Exon Skipping as a Therapeutic for Neurofibromatosis Type I

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Keywords: neurofibromatosis type 1, exon skipping, exon 17

Posted Date: July 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-751331/v1>

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Exon Skipping as a Therapeutic for Neurofibromatosis Type I

**Running Title: Targeted exon skipping of *NF1* exon 17 as a Therapeutic for
Neurofibromatosis Type I**

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ABSTRACT:

We investigated the feasibility of utilizing an exon skipping approach as a genotype-dependent therapeutic for neurofibromatosis type 1 (NF1) by determining which *NF1* exons might be skipped while maintaining neurofibromin function. Human neurofibromin is well-known as a GTPase activating protein (GAP), but outside of its GAP-related domain (GRD), it is unclear how critical other regions are for function. Initial *in silico* analysis predicted exons that can be skipped with minimal loss of neurofibromin function. Utilizing an *Nf1* cDNA expression system, we performed a functional screen to determine the effects of exon skipping on *in vitro* neurofibromin expression and GRD function. Mutant neurofibromin with induced loss of single exons 12, 17, 25, 41, 47, or 52 maintained significant GRD function in at least two Ras activity assays. Exons 18/19, 20 and 28 are critical for GRD function; deletion of exons 20, 41, or 47 led to significantly lower levels of neurofibromin. As predicted by *in silico* analysis, skipping of exons 17 or 52 resulted in both the highest neurofibromin levels and the greatest suppression of Ras activity. Assessment of NF1 patient databases indicates that pathogenic variants resulting in deletion or skipping of exons 17, 25, and 52 have not been reported; and truncating pathogenic variants in each exon account for ~0.91, 0.94, and 0.25% of unrelated NF1 cases, respectively. We designed antisense phosphodiamitrate morpholino oligos (PMOs) to skip exon 17 and evaluated effects in human cell lines engineered with a patient-specific truncating pathogenic variant, c.1885G>A. We down-selected oligos that efficiently caused skipping of exon 17 and restored NF1 expression and function. Further, homozygous deletion of exon 17 in a novel mouse model is compatible with viable and grossly healthy animals with normal lifespan and no tumor development, providing proof-of-concept that exon 17 is not essential for murine neurofibromin function. Mild phenotypes observed include abnormal nesting behavior and lymphoid hyperplasia with increased numbers of both B- and T-cells. Hence, exon skipping should be further investigated as a therapeutic approach for NF1 patients with pathogenic variants in exon 17.

Author Summary: Advances in biotechnology have led to the development of various pathogenic variant-directed therapies. Among those, exon skipping has been applied to several diseases. The ability of antisense oligonucleotides (ASOs) to alter pre-mRNA splicing has been exploited to correct both gain-of-function and loss-of-function genetic variants that cause disease. The most advanced exon skipping therapies has been developed for the severe muscle wasting disease Duchenne muscular dystrophy (DMD), where ASOs targeting dystrophin exon 51 and exon 53 have been conditionally approved by the FDA. It is currently unknown if a similar approach would be therapeutic for patients with NF1. We evaluated (*in silico*, *in vitro*, and *in vivo*), which neurofibromin exons might be skipped while still maintaining protein reading frame and function. We developed a cDNA screen to evaluate various neurofibromin constructs for neurofibromin abundance and ability to inhibit Ras signalling. We designed ASOs to skip exon 17 and evaluated them in human cell lines that we generated via CRISPR/Cas9 with a patient-specific truncating pathogenic variant (c.1885G>A) in this exon. Our results show that ASOs targeting exon 17 restored both NF1 expression and function. We also developed a mouse that is homozygous for loss of exon 17 that is both viable and fertile with normal lifespan and no tumor development. Hence, we have developed the tools for comprehensively assaying neurofibromin exons and have provided proof-of-concept that we can skip specific exons and maintain function. Assessment of ASOs *in vivo* will require development of novel precision mouse models and delivery methods specific to affected tissues.

INTRODUCTION:

The *NF1* gene spans more than 282,000 bases of genomic DNA and encodes neurofibromin, a large, multidomain protein (1). Individuals with pathogenic variants of *NF1* develop neurofibromatosis type I (NF1). Loss of function or lack of NF1 protein leads to a phenotype affecting the skin (café-au-lait macules, axillary freckling, hyperpigmentation,

cutaneous neurofibromas), the eye (Lisch nodules and optic glioma), skeleton (dysplasias and scoliosis) and peripheral and central nervous system (PNS and CNS) (cognitive disabilities, motor delays, gliomas, neurofibromas). Malignant peripheral nerve sheath tumors (MPNST) may also develop, with poor prognosis. Mutation analysis has been available for diagnostic purposes for the past 20 years. Almost 2,900 pathogenic variants have been reported in the Human Gene Mutation Database (<http://www.hgmd.org>). While there are few hotspots, as pathogenic variants are found all along the gene, most lead to lack of protein expression due to gene deletion, premature stop gain, frameshift, or abnormal splicing.

Despite its high prevalence (occurring in approximately 1 of 2000-3000 births (2, 3)), there are few effective therapeutics for NF1. Most currently available drugs being tested to treat NF1 are targeted at tumors and have focused on blocking Ras signaling or interfering with intercellular communication. MEK inhibitors such as selumetinib have demonstrated effectiveness for patients that respond and can tolerate treatment; however, not all patients benefit, plexiform neurofibromas do not completely disappear, and there can be significant side effects (4). Additional treatments that can be used alone or in conjunction with MEK inhibitors are therefore needed. One possible therapeutic class includes exon skipping, which utilizes specific ASOs to bind the target pre-mRNA through base pairing in a way that induces altered RNA splicing, causing the splicing machinery to “skip” one or more exons carrying a pathological variant. The resulting mRNAs are then translated into shortened proteins that – in the case of a successful therapy – are able to compensate the loss of critical function as a consequence of the genetic change. Antisense directed gene therapy for exon skipping has been successfully tested for the treatment of a number of diseases (5), most notably Duchenne muscular dystrophy (DMD)(6). The FDA has approved two exon skipping therapies for DMD, Eteplirsen (brand name Exondys 51), for ASO-based dystrophin exon 51 skipping (7, 8) as well as Golodirsen (Vyondys 53) for ASO-based dystrophin exon 53 skipping (9).

To evaluate the feasibility and potential utility of exon skipping as a therapeutic for NF1, we must first determine which regions, if any, can be removed or skipped while retaining crucial function of the neurofibromin protein. We evaluated *NF1* *in silico*, *in vitro*, and *in vivo* to test the effects of deletion/skipping of specific exons on neurofibromin function. *In silico* characterization, including prioritization of exons for which there have been no reports of NF1 patients with a given exon deletion, allowed us to focus on a set of exons to test *in vitro*. We evaluated cDNAs modeling specific exon deletions for neurofibromin levels and Ras activity through GTP-Ras levels, pERK/ERK ratios, and ELK1 transcriptional activity in neurofibromin null HEK293 cells. As suggested by our *in silico* analysis and confirmed by our *in vitro* data, skipping of exons 17 or 52 results in both the highest neurofibromin levels and the most suppression of Ras activity. Assessment of the UAB Medical Genomic Laboratory's NF1 patient database indicates that truncating variants within exons 17 and 52 predispose to a classic progressive phenotype. Pathogenic variants in exon 17 and 52 occur in ~0.91% and ~0.25% of unrelated NF1 patients, respectively. We designed ASOs to skip exon 17 and evaluated them in WT and mutant human cell lines. We down-selected oligos that are most efficient at skipping and restoration of neurofibromin expression and function. We further tested the effects of deletion of exon 17 (DelE17) *in vivo* in a nullizygous mouse model to show that this exon is not required for at least partial neurofibromin function. DelE17 results in a viable adult mouse with normal life span and no tumors. Collectively, our data provides a proof-of-concept that exon 17 is not essential for neurofibromin function and may be targeted for exon skipping therapeutics.

RESULTS

***In silico* analysis and prioritization of exons:** Figure 1 represents *NF1* exons and various protein domains, including the GAP-related domain (GRD) encoded by exons 27-35. Initial evaluation of the *NF1* transcript identified 25 single and an additional 18 consecutive exon pairs

that could be skipped while maintaining the translational reading frame (covering 43 of 58 exons). This represents a significant portion (74%) of the transcript that is potentially available for exon skipping therapeutics.

We searched the literature and publicly available datasets (LOVD and HGMD) for reports of NF1 patients with identified pathogenic variants that produced exon skipping or deleted exons in the mature mRNA. Analysis indicates that 49 out of the 58 individual exons can be found deleted or skipped in patient transcripts. From those that can be deleted while maintaining the reading frame, only four exons did not appear in our search, namely exons 17, 25, 31 and 52. As exon 31 also lacks pathogenic variants (none have been reported in databases), we excluded this exon from further analysis (denoted by black boxes in Figure 1), as it would not be a therapeutic target for exon skipping. Exon 31 is alternatively spliced and is present in isoform 2 but not in isoform 1. The remaining exons (17, 25, and 52) were prioritized for *in vitro* analysis. Of the consecutive exon pairs that might be skipped in combination and retain reading frame (6/7, 7/8, 15/16, 18/19, 29/30, 37/38, 42/43, 44/45, 50/51, 56/57) only 6/7 skipping/deletion has been reported in an NF1 patient (documented in the UAB Medical Genomics Laboratory but not in public databases). Our findings for single exons are summarized in Figure 1 (row “Patients”) and Supplementary Table 1 (“Exon Deletions - Sources and Phenotypes - Supp Mat.xlsx”).

Next, we evaluated exon length, since longer exons encode larger portions of the protein (see Figure 1, row “Length (nts)”). For instance, the longest exon is exon 21, with 441 nucleotides and was found deleted in NF1 patients, suggesting that it is essential. Additional long exons had already been excluded due to their skipping introducing a frameshift or known pathogenicity from reported NF1 patients. Prioritized exons 17, 25, and 52, for which no patients have been reported with skips or deletions, have similar lengths between 117 and 156 nucleotides, with a median of 135 nucleotides.

Since protein function is often associated with protein post-translational modifications (PTMs), we gathered information about the exon localization of experimentally verified PTMs, in particular phosphorylation, ubiquitination, and acetylation (Sources: Phosphosite Plus, UniProtKB, and Kinexus). Given that phosphorylation is the most common PTM, we consider phosphorylation as likely more important for NF1 function than other PTMs. Consequently, exons containing residues that have been experimentally verified to be phosphorylated are highlighted (Figure 1, row “PTMs”, marked dark red), while numbers refer to the number of modified residues in the respective exon. Most phosphorylation data were obtained through high throughput proteomic mass spectrometry, and neither the function of phosphorylated residues nor the responsible kinase are known. Six phosphorylation sites have been reported for exon 52, including positions T2554 by PKA(10) and Y2556(11) with known functional roles; pathogenic missense variants have not been reported at these sites. There are no known PTMs of residues in exon 25 and exon 17 carries one potential phosphorylation site.

