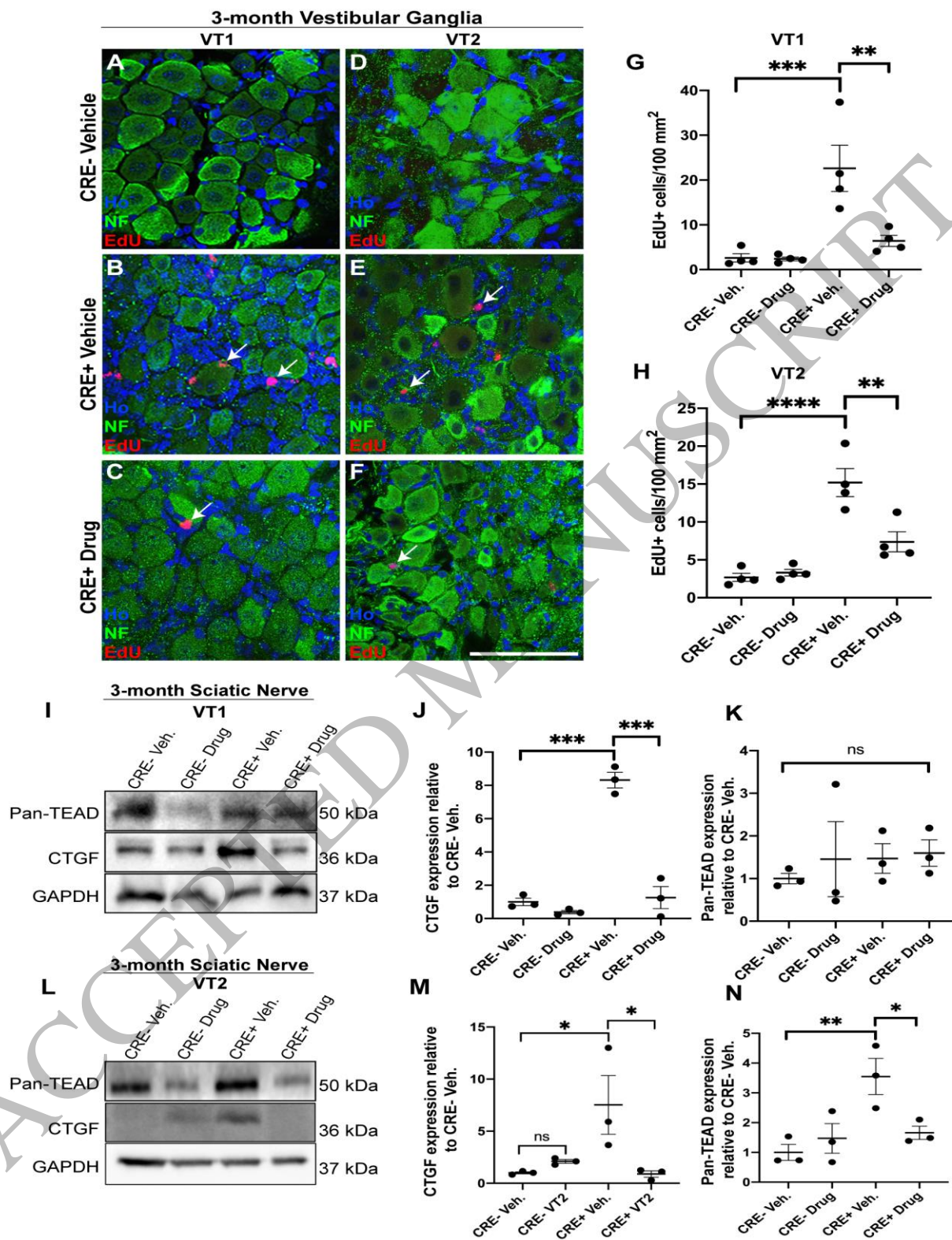


Figure 2  
162x247 mm (x DPI)

