Supplementary Information

Metastatic recurrence of Group 3 Medulloblastoma is driven by PRUNE-1 through targeting NME1–TGF-β–OTX2–SNAIL signaling via PTEN inhibition

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Supplementary Information, Figure legends

Supplementary Information, Figure S1. Overexpression of PRUNE-1 and NME1 in metastatic MB_{Group3}.

(A) RNA log^2 expression analysis of PRUNE-1 levels of primary MB samples across different publically available datasets, compared with normal cerebellum (N cerebellum). Data from four independent public-domain MB gene-expression datasets are shown: Prof. Delattre, Paris, France; Prof. Gilbertson, St. Jude Children's Research Hospital, Memphis USA; Prof. Kool, DKFZ, Heidelberg, Germany; and Prof. Roth, BC Cancer Agency, Vancouver, British Columbia, Canada. Overexpression of PRUNE-1 in MB samples is shown

($p=1.6e^{-09}$). **(B)** Event-free survival (EFS) Kaplan-Meier–Cox Regression statistical analyses of estimated survival times according to MB tumour molecular subgroup distribution from our collection at Santobono Pausillon Hospital, Naples, Italy. MB_{Group3} (n = 7) patients showed the worst survival compared with those of MB_{SHH} (n = 5) and MB_{WNT} (n = 4), who showed some degree of overlapping data. Here, MB_{Group4} (n = 6) also performed better in the survival analysis, most probably due to the limited number of 'events' of these two main groups in this tumour collection (p=0.04). **(C)** RNA log^2 expression of NME1/ NME2 across MB primary tumours (i.e., M1 [n=176] vs. M0 [n=397]) in the publically available Cavalli dataset (n=573 tumours). There were higher NME1/ NME2 expression levels in metastatic M1 compared to M0 samples ($p=0.6.9e^{-03}$). **(D, E)** Representative Western blots with an anti-PRUNE-1 antibody (**D**), and anti-NME1 and anti-phospho-(Ser120-122-125)-NME1 antibodies (**E**), as previously described (Carotenuto et al., 2013; Garzia et al., 2008) of cell lysates from immortalised MB_{Group3} and MB_{SHH} cell lines, as indicated. Higher protein levels of PRUNE-1, phospho-(Ser-120-122-153)-NME1 and NME1 were seen for MB_{Group3} cell lines compared to MB_{SHH} cell lines. β-Actin levels were used as the loading control. **(F)** Gene expression correlation analysis for PRUNE-1 and NME1-2 in the Cavalli dataset subset of metastatic samples (n = 176). Statistically significant positive correlation was shown between PRUNE-1 and NME1/ NME2 ($r = 0.23$, $p = 3.2e^{-03}$).

Supplementary Information, Figure S2. Correlations between PRUNE-1 and OTX2 expression in MB tumour samples in the context of TGF-β enhancement or inhibition.

(A-C) Gene expression correlation analyses between transcripts for PRUNE-1 and OTX2 (**A**), TGF-β1 (TGFB1) and OTX2 (**B**), and TGF-β receptor 1 (TGFBR1) and OTX2 (**C**), in MB samples from the Cavalli dataset (n=763 tumour samples). Statistically significant positive correlations were shown between these genes ($r=0.455$, $p=3.2e^{-40}$; $r=0.361$, $p=6.3e^{-25}$;

r=0.286, p=8.5e^{-16}, respectively). **(D)** RNA log^2 expression analysis of TGF-β1 (TGFB1, upper panels) and serine-threonine kinase TGF-β receptor Type 1 (TGFBR1, bottom panels) levels of primary MB samples across the indicated publically available datasets. Higher expression levels of TGFB1 and TGFBR1 were seen for MB samples compared to normal cerebellum samples (N CB; Roth dataset, n=9; TGFB1, p=6.1e^{-03}; TGFBR1, p=7.5e^{-15}). When grouped according to molecular classification or metastatic stage (Cavalli dataset), expression analysis of TGFB1 and TGFBR1 showed higher mRNA levels in MB_{Group3} samples for TGFB1 (p=1.7e^{-49}) and in MB_{Group3} and MB_{WNT} samples for TGFBR1 (p=3.9e^{-34}), and in metastatic M1 samples for both (TGFB1, p=3.0e^{-04}; TGFBR1, p=0.03). **(E)** RNA log^2 expression of both R-SMADs (i.e., SMAD2, SMAD3) and co-SMAD (i.e., SMAD4) across primary MB_{Group3} tumour samples grouped according to indicated molecular subtypes for the publically available Cavalli dataset (n=109). Higher expression levels were seen for SMAD2 in metastatic MB_{Group3}-α and MB_{Group3}-γ (p=2.1e^{-05}), for SMAD3 in MB_{Group3}-γ (p=2e^{-05}), and SMAD4 in MB_{Group3}-α (p=6.7e^{-03}).

Supplementary Information, Figure S3. Correlation studies between PRUNE-1, MYC (c-MYC, N-MYC) and OTX2 in MB tumour samples

(A, B) RNA log^2 expression of c-MYC **(A)** and N-MYC **(B)** derived from transcriptome analyses from the publically available Cavalli dataset (n=763; c-MYC, p=1.1e^{-158}; N-MYC, p=3.5e^{-132}), grouped according to the molecular and subtypes disease variants. C-MYC is highly expressed in MB_{Group3} and MB_{WNT} groups, while N-MYC is highly expressed in MB_{SHH} and MB_{WNT} groups. **(C)** RNA log^2 expression of N-MYC across MB primary tumours (i.e., M1 [n = 176] *vs*. M0 [n = 397]) in the publically available Cavalli dataset (n=573 tumours). Higher N-MYC expression levels were seen for non-metastatic M0 than for metastatic M1 samples (p=5.7e^{-04}). **(D-H)** Representative Western blots (D, E, F, G, H) with

indicated antibodies. **(D)** Cell protein lysates from immortalised MB_{Group3} and MB_{SHH} cell lines (as indicated). Higher OTX2 protein levels were increased in MB_{Group3} compared to MB_{SHH} cell lines. **(E)** Cell protein lysates from MB_{Group3} D283-Med cells transiently transfected with the SMAD2 plasmid. OTX2 was up-regulated (1.4-fold) in these cells previously transfected with a SMAD2-encoding plasmid compared to empty vector (E.V.) as control. **(F)** Cell protein lysates from HEK-293T transfected with plasmids containing SMAD3 and SMAD4 in combination with PRUNE-1 or NME1 (related to Figure 2F). **(G)** Cell protein lysates from MB_{Group3} D283-Med cells transiently transfected with the NME1 plasmid. Protein levels of phospho-SMAD2 were decreased (0.6-fold) in D283-Med cells transfected with NME1 compared to empty vector (E.V.) as control. **(H)** Cell protein lysates from MB_{Group3} D283-Med cells transiently transfected with the STRAP plasmid. The protein levels of OTX2 remain unchanged in D283-Med cells transfected with STRAP. **(I)** Immunohistochemical staining on sequential sections of eight primary tumour tissues from MB_{Group3} patients in our tissue cohort bank. The antibodies used were against EMT markers (i.e., anti-N-Cadherin, anti-E-Cadherin). Positive immunoreactivity to N-Cadherin and negativity to E-Cadherin are seen close to tumour borders in the same tissue sections, indicating EMT is present in these MB_{Group3} samples. **(L)** Representative Western blots with indicated antibodies. Cell protein lysates from MB_{Group3} D283-Med cells after transfection with full-length PRUNE-1. Protein levels of phospho-Ser473-AKT were increased (1.4-fold) compared to empty vector (E.V.). Where indicated, β-Actin and α-Tubulin levels were used as the loading controls.

