Feasibility of using NF1-GRD and AAV for gene replacement therapy in NF1-associated tumors

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Abstract
Neurofibromatosis type 1, including the highly aggressive malignant peripheral nerve sheath tumors (MPNSTs), is featured by the loss of functional neurofibromin 1 (NF1) protein resulting from genetic alterations. A major function of NF1 is suppressing Ras activities, which is conveyed by an intrinsic GTPase-activating protein-related domain (GRD). In this study, we explored the feasibility of restoring Ras GTPase via exogenous expression of various GRD constructs, via gene delivery using a panel of adeno-associated virus (AAV) vectors in MPNST and human Schwann cells (HSCs). We demonstrated that several AAV serotypes achieved favorable transduction efficacies in those cells and a membrane-targeting GRD fused with an H-Ras C-terminal motif (C10) dramatically inhibited the Ras pathway and MPNST cells in a NF1-specific manner. Our results opened up a venue of gene replacement therapy in NF1-related tumors.

Introduction
The RASopathy neurofibromatosis 1 is an autosomal dominant hereditary cancer syndrome that affects ~1:3000 individuals [1, 2]. Neurofibromatosis 1 is caused by mutations of the neurofibromin 1 (NF1) tumor suppressor gene at 17q11.2, which encodes the GTPase-activating protein (GAP) NF1 that catalyzes the inactivation of Ras by accelerating GTP hydrolysis to GDP [3, 4]. In affected individuals, truncation or loss of NF1 results in constitutively activated Ras with subsequent activation of the RAF-MEK-ERK cascade. This Ras hyperactivation supports the frequent development of multiple benign tumors, as in plexiform and cutaneous neurofibromas, and less frequently malignancies.

A hallmark of neurofibromatosis 1 is the presence of benign plexiform neurofibromas (pNFs) in which biallelic-inactivating mutations in the NF1 gene in Schwann cells occur, thus providing a selective growth advantage to a normal Schwann cell [2, 5]. About 8–13% of pNFs undergo cancerous transformation into a malignant peripheral nerve sheath tumor (MPNST) through a course of molecular evolution during which accumulated genetic mutations in CDKN2A/B, TP53, and other genes, as well as epigenetic alternations affect the regulation of multiple cellular processes [2, 6]. This transformation evolves over many years and typically affects young adults with neurofibromatosis 1 in their 20s–30s, with MPNSTs representing the leading cause of mortality in this patient population [7, 8].

The treatment of MPNSTs has unfortunately been extremely challenging. To date, surgery is the only treatment modality proven to have survival benefit for MPNSTs. However, even when maximal surgery with wide surgical margins is feasible, these tumors are almost never curable.
and about 50% of patients will succumb to this condition within 5 years after initial diagnosis, reflecting the urgent need for new and more effective therapeutics for this cancer.

One very promising but largely unexplored concept in the treatment of NF1-related MPNSTs is gene therapy. Over the past years, delivery vectors based on recombinant adenovirus-associated virus (AAVs) have shown great promise and achieved clinically meaningful long-term gene expression leading to regulatory approval for some conditions such as hemophilia, biallelic RPE65 mutation-mediated inherited retinal dystrophy, and spinal muscular atrophy type I [10–12].

The recombinant AAV is a non-pathogenic, non-replicating parvovirus, because rep and cap genes are cloned in a trans-plasmid without inverted terminal repeats. To date, more than 100 natural occurring human and non-human primate AAV serotypes have been identified [13]. Unlike lentivirus (LV), it does not cause any human disease and has a reduced carcinogenicity, because it only rarely integrates into the genome of the host cell. It is capable of infecting both dividing and quiescent cells with a low host immune recognition. Notably, advancements in our understanding of AAV capsid structure have facilitated the rational design of AAV capsids to restrict or redirect viral tropism and transduction, and considerable progress in both AAV capsid library development and screening methodology has enabled directed evolution of AAV capsids, which will ultimately ensure that transgene expression is reproducible, robust, and occurs over an extended period [13–15].