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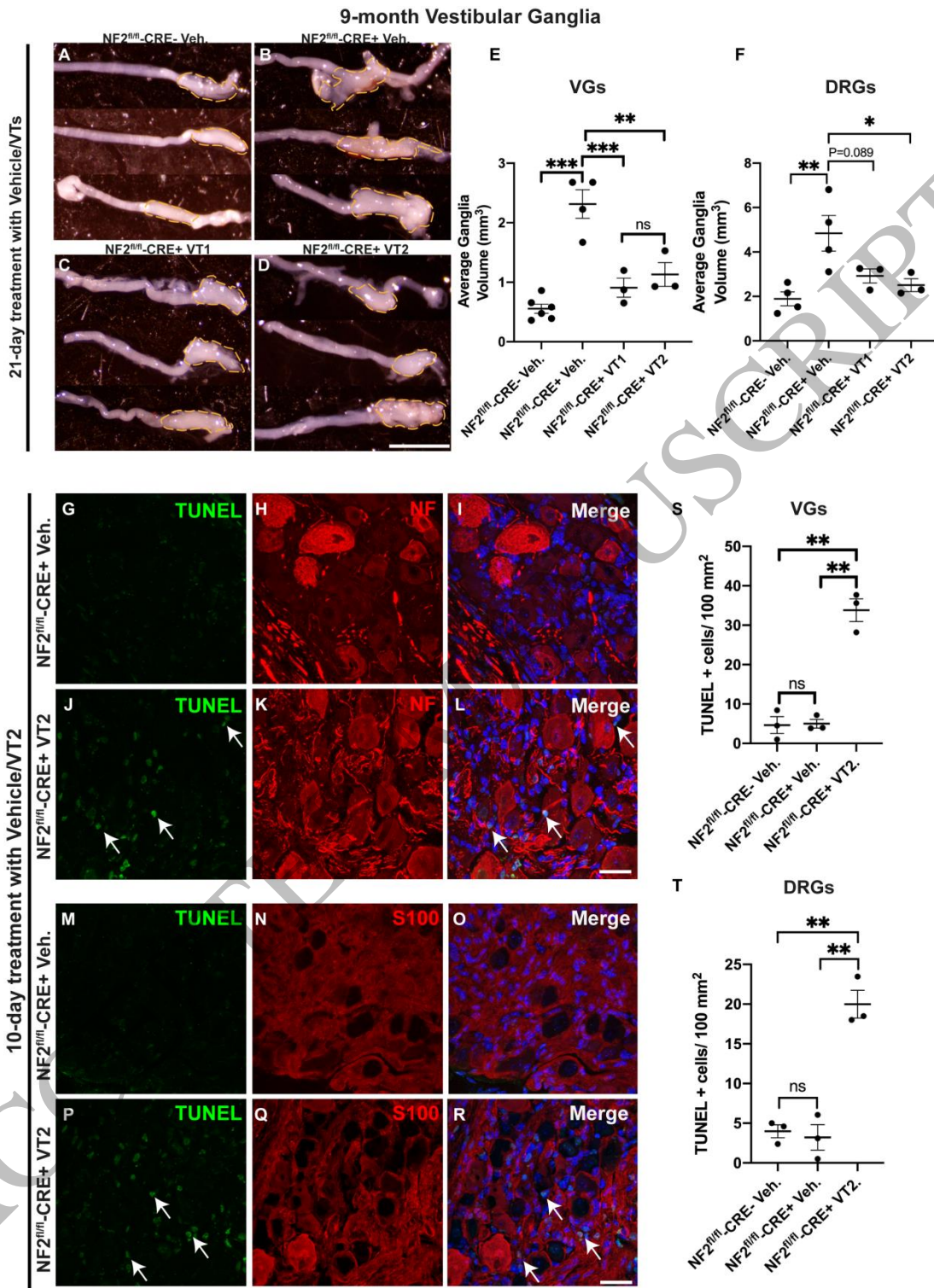
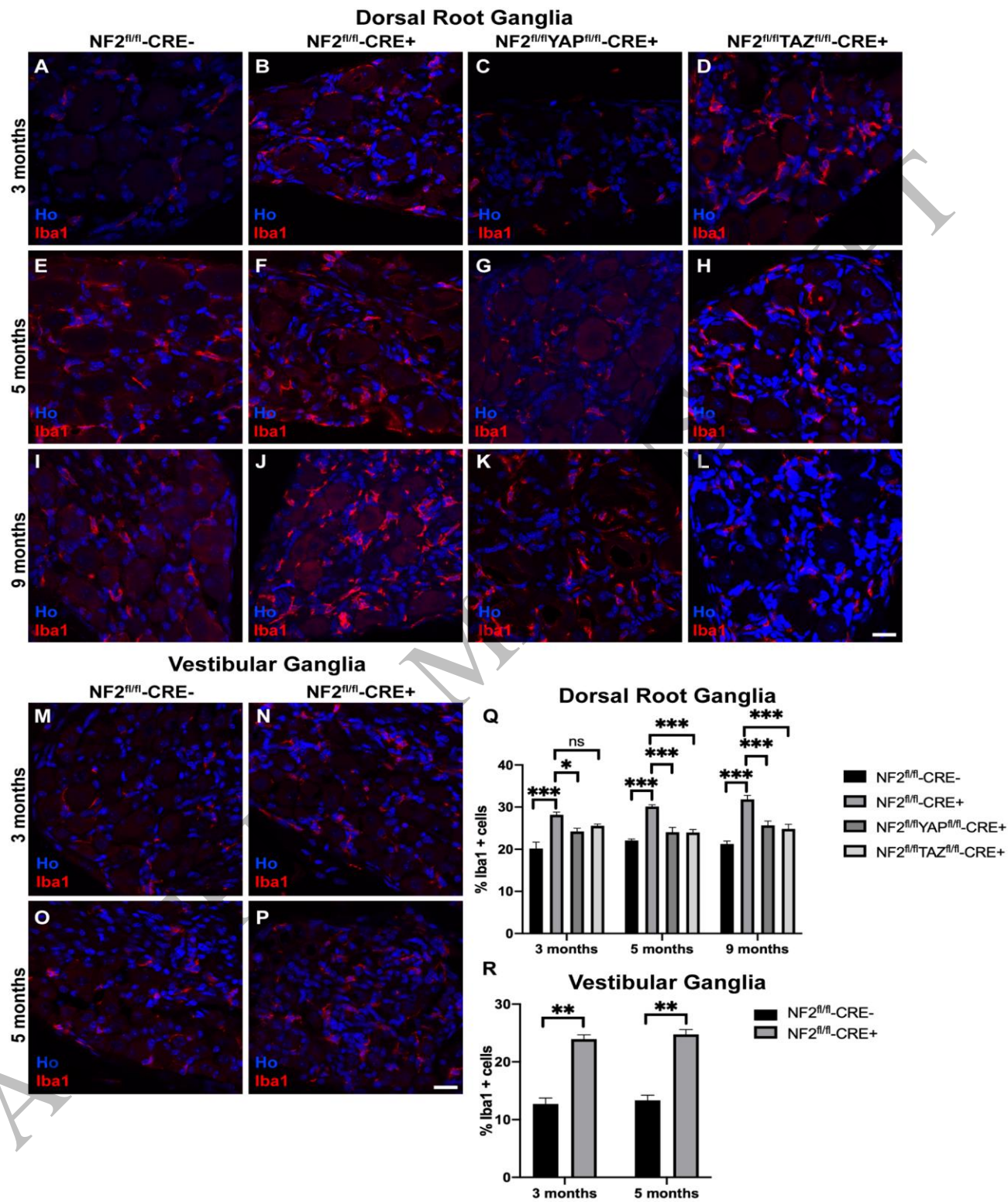