Supplementary Information, Figure S4. Overexpression of PRUNE-1 in MB_{Group3} is accomplished through down-regulation of PTEN and activation of epithelial-mesenchimal transition.

Immunohistochemical staining **(A-B)** and FISH analysis **(B)**. The histopathological grading system shown describes the levels of expression and distribution of the markers within the tumour sections according to guidelines described previously (Louis et al., 2014). Magnification: 40×. **(A)** Paraffin-embedded tumour sections from MB patients representative of the different molecular subgroups within our tissue cohort bank (as indicated). The antibodies used were against PRUNE-1 and PTEN. An inverse correlation between PRUNE-1 and PTEN levels was shown for $MB_{Group3/4}$ (i.e., pMB6, pMB7, pMB8, pMB9). **(B)** Fifteen MB_{Group3} tumours. Antibodies used were against PRUNE-1 and PTEN, to determine their levels of expression in the tumours. The same tissues were analysed for c-MYC copy numbers using FISH analysis (patients MB2, MB3, MB12, MB13, MB16, MB21; as described in Supplementary Information) and using real-time PCR assays (patients from MB44 to MB54) as described previously (Mastronuzzi et al., 2014). In several tumour samples, dichotomy of inverse expression of these two markers can be detected (PRUNE-1 high, +++; PTEN low, -/+; and *vice versa*). PRUNE-1 and PTEN staining were performed for the same cells/ tissues (3 μm apart). **(C)** Representative affinity chromatography using the indicated antibodies of protein lysates from D283-Med cells, which showed the HIS-tagged-Carboxyl-Terminal-PRUNE-1 domain and NME1 protein interaction in this cellular model of MB_{Group3}. **(D)** Representative Western blot with the indicated antibodies of protein lysates from MB_{Group3} D283-Med cells treated with CDK1 inhibitor D4476 (30 μM) or vehicle (DMSO). OTX2 protein levels decreased (0.1-fold) after this treatment. β-Actin was used as the loading control. **(E)** Quantitative immunofluorescence analyses on paraffin-embedded cerebellar tumours generated by implanting D425-Med cells previously infected with AdV-Mock and AdV-CPP. The intensity of staining for each antibody was measured in each cells, normalized to control (AdV-Mock) and expressed as fold increase/decrease. More then 100 cells were counted. CTCF=Corrected Total Cell Fluorescence.

Supplementary Information, Figure S5. AA7.1 impairs tumour progression *in vitro* in MB_{Group3} by targeting PRUNE-1.

(A) Time-course for proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) with MB_{Group3} cells (D283-Med, D425-Med cells) after infection with adenoviral particle carrying empty vector (AdV-Mock) or sh-RNA for PRUNE-1 silencing (AdV-sh-PRUNE-1). Data are means ±SD. There was statistically significant ($p < 0.05$) impairment of cell proliferation in D283-Med cells upon PRUNE-1 silencing, compared to the AdV-Mock control. **(B)** WaterLOGSY nuclear magnetic resonance signal intensities for proton resonances of AA7.1 with PRUNE-1 (1-393) and its D364A, L359R and D364A/ L359R mutations (as indicated) as a function of ligand concentration. The curves represent the best fits for the data. Mutations D364A and L359R both affected the interaction with AA7.1, giving a K_d of 37.74 (±0.15) μM. **(C)** Representative Western blots using protein lysates from MB_{Group3} D425-Med cells treated with protein synthesis inhibitor cycloheximide (CHX) alone or in combination with 100 μM AA7.1 at different times of treatment. Maximum effects on newly synthesised PRUNE-1 protein degradation were seen after 8 h AA7.1 treatment. Data are representative of three independent assays. **(D)** Analyses of MB_{Group3} D425-Med cells at 48 h from adenoviral infection with AdV-sh-Prune-1 or treatment with 100 μM AA7.1 (AdV-Mock and PBS as vehicle controls, respectively) stained with Annexin V-FITC and propidium iodide. Three cell populations were present: viable cells (dark grey), weakly stained with propidium iodide and Annexin V-FITC; secondary apoptotic cells (black), strongly stained with propidium iodide and Annexin V-FITC; and early apoptotic cells (light grey), weakly stained with propidium iodide and strongly stained with Annexin V-FITC. Silencing of Prune-1 induced by the adenoviral infection (AdV-sh-Prune-1) in D425-Med cells resulted in significantly increased

apoptotic cell populations, compared to cells treated with AA7.1. Data are from triplicate assays that were repeated twice. **(E)** Quantitative immunofluorescence analyses on paraffin-embedded cerebellar tumours generated by implanting D425-Med cells. The intensity of staining for each antibody was measured in each cells, normalized to control (AdV-Mock) and expressed as fold increase/decrease. More then 100 cells were counted. CTCF=Corrected Total Cell Fluorescence.

Supplementary Information, Tables

Supplementary Information, Table S1. ΔcT values for expression in the data analysis, as represented in Figures 1D, E, 5E.

Upper panel: PRUNE-1 expression levels at diagnosis in our cohort of primary MB samples were correlated to metastatic stage (M-stage) of patients recorded according to the five-tiered Chang classification (M+, n= 8; M0, n=26). The normal cerebellum control samples (n = 13) were from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, USA. The analysis was performed using real-time-PCR with SYBR-green technology, and the relative expression values are shown as $2^{-\Delta Ct}$ (related to Figure 1D). Similar data also shown for MB_{Group3} (n=5) and MB_{Group4} (n=5) within our cohort dataset, as determined by real-time-PCR using Taqman probe (Applied Biosystems, ThermoScientific; Human, 58497; Hs00930871_g1) (related to Figure 1E). **Lower panel:** Time-course expression analysis of AA7.1 (100 μM) *versus* PBS treatment of MB_{Group3} D425-Med cells, for PRUNE-1, OTX2, SNAIL and PTEN. The analysis was performed using real-time-PCR with SYBR-green technology, and relative expression values are shown as $2^{-\Delta Ct}$ (related to Figure 5F).