Gene delivery vectors based on AAVs have a packaging capacity of up to ~4.7 kb at near wild-type (WT) titers and infectivity; beyond this size, packaging efficiency markedly decreases and genomes with 5′-truncations become encapsidated [14]. Although NF1 cDNA of 8.5 kb is too large for AAV vectors, the NF1-GAP-related domain (NF1-GRD), a ~1-kb small subunit of the gene, is presumably solely responsible for deactivating Ras activity [16, 17], thus making neurofibromatosis 1 uniquely suitable for AAV-based gene delivery.

In this study, we have screened a panel of available AAV vectors in MPNST and primary Schwann cells to profile transduction efficiencies of different serotypes, in order to reveal potential templates for future engineering of the AAV capsid. We also tested the inhibition of the Ras pathway in NF1-related MPNST cells using GRD optimized for membrane targeting.

Materials and methods

Tissue culture and cell lines

Human NF1-associated MPNST cell lines NF90.8 and ST88–14 were provided by Dr Michael Tainsky (Wayne University, Detroit, MI) and sNF96.2 was purchased from ATCC (Manassas, VA). NP90–8, ST88–14, sNF96.2, and STS26T MPNST cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (ATCC) supplemented with 10% fetal bovine serum (FBS) (Sigma) and penicillin/streptomycin (Gibco). These cell lines were not authenticated. Human Schwann cells (HSCs) isolated from spinal nerve were purchased from ScienCell Research Laboratories and maintained in DMEM media supplemented with 3% FBS, penicillin/streptomycin, and heregulin-1 (PeproTech) 20 ng/ml and 2 μM forskolin (Sigma). All cells were tested and found free of mycoplasma contamination.

Reagents and antibodies

The rabbit anti-phospho-Erk1/2 (p44/42 MAPK) (Thr202/ Tyr204) antibody (#9101, Lot 23) and anti-Erk1/2 (p44/42 MAPK) antibody (#9102, Lot 27) were purchased from Cell Signaling Technologies. Rabbit anti-NF1 antibody (A300–140A, Lot 3) was purchased from Bethyl Laboratories, mouse anti-hemagglutinin (HA) antibody (26183, Lot RJ241582) was purchased from Invitrogen, and anti-β-Actin (C-11, SC-1615HRP, Lot G3015) horseradish peroxidase antibody was purchased from Santa Cruz Biotech. Active Ras Detection Kit (#8821, antibody Lot 7) was purchased from Cell Signaling Technology. NucBlue Live Ready Probes was purchased from Invitrogen.

NF1-GRD constructs

Human NF1 transcript variant 2 (NM_000267.3) of 2818 amino acids (AAs) was used as the template sequence. The GRD sequence of NF1 (AA 1172–1538), as described before [18], was cloned with a C-terminal 2xHA tag into the LV pFUGW and pscAAV-MCS (Cell Biolabs, VPK-430) vectors. To create a membrane-targeting GRD construct (GRD-C10), a 2xHA sequence was fused to the N terminus of GRD and a sequence encoding the H-Ras C-terminal 10 AA (GCMSCKCVLS) containing the CAAX motif was attached in frame to the GRD C terminus.

Lentiviral production and transduction

Enhanced green fluorescent protein (EGFP) or GRD-2HA was cloned in pFUGW vector and the plasmid was transfected along with CMV∆R8.91 and pMD.G in 293T cells by Lipofectamine 2000 (Invitrogen). Virus was collected after 48 h and infected MPNST cells by incubating with 8 μg/ml polybrene (Sigma).

AAV plasmids

pscAAV was purchased from Cell Biolabs, Inc. We obtained 13 hybrid pAAV-Rep-Cap (pAAV-RC) vectors,
which encode the rep of AAV2 and variable cap genes of different serotypes. Among them, pAAV-RC2 was purchased from Stratagene, pAAV-RC3B, pAAV-RC4, pAAV-RC6, and pAAV-DJ were purchased from Cell Biolabs, and pAAV2/1, pAAV2/5 JC, pAAV2/7, pAAV2/8, pAAV9n, pAAV2/rh10, pAAV2/hu11, and pAAV2/rh32.33 were obtained from Penn Vector Core of the University of Pennsylvania. For AAV packaging, the rHelper plasmid (Stratagene) and AAVpro-293T cells (Clontech) were used.