Figure 3  
200x269 mm (x DPI)

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Figure 4  
174x234 mm ( x DPI)

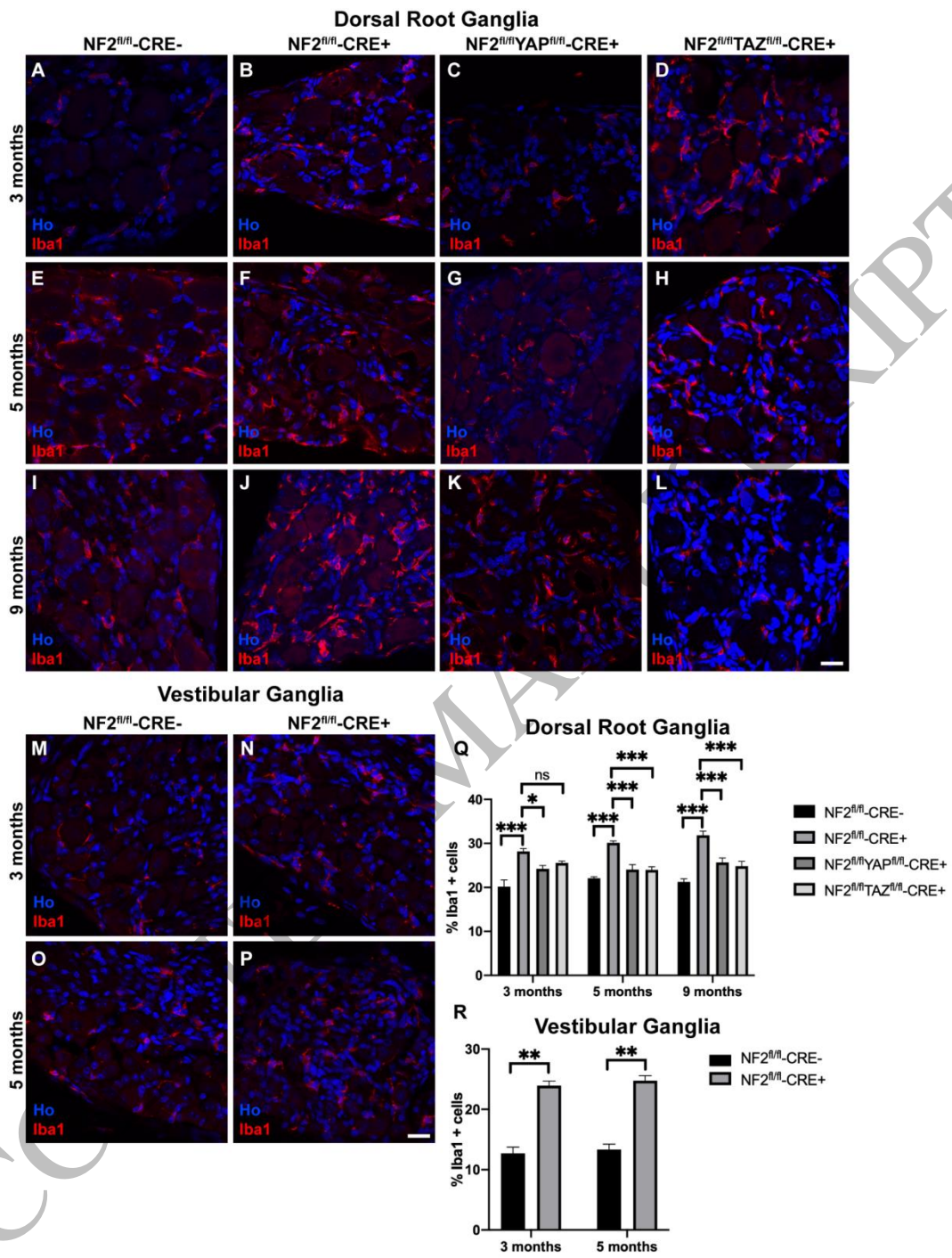