Supplementary Information, Table S2. Mini-ontology analysis of the three independent gene signatures in MB. The analysis was performed using GO terms involved in neurogenesis or neuron differentiation (GO:0030182, GO:0048666, GO:0050767, GO:0022008) using three of four probes for PRUNE-1 (see Material and Methods). The analysis indicated *OTX2* as most likely to be statistically regulated in the high PRUNE-1 expression category in the main three gene-expression MB signatures (publically available: Kool, Gilbertson, Magic-Northcott). The other genes (*CYFIP1*, *GLI2*) were inversely correlated to PRUNE-1 expression (i.e., negative [–] scores). The *AMIGO1*

gene showed minimal up-regulation, as measured by fold-change values within these expression signatures. This analysis strengthens the overall correlation between PRUNE-1 and OTX2 mRNA expression in MB.

Supplementary Information, Table S4: Bioluminescence analysis from *in-vivo* mouse trials.

Upper panel (*in-vivo* trial with AdV-CPP): *In-vivo* bioluminescence analysis from cerebellum as the region of interest in nude mice (n = 6) implanted with MB_{Group3} cells (1 $\times 10^5$) stably expressing the firefly luciferase gene (D425-Luc cells), 24 h after infection *in vitro* with multiplicity of infection 100 of adenoviral particles type 5 carrying CPP (AdV-CPP), and empty viral particles (AdV-Mock) as control. Tumorigenesis was followed *in vivo* for up to 28 days after tumour implantation (related to Figure 4D).

Lower panel (*in-vivo* trial with AA7.1): As for upper panel, initially with mice (n = 12) implanted with D425-Luc MB_{Group3} cells (1 $\times 10^5$). Fourteen days after primary tumours were established (from implantation; day 0), the mice were grouped based on bioluminescence values, and treated *in vivo* with AA7.1 (60 mg/kg every 2 days), or with PBS as vehicle control, for 14 further days (related to Figure 5B).

Supplementary Information, Table S3. Gene expression correlation analyses between MYC family members (i.e., c-MYC, N-MYC) and PRUNE-1, TGFB1 and OTX2.

Gene expression correlation analysis for c-MYC and N-MYC relating to PRUNE-1, TGF-β1 (TGFB1) and OTX2 transcripts in MB samples from the Cavalli dataset (n=763 tumour samples).

Supplementary Information, Table S5. Haematological and biochemical analyses of AA7.1 toxicity *in vivo*.

Haematological and biochemical markers of hepatic and renal functions from Balb/c mice treated *in vivo* with increasing doses of AA7.1 (15 to 60 mg/kg) administered once daily for 1 week (toxicity study), and from nude xenograft mice implanted with D425-Luc and treated *in vivo* with AA7.1 (60 mg/kg every 2 days) (pre-clinical trial). Mice treated with PBS were used as vehicle controls. Data are expressed as means from three different mice. No significant differences were detected between mice treated with AA7.1 and PBS.

Supplemental Materials and Methods

Mini-gene-ontology analysis

The median expression value of PRUNE-1 (Probe-set identifiers in Magic–Northcott: 7905299; in Kool and Gilbertson: 209586) was used within the three gene-expression MB signatures publically available (Kool [GSE10327], Gilbertson [GSE37418], Magic–Northcott [GSE37382]). Evaluation was carried out for expression of genes associated with the Gene Ontology (GO) terms of 'neurogenesis' or 'neural development' available at MSigDB (software.broadinstitute.org/gsea/msigdb) within the three gene-expression datasets (GSE37382, GSE10327, GSE37418), using the MsigDB website (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi?&species=hs). GO:0022008 (NEUROGENESIS), GO:0050767 (REGULATION OF NEUROGENESIS), GO:0048666 (NEURON DEVELOPMENT) and GO:0030182 (NEURON DIFFERENTIATION) were used in the searches. In three out of the four GO terms, *OTX2* was significantly expressed with and co-regulated by *PRUNE-1*

(Supplementary Information, Table S2). These results followed statistical analysis with Bonferroni correction (Kim, 2015)

Cell culture procedures (human primary and immortalised MB cells, murine medullospheres)

The D425-Med and DAOY cell lines were grown in minimum essential medium (Euroclone, Milan, Italy), and the D341-Med and D283-Med cells were grown in Iscove's modified Dulbecco's medium (Euroclone, Milan, Italy). All of the experimental procedures were performed on adherent populations of D283-Med cells. UW288 and HEK-293T cells were grown in Dulbecco's modified Eagle's medium (Euroclone, Milan, Italy), and ONS76 cells were grown in RPMI (Euroclone, Milan, Italy). The primary MB cells derived from different MB subgroups ($pMB3_{MB\text{-}SHH}$, $pMB4_{MB\text{-}WNT}$, $pMB6_{MB\text{-}Group4}$, $pMB7_{MB\text{-}Group3}$) were grown in a 1:1 (v/v) mixture of minimum essential medium and Dulbecco's modified Eagle's medium. The primary medullospheres derived from patients ($pMB8_{MB\text{-}Group4}$, $pMB9_{MB\text{-}Group3}$) were cultured in in Neurobasal A (Gibco) augmented with B27 supplement (Gibco), 20 ng/mL epidermal growth factor (Sigma), 20 ng/mL fibroblast growth factor (Invitrogen), and 100 U/mL penicillin/streptomycin. The D341-Med cells were grown in medium supplemented with 20% FBS, and all of the other media were supplemented with 10% (v/v) FBS (Euroclone), 2 mM L-Glutamine (Euroclone), and 1% (v/v) antibiotics (10,000 U/mL penicillin, 10 mg/mL streptomycin, Euroclone). Cells were maintained at 37 °C at 5% CO_2 in a humidified atmosphere.

Viral infection, transient DNA transfection and AA7.1 treatment procedures

Lentiviral infection was performed to generate D425-Med cells that stably expressed the firefly luciferase gene (D425-Luc). In detail, D425-Med cells (2×10^4) were plated in

complete medium into 1 well of a 24-well plate for 24 h. The medium was then replaced with 500 μL fresh complete medium containing hexadimethrine bromide (or Polybrene; Sigma-Aldrich) to a final concentration of 4 μg/mL. The appropriate amount of lentiviral particles (MOI, 50) encoding the firefly luciferase gene (RediFect Red-Fluc-Puromycin; CLS960002, Perkin Elmer, Waltham, MA, USA) was added to the medium. After 3 days, the medium was replaced and puromycin dihydrochloride (A1113802, Thermo Fisher) was added to a final concentration of 0.5 μg/mL, to select only the transduced cells.

