Functional assays combined with pre-mRNA splicing analysis improve variant classification and diagnostics for individuals with Neurofibromatosis type 1 and Legius syndrome.

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Abstract

Neurofibromatosis type 1 (NF1) and Legius syndrome (LS) are caused by inactivating variants in *NF1* and *SPRED1*. *NF1* encodes neurofibromin (NF), a GTPase activating protein (GAP) for RAS, that interacts with the *SPRED1* product, Sprouty-related protein with an EVH (Ena/Vasp homology) domain 1 (SPRED1). Establishing a clinical and molecular diagnosis of NF1 or LS can be challenging due to the phenotypic diversity, the size and complexity of the *NF1* and *SPRED1* loci and uncertainty over the effects of variants on pre-mRNA splicing and NF/SPRED1 function. The purpose of this work was to improve *NF1* and *SPRED1* variant classification. To help establish the pathogenicity of *NF1* and *SPRED1* variants identified in individuals with NF1 or LS, we employed 4 assays: (i) analysis of patient RNA by RT-PCR; (ii) *in vitro* exon trap analysis of *NF1* pre-mRNA splicing; (iii) *in vitro* analysis of NF RAS GAP activity; and (iv) *in vitro* analysis of the NF-SPRED1 interaction. In 69/105 (66%) cases we obtained evidence to support variant pathogenicity according to American College of Medical Genetics guidelines, demonstrating the utility of functional approaches for *NF1* and *SPRED1* variant classification and NF and LS diagnostics.

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Abstract

Neurofibromatosis type 1 (NF1) and Legius syndrome (LS) are caused by inactivating variants in *NF1* and *SPRED1* . *NF1*encodes neurofibromin (NF), a GTPase activating protein (GAP) for RAS, that interacts with the *SPRED1* product, Sprouty-related protein with an EVH (Ena/Vasp homology) domain 1 (SPRED1). Establishing a clinical and molecular diagnosis of NF1 or LS can be challenging due to the phenotypic diversity, the size and complexity of the *NF1* and*SPRED1* loci and uncertainty over the effects of variants on pre-mRNA splicing and NF/SPRED1 function. The purpose of this work was to improve *NF1* and *SPRED1* variant classification.

To help establish the pathogenicity of *NF1* and *SPRED1*variants identified in individuals with NF1 or LS, we employed 4 assays: (i) analysis of patient RNA by RT-PCR; (ii) *in vitro* exon trap analysis of *NF1* pre-mRNA splicing; (iii) *in vitro* analysis of NF RAS GAP activity; and (iv) *in vitro* analysis of the NF-SPRED1 interaction. In 69/105 (66%) cases we obtained evidence to support variant pathogenicity according to American College of Medical Genetics guidelines, demonstrating the utility of functional approaches for *NF1* and *SPRED1* variant classification and NF and LS diagnostics.

Keywords

Neurofibromatosis type 1, Legius syndrome, neurofibromin, SPRED1, functional genetics

Introduction

Variant classification is key to diagnosing genetic disorders. Clinical and genetic investigations, *in silico* predictions, population data, and analysis of pre-mRNA splicing and protein structure and function all help establish variant pathogenicity.

Neurofibromatosis type 1 (NF1; MIM# 162200) is an autosomal dominant disorder characterized by cafe-au-lâit macules, Lisch nodules, axillary freckling, cutaneous neurofibromas and a wide range of patient specific symptoms [Peltonen et al., 2012; Jett and Friedman, 2010; Ly and Blakeley, 2019]. NF1 has an incidence of 1/3500 and is caused by inactivation of the *NF1* tumour suppressor [Ratner and Miller, 2015]. The canonical 12 kb *NF1* mRNA transcript, NM_000267.3, encodes a 2818 amino acid (320 kDa) GTPase activating protein (GAP) called neurofibromin (NF). Loss or inactivation of *NF1* results in increased RAS signaling and the development of lesions characteristic for NF1 [Cichowski and Jacks, 2001].

Legius syndrome (LS; MIM# 611431) is an autosomal dominant disorder characterized by cafe-au-lâit macules, axillary and inguinal freckling, lipomas, as well as macrocephaly, learning disabilities and developmental delay [Brems et al., 2007]. LS has an incidence of 1/75000 and is caused by inactivation of *SPRED1* that encodes the Sprouty-related protein with an EVH (Ena/Vasp homology) domain 1 (SPRED1). SPRED1 recruits NF to the plasma membrane where it stimulates GTP hydrolysis by membrane-bound RAS [Stowe et al., 2012]. The functional relationship between NF and SPRED1 helps explain the phenotypic overlap between NF1 and LS. Indeed, while some amino acid substitutions impair NF RAS GAP activity to cause NF1, other changes that do not affect RAS GAP activity cause NF1 by disrupting the interaction with SPRED1 [Dunzendorfer-Matt et al., 2016]. Similarly, changes to SPRED1 disrupt the interaction with NF and cause LS [Hirata et al., 2016; Führer et al., 2019; Yan et al., 2020].