**AAV production and purification**

pAAV-Rep-Cap, pscAAV, and rHelper plasmids in equal amount were transfected in AAVpro-293T cells by Lipofectamine 2000 in DMEM media supplemented with 10% FBS. After 3 days, cells were collected and AAVs were purified by AAVpro Purification Kit of Clontech following the manufacturer’s instruction. Viral titers were determined by AAVpro Titration Kit from Clontech using real-time PCR.

**AAV transduction and fluorescence microscopy**

In a flat-bottomed 96-well plate, 5000 of HSC or MPNST cells were plated in each well. AAV vectors with EGFP were incubated at a multiplicity of infection (MOI) 1000 or MOI 5000 in 60 μl in the regular growth media for 5 days. Medium was then changed to FreeStyle 293 Expression medium (Gibco) without Phenol Red added with NucBlue Live Cell Stain Ready Probes (Invitrogen), which stained the nuclei to blue fluorescence. The cells have reached complete confluence and the green fluorescence signals were quantified on a PerkinElmer VICTOR3 Multilabel Counter with a green fluorescence (485/535 nm) filter set. EGFP signals were subsequently examined on an immunofluorescence microscope. Three biological samples were measured in each experiment.

**Immunofluorescence staining**

The staining followed the procedure described before [19]. Cells were grown in medium on chamber slides (Nunc) and treated as indicated. Cells were washed with phosphate-buffered saline (PBS) and fixed for 10 min with 4% paraformaldehyde solution, and permeated with methanol for 2 min with three washes in PBS in between and after. The slides were first blocked by 10% goat serum in PBS for 1 h at room temperature (RT) and incubated with the first antibody, and subsequently with AlexaFluor 488 (Green) secondary antibody (Invitrogen) in 10% goat serum in PBS at RT. They were then washed three times in PBS in between and after. After staining, the slides were covered with mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and examined on a fluorescence microscope.

**Western blottings**

Cells were lysed in buffer as described previously [19, 20]. Briefly, cells were suspended in lysis buffer (10 mM Tris-HCl pH 7.5, 130 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM sodium phosphate pH 7.5, 10 mM sodium pyrophosphate pH 7.0, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail [Roche]) and kept on ice for 10 min before being centrifuged. Cell lysates were heated for 5 min with LDS Sample Buffer (Invitrogen) supplemented with 100 mM dithiothreitol (DTT) before loading onto a 4–12% NuPAGE Bis-Tris Gel (Invitrogen). After transfer to a polyvinylidene difluoride membrane (Bio-Rad), immunostaining was performed according to standard procedure. Signals were visualized by the Super Signal chemiluminescent system (Pierce, Rockford, IL). Experiments were repeated twice.

**Assay of active Ras**

Ras activities in MPNST cells were examined by the Active Ras Detection Kit (#8821, Cell Signaling Technology) following the manufacturer’s instruction. Briefly, the cell lysates were incubated with GST-Raf1-RBD and glutathione resin. After wash and centrifugation, the bound fractions of Ras were dissociated and denatured in SDS sample buffer with DTT and examined by anti-Ras western blotting. Pre-incubating the lysates with 0.1 mM GTPγS or 1 mM GDP before binding with GST-Raf1-RBD created positive or negative control, respectively. Experiments were repeated twice.

**Cell growth assay**

The viable cells were measured with Cell Counting Kit-8 (Dojindo Laboratories, Japan) containing WST-8 tetrazolium salt at 450 nm on a PerkinElmer VICTOR3 plate reader. Three biological samples were measured in each experiment.

**Flow cytometry**

**Analysis of EGFP-positive cells.** NF90–8 cells in six-well plates were incubated with AAV-DJ-EGFP at MOI 1000 or 5000 for 36 h. Cells were collected by trypsinization, counterstained by incubating with 28 mM DAPI for 5 min in the flow buffer (PBS with 4% FBS and 1 mM EDTA), washed, and analyzed by a BD FACS Aria Fusion at the
FITC channel. Control was untransfected cells. Three independent samples were measured and data were analyzed by two-tailed t-test.