PredictProtein results for neurofibromin complemented our first evaluation of exons for therapeutic exon skipping. Figure 1 summarizes the results of predicted features, including solvent accessibility, quantified in terms of number of residues predicted to be exposed (row “Accessibility”), disorder status in terms of number of residues predicted to be exposed (row “Disorder”), percentage of Non-ORDinary secondary structure contributed by each exon (row “NORS”), the average conservation score over all residues associated with an exon (row “Average Conservation”) and the number of maximally conserved residues (row “Maximum Conservation”). With respect to solvent accessibility, the three prioritized exon candidates (exons 17, 25, and 52) are very similar (between 9 and 13 residues are classified as exposed), while individual exon contributions to neurofibromin’s surface can be significantly higher (e.g., exon 21 is associated with 46 residues predicted to be exposed). While most exons are fully ordered (including exons 17 and 25), a few have residues classified as disordered. Among these, exons

13, 21, 51, 52, 57, and 58 generate more than 19 such residues, while the only two long (≥ 30 residues) intrinsically disordered regions (IDRs) are produced by exons 50-51 and 56-57. Of note, IDRs allow a protein to adopt an ensemble of different conformations, which are thought to be in dynamic equilibrium under physiological conditions (12). Only exons 50-53 and 57-58 contribute to a predicted non-ordinary secondary structure. Lastly, the obtained conservation scores strongly suggest that exon 25 is likely not suitable for exon skipping, with a high score of 8.1. In contrast, exons 17 and 52 have low conservation scores of 1.2 and 2.2, respectively.

Following this first round of analysis of NF1, we selected individual exons to model what might happen if they were deleted. All exons that produce a frameshift when deleted/skipped, were discarded. Likewise, all exons reported as deleted in the mature transcript in at least one NF1 patient were deprioritized. This reduced the number of candidates for single exon skipping-based therapy to three: exons 17, 25, and 52. For our additional in-depth *in silico* analysis, we chose an additional eight single exons, namely exons 9, 12, 20, 21, 28, 36, 41 and 47, all of which retain reading frame when being skipped but are found in patients with an NF1 phenotype. Our selection of the additional eight exon skipping scenarios was partially based on providing controls, i.e., exons that, when skipped, are known to produce non-functional proteins, e.g., exon 28 that encodes part of the GRD and the critical R1276 “arginine finger” amino acid that binds Ras-GTP (13, 14). Moreover, from the set of consecutive exon pairs we included exons 18/19 in our analysis. By further studying these proteins, we expected to gain additional information about the effect that exon skipping might have on neurofibromin function.

Our assessment of the likelihood that skipping an individual exon or exon pair may have a therapeutic effect is based on several factors. Data are summarized in Figure 2 and detailed in Supplementary Table 3. Exon 17 appears most promising as a therapeutic target for exon skipping, as changes to secondary structure, solvent accessibility, order, protein binding sites and PTMs would be minimal. Effects of exon 17 loss on the function of the Cystein Serine-Rich

Domain (CSRD) are unknown. Exon 52 may also be a good candidate due to minimal predicted changes in secondary structure, solvent accessibility, order, and protein binding sites; however, the effects of the loss of the PTMs and its encoded nuclear localization signal (NLS) (15) are unknown. All other exons have relatively high average conservation scores (in particular, exons 25, 28, 36, and 41) and/or a large number of maximally conserved residues (such as 18/19, 21, 25, and 47), indicating crucial function. The secondary and tertiary structure of the protein may change dramatically when skipping exons 18/19, 20, 28, 41, and 47. Deletion of exon 21 would result in loss of PTMs and predicted protein binding sites. Finally, the skipping of exons 9, 12, 20 and 21 may result in proteins with less flexibility, and hence some loss of function.

Testing in cDNA Assay System: To both evaluate the *in silico* predictions and determine the functional effects of exon skipping on neurofibromin, we created and tested *Nf1* cDNAs with deletions of exons 9, 12, 17, 20, 21, 25, 28, 36, 41, 47, and 52. We also evaluated deletion of both exons 18/19 consecutively. We used synthetic gene fragments to create these deletions and cloned them into a mouse *Nf1* cDNA plasmid. All clones were validated by sequencing the entire cDNA region. All cDNAs representing the various exon skips were evaluated in four different functional assays.

First, we determined the level of NF1 protein in *NF1* null HEK293 cells when transiently transfected with a constant amount of cDNA (1 µg). A representative Western blot probed with NF1 antibody is shown in Figure 3A, as are tubulin blots (used as loading control). A minimum of three separate experiments was quantified and is depicted in Figure 3B as NF1/tubulin; all data are normalized to the WT cDNA such that data can be combined across experiments and blots. Loss of some specific exons leads to significantly decreased NF1 levels in comparison to WT levels via t-test ($p < 0.05$ and indicated with a red “**” in Figure 3B), presumably due to loss of protein stability and/or increased protein degradation. This is especially apparent for deletion of

exons 20, 21, 41 and 47, which show the lowest levels of NF1 protein. Loss of other exons, such as 9 and 17, has little effect on protein levels; while loss of exon 52 leads to increased NF1 protein levels, though this increase is not significant.

Second, we evaluated levels of GTP-Ras (Figure 4A). All samples were normalized to WT control and evaluated in at least three experiments. GTP-Ras levels of all mutant cDNAs were statistically compared by t-test to that of empty vector (EV) plasmid with no cDNA. Mutant cDNAs lacking exons 17, 25, 41, 47, or 52 were significantly more active ($p < 0.05$) than EV, as they displayed at least some ability to suppress levels of GTP-Ras. Those that are statistically less active than EV were cDNAs lacking exons 18/19, 20, or 28 ($p < 0.05$).

Third, we evaluated p-ERK/ERK ratios (Figure 4B and C). All samples were normalized to WT cDNA. p-ERK/ERK levels of all mutant cDNAs were compared to that of EV by t-test. cDNAs lacking exons 12, 17, 18/19, 20, 41, 47, or 52 retained the ability to suppress levels of p-ERK and performed significantly better than EV ($p < 0.05$).

Fourth, we evaluated ELK-1 luciferase activity (Figure 4D). All samples were normalized to WT cDNA control and evaluated in at least three experiments. Luciferase levels of all mutant cDNAs were compared to that of EV cDNA by t-test. Mutant cDNAs that were significantly better than EV cDNA (i.e., those that retain ability to suppress levels of ELK-1) were those lacking exons 9, 12, 17, 21, 25, 36, 41, 47, or 52.

NF1 exon-specific human population data: We reviewed the UAB Medical Genomics Laboratory (MGL) dataset to verify the prevalence of pathogenic variants affecting those exons with no deletion reported in the public domain, i.e., exons 17, 25 and 52. In addition, we summarized the aggregated phenotypic features associated with these pathogenic variants.

In the MGL data set, 74/8090 unrelated probands carry a truncating pathogenic variant in exon 17 (either nonsense, frameshift or out of frame splice mutation), therefore ~0.91% of the

unrelated NF1 patients are expected to carry a truncating variant affecting exon 17. No variants that result in skipping or deletion of exon 17 only were detected. In addition, eight different missense variants were observed in exon 17, with three classified as likely benign (p.Ile634Thr, p.His647Tyr and p.Arg659Gln) and five of uncertain significance (p.Asp646Tyr; p.Pro654Ser; p.Leu658Phe; p.Lys662Arg and p.Gly663Arg). Four pathogenic variants in exon 17 are recurrent: c.1885G>A, which results in out of frame missplicing r.1846_1886del; p.Gln616GlyfrTer4 and is found in 40 unrelated probands, and c.1846C>T, p.Gln616Ter, which was found in three unrelated probands. Furthermore, c.1882delT and c.1882dupT were both observed twice as well (recurrence due to presence of a stretch of 6 T's). Patients carrying a truncating pathogenic variant in exon 17 presented with a variable number of features typically associated with NF1 in an age-dependent manner, including pigmentary features (CALMs, skinfold freckling (Supplementary Table 4)).

A total of 76/8090 (~0.94%) unrelated probands in the MGL dataset carry a truncating variant in exon 25 (either nonsense, frameshift or out of frame splice variant) and another 26 carry one of twelve different missense variants that are either of uncertain significance, likely pathogenic or pathogenic according to recommendations by (16). Six probands carry one of two different observed exonic splice variants mimicking a missense variant but leading to out-of-frame missplicing, i.e., 3277G>A (r.3275_3314del) and 3304T>G (r.3304_3314del); two probands carried an exonic splice variant mimicking a nonsense variant but leading to out-of-frame missplicing, i.e., 3313A>T (r.3275_3314del); three probands carried an exonic splice variant mimicking a missense variant but leading to in-frame missplicing, i.e., 3212C>T (r.3211_3314del). Fifteen probands carry one of four different splice variants affecting the AG/GT splice sites flanking the exon leading to out-of-frame missplicing (and *not* just in-frame skipping of the exon 25 and therefore also considered truncating variants), i.e., 3198-2A>G (r.3198_3199del); 3198-2A>T (r.3198_3199del); 33314+1G>A (r.3275_3314del) and 3314+2T>A (r.3275_3314del). No

273 variants resulting in skipping *or* single exon deletion of exon 25 were found. Splicing of exon 25
274 is complex, as multiple exonic substitutions affect splicing and substitutions at the canonical
275 AG/GT splice sites lead to activation of cryptic exonic splice donor/acceptor sites and out of frame
276 missplicing. The phenotype in the individuals with the likely pathogenic/pathogenic missense
277 variants was CALMs with or without freckling, with or without learning disabilities, but we had only
278 a single individual older than 19 years; therefore the understanding of the potential phenotype
279 associated with any of these missenses is limited. The phenotype associated with the presence
280 of a truncating pathogenic variant is a classic one predisposing to the full variety of features, as
281 expected (Supplementary Table 4).