Figure 5  
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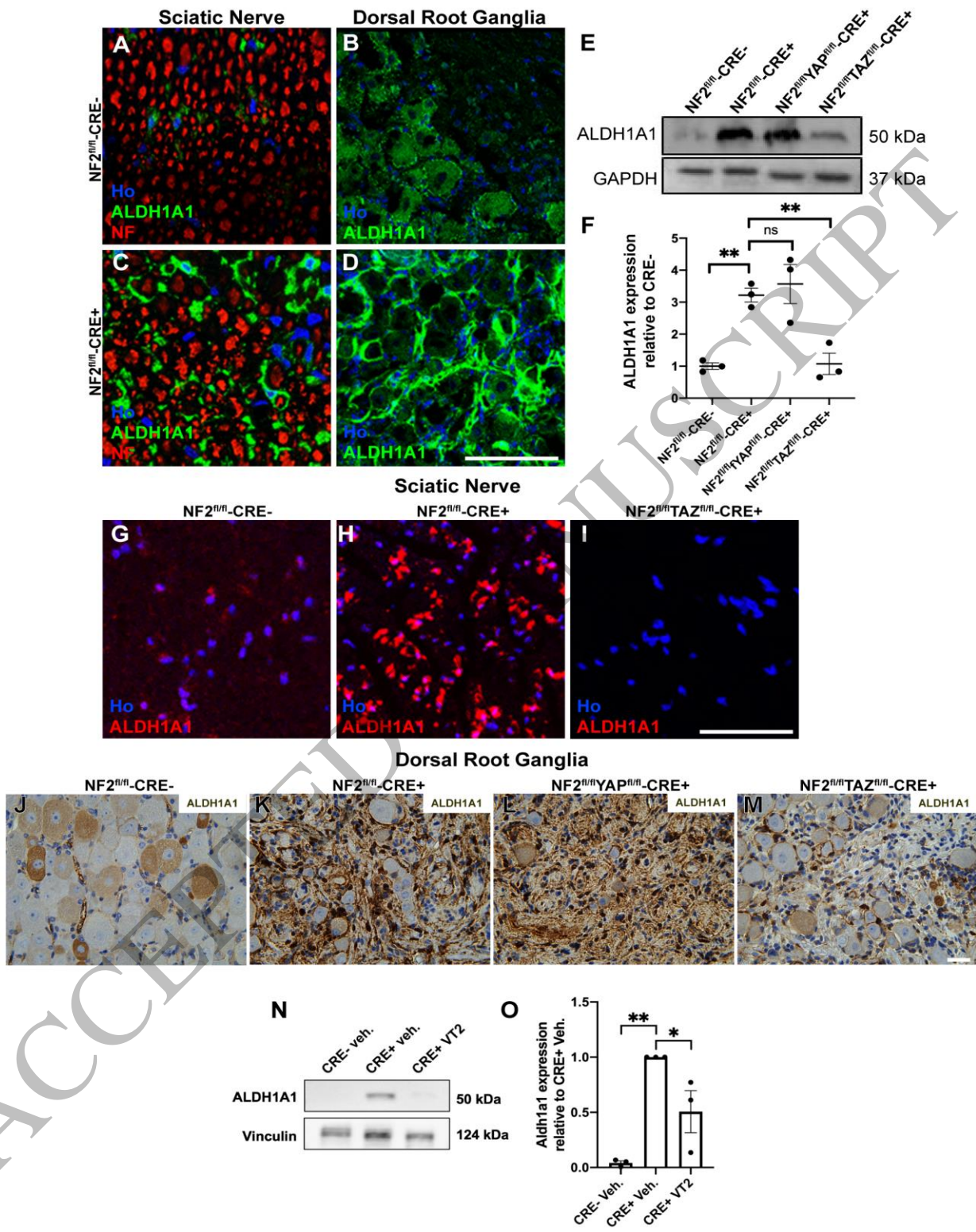


Figure 6  
174x228 mm ( x DPI)