Molecular genetic analysis can establish a diagnosis of NF1 or LS: the identification of an inactivating change, such as a frameshift or nonsense variant provides strong evidence to support pathogenicity. However, variants that could affect pre-mRNA splicing or introduce damaging changes into the NF or SPRED1 proteins are more difficult to classify. In our center, DNA-based molecular screening identifies a variant in *NF1* or *SPRED1* for which pathogenicity is uncertain in 20% of cases [van Minkelen et al., 2014]. The American College of

Medical Genetics and Genomics (ACMG) has provided guidelines for the interpretation of genetic variants [Richards et al., 2015]. Strong evidence for classifying a variant as pathogenic can be obtained by performing functional experiments (ACMG criterium PS3) [Hirata et al., 2016; Yan et al., 2020; Zatkova et al., 2004; Ars et al., 2003; Messiaen et al., 2000; Thomas et al., 2012]. We initiated functional assessment of pre-mRNA splicing and NF-SPRED1 function for variants of uncertain clinical significance (VUS) identified in our cohort of individuals with NF1 or LS, and implemented these tests in our diagnostic laboratory. Establishing variant pathogenicity would provide individuals with certainty regarding their affection status and follow-up, and would facilitate prenatal diagnostics.

We investigated the effects of 34 variants on *NF1* pre-mRNA splicing and 66 variants on NF and SPRED1 function. The combination of RNA and protein studies enabled us to fully investigate the effects of the different variants. For some variants, mRNA splicing analysis was required to identify the correct protein variant to test in the functional assays. In other cases, the identification of defects in*NF1* pre-mRNA splicing made functional testing of putative missense changes redundant. The results of the RNA and functional experiments, together with clinical and genetic data, were used to classify the variants, following ACMG guidelines.

Materials and Methods

Editorial Policies and Ethical Considerations

Informed consent was provided by all subjects, as required by the institutional review board of the Erasmus Medical Center, and according to standard diagnostic protocols.

Patient assessment and selection of variants for testing

The Erasmus MC Department of Clinical Genetics NF1/LS cohort consists of >4900 index cases suspected of NF1 or LS based on the international clinical diagnostic criteria [Legius et al., 2021] for whom DNA has been submitted for genetic testing of *NF1* and/or*SPRED1* . Prior to functional testing, variants were classified using the available clinical and genetic data. Nomenclature for all the reported variants is according to HGVS guidelines [HGVS, 2016]. For assay validation, we included 8 variants that had been classified as pathogenic and/or subjected to functional evaluation previously: NF1 p.Leu90Pro [Xiao et al., 2018], p.Met992del [Koczkowska et al., 2020], p.Asp1217Tyr [Hirata et al., 2016], p.Arg1276Gly [Mattocks et al., 2004] and p.Lys1423Glu [Thomas et al., 2012, Koczkowska et al., 2019]; and SPRED1 p.Val44Asp, p.Thr102Met [Hirata et al., 2016] and p.Ser105Ala [Yan et al., 2020].

Constructs, antibodies and cell-lines

NF1 minigene exon trap constructs and NF expression plasmids were generated using standard cloning techniques [Sambrook et al., 1989], Gibson assembly [Gibson et al., 2009] and/or site-directed mutagenesis (see Supplementary Information for details). All constructs were verified by sequencing of the complete insert. Nucleotide and amino acid numbering are according to *NF1* transcript NM_000267.3 and SPRED1 transcript NM_152594.2, unless specified otherwise.

Antibodies were from Cell Signaling Technology (Danvers, U.S.A.)(rabbit anti-HA; mouse anti-HA; 9B11 mouse anti-myc), Invitrogen (mouse anti-V5) Sigma-Aldrich (St. Louis, U.S.A.) (mouse and rabbit anti-FLAG) and Li-Cor Biosciences (Lincoln, U.S.A.)(goat anti-rabbit 680 nm and goat anti-mouse 800 nm conjugates). Anti-FLAG affinity beads were from Sigma-Aldrich, glutathione-sepharose was from GE Healthcare (Uppsala, Sweden).