To generate the adenoviral particles carrying sh-RNA for PRUNE-1, a hairpin sequence in the expression cassette of the shuttle vector Ad5-pVQ-K-NpA was cloned (i.e., CCCAGTATCTGTCATGGAACTGTAGTGAAGCCACAGATGTACAGTTCCA TGACAGATACTGGT). The correct sequences were confirmed by automatic DNA sequencing. Virus generation and amplification were performed using ViraQuest (North Liberty, IA, USA).

To generate the adenoviral particles carrying the cell competitive permeable peptide (CPP), the NME1 region (amino acids 115-128; peptide sequence: NIIHGSDSVESAEK) was 5′-XhoI, 3′-HindIII synthesised downstream in-frame to the HA epitope sequence, and upstream of the TAT protein sequence, as previously described by Carotenuto et al. (2013). Briefly, the sequence coding CPP was directly cloned into the VQ Ad5CMV K-NpA shuttle vector (ViraQuest, North Liberty, IA, USA), which provided its recombination and the CPP adenovirus construct (AdV-CPP) at 10^{12} virus particles/mL. Infection with these recombinant virus particles was accomplished by exposing the cells at MOI 100, following the Viraquest protocols (available at: http: http://viraquest.com).

Briefly, the infection with recombinant viruses was accomplished by exposing the cells (Multiplicity of Infection, MOI, 100) to adenovirus in 500 μL complete cell culture medium

for 1 h, followed by addition of further media. For the assays on MB cells (D283-Med, D425-Med cells) MOI 100 was used with 72 h infection.

D283-Med and HEK-293T cells were transiently transfected with the plasmid DNA constructs (i.e., pGL4.14-4XSBE-CMV-luc2/Hygro [4× SBE, composed of the sequence 5'-CAGACA-3'], pCS-3XFLAG-Amino-Terminus-PRUNE-1 [1-333 amino-acid residues], pCS-3XFLAG-Full-Length-PRUNE-1 [1-453 amino-acid residues], pCMV5B-SMAD4-HA, pCMV5B-SMAD2-HA, pCDNA-SMAD3-FLAG, pcDNA-HA-NME1) using TransIT-LT1 Transfection Reagent (Mirus Bio LLC, #MIR2300) according to the manufacturer instructions.

For AA7.1 treatment, D283-Med, D425-Med, D341-Med, pMB6, pMB7, pMB8 and pMB9 were treated with 100 μM AA7.1 for 6 h (with AA7.1 replacement every 3 h).

Annexin V and propidium iodide labelling

D425-Med cells were plated and infected with empty adenoviral particles (AdV-Mock) and those carrying the sh-PRUNE-1 sequence (AdV-sh-PRUNE-1) at MOI 100, or were treated with AA7.1 (100 μM) and PBS (vehicle). After 48 h, Annexin V and propidium iodide staining were performed following. Briefly, the cells (5×10^5) were collected by centrifugation and incubated with Annexin V-FITC (51-65874X; BD Bioscience) and 5 μL propidium iodide solution (P4864; Sigma-Aldrich) for 15 min in the dark at room temperature. The analyses were performed using flow cytometry (excitation, 488 nm; emission, 350 nm) using FITC signal detector for Annexin V-FITC binding, and by the phycoerythrin emission signal detector for propidium iodide staining.

Radiation treatment

D425-Med cells were treated with 100 μM AA7.1 for 6 h (with AA7.1 replacement every 3 h) or PBS as vehicle (control). Then the irradiation was performed on these pre-treated D425-Med cells using a biological X-ray irradiator, at 160 kV, 25 mA and 0.3 mm Cu filter (RS-2000; X-ray Cell Irradiator, Rad Source Technologies). We applied single doses of 10 Gy at a dose rate of 1.2 Gy/min prior to the proliferation experiments performed with the XCELLingence system, as discussed before.

Cell proliferation with the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay

The proliferation assays were performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). The D283-Med and D425-Med cells (2×10^3/well) were plated into 96-well plates and infected with empty adenoviral particles (AdV-Mock) or adenoviral particles carrying the sh-PRUNE-1 sequence (AdV-sh-PRUNE-1) at MOI 100 for 72 h. Each experimental point was assessed in triplicate. For each experimental point, 20 μL (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) solution (1.90 mg/mL) was added to each well, and the cells were incubated for 24, 48 and 72 h at 37 °C. Absorbance was then measured at 490 nm using a microtitre plate reader (Victor3 1420 Multilabel Counter; Perkin Elmer). For each experimental point, the means of the absorbance and their standard deviation (SD) were calculated. A total of three independent sets of experiments were performed.

Antibodies

The following sources were used for the antibodies (dilution; Cat N°). Cell Signaling: anti-phospho-Ser423/425-Smad3 (1:500; 9520), anti-phospho-Ser467-Smad2 (1:1000; 3108); anti-PTEN (1:1000; 9552); anti-phospho-Ser473-Akt (1:500; 4060); anti-N-Cadherin (1:1000; 33-

3900); anti-Caspase-8 (1:1000, 9746); phospho-FADD (Ser194) antibody (human specific) (1:500, 2781). Abcam: anti-α-Tubulin (1:3000; Ab15246); anti-Smad2/3 (1:200; ab63672); anti-OTX2 (1:10000; Ab130238). Sigma: anti-β-Actin (1:10000; A5441); anti-Flag (1:5000; F3165). Roche: anti-HA (1:500; 1583816). Quiagen: anti-His (1:500; 34660). BD Transduction Laboratories: anti-E-Cadherin (1:500; 610181); Santa Cruz Biotechnology: anti-NME1 (1:500; sc-343); anti-Nestin (1:1000, sc-21247, SantaCruz). Dako: anti-glial fibrillary acidic protein (GFAP; 1:1000, z0334), anti-KI67 (1:1000, m7240). BioLegend: anti-purified_Tubulin-beta-3_TUBB3 TUJ1 (1:1000, MMS-435P). The following in-house antibodies were produced: rabbit polyclonal anti-PRUNE-1 (C45; 1:500); rabbit polyclonal anti-phospho-NME1 (pS120-pS122-pS125; 1:500) (Garzia et al., 2008).