Staining of GRD-2HA and GRD-C10-2HA transfected by AAV-DJ. NF90–8 cells in six-well plates were incubated with AAV-DJ control, AAV-DJ-GRD-2HA, or AAV-DJ-GRD-C10-2HA at MOI 5000 for 36 h. Cells were trypsinized, fixed by the IC fixation buffer (Invitrogen, #00–8222), permeabilized by the permeabilization buffer (Invitrogen, #00–8333), and incubated with anti-HA antibody conjugated with AlexaFluor 647 (R&D Systems, #IC6875R) at 5 µl/10⁶ cells in 100 µl permeabilization buffer for 45 min at RT. Cells were then washed twice in 2 ml permeabilization buffer, resuspended in 0.5 ml flow buffer, and analyzed by a BD FACSAria Fusion. Data were obtained from three independent samples and analyzed by two-tailed t-test.

Propidium iodide (PI) staining. Cells were plated in six-well plates and transduced with indicated AAVs. After 2 days, cells were trypsinized, washed, pelleted, and fixed with 70% ethanol at the final concentration on ice for 15 min. Cells were then centrifuged and incubated with 500 µl PI solution in PBS (50 µg/ml PI, 0.1 mg/ml RNase A, 0.05% Tritin X-100) at 37 °C for 40 min. Subsequently, cells were washed and resuspended in PBS, and analyzed with a BD FACSymphony A3 flow cytometer using the PE-Gr-A channel. Results were analyzed with FlowJo version 10.

Statistical analysis

The results are presented as a mean value ± SD. Data were analyzed by GraphPad Prism 5.0. The p-values were determined by a Mantel-Cox test. A p-value < 0.05 was accepted as statistically significant. Sample sizes were chosen to achieve statistical significance.

Results

NF1-GRD suppressed the RAS activity and the growth of MPNST cells

Expressing the GRD domain of NF1 could restore normal growth in NF1−/− hematopoietic cells, fibroblasts, and neural stem cells [16, 17]. Likewise, the effects of the GRD have also been demonstrated in MPNST cells [21, 22].

In this study, we first subcloned the GRD domain of human NF1 into the pFUGW LV vector with a C-terminal double HA tag (2HA) for sensitive in vitro and in vivo detection. Human MPNST cells NF90.8, sNF96.2, and ST88–14 were derived from patients with neurofibromatosis 1 and lacked the WT NF1 gene, whereas both STS26T cells derived from a sporadic MPNST and primary HSCs isolated from spinal nerve expressed functional NF1 [23]. Figure 1a confirmed the expression status of NF1 protein in these cells.

**Fig. 1** Overexpression of GRD suppressed MPNST cells. a NF1 expression in MPNST cells. Western blotting was performed with the lysates of NF1-related MPNST cells (NF90–8, sNF96.2, and ST88–14), NF1-unrelated MPNST STS26T, and human Schwann cells (HSCs). b Overexpression of NF1-GRD reduced Ras activities in MPNST cells. ST88–14 and NF90–8 were transduced with EGFP or GRD-2HA lentivirus, and cell lysates were subjected to pulldown assay with GST-Raf-RBD. Western blotting was performed with indicated antibodies. GTPγS and GDP represent the positive or negative control, respectively. c NF1-GRD suppressed the growth of MPNST cells. MPNST cells were transduced with EGFP or GRD-2HA via lentivirus in 96-well plates and viable cells were measured after 72 h. Three biological samples were measured in each date point and P-values were evaluated by two-tailed t-test.
As shown in Fig. 1b, the GRD-2HA construct was able to significantly reduce the aberrant Ras activity in ST88-14 and NF90.8 cells in the GST-Raf1-RBD pulldown assay, suggesting that the GAP domain of NF1 compliments the inability of these cells to inactivate RAS. The replacement of the NF1-GRD via lentiviral transduction significantly suppressed the growth of NF90.8, sNF96.2, and ST88-14 cells, compared with the EGFP construct as control (Fig. 1c).

Next, we compared the transduction efficacy of a panel of commercially available AAV vectors in MPNST and primary HSCs by expressing EGFP and quantifying the green fluorescent signals. We used 13 hybrid pAAV-Rep-Cap (pAAV-RC) vectors, which encode the rep of AAV2 and variable cap genes of different serotypes, including AAV1 (pAAV2/1), AAV2 (pAAV2-RC), AAV3B (pAAV-RC3B), AAV4 (pAAV-RC4), AAV5 (pAAV2/5 JC), AAV6 (pAAV-RC6), AAV7 (pAAV2/7), AAV8 (pAAV2/8), AAV9 (pAAV9n), AAV10 (pAAV2/rh10), AAV11 (pAAV2/hu11), and AAV32/33 (pAAV2/rh32.33), as well as the synthetic AAV-DJ (pAAV-DJ) [24–28], along with the pHelper and self-complimentary pscAAV (pscAAV-MCS) expression vector to enhance the transduction [29].