HEK 293T and COS-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)(Lonza, Verviers, Belgium) containing 10% fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified 37°C, 5 - 10% CO_2 incubator.

Assessment of the effects of NF1 variants on NF1 pre-mRNA splicing in patient material

Reverse transcriptase (RT) PCR was performed on 1 - 2 μg total RNA as described in the Supplementary Information.

In vitro assessment of the effects of NF1 variants on NF1 pre-mRNA splicing

Exon trap experiments were performed as described previously [Dufner-Almeida et al., 2019; Halim et al., 2017]. See Supplementary Information for details.

In vitro assessment of RAS GAP activity

To estimate RAS GAP activity, HA-H-RAS together with wild-type NF or variant NF were expressed in COS-7 or HEK 293T cells. GTP-bound RAS was subsequently isolated using glutathione-agarose beads coated with recombinant GST-RAF-RBD [van Triest et al., 2001]. See Supplementary Information for details.

In vitro assessment of the NF1-SPRED interaction

To investigate the NF-SPRED interaction, FLAG-SPRED1 together with wild-type or variant NF were co-expressed in HEK 293T cells. NF-SPRED1 complexes were immunoprecipitated using anti-FLAG affinity beads (Sigma-Aldrich). See Supplementary Information for details.

Results

Assessment of the effects of NF1 variants on NF1 pre-mRNA splicing

We investigated the effects on *NF1* pre-mRNA splicing of 34 variants that had been identified in individuals suspected of NF1 (Figure 1). In 13 cases, the results of the *in vitro* exon trap experiments were confirmed by analysis of RNA from a blood sample from the corresponding individual; in 4 cases subject RNA was analyzed directly, without performing exon trapping (Figure 1B). For the remaining subjects, RNA was not available.

We detected one or more abnormal splice products, either *in vitro* , in subject RNA, or in both for 25 variants (74 %). Exon skipping (type I defect) [Anna and Monika, 2018] was observed for 18 variants; 2 variants resulted in incorporation of a pseudo-exon causing premature truncation of the *NF1* ORF (type II defect); utilization of a non-canonical splice site, resulting in exon truncation (type III defect) was observed for 4 variants; and 3 variants caused intron retention (type IV defect). In 5 cases, abnormal splicing resulted in an in-frame deletion (Supplementary Information, Table S1; Figure 1C). Of these, the c.288+3 A>T, r.205_288del, p.(Arg69_Gly96del) and c.2710A>T, r.2707_2850del, p.(Cys904_Val951del) deletions were selected to determine whether the deletion affected NF activity (see below). In 7 cases, a missense change completely prevented canonical *NF1* pre-mRNA splicing, making assessment of NF function redundant. In 9 cases, we did not observe an effect of the variant on*NF1* pre-mRNA splicing, either *in vitro* or in subject RNA (Supplementary Information, Table S1). These cases included 5 missense variants, of which 2 could be subjected to functional assessment (see below). In total, analysis of *NF1* pre-mRNA splicing assisted in the classification of 25 variants as likely pathogenic (Supplementary Information, Table S1).

In vitro assessment of the effects of NF1 variants on NF RAS GAP activity

The ability of the NF GAP-related domain (GRD; amino acids 1180 - 1504) to inactivate RAS can be determined by measuring the amount of active, GTP-bound RAS in the presence of the NF GRD. High levels of RAS-GTP indicate that the activity of the NF GRD is impaired and can help classify a variant as pathogenic [Thomas et al., 2012]. We identified the *NF1* c.3829G>C, p.(Gly1277Arg) and c.3651T>A, p.(Asp1217Glu) variants in the NF GRD in 2 individuals with NF1. We introduced both variants and the pathogenic*NF1* c.3826C>G, p.(Arg1276Gly) variant into a NF V5-p.1180_1504 expression construct [Thomas et al. 20122] and determined the RAS GAP activity of the variant proteins in transfected cells using a pull-down assay for GTP-bound RAS (Figure 2). Neither the p.Arg1276Gly variant nor the p.Gly1277Arg variant had RAS GAP activity, as estimated from the levels of GTP-bound RAS (RAS GTP) in the pull-down fraction (Figure 2C), supporting the likely pathogenicity of the*NF1* c.3829G>C, p.(Gly1277Arg) substitution.