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2  
3



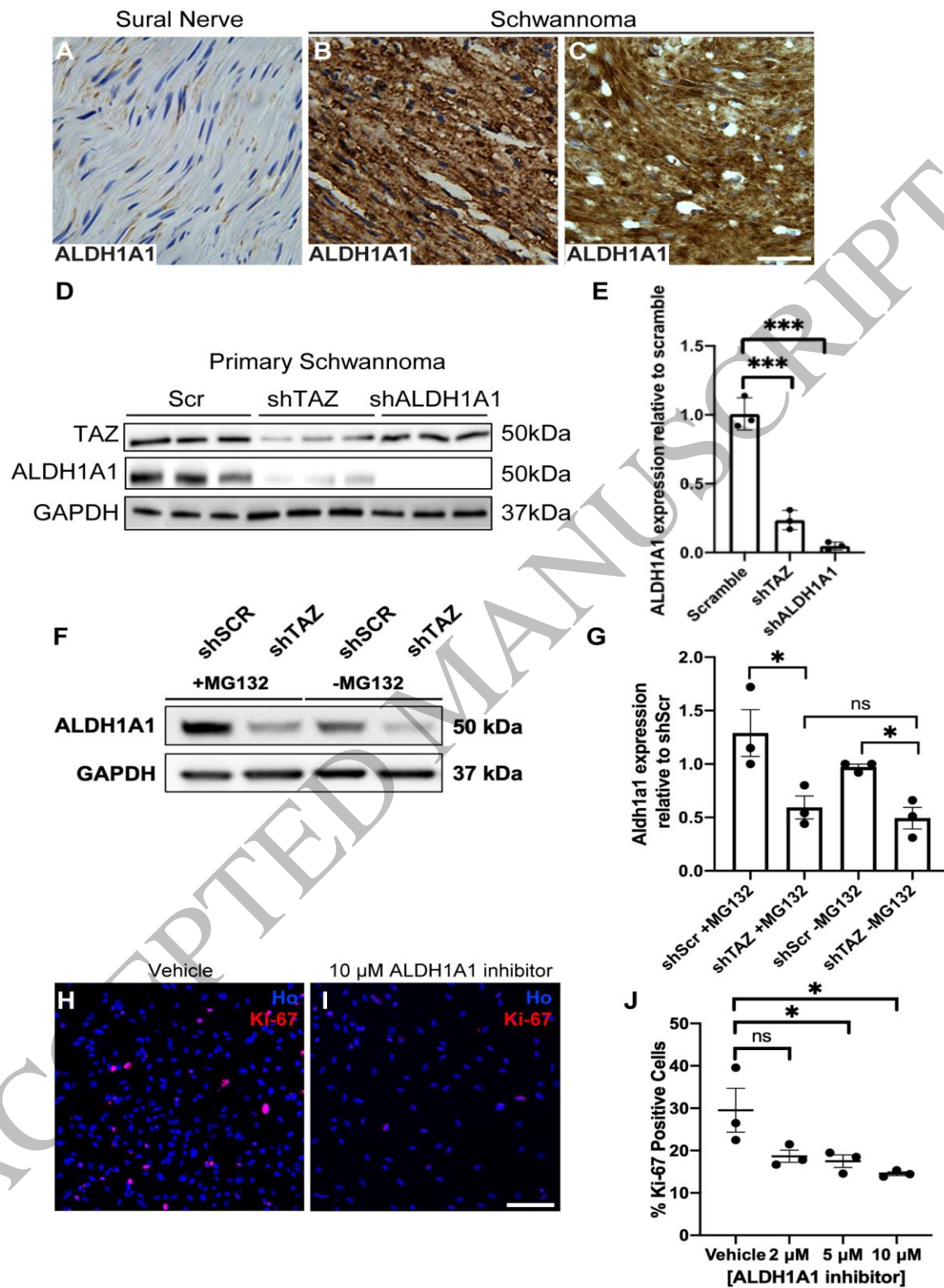


Figure 7  
159x225 mm ( x DPI)