The pscAAV vector uses the cytomegalovirus promoter to express the transgene. After production, purification and quantification of AAVs, 5000 ST88-14, NF90-8, or sNF96.2 cells were plated in 96-well plates and incubated with indicated AAVs at MOI 1000 or 5000. Green fluorescent signals were measured after 5 days. Cells were allowed to grow 5 days to reach complete confluence as examined optically by microscope. Green fluorescent signals in each well were quantified by a plate reader, which reflected the expression levels of EGFP in the transduced cells. At an MOI of 5000, AAV2, 3B, and DJ demonstrated superior transduction of EGFP in ST88-14 cells, whereas in NF90-8 cells, AAV1, 2, 3B, and DJ showed significant transduction in contrast to other AAVs (Fig. 2a, c). Cells incubated at MOI of 1000 followed a similar trend. The results were consistent with the observation of the fluorescence microscopy, where all the cells were photographed and either selectively shown in Fig. 2b, d or not shown. In sNF96.2 cells, AAV1, 2, 3B, and DJ again stood out both at
Using flow cytometry, we measured in average 98.7% and 99.1% NF90–8 cells were positive of EGFP at MOI 1000 and 5000 of AAV-DJ-EGFP, respectively, with higher expression levels observed at MOI 5000 (Supplementary Fig. 1). It has been established that NF1-related MPNSTs originate from NF1-haploid Schwann cells [2, 30]. Restoring NF1 function in those NF1-haploid Schwann cells via gene replacement therapy could thus also benefit patients before the malignant transformation occurs and possibly prevent this often fatal complication. To test this, we transduced primary HSCs that are positive of S-100 staining (Fig. 3c) with 13 AAV vectors encoding EGFP and found AAV 1, 2, 6, and DJ delivered EGFP most efficaciously (Fig. 3d, e).

Next, we tested GRD constructs with AAV-DJ, one of the consistently efficacious AAV vectors among MPNST and HSCs. As R as proteins are attached to the cellular membrane through prenylation and palmitoylation of cysteine residues including a CAAX motif located in the C-terminal hypervariable region (HVR) that confers targeting to the plasma membrane [31–33], we created a GRD construct fused with the 10 AAs of H-Ras C terminus (C10) containing the palmitoylation sites and CAAX motif, with a double HA tag at the N terminus (called ‘GRD-C10’). When transduced by AAV-DJ in NF90–8 cells, GRD-C10 drastically outperformed GRD in suppressing the phosphorylation of Erk1/2 (pErk1/2) in that no pErk1/2 signal was detected with GRD-C10 compared to a reduced, but still detectable, pErk1/2 signal with GRD, despite a relatively low GRD-C10 expression level in those cells (Fig. 4a).

Using intracellular anti-HA staining and flow cytometry, we found that on average 13.2% and 9.8% of NF90–8 cells were stained positive 36 h after transfected by AAV-DJ-GRD and AAV-DJ-GRD-C10 at MOI 5000, respectively (Supplementary Fig. 2). Immuno-fluorescence staining of HA demonstrated the membrane-targeting of GRD-C10.
The major challenge facing therapies using AAVs is the poor efficacy in delivery, to which many efforts have been directed in modifying and optimizing the AAV *cap* gene [34]. For example, aiming to improve targeting and reduce anti-AAV immunity, recombinant AAV-DJ was created via DNA shuffling and immunoselection from *cap* genes of eight natural serotypes and markedly outperformed all the parental AAV vectors in vitro [28]. Further engineering of AAV-DJ resulted in drastically improved delivery in various mice tissues [28, 35]. In this study, we analyzed the transduction efficacies of recombinant vectors distinct from each other only on capsids of 12 natural serotypes of human and monkey origins, and the synthetic AAV-DJ in various MPNSTs and HSCs. The consensual AAV1, 2, 3B, and DJ, as well as AAV6, which transduced HSCs and MPNST cell line NF90–8 particularly efficiently, can be used for further in vivo testing and should be included as templates in future engineering efforts to improve targeting of MPNSTs and HSCs.