Expression levels of the wild-type and variant NF V5-p.1180_1504 proteins were low. To facilitate detection, we modified the NF V5-p.1180_1504 construct by altering the sequence preceding the initiation codon to correspond to the Kozak consensus sequence and by introducing an extra C-terminal V5-epitope tag. We observed robust expression of the resulting wild-type NF V5-p.1180_1504-V5 protein and derived 13 *NF1* variants identified in individuals in our NF1 patient cohort in the NF V5-p.1180_1504-V5 expression construct (Figure 2B). In 5 cases, the variant lacked RAS GAP activity: levels of GTP-bound RAS (RAS-GTP) were not significantly different to those in the absence of NF V5-p.1180_1504-V5 ($P > 0.025$; Student's paired t-test with Bonferroni correction; shown in red in Figure 2D). In 2 cases, RAS GAP activity was significantly reduced compared to wild-type NF V5-p.1180_1504-V5, but significantly increased compared to the absence of NF V5-p.1180_1504-V5 ($P < 0.025$; Student's paired t-test with Bonferroni correction; orange in Figure 2D), suggesting that these variants impaired RAS GAP activity but did not inactivate the GRD completely. The remaining 6 variants retained full RAS GAP activity ($P > 0.025$; Student's paired t-test with Bonferroni correction; black in Figure 2D).

In vitro assessment of the effects of NF1 variants on NF p.1_2069 RAS GAP activity

Many *NF1* VUS identified in our cohort mapped outside the NF GRD. Attempts to introduce nucleotide changes into a full-length *NF1*expression construct were unsuccessful. However, we were able to introduce variants into 2 expression constructs that encoded the N-terminal 2069 amino acids of NF (Figure 3A). The only difference between these 2 constructs was the inclusion of sequences corresponding to a neuron-specific *NF1* transcript encoding a 10 amino acid insertion [Geist and Gutmann, 1996]. We referred to the proteins expressed from these constructs as NF p.2069myc, corresponding to reference transcript NM_000267.3, and NF p.420ins10myc, corresponding to the transcript encoding the insertion. We did not detect significant differences between the wild-type p.2069myc and p.420ins10myc proteins. Both were expressed at similar levels and exhibited equivalent RAS GAP activity.

We introduced 66 *NF1* variants in these constructs, including 13 variants previously tested in the NF V5-p.1180_1504 or NF V5-p.1180_1594-V5 constructs, and estimated the RAS GAP activity of all the 66 variant proteins (Figure 3B; Supplementary Information, Table S2). Some were as active as the corresponding wild-type NF protein, some had severely attenuated RAS GAP activity and others had intermediate levels of activity. This made it difficult to assign an exact cut-off value to distinguish pathogenic, inactivating variants. Therefore, we devised an empirical scheme to categorize the variants. We compared the mean RAS GAP activities of the variants, and whether these were reduced relative to the wild-type ($P < 0.05$, Student's paired$t$ -test). If the mean estimated RAS GAP activity was $< 50\%$ of the wild-type and if this reduction was statistically significant, then we considered it evidence for disruption of NF RAS GAP activity and supporting evidence for pathogenicity (ACMG criteria PS3). This was the case for 25 variants (Figure 3B; variants indicated in red: mean activity $< 50\%$ of wild-type NF; Student's paired$t$ -test $P < 0.05$), 19 of which mapped to the NF1 GRD. In 12 cases, the results with the NF p.1180_1504-V5 and V5-p.1180_-1504-V5 proteins were confirmed. We did not detect significant impairment of RAS GAP activity by the NF p.420ins10myc p.Thr1199Ile variant, in contrast to the reduction associated with the NF V5-p.1180_1504-V5 p.Thr1199Ile variant (compare Figures 2D and 3B, and see Discussion).

In 16 cases, RAS GAP activity was significantly reduced compared to the wild-type control ($P > 0.05$; Student's paired t-test), but was $> 50\%$ of the wild-type value (Figure 3B; variants indicated in orange). We considered this insufficient evidence to support pathogenicity. The remaining variants did not show evidence for impaired RAS GAP activity: mean activity was not significantly different to wild-type NF ($P > 0.05$; Student's paired t-test; Figure 3B, variants indicated in black).

In vitro assessment of the effects of NF1 and SPRED1 variants on the NF-SPRED1 interaction

Pathogenic *NF1* variants can disrupt the interaction between NF and SPRED1 without affecting NF RAS GAP activity [8]. The wild-type NF p.2069myc and p.420ins10myc proteins could be immunoprecipitated (IP) together with co-expressed wild-type FLAG-SPRED1 (Figure 4A) and we used this coimmunoprecipitation (coIP) assay to determine whether 59*NF1* and 5 *SPRED1* variants affected the NF-SPRED1 interaction.