282 A total of 21/8090 (~0.25%) unrelated probands carry a truncating pathogenic variant in
283 exon 52: fourteen probands carry a frameshift variant; three carry a nonsense; four carry a
284 truncating splice variant: c.7606C>T, identified in two probands, mimicks a nonsense; and
285 c.7610A>T mimicks a missense, but both lead to out of frame missplicing (r.7605_7675del71);
286 and c.7675+1del results in out of frame skipping of the last nucleotide (G) of the exon. Another
287 two probands carry the c.7675+1G>A pathogenic variant which results in both in-frame skipping
288 of exon 52 in the majority of transcripts, *and* in-frame insertion of 39 nts from the intron 52,
289 incorporating a premature termination codon in a small fraction of the transcripts
290 (r.7675_7676ins7675+1_7675+39), making the longer transcript prone to nonsense mediated
291 decay. One >14-<18-year-old proband carrying the c.7675+1G>A presented with <6 CALMs and
292 symptomatic spinal neurofibromas; the second proband presented with ~10 CALMs at the time
293 of testing (<24-months) and his mother (>26-year) had >6 CALMs and bilateral inguinal freckling
294 only. Proband (n=21) with a truncating variant affecting exon 52 presented with pigmentary
295 features (CALMs and/or freckling) and neurofibromas. No symptomatic optic pathway gliomas
296 (but 1 asymptomatic) or malignancies were observed, but the dataset is limited (0/7 and 0/18).
297 Only 6/21 probands with phenotypic data available were ≥18 years at the time of data collection.

Aggregated phenotypic data on the probands carrying a truncating variant in exon 52 are summarized in Supplementary Table 4.

ASO Design and Efficiency:

We utilized ESE Finder to evaluate exon 17 and flanking intronic sequence to allow the design of ASOs to target exonic splice enhancer (ESE) motifs (Figure 5A). Pre-mRNA secondary structure of exon 17 and 250bps of flanking introns were modeled using mFold Software (Figure 5B with the target sites of two of the designed ASOs highlighted). We selected and evaluated four 25-mers and nine 28mers ASOs with phosphorodiamidate morpholino oligomer (PMO) chemistry, based upon positioning relative to ESEs, % GC content, predicted Gibbs free energy of binding to the predicted secondary structure, % of ASO target sequence in open loop conformation and number of ASO ends predicted to bind to single stranded sequence (summarized in Table 1). PMOs were transfected at a 2 μ M dose using 6 μ M Endoportor into WT HEK293 cells. RNA was harvested 24 hour post treatment and cDNA generated and subjected to nested PCR. Amplicons were separated on 3% w/v agarose gels and quantified using densitometric analysis using Image J (Figure 5C-D). The identity of both full length and skipped amplicons was confirmed by sequencing (results not shown). The 25mers that produced the highest levels of exon 17 skipping were those targeting +70+94 (24.1% $\pm$ 0.5) and +108+132 (20.3% $\pm$ 1.1), and the most efficacious 28mers targeted +67+94 (39.7% $\pm$ 0.5), +69+96 (34.1% $\pm$ 0.9), +68+95 (28.7% $\pm$ 0.1), +70+97 (27.5% $\pm$ 1.291) and +108+135 (20.2% $\pm$ 3.5). All results based upon N=3 except +69+96 N=9, with Standard Error of the Mean indicated by error bars (SEM). These ASOs are predicted to bind with high energy to sequences within the pre-mRNA that has open conformation.

Creation and Characterization of Precision Cell Line Models: Precision model systems were needed to evaluate the efficacy of each ASO's ability to restore neurofibromin expression and functional ability to inhibit Ras signaling. Cell lines containing the recurrent and inactivating

323 *NF1* pathogenic variant c.1885G>A, which creates a cryptic splice acceptor site and deletion of
324 r.1846-1886 resulting in a frameshift that is likely susceptible to nonsense mediated decay within
325 exon 17, were generated via CRISPR/Cas9 gene editing (Suppl Figure 1A). Multiple clones were
326 isolated (A15, B6, B48, and B52) and characterized by sub-cloning and sequencing, evaluation
327 of neurofibromin RNA, protein, and Ras signaling. Clones had varying ratios of *NF1* WT and
328 variant alleles (Figure S1B). In particular, clone A15 showed only variant sequence. Based on
329 presence of WT or variant alleles, RT-PCR products showed varying levels of WT and variant
330 (deletion) transcripts in each clone (Figure S1 C). Again, clone A15 showed only variant
331 transcript. Neurofibromin protein levels based on Western blot for each clone were as anticipated
332 based on subcloning and RT results (Figure S1D and E), clone A15 showed no neurofibromin
333 protein. Readouts of Ras signaling including both GTP-Ras levels as well as pERK/ERK ratios
334 were also as anticipated and elevated for clone A15 (Supplementary Figure 1 D, F, and G).

335 **ASO Efficacy:** We began evaluating skipping efficiency in our *NF1* mutant cell line clone A15
336 homozygous for c.1885G>A. PMOs include: hNF1.e17 [+79, +106], later replaced with more
337 efficacious PMOs, hNF1.e17 [+67, +94] and hNF1.e17 [+69, +96]. Our first analysis examined
338 skipping efficiency at a single 2 μ M dose (Figure 5E). In this assay, hNF1.e17 [+79, +106] leads
339 to significantly more skipping than hNF1.e17 [+67, +94] and hNF1.e17 [+69, +96]. We then
340 evaluated efficacy of the skipping in terms of restoration of neurofibromin expression and function
341 in Ras signaling inhibition. PMOs were utilized in dose response from 0.08 μ M to 20 μ M and
342 included: Std Ctrl Oligo, hNF1.e17 [+79, +106] (Figure 6 A, B, E, F), hNF1.e17 [+67, +94] (Figure
343 6 C, G), and hNF1.e17 [+69, +96] (Figure 6 D, H). All ASOs showed a dose-response effect and
344 were able to restore neurofibromin expression to up to 40% WT levels (Figure 6 A-D) and activity
345 in terms of repression of pERK/ERK ratios by 2-3 fold (Figure 6 E-H). To determine which ASO
346 is most potent, we treated cells with each PMO at a single dose (2 μ M) and ran assays
347 simultaneously (Figure 6 I-L). The three PMOs have relatively similar efficacies at this 2 μ M dose,

but hNF1.e17 [+67, +94] can restore the most neurofibromin expression and yields the lowest pERK/ERK ratio, though this is not statistically significant. In conclusion, we have designed ASOs that are able to effect exon skipping of Exon 17 to ameliorate the increased Ras-signaling caused by the pathogenic variant.

Mouse model DelE17: To test the essentiality of exon 17, we created mice that carry *Nf1* alleles with exon 17 completely deleted (DelE17) (Figure 7). Two alleles were generated: one removes from -2 base pairs 5' of exon 17 to +1 base pair 3' of exon 17; c.1845-2_2007+1del; p.Q616_M669del54. The second allele removes from -9 base pairs 5' of exon 17 to +7 base pairs 3' of exon 17 and inserts 5 bp c.1845-9_2007+7insGACAC; p.Q616_M669del54. The DNA sequence is depicted in Figure 7A and RT-PCR with primers flanking the deletion indicates that the mutant transcript is shorter than the WT transcript (depicted in Figure 7B). Sequencing of RT-PCR product reveals deletion of exon 17 RNA sequences. Identical results were obtained from both independent lines; therefore, we combined them. When bred to homozygosity, mice with either of these deletions are viable. This contrasts with almost all other mouse models harboring *Nf1* pathogenic variant alleles in which *Nf1* nullizygosity is lethal by E13.5. Likewise, the G848R nullizygous mouse is also viable (17, 18). This provides proof of concept that exon 17 is not essential for neurofibromin function in the mouse, or at least that its loss results in a partially functional protein. We have aged a cohort of these nullizygous mice out past 20 months and have yet to observe any tumor development (N=11; 5 females and 6 males). We also have at least 30 additional nullizygous mice (15 males and 15 females) between 12 and 20 months of age with no signs of tumor development. The nullizygous mice appear grossly the same as wild type controls.

We established multiple independent cohorts of mice for further study. We evaluated baseline activity in an open field assay with six male and six female mice of both WT and null

genotypes at 5-7 months of age and were unable to detect differences in overall activity or activity spent in the center or outer portions of the field (Figure 7C). Differences are undetectable even when evaluated by sex. We also evaluated nest building behavior in 9 WT females, 9 WT males, 9 null females, and 8 null males at 6-14 months of age (Figure 7D). Overall, exon 17 null mice build significantly poorer nests than WT mice. Significance holds for male mice, but not for female mice since WT female mice show relatively poor nest building in this cohort.

A separate cohort of 8-month-old mice (3 male and 3 female nullizygous) were submitted for diagnostic necropsy and histology. We evaluated heart, lungs, kidney, pancreas, stomach and small intestine, large intestine, liver/spleen, reproductive tissue, and brain/spinal cord by H&E. Initial examination was grossly unremarkable. More detailed microscopic analysis resulted in observation of lymphoid hyperplasia in the spleen (Figure 7E),. Lymphocytic infiltrate was also noted to variable degrees in the liver, kidney, lungs, and GI tract. Another cohort of 3 male and 3 female mice of each genotype (14 months) was utilized to explore the hyperplasia. Spleen, thymus, and lymph nodes were harvested; spleen and thymus weights did not differ by genotype in this cohort (Figure 7F). Single cells suspensions of spleen, thymus, and lymph nodes were utilized for FACs analysis of T cells and B cells. Consistent with histopathologic observation of lymphoid hyperplasia, splenic CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, and CD19⁺ B cells were significantly increased in proportion in null mice (Figure 7G). Furthermore, thymic CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, CD4 and CD8 double positive T cells, and CD19⁺ B cells were significantly increased in null mice (Figure 7H). There were no significant differences between WT and exon 17 null mice in T and B cell numbers in the mesenteric lymph nodes.

Yet another cohort of 3 WT (2 female; 1 male) and 3 null (2 female; 1 male) animals at 11-15 months were utilized to investigate Ras signaling in whole brain (Figure 8 I and J). Neurofibromin levels were approximately equal for both genotypes suggesting that despite loss of exon 17 the protein product remains stable. pERK/ERK ratios were not significantly different

between genotypes, though the null mice may have slightly elevated levels. We saw no differences in pAKT/AKT or pS6/S6 levels. Hence there were no significant Ras signaling differences.

DISCUSSION

Exon skipping strategies have not been applied to NF1 to date. Cryptic splice sites created by deep intronic mutations within *NF1* (affecting 2-3.5% of patients) can be silenced *in vitro* (19, 20). ASOs were used to successfully target newly created 5' splice sites thereby restoring normal splicing in fibroblasts and lymphoblast cell lines with one of three different deep intronic pathogenic variants (c.288+2025T>G, c.5749+332A>G, and c.7908-321C>G) (19). This study showed antisense-dependent decrease in Ras-GTP levels, which is consistent with the restoration of neurofibromin function. The second study assessed c.3198-314G>A and noted leakiness of the splicing mechanism that generated a proportion of correctly spliced transcripts and demonstrated correction of the splicing defect by using specific ASOs (20). While repression of a cryptic intronic splice site has the distinct advantage that no coding part of neurofibromin is removed, a new ASO therapy must be designed and tested for each pathogenic variant.