Although AAV has not been tested with MPNST cells in prior publications, Hoyng et al. [36] reported the transduction of primary HSCs and rat Schwann cells with AAV serotypes 1–9 carrying GFP in culture and in nerve segments. In that study, AAV2 and AAV6 outperformed other vectors in cultured HSC, which is largely consistent with our finding in Fig. 3d.

Discussion

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**Fig. 4** Suppression of MPNST cells by GRD and GRD-C10 transduced by AAV-DJ. a Transduction of GRD-2HA and 2HA-GRD-C10 in NF90–8 cells by AAV-DJ. NF90–8 cells were transduced at MOI 5000 by AAV-DJ carrying EGFP, GRD-2HA, or 2HA-GRD-C10. Anti-2HA western blotting showed the expression of GRD-2HA and 2HA-GRD-C10, and anti-pERK1/2 blotting demonstrated the complete inhibition of ERK1/2 (p42/44) phosphorylation by GRD-C10. Anti-HA western blotting showed the expression of GRD-2HA or 2HA-GRD-C10 constructs. Anti-β-Actin was used as control of housekeeping gene. b Anti-HA immunofluorescence images of NF90–8 cells transduced with GRD-2HA or 2HA-GRD-C10 by AAV-DJ. Nuclei were stained by DAPI in blue. c–e Inhibition of MPNST cells by GRD and GRD-C10 transduced by AAV-DJ. NF90–8, ST88–14, and sNF96.2 cells were plated in 96-well plates and incubated with AAV-DJ carrying EGFP, GRD-2HA, or 2HA-GRD-C10 constructs at MOI 5000. After 4 days, viable cells were measured. Three biological samples were measured in each data point and *P*-values were evaluated by two-tailed *t*-test. f, g NF90–8 and ST88–14 cells were transduced by control AAV-DJ or AAV-DJ-2HA-GRD-C10 at MOI 5000. After 2 days, cells were collected, stained by propidium iodide, and analyzed by flow cytometry (*n* = 3). Fractions were calculated and *P*-values were evaluated by two-tailed *t*-test.
NF1 is a large protein with various domains of complex functionalities, including cysteine-serine-rich domain, tubulin-binding domain, GRD, Sec14, PH-like domain, and FAK-binding region [37]. A GRD version of 333 AAs (NF1–333, AA 1198–1530) has been extensively investigated, which revealed a central minimal GAP domain of 230 AA (AA 1248–1477) and flanking extra regions that also appeared to mediate important GAP-related functions and interactions [37–39]. In physiological conditions, NF1 protein is recruited to the plasma membrane by interacting with Spred1, which has been mapped between Spred1 EVH1 domain and GRD’s N-terminal (AA 1202–1217) and C-terminal (AA 1511–1530) extra regions [40–42]. In this study, we used a slightly larger GRD sequence (AA 1172–1538) in our construct with a double HA tag [18]. This construct presented a diffused subcellular distribution in MPNST cells upon viral transduction. The C-terminal HVRs of Ras proteins with the CAAX motif contain cysteine residues subject to prenylation and palmitoylation and confer the targeting to the plasma membrane. For example, the C-terminal 11 AA, 10 AA, and 20 AA of respective N-Ras, H-Ras, and K-Ras4B have been shown to re-target an exogenously expressed protein to the plasma membrane [31, 33]. In order to improve the targeting on membrane-associated Ras proteins, GRD was fused with an H-Ras C10 sequence containing cysteine 181 and 184, as well as the CAAX motif. This GRD-C10 construct drastically enhanced GRD’s potency in suppressing pErk1/2 and the growth of NF1-related MPNST cells with remarkable specificity while NF1-unrelated MPNST cells and HSC remained unaffected. Thus, given its potency and specificity, GRD-C10 would be therapeutically well suited as a treatment agent in patients with NF1-related MPNSTs and also as a tumor preventative in NF1-haploid individuals in whom tumors have not developed yet. In such a therapy, a noticeable efficacy would entail almost all of the MPNST cells or the majority of the NF1-haploid Schwann cells to receive the GRD-C10 transgene, since the non-transduced populations exhibit a clear growth advantage. With the current available tools for in vivo gene delivery, this would be only feasible with a drastically improved AAV vector for MPNST or Schwann cells, which could be achieved through protein engineering of the AAV capsids. We further reason that a gene replacement therapy may have an especially good chance of success as a preventative measure before the malignant transformation to MPNST occurs by repeatedly targeting NF1-haploid Schwann cells that grow less aggressively than MPNST cells. Alternatively, approaches to upregulate the expression of NF1 on the intact allele in NF1-haploid cells can also be considered.
e.g., via techniques with specific recognition such as CRISPR-dCas9-VPR, where the nuclease-null Cas9 is fused with a tripartite activator, VP64-p65-Rta (VPR) [43]. However, although the latter approach could produce more copies of the full-length NFI, it will also face acute limitations in the cloning capacity and transduction efficiency of AAV vectors.