We compared the NF p.2069myc and p.420ins10myc wild-type and variant signals in the IP fractions (Figure 4B) and categorized the variants using the same criteria as for the RAS GAP assay: a significant reduction ($P < 0.05$, Student's paired t-test) of $> 50\%$ in the mean NF signal in the IP fraction was evidence for an effect of a variant on the NF-SPRED1 interaction, and supported pathogenicity.

The NF-SPRED1 interaction was reduced $> 50\%$ for 26*NF1* and 2 *SPRED1* variants, including the *NF1*p.Asp1217Tyr and *SPRED1* p.Val44Asp variants previously shown to disrupt the NF-SPRED1 interaction [Hirata et al., 2016] (Figures 4B, variants indicated in red). In 9 cases there was a significant reduction in the NF signal in the IP fraction, but the mean value was $> 50\%$ of the wild-type and therefore not sufficient evidence to support pathogenicity (Figure 4B, variants indicated in orange). The remaining variants did not show significant differences from the wild-type controls ($P > 0.05$, Student's paired t-test; Figures 4B, indicated in black).

In vitro assessment of the effects of NF1 and SPRED1 variants on the expression and stability of NF and SPRED1

The estimated RAS GAP activity and the estimated strength of the NF-SPRED1 interaction might reflect differences in the expression and/or stability of the variant proteins. To investigate effects on NF and SPRED1 stability, we compared the wild-type and variant signals in the cell lysates by immunoblotting. In 12 cases, the mean expression of a variant was reduced to below 50% of the wild-type (Figure 4C, indicated in red). Of these, 6 also showed a significant reduction of $> 50\%$ in either RAS GAP activity, SPRED1 binding or both, and in 2 cases, p.Leu90Arg and p.Arg69_Gly96del, both RAS GAP activity and NF-SPRED1 binding were significantly reduced, but by $< 50\%$. The remaining variants showed either significantly reduced expression ($P < 0.05$ Student's paired t-test), but still $> 50\%$ of wild-type (that is, < 50 % reduction; Figure 4C, indicated in orange), or did not affect NF or SPRED1 expression or stability ($P > 0.05$ Student's paired t-test; Figure 4C, indicated in black).

Categorization of NF1 and SPRED1 variants, based on the results of the functional assessment

To use the results of the functional experiments to help classify the variants, we applied empirical rules to define 2 groups. The first group consisted of variants that clearly disrupted NF or SPRED1 function in the *in vitro* functional assays. We defined clear disruption as a significant reduction ($P < 0.05$, Student's paired t-test) of $> 50\%$ in either RAS GAP activity or in NF-SPRED1 binding. In addition, if the mean expression of a variant was significantly reduced by $> 50\%$, and both RAS GAP activity and SPRED1 were significantly reduced but the mean reduction was $< 50\%$, then we concluded that the effect on NF expression could be biologically relevant, and therefore that there was evidence to support pathogenicity (Supplementary Information, Tables S2 and S3). The second group did not show clear evidence for disruption of NF1 or SPRED1 function. We considered a reduction of $> 50\%$ in expression as insufficient evidence to support pathogenicity if we detected normal levels of RAS GAP activity and SPRED1 binding.

Discussion

To complement the molecular genetic test results from our diagnostic laboratory, we performed functional assessment of 105 *NF1* and*SPRED1* variants. We employed 4 assays: (i) analysis of subject RNA by RT-PCR; (ii) *in vitro* exon trap analysis of *NF1*pre-mRNA splicing; (iii) *in vitro* analysis of NF RAS GAP activity; and (iv) *in vitro* analysis of the NF-SPRED1 interaction. In 69 cases (66%) we obtained evidence to support pathogenicity (Supplementary Information, Tables S1, S2 and S3).

In contrast to laboratories that specialize in *NF1* variant detection and classification using patient RNA, our diagnostic laboratory performs molecular screening primarily on DNA samples [van Minkelen et al., 2014]. The decision to focus on DNA variant identification was originally taken to simplify the workflow and allow the laboratory to apply a standard method to variant detection for a wide range of genetic disorders. Direct analysis of RNA was not considered practical for routine screening in our setting. The *in vitro* exon trap experiments therefore provided a useful screen for identifying variants likely to affect splicing, without having to re-sample patients. We did not observe major discrepancies between the exon trap and RT-PCR