We sought to assess strategies to skip exons to mitigate intragenic *NF1* pathogenic variants that reside in non-critical regions of neurofibromin. Production of even partially functional neurofibromin could help ameliorate phenotypes. Literature in this area is non-existent. In contrast to masking cryptic splice sites, exon skipping has the possible benefit of a single therapy skipping over any pathogenic variant within the region of interest and potentially helping multiple patients with different pathogenic variants. We have systematically evaluated *NF1* *in silico*, *in vitro*, and *in vivo* to determine which, if any, exons might be skipped and retain function. We first analyzed the sequence *in silico* to make predictions and then evaluated these in an *in vitro* cDNA

system. Using the mouse cDNA, we have demonstrated the ability to express full-length functional *mNf1* cDNA. We were able to validate its GRD-related functional activity by different methods: NF1 levels, GTP-bound Ras levels, p-ERK/ERK levels, and ELK-1 transcriptional activity. Of note, using this approach, we have previously been able to evaluate the functional consequences of specific variants (21). A limitation of our study is that the function assessed here is based only on GRD activity; it is possible that variants affect alternative neurofibromin functions in other ways. For instance, NF1 has recently been shown to bind to the estrogen receptor and act as a transcriptional co-repressor, independent of Ras (22).

In general, the *in silico* data strongly predict the results of our *in vitro* assays. For example, those exons that were predicted to have the least effect by *PredictProtein* (exons 17 and 52) have the highest levels of neurofibromin and ability to suppress Ras activity *in vitro*. The exons with the highest neurofibromin levels when skipped/deleted (9, 17, and 52) have the lowest percent of residues predicted to undergo changes in secondary structure when compared to full-length neurofibromin. Conversely, exons 20, 41, and 47 were predicted to significantly alter secondary structure, and deletion of these exons led to the lowest levels of neurofibromin expression in our *in vitro* assay. Those exon deletions that were unable to lower GTP-Ras levels (18/19, 20, and 28) were all predicted to have significant changes in secondary structure. Notably, exon 28 deletion retained the least function in Ras assays. This is likely because it encodes a portion of the GRD domain that physically interacts with Ras, including the “arginine finger” residue R1276 (13, 14). Hence, we believe that our *in silico* model is predictive.

We also report here (and previously in (21)), that some cDNAs may lead to hyperactivation of GTP-Ras levels above what is seen with EV, e.g., without *NF1*. Deletion of exons 18/19, 20, and 28 leads to increased GTP-Ras levels (Figure 4A). In our previous study, we noted a similar response with a cDNA carrying the p.R681X mutation, which maps within exon 18. This variant also leads to a mouse with a more severe phenotype than a mouse with a deletion of *Nf1* (18).

This may imply that these changes could impede hydrolysis of Ras-GTP. While we do not see such evidence in our analysis of pERK/ERK levels for these clones (Figure 4B and C), exons 18/19, 20, and 28 are the only clones that were not able to suppress ELK1 transcriptional activity (Figure 4D). Hence, it is likely that these exons encode regions of NF1 that are essential to its GRD function. While this is known for exon 28, the role of exons 18/19 and 20 was previously unclear. These exons encode amino acids 670-805 within the CSRD domain and are upstream of (but not overlapping) amino acids 844-848, which have a known genotype-phenotype correlation associated with a more severe NF1 phenotype (23). Hence, this region (exons 18-21) may be critical and should not be targeted for exon skipping. These data are compatible with the recent finding that neurofibromin forms a dimer/oligomer (24, 25). While regions critical for dimerization have not yet been reported, it is possible that they overlap with these critical exons.

We also note that even though some cDNAs result in lower levels of neurofibromin expression (presumably due to loss of stability or increased proteosomal degradation) this does not equate to loss of GRD function. In fact, some of the cDNAs with lower amounts of NF1 protein have the most robust ability to inhibit Ras activity. It is unclear if this is due to the overexpression nature of our system or if loss of these exons does actually enhance inhibition of Ras activity. This has been reported for exclusion of the alternatively spliced exon 31 (26, 27). If these cDNAs indeed lose stability, but retain function, treatment with small molecules to stabilize NF1, such as protein correctors, similar to tezacaftor or lumacaftor used to stabilize the CFTR protein in cystic fibrosis, might be a promising therapy.

One cDNA resulted in higher levels of neurofibromin, namely the one representing loss of exon 52. There are at least two possible explanations for why deletion of exon 52 might result in increased neurofibromin. First, based on our *in silico* analysis with UbiProber, which computationally predicts eukaryotic ubiquitylation sites, exon 52 encodes a polyubiquitination site. NF1 is known to be regulated by proteolysis and Cul3 (28, 29); hence, it is probable that loss of

this site leads to less ubiquitination and less targeting of the protein to the proteasome for degradation. Second, deletion of exon 52 leads to loss of disordered regions of neurofibromin. Long IDRs, such as the one produced by exons 51 and 52, are also known to increase the degradation efficiency by the proteasome, and consequently regulate protein half-life (30). Hence, loss of the disordered region may diminish that targeting and result in higher levels of protein.

Our analysis of the functional effects of deletion of Exon 52 is inconclusive. While our *in vitro* data indicate that this will result in increased neurofibromin levels that maintain GRD function, the effects of the loss of the NLS and PTMs are unknown. *In vivo* assessment will be necessary to make this determination. Splice variants representing loss of exon 52 (NF1-ΔE43; note historical exon numbering) have also been reported for human but not mouse *NF1* transcripts (31). This splice variant shows relatively high expression in specific tissues: it represents 22% of transcripts in fetal liver, 15.6% in liver, 14.4% in skeletal muscle, 11.7% in kidney, 8% in placenta, and 6.3% in lung (31). These tissues are not typically associated with NF1 pathogenesis. Further, there seems to be inter-individual variations in expression of this transcript in lymphocyte cultures from NF1 patients and healthy controls.

Our initial *in silico* analysis included all constitutively expressed exons. Our *in vitro* analysis included all exons without reported exon skips or deletion in NF1 patients and was expanded to include additional exons that maintained reading frame but had some patients reported to carry such exon skipping or deletion pathogenic variants. We did not test the following single exons *in vitro* even though they would maintain frame: 2, 3, 10, 11, 13, 14, 23, 24, 32, 34, 35, 46, and 49. We also did not test 7/8, 15/16, 29/30, 37/38, 42/43, 44/45, 50/51, or 56/57 even though skips of these exons have not been reported. Of these, 29/30, 32, 34, and 35 encode the GRD and are likely essential. Our secondary analysis indicates that exons 2, 13, 42/43, and 50/51 code for PTMs, and that exons 15/16, 23, 37/38, 42/43, 44/45, and 56/57 are conserved with individual exon conservation score greater than 7, arguing their likely essentiality. This

leaves exons 3, 7/8, 10, 11, 14, 24, 46, and 49 unevaluated *in vitro*, despite possible residual function. All have no or questionable PTMs, low accessibility, low disorder, no non-ordinary secondary structure, and moderate conservation. While the report of patients with these in-frame exon skips makes them less suitable as exon skipping targets, if the resultant exon skip primarily results in lowered neurofibromin levels with functional GRD activity, then this issue might be resolved with combination therapy of ASOs and corrector-like compounds. Hence, this leaves room for further analysis in the future. These data can lead to future structure-function studies and possibly help lead to a “mini-*NF1*” gene cassette for gene replacement therapies utilizing viral vectors.

We also interrogated the UAB MGL NF1 patient dataset representing 8090 unrelated probands for pathogenic variant prevalence, potential genotype-phenotype correlations, and reports of individuals with deletions or exon skips for exons 17, 25, and 52, as these had not been previously reported. Overall, truncating variants are expected in exon 17 in ~0.91% of NF1 patients, exon 25 in ~0.94%, and exon 52 in ~0.25%. Pathogenic variants within these exons were associated with an age-dependent phenotype and included, besides pigmentary signs, neurofibromas, optic pathway glioma and osseous lesions. Finally, while no exon skips or deletions were identified for exons 17 and 25; two probands carrying a pathogenic variant at c.7675+1G>A, which results both in in-frame skipping of exon 52 in the majority of transcripts, and insertion of 39 nucleotides from the intron 52 in a small part of the transcripts (r.7675_7676ins7675+1_7675+39) and resulting in a PTC that likely undergoes NMD. It is unclear how well this particular patient variant (c.7675+1G>A) might predict ASO-induced exon skipping, as ideally the ASO would lead to transcripts that skip exon 52 but would not lead to insertion of 39 nucleotides from the intron 52 in some transcripts. More studies will be required to make this determination.

Our data and cDNA system enable us to routinely tease out differences in NF1 levels and GRD function, including increased GTP-Ras levels for various cDNAs. This is significant, as genotype-phenotype correlations have been made and it is possible that pathogenic variants that result in loss of NF1 levels might have different phenotypic correlations, in contrast to pathogenic variants that lead to loss of GRD function. Collectively our data suggest that exon skipping in *NF1* could result in at least partial GRD function. Skipping of exons 17, 41, 47, and 52 appears to retain GRD activity. Exons 17 and 52 are more promising as they also retain high levels of neurofibromin.

We designed ASOs to efficiently skip exon 17 and evaluated their effect in cell lines with both WT and inactivating *NF1* variants in exon 17. ASOs were able to skip mutant exons and restore neurofibromin expression. As predicted in all assays, skipping inactivating pathogenic variants in exon 17 both restores neurofibromin expression and function (in terms of Ras pathway inhibition).

As proof of concept, we deleted exon 17 in mouse models using CRISPR targeting. DelE17 mice are viable and grossly healthy with no tumor phenotypes; however, we did note increased numbers of T and B cells in the thymus and spleen and variable increase in lymphocytic infiltrate in the liver, kidney, and lungs. Exon 17 null mice exhibited altered behavior, specifically nesting behavior. These relatively mild phenotypes suggest that loss of exon 17 creates a mild hypomorphic allele. Lymphoproliferative defects might be anticipated if the DelE17 alleles are hypomorphic, as loss of NF1 leads to increased Ras signaling and aberrant growth in hematopoietic cells (32). Indeed, there is a well-known increased risk of myeloid leukemia in children with NF1 (33). Mouse models in which the hematopoietic system is reconstituted with *Nf1* deficient hematopoietic stem cells produce myeloproliferative symptoms associated with human juvenile chronic myelogenous leukemia as *Nf1* loss leads to chronic clonal hyperproliferation due to hypersensitivity to GM-CSF (34). NF1 also plays a documented role in

T-cell development and function. Loss of *Nf1* in T-cells leads to enhanced Ras activation and thymic and splenic hyperplasia as well as an increase in immature and mature T-cell subsets (35). Another study indicates that NF1 promotes thymocyte positive selection (36). Our data with increased numbers of B and T cells agrees with the literature indicating that NF1 maintains immune homeostasis; however, our phenotype was not as severe (indicating some NF1 function) as we did not observe leukemia-like phenotypes.