1  
2  
3

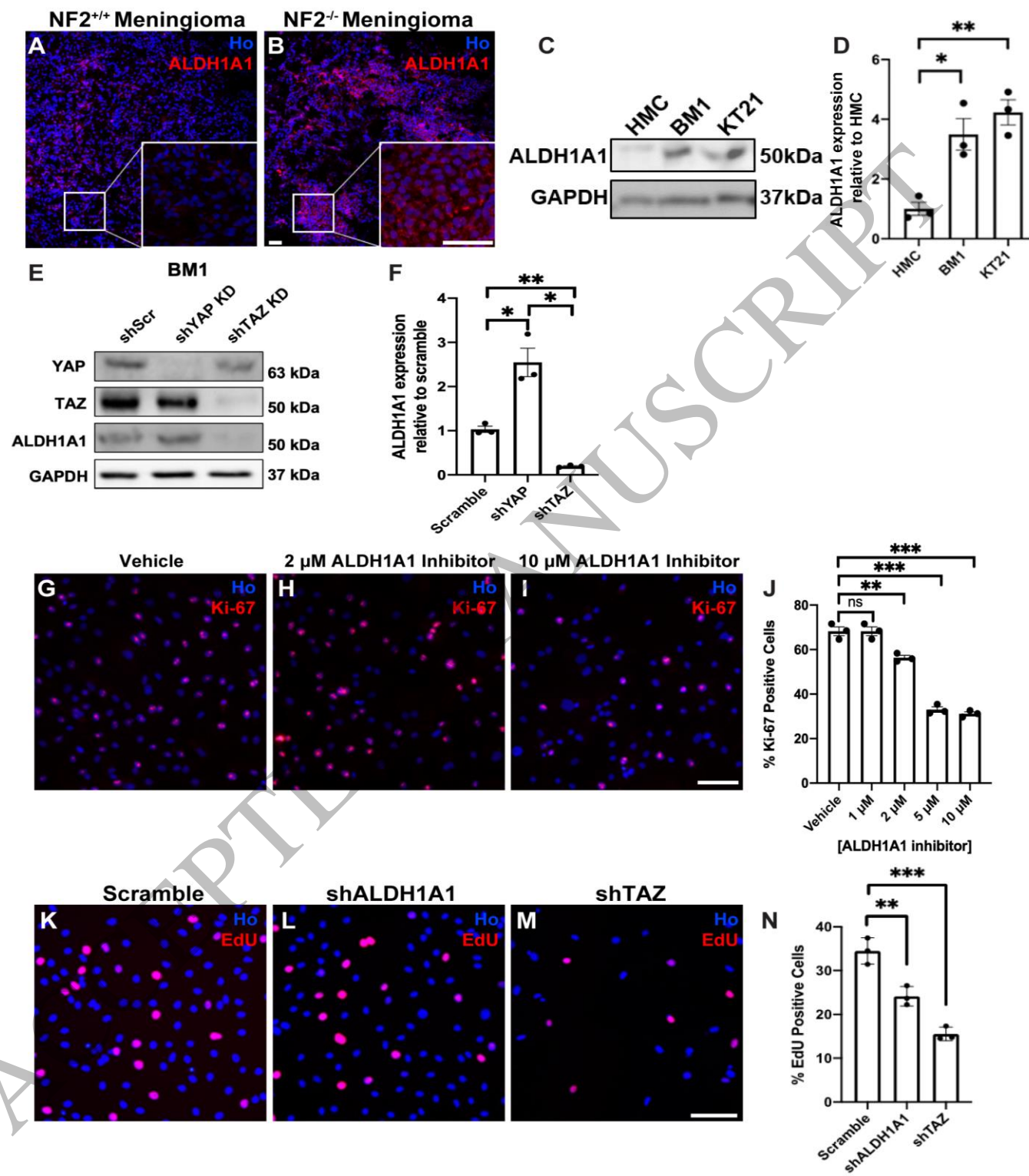


Figure 8  
179x218 mm ( x DPI)

1  
2  
3