results that would have led to a different classification for any of the variants tested, consistent with other work from our laboratory [Douben et al., in revision; Dekker et al., submitted]. Information on the observed *in vitro* effects could be provided to the clinician and the relevant individual(s) prior to taking a sample for confirmation. Moreover, the exon trap approach was a simple method to help resolve allele-specific patterns of pre-mRNA splicing that were unclear from the subject RNA data. The exon trap experiments indicated whether a variant prevented canonical splicing of an exon completely, or had only a partial effect. In 4 cases there were some minor differences between the *in vitro* and *in vivo*RNA data (Supplementary Information, Table S1). However, we did not identify cases where a variant affected splicing *in vitro* but not*in vivo* , or *vice versa* . Analysis of pre-mRNA splicing was also a useful screen for the functional assessments as it was not always obvious whether a variant was likely to affect splicing and/or protein function. In some cases, RNA analysis revealed abnormal *NF1*splicing, making functional assessment redundant, whereas in other cases, RNA analysis indicated that functional assessment of an in-frame deletion was required to help establish pathogenicity.

Compared to the exon trapping and RT-PCR experiments, assessment of NF-SPRED1 function was labour-intensive, time-consuming and the results were sometimes more difficult to analyze and interpret. Nonetheless, we obtained insight into the effects of multiple *NF1* and*SPRED1* variants on NF-SPRED1 function (Supplementary Information, Tables S2 and S3). We focused on 3 characteristics: (i) NF RAS GAP activity, (ii) the NF-SPRED1 interaction and (iii) NF/SPRED1 expression/stability, and devised an empirical scheme to categorize the variants. It is possible that some variants that disrupted NF-SPRED1 function in our *in vitro* assays might still retain sufficient activity *in vivo* to prevent NF1 or LS. Furthermore, differences in estimated activity or expression might be due to variation in transfection efficiency, cell numbers, immunoblotting artefacts or other processing errors. Therefore the results had to be interpreted with caution and in the light of the clinical and genetic evidence. Despite these caveats, we considered a $> 50\%$ reduction in either RAS GAP activity or NF-SPRED1 binding as functional evidence to support pathogenicity. We did not consider a $> 50\%$ reduction in expression/stability as sufficient evidence for pathogenicity unless it was concordant with significant disruption of both the RAS GAP activity and NF-SPRED1 interaction ($P < 0.05$, Student's paired t-test), even if the RAS GAP activity or NF-SPRED1 interaction was $> 50\%$ of the wild-type value (indicated in orange in Figures 3B and 4B and Supplementary Information, Table S2). Variants that did not show significant reductions in RAS GAP activity, or the interaction with SPRED1 remained of uncertain significance, unless other evidence was obtained to support or exclude pathogenicity.

We obtained evidence supporting pathogenicity for 43 *NF1* and 2*SPRED1* variants, including the known pathogenic variants*NF1* p.Leu90Pro, p.Met992del, p.Asp1217Tyr, p.Arg1276Gly and p.Lys1423Glu, and *SPRED1* p.Val44Asp (Supporting Information, Tables S2 and S3). None of these variants were identified more than once in the gnomAD (v2.1) database [https://gnomad.broadinstitute.org/](accessed 7/3/2022), and none were classified as benign or likely benign in Clinvar [https://www.ncbi.nlm.nih.gov/clinvar/] (accessed 7/3/2022). In all cases, the variant was identified in one or more individuals suspected of NF1 or LS. The remaining variants did not show sufficient evidence for an effect on NF or SPRED1 function to support pathogenicity, even though several are described as likely pathogenic in Clinvar (Supplementary Information, Table S2). In one case we observed a discrepancy between the results of the RAS GAP assay with the NF V5-p.1180_1504-V5 GRD and the NF p.420ins10 protein. The NF1 p.Thr1199Ile variant impaired RAS GAP activity of the GRD but did not significantly affect RAS GAP activity of the NF p.420ins10 protein (compare Figures 2D and 3B). It is possible that the NF GRD and p.420ins10 proteins have distinct sensitivities to changes in secondary structure. The extra scaffolding around the active site of the GRD provided by the p.420ins10 protein might restrict structural changes and thereby maintain RAS GAP activity.

We could not exclude pathogenicity based on the results of the functional assessment. We only interrogated 3 aspects of NF-SPRED1 function: RAS GAP activity, the NF-SPRED1 interaction and expression/stability. We did not investigate other putative functions of NF or SPRED1 [D'Angelo et al., 2006; Welti et al., 2011; Fadhlullah et al., 2019]. Furthermore, we were unable to investigate *NF1*variants that mapped distal to the C-termini of the p.2069myc and p.420ins10myc proteins. It would obviously be desirable to be able test all variants in the context of full-length NF and efforts to efficiently derive *NF1* variants in a full-length

NF1 expression construct are on-going in our laboratory. Nonetheless, despite these limitations, our work provided valuable information for individuals with NF1 and LS regarding their disease status and genetic risks, confirming the utility of functional testing for *NF1* and *SPRED1* variant classification.