Behavioral phenotypes might also be anticipated with loss of neurofibromin function. Children with NF1 often experience cognitive and behavioral difficulties; as many as 80% will manifest some delay in these domains and often have comorbid ADHD (37). Utilization of mouse models to study these deficits has been challenging. While hyperactivity or altered nest building have not been reported in any *Nf1* mouse models, several studies have shown that *Nf1* heterozygous mice display learning deficits in Morris water maze (38, 39) and that this phenotype responds to genetic and pharmacological manipulations that decrease Ras signaling (38). In addition, conditional mouse models (*Nf1* *Syn1*^{+/-} and *Nf1* *Dlx5/6*^{+/-}) in which *Nf1* has been inactivated via Cre-recombinase in either excitatory and interneurons or inhibitory neurons respectively, show spatial learning deficits in Morris water maze (40). Unfortunately, these studies utilizing water maze to show learning deficits in *Nf1* mice have not been replicated by other groups. Genetic background may play a role, as these studies utilized a C57BL/6J X 129/SvEv background which may not typically be utilized. Hence, new behavioral assays with robust NF1-driven phenotypes are required. Thus, we utilized open field and nest building as simplistic assays and report here the first altered nest building response for *Nf1* mice.

Further, some *Nf1* mutant mice may have similar phenotypes to DelE17; deletion of exon 31 (historically exon 23a; which is alternatively spliced) results in deficits in spatial learning, impaired contextual discrimination and delayed acquisition of motor skills, but does not lead to tumorigenesis (41). In theory, exon 31 deletion simply results in lower GAP activity (in comparison

to full length NF1 (26)); however, altered Ras signaling in the brain or other tissues was not evaluated in these mice. Of note is the fact that one of the nullizygous exon 31 deletion mice showed splenic hyperplasia, with expansion of red pulp and increased extramedullary hematopoiesis. Furthermore, the data from the exon 31 deletion mouse indicates that the learning deficits caused by reduced GAP activity are not a result of developmental deficits (as mice have normal embryological development and tumor suppression), but instead suggest that they result from disruption of function in the adult brain, implying that treatment for these learning disabilities may be possible. Effects of Ras-inhibitory treatment on DeIE17 mice remain to be characterized. If successful, these mice and the nest building assay will represent a robust behavioral model in which to evaluate therapeutics to improve cognitive function in individuals with NF1.

In summary, our data suggests clinical treatment by skipping NF1 exon 17 is feasible for individuals with pathogenic variants in this exon. Future studies will include the use of ASOs for exon skipping in precision mouse models with humanized exon 17 sequence.

MATERIALS AND METHODS

In silico analysis: Human *NF1* cDNA sequence was downloaded. We utilized the exon numbering nomenclature as outlined in LRG_214. Mutation nomenclature follows transcript NM_000267.3. Coding frames and exon boundaries were mapped to identify which exons could be skipped either as singletons or as two consecutive exons while still maintaining protein reading frame. Known and prospective protein domains were overlaid onto this map. Exon lengths were determined. We also evaluated the literature, the Human Gene Mutation Database (HGMD), and the Leiden Open Variation Database (LOVD) for reports of patients with known *NF1* mutations that result in skipping of exons during splicing (see Supplementary Table 1 for summary of LOVD

entries, list of publications mined for reports of NF1 patients with skipped/deleted exons and associated phenotype). In addition, we mapped experimentally verified posttranslational modifications (PTM) of NF1 as reported on UniProtKB (<https://www.uniprot.org/uniprot/P21359>), PhosphoNet (<http://www.phosphonet.ca>), and Phosphosite Plus (<https://www.phosphosite.org/>). PTMs included phosphorylation, acetylation, methylation, and ubiquitination. Further, neurofibromin (P21359-2) was analyzed using *PredictProtein* (<https://www.predictprotein.org>)(42) a web server that combines various (protein) sequence analysis and structure prediction tools. Specifically, *PredictProtein* returns predictions of protein secondary structure, solvent accessibility, disorder status, protein-protein binding, PTMs, and conservation, among others. These detailed data are summarized in Supplementary Table 2. Conservation scores were obtained via *PredictProtein* using *ConSurf* (<https://consurf.tau.ac.il>)(43). For PTM predictions based on signature sequences, *PredictProtein* calls *Prosite* (<https://prosite.expasy.org>)(44). Outputs were analyzed for all exons, individually. Note that unlike other structure prediction tools, inputs to *PredictProtein* are not limited by their sequence length, which makes this tool particularly useful given neurofibromin's length.

For a selection of 12 exons, 11 single and one pair of consecutive exons, we also ran *PredictProtein* on the amino acid sequences obtained by skipping the exon(s) (summarized in Supplementary Table 2). Resulting predictions of various features were compared to those previously obtained for neurofibromin (see above) to assess the impact that individual exon deletions have on the protein structure and functionality. Residue-specific differences were quantified and summarized. In addition, we predicted ubiquitination sites in selected exons using the tools CKSAAP_UbSite (http://systbio.cau.edu.cn/cksaap_ubsite/)(45) and UbiProber (<http://bioinfo.ncu.edu.cn/UbiProber.aspx>)(46).

cDNA expression System: We have established a heterologous cell culture expression system using a full-length *mNf1* and *NF1*-null human cell lines(21). The full-length *mNf1* cDNA produces

620 a >250 kDa neurofibromin protein that is capable of modulating Ras signaling. We created mutant
621 cDNAs representing various exon skips and assessed their ability to produce mature
622 neurofibromin and restore *Nf1* activity in *NF1*^{-/-} cells. Ras activity data for cDNAs included levels
623 of GTP-Ras, p-ERK/ERK ratios, and ELK1 transcriptional activity normalized to wild type (WT) for
624 each experiment and in comparison to empty vector (EV).

625 **Cell culture** HEK293 (WT or *NF1* +/+) cells were obtained from ATCC (CRL-1573) and cultured
626 in DMEM + 10% FBS and 1X Pen/Strep using standard culture procedures. *NF1* -/- or null
627 HEK293 cells were created through CRISPR Cas9 targeting *NF1* exon 2 (21) .

628 ***Nf1* cDNA plasmid Development:** The *Nf1* cDNA plasmid was developed by GeneCopoeia and
629 is commercially available.

630 **Transient transfections:** HEK293 WT or null cells were transfected with LipoD293 (SigmaGen
631 Lab. Cat# SL100668) or Lipofectamine 3000 (Invitrogen cat# L30000008) and cDNA at 1ug per
632 6-well dish seeded with 500,000 cells per well or 100ng/96-well seeded with 50,000 cells. Assays
633 were performed 48-72 hours later.

634 **Western blotting:** Cells were lysed with RIPA buffer and lysates were cleared by centrifugation
635 at 20,000 RPM for 20 minutes at 4C. Protein was quantitated with a Bradford assays and 50 ug
636 of protein was loaded per well for NF1 blots and 10 ug of protein was loaded for other blots. 8%
637 SDS-polyacrylamide gels were run at 100 V for 2 hours and transferred at 100 V for 2 hours onto
638 PVDF. Blots were probed overnight at 4C with primary antibody washed and probed 1 hour at
639 room temperature with secondary. Primary antibodies include N-Terminal NF1 (Cell Signaling
640 cat# D7R7D 1:1000), tubulin (Abcam cat# ab52866 1:1000), p-ERK (Cell Signaling cat# 9101
641 1:1000), and total ERK (Cell Signaling cat# 9102 1:1000). Secondary was HRP tagged from
642 Santa Cruz. Chemiluminescent substrate from Bio-Rad was used as per manufacturer's
643 protocols.

RAS-G-LISA Assay: The RAS-G-LISA assay was obtained from Cytoskeleton Inc. and was performed according to the manufacturer's instructions.

ELK-1 Transcriptional repression assay: ELK1 is a major nuclear substrate for ERK, where phosphorylation of ELK1 by kinases results in the conformational change of ELK1 and triggers its DNA binding activity. Plasmids containing the ELK-1 transactivation domain fused to GAL-4 and UAS-Luciferase constructs were a kind gift from the Roger Davis laboratory. Together, they act as a reporter system to monitor ELK1-dependent transcriptional activity and MAPK signaling. In fact, ERK suppression has been measured by the ELK reporter assay in HEK293 cells to show that SPRED1 recruits NF1 to suppress Ras activation. Both *NF1* and SPRED1 mutations in the GRD-EVH1 interaction domains reduce Ras-ERK suppression activity (47). A strong correlation among pathogenic mutations, disruption of the GRD-EVH1 interaction, and ERK suppression activity has been reported (47). Hence, *NF1* ^{-/-} HEK 293 cells were transfected with 25 ng of pGAL4 and pGal4-ELK1 plasmids, 1 ng pNL1.1TK [Nluc/TK] transfection control, along with 100 ng of respective *Nf1* mutation plasmids with Lipofectamine 3000 and plated in a 96 well plate such that each well received 50,000 cells. After 24 hours, the medium was replaced with normal growth medium. The experiment was terminated at 48 hours after transfection with reporter lysis buffer. After lysis, nanoluc and firefly luciferase readings (Relative Light Units, RLUs) were obtained using Luciferase Assay Reagent (Promega, E1500) and a BioTek Synergy 2 plate reader. Readings were normalized to NanoLuc expression and percentage change in luciferase activity in comparison to *NF1* ^{-/-} cells transfected with WT cDNA vector. We evaluated statistical deviation of each cDNA from the empty vector clone to evaluate if cDNAs retained any function. Each mutation set was done in triplicate and the entire experimental set up was repeated at least three times.

ASO Design:

668 PMOs were designed in accordance with previously published work (48). Briefly, *NF1* exon 17
669 and 50bps of flanking intron sequence was analyzed with ESE finder to identify Exonic Splicing
670 Enhancer motifs within the exon. Secondary structures of Exon 17 and 250bps of flanking
671 sequence were then predicted with *mfold*, and the model with the most favorable energetic profile
672 was selected.

673 PMOs were designed using *sFold* to assess the strength of binding to target. Candidates were
674 selected for screening based upon their % GC content, ability to mask predicted ESEs motifs,
675 their Gibbs free energy of binding and position relative to “open” regions of the predicted
676 secondary structure.

677 **ASO Treatment:**

678 Custom morpholinos were ordered from Gene Tools, LLC and reconstituted in 0.22 μ M filtered
679 ultrapure nuclease-free water, to make a 1mM stock concentration, as per the manufacturer’s
680 directions. WT HEK293 cells were cultured in 10% (v/v) FCS in DMEM (Gibco) with 1% P/S
681 (Gibco)); once cells attained ~70-80% confluency they were treated with ASOs diluted in 1ml of
682 fresh culture media at desired concentrations alongside 6 μ M of Endo-Porter (DMSO) (Gene
683 Tools LLC). Cells were incubated at 37°C, 5% CO₂ for 24 hours prior to harvest.