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References
1. Carey JC, Baty BJ, Johnson JP, Morrison T, Skolnick M, Kivlin J. The genetic aspects of neurofibromatosis. Ann N Y Acad Sci. 1986;486:45–56.
2. Staedtke V, Bai RY, Blakeley JO. Cancer of the peripheral nerve in neurofibromatosis type 1. Neurotherapeutics. 2017;14:298–306.
3. Abramowicz A, Gos M. Neurofibromin in neurofibromatosis type 1 - mutations in NF1 gene as a cause of disease. Dev Period Med. 2014;18:297–306.
4. Ratner N, Miller SJ. A RASopathy gene commonly mutated in cancer: the neurofibromatosis type 1 tumour suppressor. Nat Rev Cancer. 2015;15:290–301.
5. Mautner VF, Asauagbor FA, Dombi E, Funsterer C, Kluwe L, Wenzel R, et al. Assessment of benign tumor burden by whole-body MRI in patients with neurofibromatosis 1. Neuro Oncol. 2008;10:593–8.
6. De Raedt T, Beert E, Pasmant E, Luscan A, Brems H, Ortonne N, et al. PKR2 loss amplifies Ras-driven transcription and confers sensitivity to BRD4-based therapies. Nature. 2014;514:247–51.
7. Rodriguez FJ, Folpe AL, Giannini C, Perry A. Pathology of peripheral nerve sheath tumors: diagnostic overview and update on selected diagnostic problems. Acta Neuropathol. 2012;123:295–319.
8. Sun D, Tainsky MA, Haddad R. Oncogene mutation survey in NF1 associated pro-survival and malignancy. Transl Oncogenomics. 2012;5:1–7.
9. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci USA. 2002;99:11854–9.
10. Cearley CN, Wolfe JH. Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. Mol Ther. 2006;13:528–37.
11. Cearley CN, Vandenbergh LH, Parente MK, Carnish ER, Wilson JM, Wolfe JH. Expanded repertoire of AAV vector serotypes mediate unique patterns of transduction in mouse brain. Mol Ther. 2008;16:1710–8.
12. Deverman BE, Ravina BM, Bankiewicz KS, Paul SM, Sah DWY. Gene therapy for neurological disorders: progress and prospects. Nat Rev Drug Discov. 2018;17:641–59.
13. Lisowski L, Tay SS, Alexander IE. Adeno-associated virus serotypes for gene therapeutics. Curr Opin Pharmacol. 2015;24:59–67.
14. Kotterman MA, Schaffer DV. Engineering adeno-associated viruses for clinical gene therapy. Nat Rev Genet. 2014;15:445–51.
15. Naldini L. Gene therapy returns to centre stage. Nature. 2015;526:351–60.
16. Hiatt KK, Ingram DA, Zhang Y, Bollag G, Clapp DW. Neurofibromin GTPase-activating protein-related domains restore normal growth in NFI-/-cells. J Biol Chem. 2001;276:7240–5.
17. Dasgupta B, Gutmann DH. Neurofibromin regulates neural stem cell proliferation, survival, and astroglial differentiation in vitro and in vivo. J Neurosci. 2005;25:5584–94.
18. Morcos P, Thapar N, Tusneem N, Stacey D, Tamanai F. Identification of neurofibromin mutants that exhibit allele specificity or increased Ras affinity resulting in suppression of activated ras alleles. Mol Cell Biol. 1996;16:2496–503.
19. Bai RY, Staedtke V, Aphyrs CM, Gallia GL, Riggins GJ. Antiparasitic mebendazole shows survival benefit in 2 preclinical models of glioblastoma multiforme. Neuro Oncol. 2011;13:974–82.
20. Bai RY, Dieter P, Peschel C, Morris SW, Duyster J. Nucleophosmin-anaplastic lymphoma kinase of large-cell ana-plastic lymphoma is a constitutively active tyrosine kinase that utilizes phospholipase C-gamma to mediate its mitogenicity. Mol Cell Biol. 1998;18:6951–61.