Western blotting

The cells were washed in cold phosphate-buffered saline (PBS) and lysed in cell lysis buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 10% [v/v] glycerol, 1% [w/v] Na-deoxycholate, 1% [v/v] Triton X-100) supplemented with protease inhibitors (Roche, Basel, Switzerland). Cell lysates were cleared by centrifugation at 16,200× *g* for 10 min at room temperature, and the supernatants were removed and assayed for protein concentration using the Protein Assay Dye Reagent (Bio-Rad). Cell lysates (50 μg protein lysate) were separated using SDS-PAGE gels of different percentages, depending on the molecular weights of the proteins of interest. The proteins were then electrophoretically transferred to PVDF membranes (Millipore). After 1 h in blocking solution with 5% (w/v) dry milk fat in PBS, or 5% (w/v) bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (both of which contained 0.02% (v/v) Tween-20), the membranes were incubated with the primary antibody overnight at 4 °C, and then with the secondary antibodies for 1 h at room temperature. Primary and secondary mouse or rabbit horseradish-peroxidase-conjugated antibodies (NC

27606; ImmunoReagents, Inc) were diluted in 5% (w/v) bovine serum albumin in TBS-Tween or in 5% (w/v) milk fat in PBS-Tween, according to the manufacturer instructions. The protein bands were visualised with a chemiluminescence detection system (Pierce-Thermo Fisher Scientific Inc., IL, USA). Western blotting was performed in triplicate. The densitometry analysis was carried out using the ImageJ software programme. The peak areas of the bands were measured on the densitometry plots, and the percentages were calculated. Then, the density areas of the peaks were normalised with those of the loading controls, and the ratios for the corresponding controls (e.g., empty vector or AdV-Mock) are presented as fold-changes.

Immunohistochemistry methods on paraffin-embedded tissues

The immunohistochemistry methodologies were as presented by (Ellison et al., 2011). Paraffin sections (thickness, 3 μm) of the tumour specimens were deparaffinised in Bioclear (06-1782D; Bio-Optica) for 30 min, rehydrated in 100%, 90% then 70% ethanol, and washed with PBS and then PBS containing 0.02% Triton-X 100 (215680010; Acros Organics). After incubation in pre-warmed Target Retrieval Solution (S170084; Dako) at 97 °C for 45 min, the sections were washed with PBS and placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 15 min. The tissue sections were then blocked with Antibody Diluent Background Reducing (S302281; Dako) for 1 h at room temperature, and then incubated with the primary antibodies overnight at 4 °C in a humidified chamber. Tissue sections were washed in PBS and incubated with labelled streptavidin biotin LSAB mouse and rabbit reagents (K0672; Dako). Detection was with the Liquid DAB Substrate Chromogen System (K3468, Dako). All of the slides were counterstained with Gill's haematoxylin (Bio-Optica). The slides were then washed, dehydrated with 70%, 90% and then 100% ethanol, and mounted with cover slips using Eukitt (09-00250; Bio-Optica). Micrographs were taken with

a high definition digital microscope camera (ICC50 HD; Leica). The histopathological score grading followed the guidelines of (Louis et al., 2014).

Indirect immunofluorescence analyses

Immunofluorescence on cells

HEK-293T cells were plated and grown (1 $\times 10^3$ cells) on coverslips. FLAG-tagged-Full-Length-PRUNE-1, HA-tagged-SMAD2 and the empty vector expression plasmids were transfected using TransIT-LT1 Transfection Reagent (#MIR2300; Mirus Bio LLC). After 36 h of transfection, the cells were fixed in 4% paraformaldehyde, permeabilised for 10 min in phosphate buffer containing 0.1% Triton X-100, and incubated with a blocking solution containing 0.1% Triton X-100, 10% normal goat serum and the primary antibodies, as required. Anti-mouse Alexa Fluor 488 (ab150113; Abcam) and anti-rabbit Alexa Fluor 546 (#A10040; ThermoFisher) were used as the secondary antibodies. DNA was stained with DRAQ5 (#62254; ThermoFisher). Confocal microscopy was carried out using a laser scanning confocal microscope (LSM 510 META, Zeiss), with the 63× oil immersion objective.

Immunofluorescence on paraffin-embedded tissue sections

Paraffin sections of the tumour specimens (thickness, 3 μm) were deparaffinised and rehydrated by immersing the slides in Xylene Substitute (A5597; Sigma) (three washes for 5 min each), then serially in 100%, 95%, 70%, 50%, 30% ethanol (two washes for 10 min, for each), and deionised water (two washes for 5 min each). The washes were then followed by PBS, PBS containing 0.02% Triton-X 100 (215680010; Acros Organics), and PBS (two washes for 5 min, for each). For antigen retrieval, the slides were immersed in boiling 10 mM

sodium citrate buffer (pH 6.0) using a microwave oven, and then maintained at sub-boiling temperature for 10 min. The slides were left to cool at room temperature for 30 min. The sections were then washed by immersion in distilled water for 5 min. To block endogenous peroxidase activity, the tissue sections were placed in a solution with 3.0% hydrogen peroxide in methanol for 15 min. Then to decrease the non-specific background fluorescence, the tissues were digested by treating them with a solution containing 0.2% Trypsin (T2600000; Sigma-Aldrich) and 0.001% $CaCl_2$ for 10 min at 37 °C, using a humidified chamber. The slides were then washed in PBS, PBS containing 0.02% Triton-X 100 (215680010; Acros Organics), and PBS (two washes for 5 min, for each). The tissue sections were then blocked with 6% bovine serum albumin (A9418; Sigma), 5% FBS (ECS0180L; Euroclone), 20 mM $MgCl_2$ in PBS containing 0.02% Triton-X 100 for 1 h at room temperature, and incubated overnight with the primary antibodies at 4 °C in a humidified chamber. Tissue sections were washed in PBS and PBS containing 0.02% Triton-X100, and incubated with anti-mouse Alexa Fluor 488 (ab150113; Abcam), anti-rabbit Alexafluor 488 (150077; Abcam) and anti-rabbit Alexa Fluor 546 (#A10040; ThermoFisher), as the secondary antibodies. DNA was stained with DRAQ5 (#62254; ThermoFisher). The slides were then washed, dehydrated with 70%, 90% and 100% ethanol, and mounted with cover slips using 50% glycerol (G5150; Sigma-Aldrich). Confocal microscopy was carried out using a laser scanning confocal microscope (LSM 510 META, Zeiss), with the 63× oil immersion objective.

Fluorescent *in-situ* hybridisation

For the fluorescent *in-situ* hybridisation (FISH) analysis on paraffin-embedded tumours, the tissue sections (thickness, 4 μm) were deparaffinised in Bioclear (Bio-Optica) and rehydrated in 100% ethanol. After incubation in pre-warmed 10 mM sodium citrate, 2 mM EDTA at 80 °C for 45 min, the sections were washed in deionised H_2O and treated with pepsin (5 mg/mL,

in 0.9% NaCl) at 37 °C for 15 to 40 min. Then, the slides were washed in deionised H_2O, incubated in 10% neutral buffered formalin for 10 min, soaked in 2× saline-sodium citrate buffer, and dehydrated in 70%, 85% and then 100% ethanol.