684 **ASO Efficiency Assay:**

685 Post treatment with AONs, cells were lysed in RLT buffer and total RNA extracted using an
686 RNeasy Miniprep Kit (Qiagen, U.K.). Extracted RNA was subject to a Quantitect cDNA synthesis
687 reaction (Qiagen, U.K.). Briefly, 1 μ g RNA was added to 2 μ l gDNA wipeout and made up to 14 μ l
688 with nuclease-free water, this RNA-water mix was heated to 42°C for 2 mins and held at 4°C for
689 5 mins to remove secondary structures. Upon completion, 4 μ l 5x Quantiscript RT Buffer, 1 μ l
690 Quantiscript Reverse Transcriptase and 1 μ l of primer mix was added and the reaction was then
691 heated to 42°C for 30 mins and inactivated at 95°C for 3 mins.

Resultant cDNA was then amplified via nested PCR. Amplification was achieved with GoTaq G2 Flex (Promega, UK). The standard 25µl PCR reaction comprised 1.5mM MgCl, 0.2µM forward and Reverse Primers, 0.2µM dNTPs and 0.05u/ul GoTaq G2 Flex Polymerase. For the first round, 3µl of cDNA was added to 22µl of master mix. Primers used were Ex17_1F 5'-AATGGAGGCTCTGCTGGTTC-3' and Ex17_1R 5'-ACACTTCATCCACCCCACAC-3', yielding a full length (unskipped) amplicon of 545 bps and an exon 17 skipped amplicon of 389 bps. Thermocycling conditions: 94°C for 2 mins, 20x cycles [94°C 30s, 60°C 30s, 72°C 30s] and 72°C for 5 mins.

For the second round, 1µl of the first-round product was added to 24µl of master mix. Primers used were Ex17_2F 5'-TCTCAAGTGGTTGCGGAAA-3' and Ex17_2R 5'-CTGCTTCCTCACAGAGGTGG-3' yielding a full length (unskipped) amplicon of 365 bps and 209 bps. Thermocycling conditions: 94°C for 2 mins, 30x cycles [94°C 30s, 63°C 30s, 72°C 30s] and 72°C for 5 mins.

A 20µl aliquot of the second-round product was resolved on a 3% w/v agarose in TAE buffer at 70 V for 2 hours and imaged. The images were then subject to densitometric analysis using *ImageJ* software. Arbitrary values assigned to amplicons based upon analysis were then subject to the following calculation.

$$\% \text{ Exon Skipping} = \frac{\text{Skipped Amplicon}}{\text{Total Product (Unskipped + Skipped Amplicon)}} * 100$$

Mutant Cell Line Generation and characterization: CRISPR Guides were designed using CRISPOR (crispor.tefor.org) and a repair template was designed to generate a known patient mutation and also introduce silent changes to obliterate PAM site and generate a restriction site for screening as necessary. Exon 17 Guide reverse strand: 5'

CACATCCTACCCCGTAAAAAAGG 3' Repair template coded for recurrent: c.1885G>A, which results in out of frame missplicing r.1846_1886del; p.Gln616GlyfrTer4 and found in 40 unrelated probands. The exon 17 repair template also coded for a silent c.1875C>T to obliterate the PAM sequence and a silent c.1890A>C to generate an Hpy 188I site for cell line screening.

Guides were cloned into pX459 and transfected into HEK293 cells. HEK293 cells were chosen because this cell line is well characterized, used historically in NF1 research, easily takes up exogenous DNA, and is easy to culture and scale. This cell line is derived from human embryonic kidney cells and carries a modal chromosome number of 64 in 30% of cells; and Chromosome 17 (NF1) is present in 3-4 copies. Notably, this increased chromosome number does not affect any of the RAS or RASGAP genes. HEK293s have all three Ras isoforms. Cells were selected with puromycin, and single cell cloning was used to isolate clonal lines for screening. DNA was isolated from each clone as screened for mutations of interest via restriction digest or direct sequencing of PCR products. PCR amplification primers include: Exon 17 Forward 5' GGAAGACAACTCAAATAAGTGTTTATTCC 3' and Reverse 5' AATTTCAATTCAGAAAACAAACAGAGCACATAAAA 3'. Once clones of interest were identified, PCR products were cloned and individually sequenced to define alleles. Multiple clones containing the variant of interest (c.1885G>A) were identified and further characterized: A15, B6, B48, and B52.

Mutant mouse Generation:

CRISPR/sgRNA design and synthesis: CRISPR guides were designed using CRISPOR (crispor.tefor.org) to excise exon 17 and the canonical AG splice receptor signal in the mouse *Nf1* locus: Ex17 5' G2 (forward strand), 5'-C GGATACGTCTTCTTTCCAGC-3'; Ex17 3' G1 (reverse strand), 5'- ACAGAGGGAGTTACCTACCA-3'. Modified synthetic sgRNAs (Synthego, Inc.) were

allowed to complex with Alt-R® S.p. Cas9 Nuclease V3 (IDT, Inc.) at room temperature for 15 minutes before dilution with DMEM to a final concentration of 100 ng/ul, 100 ng/ul and 200 ng/ul of guide 1, guide 2 and Cas9, respectively.

Gonadotropins: Female C57B6J embryo donors from 3-4 weeks of age were administered 5 IU of PMSG (Sigma, St. Louis, MO, USA) on day -3 followed by 5 IU of HCG (Sigma, St. Louis, MO, USA) on day -1 to induce superovulation. Donor and recipient females were mated to stud and vasectomized males, respectively, on day -1.

Collection of embryos: At day 0.5 post-conception, superovulated donor females with copulatory plugs were humanely sacrificed using CO₂ followed by cervical dislocation. Oviducts were dissected into with sterile medium and nicked to expose the cumulus masses containing fertilized embryos. Embryos were cultured in KSOM (Millipore, Darmstadt, Germany) under 5% blood-gas prior to electroporation.

Electroporation: Fertilized embryos were placed in a 5 mm petridish parallel platinum plate electrode (Nepa Gene, Co., Chiba, Japan) and electroporated using the NEPA21 Super Electroporator in batches of 25 using the following parameters: Poring 150V, 3 ms, No. 4, Decay Rate 10%, Polarity +; Transfer 20V, 50 ms, No. 5; Decay Rate 40%, Polarity +/- . Surviving embryos were transferred as previously described.

Animal identification: Animals were identified by cage card, sex, and unique “ear tags” consecutively numbered that were affixed at weaning.

Biopsies: Tail biopsies were collected at weaning. A 5-7 mm portion of the distal segment of the tail was cut for analysis and the remainder cauterized. Genomic DNA was purified from the lysed tail samples.

Identification of founders: Founder animals were identified by PCR using primers flanking the target loci that amplified a 519 bp fragment in wild type animals (Ex17 F2: 5’-

763 ACTTGGTTTGGAGGTTGGACA-3'; Ex 17 R1 TTGCTCAGGGTTCCACAGTG). PCR samples
764 were run on 6% polyacrylamide/TBE gels at 100 V for 45 minutes before staining with ethidium
765 bromide. Mutations were identified in three out of three pups born from electroporated embryos
766 and all appeared to harbor a large deletion allele. Positive samples were confirmed by modified
767 Sanger sequencing which confirmed all three founders had all of exon 17 deleted including the
768 canonical AG splice site at the 3' end of intron 16 flanking exon 17 as well as the +1 site of intron
769 17. At least two alleles were identified; c.1845-2_2007+1del and c.1845-9_2007+7insGACAC;
770 both alleles result in p.Q616_M669del54.

771 **RT-PCR for Exon 17 in human and mouse:** WT and c.1885G>A HEK293 cells were collected,
772 and RNA was extracted using Qiagen RNeasy kits as per manufacturer's directions. RNA was
773 reverse transcribed and used in a PCR reaction with the following human primers: F 5'
774 GGGAGATTAGCTCACAAATGC 3' and R 5' GTGCTGCTCTTCCTGTTGACATC 3'. Transcripts
775 containing exon 17 result in a PCR product of 552 base pairs. Transcripts not containing exon
776 17 result in a PCR product of 396 base pairs. Mouse brains were harvested from an age-matched
777 wild type and DelE17 null mice and stored immediately in RNAlater. RNA was extracted using
778 Qiagen RNeasy kits as per manufacturer's directions. RNA was reverse transcribed and used in
779 a PCR reaction with the following primers: F 5' CTGATTCATGCAGACCCAAA 3' and R 5'
780 GACATGGGACATCCGTCTCT 3'. Transcripts containing exon 17 result in a PCR product of 972
781 base pairs. Transcripts not containing exon 17 result in a shorter PCR product.

782 **Mouse cohorts:** At least 4 independent cohorts were generated for analysis: Cohort 1 for initial
783 behavior studies (open field and nesting): 6 males and 6 females of WT and null genotypes aged
784 5 to 7 months. Cohort 2 for necropsy of 3 null males and 3 null females at 9 months. Cohort 3
785 for FACs analysis and additional nesting behavior 3 males and 3 females of WT and null
786 genotypes at 14 months. Cohort 4 of 3 WT (2 females; 1 male) and 3 null (2 females; 1 male)
787 mice for Western blots at 11-15 months.

Open Field Activity Assay was performed using standard operating procedures at the UAB small animal behavioral assessment core as previously described with the exception that we tested only one animal at a time (49). Animals were tracked in an Open Field (square box) for 4 min. The amount of time spent in the center of the arena versus the side was recorded as a measure of anxiety. Other measures included: ambulation time, rearing, self-grooming, and fecal droppings.

Nest Building Assay: Nest building was performed and scored on a 1-5 scale as previously described (50). Briefly, mice were given cotton nestlets overnight and scoring was performed on a 1-5 scale with 1 having more than 90% of the nestlet intact and 5 having a near perfect nest.

Necropsy Animals were examined prior to euthanasia and exhibited no remarkable abnormalities. Animals were euthanized following standard protocol using Barbitol prior to blunt dissection. All organ systems were examined succeeding ventral dissection and noted as grossly unremarkable. Abdominal organs were excised by pluck dissection and placed in Formalin. Brain and spinal column were placed in Formical for 24 hours then transferred to 70% EtOH. Tissues were dissected from pluck and placed into individual cassettes to be submitted to Comparative Pathology Center UAB for pathological analysis. Tissues were processed, embedded in paraffin, then 5 μ M sections were stained with hematoxylin and eosin and evaluated by a board certified veterinary anatomic pathologist.