In summary, we applied *in vitro* assays to investigate the effects of *NF1* and *SPRED1* variants on *NF1* pre-mRNA splicing and NF-SPRED1 function. We tested 100 *NF1* and 5 *SPRED1* variants and obtained evidence to support pathogenicity in 69 cases (66%)(Supplementary Information, Tables S1, S2 and S3). The results of our experiments have been submitted to the *NF1* and SPRED1 Leiden Open Variation Databases (https://databases.lovd.nl/shared/genes/NF1; https://databases.lovd.nl/shared/genes/SPRED1). Our work demonstrates that functional testing helps identify likely pathogenic *NF1* and*SPRED1* variants. Implementation of these tests in our diagnostic laboratory, together with consideration of the clinical, population, *in silico* and segregation data, has resulted in improved molecular diagnostics for individuals with NF1 and LS and facilitated appropriate monitoring, treatment and prenatal diagnostic options for family planning.

Data availability

Data and materials are available from the corresponding authors upon reasonable request.

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Author contributions

Conceptualization: MN, JJS, TvH, RvM; experimentation: HD, MH-W, MN, JL, MK-dH, MP, BvO, LvU, PE, EK; clinical investigation: YvB, MvV, RO, AW, YvI; supervision: MH-W, MN, AW, TvH, RvM; writing, review and editing: MN, AW, YvI, TvH, RvM.

Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Analysis of *NF1* pre-mRNA splicing.

(A) Schematic overview of *NF1* exon trap constructs derived as part of this study, showing the approximate location of the tested variants. Exonic sequences are shown as boxes, intronic sequences as horizontal lines; intronic sequence representing a pseudo-exon is indicated by the shaded box. SS indicates a variant also subjected to functional assessment (see Supplementary Table S2). Variant nomenclature is according to reference transcript NM_000267.3, except P: reference transcript NM_001042492.2.

(B) Approximate location of the *NF1* variants investigated with RT-PCR and Sanger sequencing in patient RNA in relation to the NF1 reference transcript (NM_000267.3).

(C) Exon trap and RT-PCR analysis of the NM_000267.3(NF1):c.2710T>A variant. Agarose gel electrophoresis of the NF1 exon 21 wild-type (WT) and c.2710T>A exon trap RT-PCR products (left) and RT-PCR products from the index patient and 3 control individuals (center). A indicates the canonical exon 21 product; B indicates the product obtained due to the cryptic splice site at c.2706 created by the c.2710T>A substitution. Electropherogram of Sanger sequence analysis of the RT-PCR products, confirming the abnormal r.2707_2850del transcript.

(D) Exon trap and RT-PCR analysis of the NM_000267.3(NF1):c.3871-3T>G and c.3974+5G>C variants. Agarose gel electrophoresis of the NF1 exon 29 wild-type (WT) and c.3871-3T>G, c.3974+5G>C and c.3871-3T>G + c.3974+5G>C exon trap RT-PCR products (indicated with the red box). Note the complete loss of the wild-type product from the constructs containing both substitutions.

Figure 2. *In vitro* functional assessment of RAS GAP activity of the NF GRD.

(A) Schematic overview of the pull-down assay for NF RAS GAP activity. Variants were first introduced into the wild-type NF expression construct by site-directed mutagenesis (SDM), co-transfected into mammalian cells in culture, together with a RAS reporter expression construct and, after 5 minutes stimulation with EGF, the cells were lysed and GTP-bound RAS subjected to GST-RAF-RBD pull-down with glutathione-agarose beads. Lysate and pull-down fractions were subsequently analyzed by immunoblotting.

(B) Schematic overview of NF (above), showing the different proposed functional domains (CSRD, orange; TBD, green; SPRED1 interaction, violet; SEC14-PH, cyan; HEAT, pink; SBD, blue) and the NF GRD expression constructs used in this study (below). Amino acid changes are given according to reference transcript NM_000267.3. Variants derived in the NF V5-p.1180_1504 expression construct [Thomas et al., 2012] are indicated in cyan; other variants were derived from the wild-type NF p.V5-1180_1504-V5 expression construct.