For the fluorescent *in-situ* hybridisation analysis on primary cell cultures, 1×10^6 cells from each of the human MB cell lines were resuspended in 7 mL hypotonic solution (75 mM KCl, pre-warmed to 37 °C). Following incubation for 10 min at 37 °C, the cells were fixed in Carnoy's solution (methanol/ glacial acetic acid; 3:1 [v/v]) and centrifuged on microscope slides using a cyto-centrifugation (Shandon Cytospin centrifuge; ThermoScientific). The slides were baked overnight at 60 °C, rinsed in 2× saline-sodium citrate buffer, and pre-treated with pepsin (0.05 mg/mL in 10 mM HCl) for 6 min at 37 °C. The slides were then washed in PBS, fixed in 1% formaldehyde/ PBS/ $MgCl_2$, and dehydrated through a series of increasing ethanol concentrations.

The FISH procedures were performed on both 4-µm-thick tissue sections and on human MB cell lines, using the following probe: IGH (14q32)/ MYC (8q24)/ CEP 8 (8p11.1-q11.1) Tri-Color Dual Fusion (Abbott-Vysis). After applying 2 µL to 10 µL of the probe mix to the slides, co-denaturation of the target and probe DNA was performed (tissue, 10 min at 80 °C; cells, 5 min at 73°C) using the Hybrite Slide Stainer (Vysis), followed by hybridisation for 18 h at 37 °C. After hybridisation, the slides were washed to remove unbound DNA (according to the manufacturer instructions), counterstained with DAPI II (Vysis), and analysed using an epifluorescence microscope (BX-61; Olympus) equipped with a triple bandpass filter set (DAPI/ Green/ Orange), a dual bandpass filter set (Green/ Orange), single bandpass filters (DAPI, Green, Orange) and the Cytovision software. Hybridisation signals on not less than 100 intact, well-preserved, and non-overlapping nuclei were evaluated by two independent investigators.

In-vitro binding assay using affinity chromatography

The methodology followed for the *in-vitro* binding assay using affinity chromatography was as presented by (Carotenuto et al., 2013).

Chemical synthesis of AA7.1

Compound AA7.1 (AA7.1 hydrochloride; (R)-1-[4-(4-methoxy-benzylamino)-pyrimidin-2-yl]-pyrrolidin-3-ol hydrochloride; $C_{16}H_{21}ClN_4O_2$) was synthesised starting from 1.84 g (21.1 mmol) (R)-3-pyrrolidinol (Aldrich, Cat. N° 382981, Lot N° 101131354) added dropwise to a solution of 2-chloro-N-(4-methoxybenzyl)pyrimidin-4-amine (4.80 g, 19.2 mmol; HDH Pharma, Morrisville, NC, USA) in dry dimethylformamide (19 mL) at room temperature under nitrogen atmosphere. The orange solution was heated to 100 °C for 8 h, at which time, thin layer chromatography with dichloromethane:methanol (9:1; v/v) indicated little starting material remaining. The reaction was cooled to room temperature. One third of the reaction solution was diluted with about the same volume of water (*ca*. 6 mL) and loaded onto a RP C-18 column (Agela, 330 g). This was purified on a chromatography system (Isco Combiflash Companion flash) using a continuous gradient from 5% CH_3CN in H_2O with 0.1% trifluoroacetic acid, to 95% CH_3CN in H_2O with 0.1% trifluoroacetic acid. The fractions were examined by HPLC, and the appropriate fractions were concentrated *in vacuo*. This provided 1.9 g product as a light-brown solid. This material was combined with 1.6 g of a previous batch that had been prepared in a similar manner, and these were dissolved in 250 mL distilled water that was then acidified with 4 N aqueous HCl. This solution was lyophilised, to recover 2.2 g (R)-1-[4-(4-methoxy-benzylamino)-pyrimidin-2-yl]-pyrrolidin-3-ol as the HCl salt (IUPAC name; AA7.1 hydrochloride).

NMR spectroscopy

Nuclear magnetic resonance (NMR) experiments were recorded at 298 K on a Varian Inova 600 MHz spectrometer, equipped with a cold probe. For chemical-shift perturbation studies, 2D [15N, 1H] HSQC spectra were acquired with 1024 (HN) × 128 (N) data points and 8 scans. STD spectra were acquired using a series of equally spaced 50-ms Gaussian-shaped pulses for selective saturation, with a total saturation time of 2 s. WaterLOGSY experiments used a 20-ms selective Gaussian 180° pulse at the water signal frequency, and an NOE mixing time of 1 s.

Cerebellum orthotopic implantation of D425-LUC in xenograft nude mice

The methodology used for cell implantation in IV ventricle was similar to that presented by (Asadzadeh et al., 2017)**.** Briefly, to establish orthotopic intracerebellar D425-Luc xenograft models, 6-week-old nude mice were anaesthetised with Ketamine/ Xylazine (87.5 mg/kg, 12.5 mg/kg, respectively). The mice were then placed in a stereotactic frame by hooking their incisors onto the frame hold. A small skin incision (length, 1 mm) and a burr hole (diameter, 0.7 mm) were created using a microsurgical drill (Fine Science Tools, Foster City, CA, USA). D425-Luc cells (1×10^5) were resuspended in 5 μL PBS and injected slowly through the burr hole into the right cerebellar hemisphere (stereotactic coordinates from Bregma: anteroposterior, 5.5 mm; right lateral, 2.1 mm; dorsoventral 5.0 mm), with a steady force over 30 s, using a 10-AL, 26-gauge Hamilton Gastight model 1701RN syringe needle (Sigma-Aldrich) that was inserted perpendicular to the cranial surface. The incision was sutured using Safil polyglycolic acid synthetic absorbable suture (6/0). The mice were kept on a warming blanket after the surgery, to help them to maintain their body temperature. Their mobility and respiratory patterns were observed continuously, and once the mice had recovered from the anaesthesia, they were place in a sterile housing cage. The mice were monitored daily for development of potential neurological deficits. Seven days after the implantation of the cells,

the mice were imaged, and tumour growth was evaluated by bioluminescence acquisition using an imaging system (IVIS 3D Illumina; Xenogen/ Caliper). For the acquisitions, the mice were anaesthetised by inhalational of isofluorane, and the D-luciferin (122799; PerkinElmer) (15 mg/mL stock) was injected intraperitoneally (100 μL per 10 g body weight). At 5 min from the luciferin injection, the mice were imaged for 1.5 min. Several acquisitions were made per mouse until each mouse reached its peak of photon emission. Then five total acquisitions (weekly, over a total of 28 days of analysis) near to the peak value for each mouse were analysed. To quantify the bioluminescence, the integrated fluxes of photons (ph/s) within each area of interest were determined using the Living Images Software Package 3.2 (Xenogen-Perkin Elmer).