FACs Analysis: Animals were euthanized according to standard protocol using Isoflurane inhalation followed by cervical dislocation. Cardiac puncture was performed to collect blood for plasma analysis. Spleen, Thymus, and Mesenteric Lymph nodes were collected and weighed. Splenocytes and Thymocytes were isolated from fresh tissues and cells were counted manually using hemocytometer. Zombie Aqua (Cat# 423101, dilution 1:1000) was used to stain for dead cells. Cell surface stains were applied and FACS analysis was performed using LSRII (Flow Cytometry Core UAB). Primary antibodies were all from Biolegend and include: CD3 PE (Cat#

100307, dilution 1:200), CD4 PacBlue (Cat# 100427, dilution 1:200), CD8 PECy7 (Cat# 100721, dilution 1:400), TCR $\gamma\delta$ PerCP Cy5.5 (Cat # 118117, dilution 1:400), NK1.1 FITC (Cat# 108705, dilution 1:100), Foxp3 AF647 (Cat # 126407, dilution 1:100), and CD19 FITC (Cat# 152403, dilution 1:200). Data was gated and analyzed using *FlowJo* software.

NF1 Genotype-Phenotype Correlations:

Comprehensive *NF1* mutation analysis, interpretation of variant pathogenicity and collection of clinical data was performed as previously described (23, 51). To describe the phenotypes, we used the same approach as previously reported (23, 51-53). For the current study, the aggregated phenotypic dataset was derived solely from the phenotypic checklists originally submitted by the referring physicians when genetic testing was requested. Individuals with missing data for a particular sign and/or symptom were classified as “unknown” or “not specified” and consequently excluded from that part of the aggregated phenotypic data. Most features were identified by physical examination; ophthalmologic examination for Lisch nodules and imaging to detect asymptomatic OPGs and spinal neurofibromas was not performed in most individuals.

Ethics Statement: All procedures were conducted with the approval of the UAB IACUC and the UAB Animal Resources Program (ARP) and follow guidelines for the care and use of laboratory animals and rodent survival surgery under approval number APN 20300. Animals are sacrificed in full accordance with the IACUC guidelines. This includes CO₂ gas inhalation followed by cervical dislocation or decapitation for rodents. These methods are consistent with the recommendation of the Panel on Euthanasia of the American Veterinary Association.

835 **Acknowledgements:**

836 **Funding Acknowledgements:** This work was supported through the Gilbert Family
837 Foundation's Gene Therapy Initiative grant number 563624 to DW. The authors would like to
838 thank members of the University of Alabama at Birmingham Transgenic and Genetically
839 Engineered Models (TGEMS) facility for creating the DelE17 mice, the Behavioral Assessment
840 Core as well as the Comprehensive Flow Cytometry Core. TGEMS is supported by NIH NCI
841 Grant P30CA13148 and NIH NIDDK Grants P30 DK074038 and P60 DK079626 (to RAK).

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844

845 **Conflict of Interest:**

846 Conflicts of Interest are as follows; the remaining co-authors do not have any conflicts of interest.

847 Bruce Korf:

- 848 • Chair, Children's Tumor Foundation Medical Advisory Committee
- 849 • Chair, External Advisory Committee for NTAP and also for NF Research Initiative
- 850 • Member, advisory committees for AstraZeneca, Springworks Therapeutics, and Inflixon

851 George Dickson has consultancies with

- 852 • Sarepta Biopharma
- 853 • AskBio
- 854 • RegenX

855 Robert Kesterson is a lead advisor for

- 856 • Inflixon

857

858 Deeann Wallis, Robert Kesterson, Laura Lambert, Bruce Korf, André Leier, Linda Popplewell and

859 George Dickson are inventors on US Patent (PCT/US20/51827) - Exon skipping to treat

860 Neurofibromatosis Type 1.

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Figures and Tables

Table 1: Summary of designed ASO characteristics displaying %GC content, number of ESE motifs covered, strength of binding, % open conformation and number of ends in open loop structures.

PMO Name	Length	PMO 5'-3'	% GC	ΔG	Number of ESE peaks covered	% in open conformation	Number of ends in open loop structures
hNF1.e17[+70;94]	25	GATCCATGGACATTTGACTGGTATT	40	-9.6	3	56%	1
hNF1.e17[+79;103]	25	ATTCTTCATGATCCATGGACATTTG	36	-5.15	5	36%	2
hNF1.e17[+85;109]	25	GTAGTAATTCTTCATGATCCATGGA	36	-1.4	6	28%	0
hNF1.e17[+108;132]	25	CCGGAGAGAGGCTCCAGGAGTACGT	64	-8.5	8	40%	1
hNF1.e17[+67;94]	28	GATCCATGGACATTTGACTGGTATTTCC	42	-10.2	4	50%	1
hNF1.e17 [+68;95]	28	TGATCCATGGACATTTGACTGGTATTTTC	39	-10.5	4	50%	0
hNF1.e17[+69;96]	28	ATGATCCATGGACATTTGACTGGTATTT	36	-10.5	4	50%	0
hNF1.e17[+70;97]	28	CATGATCCATGGACATTTGACTGGTATT	39	-10.1	3	50%	0
hNF1.e17[+76;103]	28	ATTCTTCATGATCCATGGACATTTGACT	36	-8.4	5	43%	2
hNF1.e17[+79;+106]	28	GTAATTCTTCATGATCCATGGACATTTG	36	-6.4	5	39%	1
hNF1.e17[+81;+108]	28	CTTCCGGAGAGAGGCTCCAGGAGTACGT	61	-8.7	5	32%	1
hNF1.e17[+108;+135]	28	CTTCCGGAGAGAGGCTCCAGGAGTACGT	61	-8.7	8	43%	0
hNF1.e17[+115;142]	28	CTTTCCCTTCCGGAGAGAGGCTCCAGG	61	-8.3	5	46%	1

Figure 1: *In silico* analysis of NF1.

Domain: Exons that contribute to the formation of a known (or suspected) functional protein domain. Note: The C-Terminal Domain includes the nuclear localization signal and the binding region for Syndecans.

1007 **Exon #:** Exon number. We utilized the exon numbering nomenclature as outlined in LRG_214.
 1008 Exons (E) that underwent in-depth *in silico* analysis and that were tested *in vitro* are marked with
 1009 dark border.

1010 **In-Frame?:** Single exons that, when skipped, produce an in-frame deletion (without producing a
 1011 missense mutation) are marked in green. Single exons that, when skipped, produce a frameshift,
 1012 leading to a truncated protein, are marked in red. The same holds for consecutive exon pairs that
 1013 when skipped individually lead to a frameshift but when skipped together result in an in-frame
 1014 deletion. Note: Deletion of exon 1 (marked gray) is in frame. Skipping of exons 56 and 57 (marked
 1015 gray) maintains reading frame but creates a missense mutation.

1016 **Length:** Number of nucleotides contributed by each exon. The longer the exon, the higher the
 1017 probability that it provides crucial functionality to the protein - and the darker the color code.

1018 **Patient:** We mined the LOVD 3.0 (Build 21) for reports of genomic variants with individual exons
 1019 deleted and report here the number of such entries. Moreover, we searched the literature for
 1020 patients with individual exons skipped/deleted. Here, we mark an exon in dark red if a patient with
 1021 NF1 has been reported that has that exon deleted in transcripts due to a mutation.

1022 **PTM:** Exons with known, experimentally verified post-translational modifications (PTMs), in
 1023 particular phosphorylation, ubiquitination, and acetylation (in human and/or murine tissue). We
 1024 view phosphorylation as likely more important for NF1 function than other PTMs. Consequently,
 1025 exons containing residues that have been experimentally verified to be phosphorylated are
 1026 marked in dark red, while all others are marked in pink. Numbers refer to number of modified
 1027 residues in a given exon. Note, with few exceptions, most were obtained through high throughput
 1028 proteomic mass spectrometry. Source: Phosphosite Plus, UniProtKB, and Kinexus.

1029 **Accessibility:** Number of the amino acids (associated with a given exon) that are predicted to be
 1030 exposed. Prediction obtained from PROFacc using *PredictProtein*.

1031 **Disorder:** Number of disordered residues. Amino acids are counted towards the exon that
1032 provides at least two nucleotides. Prediction obtained from MD (MetaDisorder). MD results are
1033 provided as part of the *PredictProtein* output. MD includes four predictors, namely PROFbval,
1034 DISOPRED2, Ucon and NORSnet. We summarize the output (MD2st; the two-state prediction by
1035 MD) for each exon.

1036 **NORS:** Percentage of Non-ORdinary Secondary structure, i.e. unstructured loops, contributed by
1037 each exon. This is obtained using NORSnet.

1038 **Average Conservation:** Average conservation score for each exon as calculated by ConSurf.

1039 **Maximum Conservation:** Number of amino acids with the highest conservation score for each
1040 exon as obtained from ConSurf.

1041 * Exon 31 is alternatively spliced and pathogenic variants have not been reported in databases.

1042 Black boxes indicate that Exon 31 was not analyzed in silico.

1043 Bold outlined boxes indicate exons selected for cDNA screen

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1048 **Figure 2: *PredictProtein* Results:** Summary of *in silico* analysis for selected exons:

1049 **Exon(s) skipped:** Number of the exon skipped according to continuous 1..58 exon numbering
1050 outlined in LRG_214.

1051 **Predicted secondary structure (%):** The percentage of residues in the remaining protein
1052 (NF1delEX) that are predicted to undergo a change in secondary structure when compared to
1053 (full length) human neurofibromin.

1054 **Highest reliability (secondary structure):** Predictions of secondary structures have a reliability
1055 score assigned. Here we report the highest reliability reported for any such prediction.

1056 **Predicted solvent accessibility (%):** The percentage of residues in the remaining protein
1057 (NF1delEX) that are predicted to undergo a change in solvent accessibility when compared to
1058 predictions for (full length) human neurofibromin.

1059 **Highest reliability (solvent accessibility):** Predictions of solvent accessibility have a reliability
1060 score assigned. Here we report the highest reliability reported for any prediction.

1061 **Surface contributed by exon ($\text{\AA}^2$):** Predicted solvent accessibility in squared angstrom attributed
1062 to the amino acids that have been translated from the exon(s).

1063 **Change of surface area ($\text{\AA}^2$):** Predicted total solvent accessibility in squared angstrom of human
1064 full length (fl) neurofibromin minus the predicted surface area contributed by the skipped exon(s)
1065 minus the predicted solvent accessibility of the protein with skipped exon. A positive value means
1066 that the surface of fl is larger than the surface of the protein with the skipped exon.

1067 **Predicted O -> D (#):** Number of residues in the shortened protein (NF1delEX) predicted to
1068 change status from ordered to disordered, when compared to full length neurofibromin.