21. Bodempudi V, Yamoutoor F, Pan W, Dudek AZ, Esfandyari T, Piedra M, et al. Ral overactivation in malignant peripheral nerve sheath tumors. Mol Cell Biol. 2009:29:3964–74.
22. Reuss DE, Mucha J, Hagenlocher C, Ehemann V, Kluwe L, Mautner V, et al. Sensitivity of malignant peripheral nerve sheath tumors to TRAIL is augmented by loss of NF1 through modulation of MYC/MAD and is potentiated by curcumin through induction of ROS. PLoS ONE. 2013;8:e57152.
23. Sun D, Tainsky MA, Haddad R. Oncogene mutation survey in MPNST cell lines enhances the dominant role of hyperactive Ras in NF1 associated pro-survival and malignancy. Transl Oncogenomics. 2012;5:1–7.
24. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci USA. 2002;99:11854–9.
31. Hancock JF, Cadwallader K, Paterson H, Marshall CJ. A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. EMBO J. 1991;10:4033–9.
32. Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in human disease. Cell. 2017;170:17–33.
33. Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, et al. Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. Cell. 1999;98:69–80.
34. Weinmann J, Grimm D. Next-generation AAV vectors for clinical use: an ever-accelerating race. Virus Genes. 2017;53:707–13.
35. Mao Y, Wang X, Yan R, Hu W, Li A, Wang S, et al. Single point mutation in adeno-associated viral vectors -DJ capsid leads to improvement for gene delivery in vivo. BMC Biotechnol. 2016;16:1.
36. Hoyng SA, De Winter F, Gnavi S, van Egmond L, Attwell CL, Tannemaat MR, et al. Gene delivery to rat and human Schwann cells and nerve segments: a comparison of AAV 1-9 and lentiviral vectors. Gene Ther. 2015;22:767–80.
37. Scheffzek K, Shivalingaiah G. Ras-Specific GTPase-Activating Proteins: Structures, Mechanisms, and Interactions. Cold Spring Harb Perspect Med. 2018:9:a031500.
38. Ahmadian MR, Wiesmuller L, Laibahn A, Bischoff FR, Wittinghofer A. Structural differences in the minimal catalytic domains of the GTPase-activating proteins p120GAP and neurofibromin. J Biol Chem. 1996;271:16409–15.
39. Scheffzek K, Ahmadian MR, Wiesmuller L, Kabsch W, Stege P, Schmitz F, et al. Structural analysis of the GAP-related domain from neurofibromin and its implications. EMBO J. 1998;17:4313–27.
40. Stowe IB, Mercado EL, Stowe TR, Bell EL, Oses-Prieto JA, Hernandez H, et al. A shared molecular mechanism underlies the human rasopathies Legius syndrome and Neurofibromatosis-1. Genes Dev. 2012;26:1421–6.
41. Hirata Y, Brems H, Suzuki M, Kanamori M, Okada M, Morita R, et al. Interaction between a domain of the negative regulator of the Ras-ERK pathway, SPRED1 protein, and the GTPase-activating protein-related domain of Neurofibromin is implicated in Legius Syndrome and neurofibromatosis type 1. J Biol Chem. 2016;291:3124–34.
42. Dunzendorfer-Matt T, Mercado EL, Maly K, McCormick F, Scheffzek K. The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation. Proc Natl Acad Sci USA. 2016;113:7497–502.
43. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, PRI E, et al. Highly efficient Cas9-mediated transcriptional programming. Nat Methods. 2015;12:326–8.