(C) Relative RAS GAP activity of the NF p.V5-1180_1504 variants. NF V5-p.1180_1504 variants were co-expressed with HA-H-RAS and RAS GAP activity was estimated in a pull-down assay using recombinant GST-RAF-RBD to specifically bind RAS-GTP. The signals for HA-H-RAS in the pull-down fractions were determined in 3 independent experiments. The mean estimated RAS GAP activity is shown relative to the wild-type (V5-GRD); = 1.0). Error bars represent the standard error of the mean. Variants showing significantly reduced RAS GAP activity are indicated in red (see main text for details).

(D) Relative RAS GAP activity of the NF p.V5-1180_1504 variants. Pull-down assay was performed as in (C); RAS GAP activity was estimated relative to the wild-type control (V5-GRD-V5). Variants showing no evidence for RAS GAP activity are indicated in red; variants with reduced RAS GAP activity are shown in orange; active variants are indicated in black (see main text for details). Quantification data of at least 3 independent experiments is shown.

(E) Representative immunoblot showing the GST-RAF-RBD pull-down fractions.

(F) Cell lysate fractions corresponding to the samples shown in (E).

Figure 3. *In vitro* functional assessment of NF RAS GAP activity.

(A) Schematic overview of neurofibromin (NF) (top) and the expressed truncated NF proteins used for the functional experiments (below). The C-terminal region absent from the expressed NF p.1_2069-myc and p.420ins10-myc proteins is indicated and the approximate positions of the different variants are shown, including the 10 amino acid insertion (420ins10) that differentiates between the two expressed wild-type proteins. Variants derived in the NF p.1_2069ins10-myc construct are indicated in cyan; variants derived in the NF p.1_2069-myc construct are shown in black. To determine the RAS GAP activity of the variants, the scheme shown in Figure 2A was used. Briefly, NF p.1_2069-myc and NF p.1_2069ins10-myc variants were co-expressed with HA-H-RAS. RAS GAP activity was estimated in a pull-down assay using recombinant GST-RAF-RBD in at least 4 independent experiments, relative to the wild-type NF p.1_2069-myc or NF p.1_2069ins10-myc (indicated in cyan) proteins.

(B) Quantification of *NF1* variant RAS GAP activity. Variants showing evidence of disruption of RAS GAP activity ($> 50\%$ reduction in activity; $P < 0.05$) are indicated in red; variants with reduced RAS GAP activity are shown in orange ($< 50\%$ reduction; $P < 0.05$); active variants (no evidence for reduced RAS GAP activity)($P > 0.05$) are indicated in black (see main text for details). IT/RC: NF1 p.Ile1799Thr/Arg1809Cys (double *cis* variant).

(C) Representative immunoblot showing the lysate (above) and GST-RAF-RBD pull-down (below) fractions.

Figure 4. Functional assessment of the effects of *NF1*and *SPRED1* variants on NF-SPRED1 interaction and NF expression.

(A) Schematic overview of the *in vitro* functional assessment of the NF1-SPRED1 interaction. Variants were introduced into the wild-type (WT) expression construct by site-directed mutagenesis (SDM) and the NF p.1_2069-myc or p.1_2069ins10-myc and FLAG-SPRED1 expression constructs co-transfected into

mammalian cells. NF-SPRED1 complexes were isolated by immunoprecipitation (IP) using anti-FLAG agarose beads. Lysate and IP fractions were subsequently analyzed by immunoblotting. Signals for the variants relative to the WT proteins (NF p.1_2069-myc, NF p.1_2069ins10-myc or FLAG-SPRED1) were determined in at least 3 independent experiments.

(B) Quantification of the IP signals for the *NF1* (left) and*SPRED1* (right) variants. Variants showing a reduced interaction ($>$ 50% reduction in signal in the coIP fraction; $P<$ 0.05) are indicated in red; variants showing $<$ 50% reduction ($P <$ 0.05) are shown in orange; variants showing a comparable interaction to wild-type NF-SPRED1 ($P>$ 0.05) are indicated in black (see main text for details). IT/RC: NF1 p.Ile1799Thr/Arg1809Cys (double *cis* variant).

(C) Quantification of the signals for the expressed *NF1* (left) and *SPRED1* (right) variants. Variants showing $>$ 50% reduction ($P <$ 0.05) are indicated in red; variants showing $<$ 50% reduction ($P <$ 0.05) are shown in orange; variants expressed at levels comparable to the wild-type control ($P >$ 0.05) are indicated in black (see main text for details). IT/RC: NF1 p.Ile1799Thr/Arg1809Cys (double,*cis* variant).

(D) Representative immunoblot showing the anti-FLAG IP (above) and lysate (below) fractions for *NF1* variants.