For AA7.1 *in-vivo* treatment, starting after 14 days from tumour implantation (i.e., once the tumours were established) the mice (grouped according to their bioluminescence values) were injected intraperitoneally with AA7.1 at 60 mg/kg every 2 days, or with PBS as the vehicle for the control group. After 28 days from tumour implantation (i.e., after two weeks of AA7.1 treatment), the mice were sacrificed due to their enhanced tumour burden, and the organs were collected for immunohistochemistry and immunofluorescence.

Tumour tissues and patient clinical features

In total, 44 children with MB were enrolled at Santobono Hospital in Naples, Italy (see Table 1), with their tumours sampled, and their disease progression and clinical features followed. All of the children diagnosed with MB who attended the Neurosurgery Unit at Santobono-Pausilipon Children's Hospital between January 2004 and December 2013 were enrolled. Clinical features of the patient are included, as of September 2017. The median age at diagnosis was 6.00 years (range, 0.42-15.33 years), with a male female ratio of 0.76. All of the diagnoses were confirmed by central neuropathology review at 'La Sapienza' University

of Rome. Eighteen patients were considered standard risk, with median age at diagnosis of 7.29 years (range, 3.58-15.33 years) and male to female ratio of 0.5. Twenty-six patients were considered high risk, with median age at diagnosis of 3.41 years (range, 0.42-12.00 years) and male to female ratio of 1.

Eighteen patients had metastatic disease at diagnosis. Surgical resection or biopsy was performed for each patient, which was designed to provide maximal safe resection, with all procedures carried out with parent and/or patient consent. Standard-risk patients were treated according to the PNET 4 protocol, with conventional radiotherapy up to 2008, and with reduced cranio-spinal prophylactic radiotherapy from 2009. High-risk patients were treated with pre-irradiation chemotherapy, radiotherapy (when feasible, according to age), and high-dose chemotherapy with autologous stem-cell transplantation.

Gadolinium-enhanced spinal and brain magnetic resonance imaging and lumbar cerebrospinal fluid cytology were used to evaluate the tumour extent preoperatively. After surgery, residual tumour was evaluated using brain magnetic resonance imaging. Patients were staged according to the Chang classification system (Harisiadis and Chang, 1977). Patients who were <3 years of age, metastatic at diagnosis, or with residual disease >1.5 cm^2 were considered eligible for high-risk protocols. Prospective tumour sample collection for biological research was initiated in 2006. Tumour samples were freshly frozen and stored at the CEINGE Research Centre (Naples, Italy).

RNA extraction, determination of RNA quality, cDNA synthesis, and quantitative real-time PCR.

Freshly frozen tissues were available for 36 patients (66.7%), and RNA was extracted for all of these tissues. Efficient disruption and homogenisation of the starting materials (approximately 50 mg per sample) was performed in RLT buffer (79216; Qiagen) using

TissueLyser, with stainless steel beads (mean diameter, 5 mm) for 2 min at 30 Hz. The following RNA extractions were performed in combination with QIAGEN RNeasyMini sample purification kits and following the manufacturer instructions (1053394, 79254, 1018013; Qiagen). To elute the purified RNA, the spin column membrane was incubated with 45 µL RNase-free water, and centrifuged for 1 min at 10,000× *g* at 4 °C. The RNA concentrations were measured using a Nanodrop 1000 instrument (Nanodrop), while the RNA quality and integrity were assessed using LabChip microfluidic technology nucleic-acid electrophoresis (Bio-Rad Experion). The same procedures were applied for RNA extraction from D283-Med cells previously infected with AdV-Mock or AdV-sh-Prune-1. Reverse transcription was carried out with iScript cDNA RT-PCR synthesis kits. The procedure followed the BioRad protocol. The cDNA preparation was through the cycling method, as follows: incubation of the complete reaction mix for 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, and hold at 4 °C. The cDNA was then stored at -20 °C until used for the real-time PCR. The reverse transcriptase products (cDNA) were amplified by quantitative real-time PCR using a real-time PCR system (7900; Applied Biosystems, Foster City, CA, USA). The relative expression of the target genes was determined using the $2^{-\Delta Ct}$ method. All of the data are presented as means ±standard error of two to three replicate experiments. PRUNE-1 expression was detected using a TaqMan probe (Human-58497- Hs00930871_g1; Applied Biosystems-ThermoScientific).

Molecular subgrouping procedures.

The primer sequences used for molecular subgrouping testing corresponded to the gene names described by (Northcott et al., 2012b): WNT: *DKK2-TNC-WIF*; SHH: *EYA1-SFRP1-HHIP*; Group 3: *IMPG2-GABRA5-NRL-NRPR3*; Group 4: *EOMES-KHDRBS2-UNCD5*. The *ACTB* gene (β-actin) was also included in the analyses set as a housekeeping gene, for biological

normalisation purposes. Gene expression data were analysed using SYBR Green PCR amplification assays on a real-time PCR system (7900HT; Applied Biosytems), using 10 ng cDNA per sample. The relative quantities for each gene of interest within each experiment were subjected to technical normalisation using the counts obtained for the housekeeping gene. The normalised data were log-2 transformed and then used as the input for class prediction analysis. The expression profiles for the same genes generated from four 'normal' cerebella (Garzia et al., 2009) in the same experiment were used to set the reference threshold of expression. The control normal cerebellum samples (n=13) were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders (University of Maryland, USA). A series of MB with known subgroup definition were used to validate the real-time PCR analysis (n = 14; previously determined by Affymetrix SNP6 arrays analysis), according to (Northcott et al., 2012a).

Oligonucleotide sequence (forward, reverse) of the primers used in this study, including those used to determine the molecular classification for the MB genetic groups.

h-EYA1	Forward	AGCCTATGCCACGTACCCAC
	Reverse	TCAGTCTTGATGCCTGCCC
h-TNC	Forward	CATCTGCAACGAGGGCTACA
	Reverse	TCCGTCACTTCTGTCACAACG
h-EOMES	Forward	TGATCATTACGAAACAGGGCAG
	Reverse	TGTAGTGGGCAGTGGGATTGA
h-WIF1	Forward	GACCTGTTTCTACCCTGGAAAATGT
	Reverse	GGTTGTGGGCATTTGCTGAT
h-DKK2	Forward	CGGAGAAAAAGAAGCGCTG
	Reverse	AACTGGGATACAGATGCCATTATTG
h-SFRP1	Forward	CCCAATGCCACCGAAGC
	Reverse	GATGGCCTCAGATTTCAACTCG
h-KHDRBS2	Forward	TCAGAATAGCTCCCACAGCTCC
	Reverse	TGAGAACACCTCGTCCAGGTG
h-HHIP	Forward	TGCTGCCTGCGGAGTGA
	Reverse	CCCACATTCTGTGTTGTTGGTAAC
h-NPR3	Forward	ATCTGGAAGACATCGTGCGC
	Reverse	TGCTCCGGATGGTGTCACT
h-NRL	Forward	ATGGCTACTACCCAGGGAGC