1069 **Predicted D -> O (#):** Number of residues in the shortened protein (NF1delEX) predicted to
1070 change status from disordered to ordered, when compared to full length neurofibromin.

1071 **Highest reliability (Ordered/Disordered):** Predictions of status (ordered versus disordered)
1072 have a reliability score assigned. Here we report the highest reliability reported for any prediction.

1073 **Predicted P-P binding sites (#):** Number of predicted Protein-Protein binding sites as predicted
1074 by PROFisis (part of *PredictProtein*).

1075 **Predicted PTMs:** Number of PTMs as predicted from Prosite as part of *PredictProtein*,
1076 CKSAAP_UbSite and UbiProber (with score >0.8). This also includes PTMs that are not on

residues formed by the exon but likely affected by the exon skipping, if the recognition sequence is right adjacent to the skipped region.

Avg. conservation score: Average conservation score for each exon as calculated by *ConSurf*.

Maximally Conserved residues (#): Number of amino acids with the highest conservation score for each exon as obtained from *ConSurf*.

Figure 3: Functional analysis of *mNf1* cDNAs with selected exon skips: Nf1 levels. A. Representative Western blot of NF1 and tubulin levels B. Quantitation of NF1/tubulin ratios normalized to WT ratio ; N≥3. Error bars represent SEM. Resultant neurofibromin abundance for each cDNA was compared to WT levels by t-test. Red asterisk indicates p<0.05.

Figure 4: Functional analysis of *mNf1* cDNAs with selected exon skips: GTP-Ras levels, p-ERK/ERK ratios, and ELK-1 transcriptional activity. A. GTP-RAS levels normalized to WT and compared to EV control. N>3. B. Representative Western blot of p-ERK/ERK ratios. C. Quantitation of p-ERK/ERK ratios normalized to WT and compared to EV control; N≥3. and D. ELK1 transcriptional activity normalized to WT and compared to EV control; N≥3. Error bars represent SEM.

Figure 5: Exon 17 PMO Design and Efficiency. A. ESE finder analysis of *NF1* exon 17 with 50 nucleotides from each flanking intron (ESEfinder, n.d.). ESEfinder shows the ESE-binding motifs for indicated SR proteins that are above the threshold level in graphical format. The height of the bars indicates the motif scores generated by the tool. The width of the bar represents the length of the motif (6, 7, or 8 nucleotides). The different colors represent the different SR proteins. B.

mFold analysis of exon 17 *NF1* pre-mRNA with 250 nucleotides of flanking intronic sequence, showing the most bio-energetically favorable folded structure, with two designed ASOs mapped as examples. C-D. Gel Electrophoresis and Densitometric Assessment of *NF1* Exon Skipping, for four 25mer and nine 28mer ASOs with a phosphorodiamidate morpholino oligomer (PMO) chemistry. ASOs were transfected at a 2 μ M dose using 6 μ M Endoport[®] into wild type HEK293 cells, 24-hour post treatment cDNA was generated and subjected to nested PCR. Amplicons were separated on 3% w/v agarose gels, yielding full length and exon 17 skipped amplicons of 365 bps and 209 bps respectively (representative results shown in C) and quantified using densitometric analysis using ImageJ (D). E. The same process described was repeated for three 28mer ASOs in the A15 HEK293T culture (mutation c.1885G>A) and the exon skipping quantified.

Figure 6: PMO Efficacy. *NF1* restoration efficacy was evaluated in mutant cell line clone A15 with c.1885G>A. A-H Dose response for select PMOs. A, B, E, and F were treated with PMO hNF1.e17 [+79; +106]. C and G were treated with hNF1.e17 [+67, +94] and D and H were treated with hNF1.e17 [+69,+96]. A-D *NF1*/actin levels after treatment with PMOs. A. Representative Western blot showing restoration of neurofibromin expression (in comparison to actin expression) after treatment of cells with indicated doses of PMO. B, C, and D Quantitation of neurofibromin/actin levels of at least three separate experiments after treatment of cells with indicated doses of PMOs. E. Representative Western blot showing restoration of suppression of pERK/ERK ratios after treatment of cells with indicated doses of PMOs. F,G, and H Quantitation of pERK/ERK ratios of at least three separate experiments after treatment of cells with indicated doses of PMOs. I-L. Comparison of PMOs at single 2 μ M dose. I. Representative Western blot of *NF1*/actin levels. J. Quantitation of neurofibromin/actin levels of at least three separate experiments after treatment of cells with indicated PMOs. K. Representative Western blot showing restoration of suppression of pERK/ERK ratios after treatment of cells with indicated

PMOs. L. Quantitation of pERK/ERK ratios of at least three separate experiments after treatment of cells with indicated PMOs. Error bars represent SEM.

Figure 7: Creation and phenotyping of Nf1 exon 17 deletion mice. A. Schematic view of murine *Nf1* genomic region with intron and exon boundaries. Blue bar represents Exon 17. Top sequences depict wild type allele. Exons are bolded and underlined with canonical splice sites in red text. The bottom sequences show the sequence of the deletion alleles. B. RT-PCR products from WT and a DelE17 nullizygous brain. The null brain has a shortened RT-PCR product that corresponds to the loss of exon 17. C. Open field activity of WT and null mice based on time spent in center of the field and outer edges. Left side shows all mice, middle shows data from males and right shows data from female mice. D. Nest building scores for all mice, male mice, and female mice. E. Micrograph showing lymphoid hyperplasia with prominent germinal centers in the spleen denoted with asterisk in top panel (20X magnification) and outlined in red and labeled GC in bottom panel (40X magnification). F. Spleen and thymus weights of WT and null mice subsequently used for FACs analysis. G. FACs analysis of splenocytes from WT and null mice. H. FACs analysis of thymocytes from WT and null mice. I. Representative western blots showing neurofibromin levels and Ras signaling in brain tissue of 3 WT and 3 null mice. J. Densitometric analysis of NF1 and Ras signaling in brain by WT and null genotype. Asterisks indicate $p < 0.05$. Error bars represent SEM.

Supplementary Data

Supplementary Table 1: Exon Deletions- Sources and Phenotypes. The Reported Phenotypes Tab tabulates exon numbers and the specific RNA change identified as well as the resulting protein change with the original reference and any reported phenotypic data. The LOVD 3.0 entries tab list the exon numbers and the specific RNA change identified as well as the resulting protein change along with DB-ID, cellular origin, LOVD individual ID, LOVD link, publication reference and a link to the reference. The Literature tab lists the references in alphabetical order and provides a link to the reference.

Supplementary Table 2: Protein Predict Results provides all the results from Protein Predict for predictions of full length neurofibromin and for each potential protein with deleted exons of interest.

Supplementary Table 3: Summary assessment of selected exons as targets for therapeutic exon skipping based on data from *PredictProtein* detailed in Suppl Table 2.

Supplementary Table 4: Aggregated phenotypic features in probands carrying a truncating NF1 mutation in exon 17[12b], 25[19b] and 52[43].

Supplementary Figure 1: Characterization of Precision Cell Line Models for c.1885G>A in NF1 Exon 17 in HEK293 cell lines. A. Diagram depicting wild type genomic sequence surrounding 5' region of NF1 exon 17. Exon 17 is denoted with a gray bar and alterations are indicated with gray rectangles. B. Table summarizing sequencing results from subclones of four different single cell isolates containing c.1885G>A: A15, B6, B48, and B52 indicating various ratios of WT and null alleles. C. RT-PCR products of each cell line displaying both WT allele (denoted with green arrow) and mutant deletion allele (denoted with red arrow) indicating the anticipated altered splicing in variant cell lines in comparison to WT. D. Example Western blots showing

1170 representative neurofibromin, actin, pERK, and total ERK expression for each variant cell line
1171 indicated in comparison to WT HEK293 cells (+/+) and null HEK293 cells (-/-) where exon 2 has
1172 been targeted. E. Quantitation of neurofibromin/actin ratios for the various cell lines. For
1173 comparison between cells, WT cells were set at 1 and variant cell lines were normalized based
1174 on WT. N=3; error bars represent SEM. F. Quantitation of GTP-Ras level via Ras-GLISA for the
1175 various cell lines N=3; error bars represent SEM. For comparison between cells, WT cells were
1176 set at 1 and variant cell lines were normalized based on WT GTP-Ras levels. G. Quantitation of
1177 pERK/ERK ratios for the various cell lines N=3; error bars represent SEM. Again, WT pERK/ERK
1178 ratios were set at 1 and all other lines normalized to WT levels.

1179

Figures

Figure 1

In silico analysis of NF1.

Protein Predict - Results												
Exon(s) skipped	9	12	17	18/19	20	21	25	28	36	41	47	52
Predicted secondary structure (%)	4	3	2	3	3	5	4	4	4	3	3	3
Highest reliability	low	low	low	high	very high	low	medium	very high	low	high	high	low
Predicted solvent accessibility (%)	13	8	7	9	8	14	12	9	12	8	10	9
Highest reliability	low	low	low	medium	very high	medium	low	medium	low	low	low	low
Surface contributed by exon (Å²)	1686	1303	1363	2725	1585	6052	1458	1230	1352	1107	1438	2319
Change of surface area (Å²)	2746	1613	-143	-154	-601	3022	3377	2083	2894	463	3918	988
Predicted O -> D (#)	6	5	1	1	2	5	2	11	1	2	3	3
Predicted D -> O (#)	37	35	17	27	21	25	23	11	29	14	29	18
Highest reliability	medium	medium	very low	low	high	medium	very low	low	very low	very low	very low	low
Predicted P-P binding sites (#)	0	0	1	0	1	8	1	0	3	0	1	0
Predicted PTMs (#)	3	2	4	4	3	9	0	3	3	3	2	5
Avg. conservation score	5.9	4.9	1.2	6.4	4.5	5.2	8.1	8.4	7.4	7.5	6.8	2.2
Maximally conserved residues (#)	8	5	0	23	5	27	21	34	12	22	18	0

Figure 2

PredictProtein Results: Summary of in silico analysis for selected exons

Figure 3

Functional analysis of mNf1 cDNAs with selected exon skips: Nf1 levels.

Figure 4

Functional analysis of mNf1 cDNAs with selected exon skips: GTP-Ras levels, p-ERK/ERK ratios, and ELK-1 transcriptional activity.

Figure 5

Exon 17 PMO Design and Efficiency.

Figure 6

PMO Efficacy.

Figure 7

Creation and phenotyping of Nf1 exon 17 deletion mice.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SuppFig1.pptx](#)
- [SupplTable1ExonDeletionsSourcesandPhenotypesv2.xlsx](#)
- [SupplTable2ProteinPredictResultsv2.xlsx](#)
- [SupplTable3.pdf](#)
- [SupplTable4.pdf](#)