	Reverse	TTTAGCTCCCGCACAGACAT
h-IMPG2	Forward	GCTTTCACCACCAGCACCTT
	Reverse	TTTCCTTGTAGCCTGGTAACCC
h-UNC5D	Forward	CTTCTGCATTGACAGGTGGCT
	Reverse	TGGCAGAATTCAGGAGCAGG
h-GABRA5	Forward	AACTGGTTGGCATCTGTGAAAA
	Reverse	TCATGATCAAAAACCTCCTTCTCTTT
h-β-ACTIN	Forward	GACCCAGATCATGTTTGAGACCTT
	Reverse	CCAGAGGCGTACAGGGATAGC
h-PRUNE-1	Forward	CTGGCCAACTCACCCTCATC
	Reverse	TGCCTCCTCTAGGGCTGTGT
h-OTX2	Forward	GGCACTGAAAATCAACTTGCC
	Reverse	CACTTCCCGAGCTGGAGATG
h-SNAIL	Forward	CCCCAATCGGAAGCCTAACT
	Reverse	CGTAGGGCTGCTGGAAGGTA
h-PTEN	Forward	CAAGATGATGTTTGAAACTAT
	Reverse	CCTTTAGCTGGCAGACCACAA

Kaplan Meyer and COX regression analysis

The univariable distribution of the metric variables was described by the median and the range. The distributions of the event-tree survival (EFS) were calculated according to the Kaplan–Meier method. EFS was calculated from the date of diagnosis until an 'event' (i.e., the date of first progression, relapse, occurrence of secondary malignancy, death of any cause, or last contact for patients without an event). The MB_{Group3} and MB_{Group4} patients were stratified into two groups on the basis of their PRUNE-1 mRNA levels at diagnosis, as determined by real-time-PCR, using the median PRUNE-1 expression as the cut-off value, as follows: low PRUNE-1, bottom 50th percentile; high PRUNE-1, top 50th percentile. The expression values are reported in Table S1.

Next-generation sequencing (NGS)

Next-generation sequencing studies (NGS) were performed using whole exome sequencing methodologies (WES), to determine the mutational status of the primary cell lines of MB_{Group3} and MB_{Group4} (pMB7 and pMB6 respectively).

In more detail, 1×10^6 cells/sample were used to isolate total DNA using MasterPure DNA purification kits (Epicentre, Madison, WI, USA). Next, the exome libraries were enriched using Nextera Rapid Capture Expanded Exome kits (Illumina, San Diego, CA, USA). This system (50 ng genomic DNA/sample) allows the capture of a total target of about 62 Mb of genomic sequences, including 212,158 targeted exonic regions (as annotated in the RefSeq, CCDS, ENSEMBL, GENCODE databases), 5'-UTRs and 3'-UTRs (as annotated in RefSeq), and 77% of the predicted microRNA coding genes (miRBase). NGS reactions were carried out on an Illumina HiSeq 1500 instrument (Illumina, San Diego, CA, USA) in a single 100-cycle paired-end run, as defined in the Illumina protocol. This analysis using the mutation status in the coding region determined by exonic non-polymorphic variants was determined. More than 80 $\times10^6$ reads/sample were obtained, 96% of which were high-quality filtered reads. After the alignment on the human genome reference sequence (GRCh37/hg19), a mean of 70,460 variants/sample were identified. In detail, there were 76,924 total variants (71,326 single nucleotide variants; 5,598 small insertion/ deletions) in pMB6 cells, and 79,734 total variants (73,646 single nucleotide variants; 6,088 small insertion/ deletions) in pMB7 cells.

Discussion of the NGS studies

Using WES data, we found 1774 'coding non-synonymous homozygous variants' (NSVs) in common with metastatic $MB_{Group3/4}$ patients (pMB7, pMB6, respectively). To identify from among these NSVs those specific alterations with potential roles in metastatic MB dissemination, we verified the presence of NSVs deleterious for protein functions, and predicted loss of function mutations using *in-silico* 'sorting intolerant from tolerant' (SIFT), 'polymorphism phenotyping' (PolyPhen) bioinformatic tools, together with the ClinVar archives database (http://www.ncbi.nlm.nih.gov/clinvar/).

In all, 41 common variants were selected that were predicted to be 'deleterious', 'probably

damaging' and 'not benign' using SIFT, PolyPhen and ClinVar, respectively (prediction software available through public domains), to generate a protein interaction network via the Search Tool for the Retrieval of Interacting Genes/ Proteins (STRING) database, together with NME1 and PTEN, the functions of which were inhibited in metastatic MB due to PRUNE-1 overexpression. As shown in Figure 6E, using these 41 deleterious, NSVs common to pMB6 and pMB7 patient-derived MB cells provided a protein network with 68 nodes (combined score set to 0.4; representing proteins connected via 209 edges, by virtue of protein-protein associations). This approach allowed us to select only those NSVs (Table 2, n=23) that take part in this gene network (as shown in Figure 6E) which underlie the pro-metastatic genes where their loss-of-function mutations are responsible for metastatic dissemination, following the axis identified here (i.e., PRUNE1/ TGF-β/ OTX2/ PTEN). ThESE data represent the metastatic molecular networks that occur in metastatic $MB_{Group3/4}$. Further analyses using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database as a bioinformatic resource showed that the proteins identified in the network are involved in focal adhesion (pathway ID:5200), tight junctions (pathway ID:4530) and natural-killer-cell-mediated cytotoxicity (pathway ID:4650). This suggested that their loss-of function might be responsible for metastatic spread, due to loss of cell adhesion and de-regulation of the innate immune response involving natural killer cells (Lee et al., 2015). Moreover, the same proteins were also defined as having roles in regulation of cell migration (GO.0030334), of cell-cell or cell-matrix adhesion (GO.0045785, GO.0022407 and GO.0001952), of neuron death (GO.1901214) and of the cell cycle (GO.0051726) whereby the loss of these could mediate cell dissemination and MB progression. Interestingly, there were also proteins with roles in 'positive regulation of myeloid leukocyte differentiation' (GO.0002763, GO.0002761). This is of importance, because these new MB metastatic 'drivers' with loss-of-function mutations might also take part in the cross-talk between tumorigenic and immune

cells in the tumour microenvironment, as previously described to be of importance to tackle TGF-β signalling (Pickup et al., 2013).

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