Supplementary Online Content

Messiaen L, Yao S, Brems H, et al. Clinical and Mutational Spectrum of Neurofibromatosis Type 1-like Syndrome. *JAMA*. 2009;302(19):2111-2118.

This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Supplemental Methods and Results

Participants and Methods

Mutation analysis

NF1 mutational testing was carried out as previously described.[1-3]

All 7 *SPRED1* exons (reference sequence NM_152594.2), as well as the previously described exon 1 currently known not to be part of the transcript (reference sequence NM_152594.1), were amplified from genomic DNA (primer sequences provided in eTable 1) and sequenced bidirectionally using the ABI BigDye Terminator Sequencing kit on the ABI3100 instrument. In addition, to screen for the presence of a common intragenic Copy Number Change (CNC), we analyzed a random subpopulation of 450/1318 patients by RT-PCR of the entire coding region starting from an aliquot of RNA, previously extracted from puromycin-treated short-term lymphocyte cultures as described (primer sequences provided in eTable 1). We also performed MLPA (MRC-Holland), with probes interrogating all *SPRED1* exons (primer sequences available upon request), in 74 patients previously analyzed by RT-PCR and not showing any polymorphisms in the coding region and in 7 familial patients with >5 CALM +/– freckling and no other NF1-criteria fulfilled in whom no *SPRED1* mutations were found after gDNA-based sequencing because these 7 patients had the specific familial phenotype initially reported in *SPRED1* mutation positive patients.[4] Nucleotide numbering is based on cDNA sequence NM_152594.2 with the A of the start codon marked as +1. All nonsense mutations, out-of-frame deletions and insertions were considered pathogenic. Missense mutations were analyzed for their potential effect on splicing by RT-PCR analysis and sequencing of the relevant regions. All missense mutations were absent in 200 normal control chromosomes. A missense mutation was considered to be a rare benign variant when (1) in vitro studies showed no functional defect in down-regulation of the RAS-MAPK pathway (see below) and (2) the missense mutation was found in an unaffected relative. Missense mutations showing no functional defect in down-regulation of the RAS-MAPK pathway in vitro and no relatives available, were classified as probably rare benign variants when the amino acid was not evolutionary conserved in at least 2 of the following species *Pan troglodytes, Mus musculus, Rattus norvegicus, Canis familiaris, Gallus gallus, Xenopus tropicalis, Tetraodon nigroviridis* and

Drosophila melanogaster, with additional supportive data provided by either PolyPhen (http://genetics.bwh.harvard.edu/pph) or SIFT (Sorting Intolerant from Tolerant; http://sift.jcvi.org).

Functional analysis of missense mutations

Neurite outgrowth of PC12 cells in vitro after stimulation with Nerve Growth Factor (NGF) is highly dependent on MAPK activity.[5] In vitro overexpression of wild-type SPRED1 in PC12 cells will inhibit the MAPK pathway and decrease the number of cells showing neurite outgrowth. Overexpression of SPRED1 inactivating mutants will not be able to down-regulate the MAPK activity and will result in a large number of cells with neurite outgrowth.

Wild-type *SPRED1* cDNA was cloned into a pcDNA3.1 (Invitrogen) construct encoding an N-terminal Flag-tag. *SPRED1* mutants were generated by PCR-directed mutagenesis and verified by sequencing. Wild-type and mutant *SPRED1* constructs were subcloned in a pmax vector (AmaxaBiosystems).

Rat pheochromocytoma-derived PC12 cells, maintained in DMEM with 10% fetal bovine serum and 5% horse serum, were seeded in plates coated with Cellmatrix type I-P (Nitta Gelatin), and co-transfected with pEGFP empty vector (1.0 μg) and Flag-tagged wild-type or mutant SPRED1 (1.0 μg), using Lipofectamin 2000 Reagent (Invitrogen). Twenty-four hours after transfection, PC12 cells were further cultured for an additional 72 hours in the presence or absence of NGF (100 ng/ml), then examined using fluorescent microscopy. Cells with processes longer than 1.5 times the diameter of the cell body were considered as neurite outgrowth positive.[6]

The Elk-1 transcription factor is a substrate for phosphorylation by the activated MAPK pathway. Upon phosphorylation of Elk-1 the transcription of its target genes will be activated after binding of Elk-1 to specific sites in the promoter region. Elk-1 activation was measured by the GAL4 DNA binding domain (DB)/ Elk-1 fusion system (pFA-Elk-1) according to the manufacturer's instructions (PathDetect in vivo signal transduction pathway trans-reporting system, Stratagene). In all Elk-1 reporter assays, 2×10^5 HEK293T cells (ATCC, cat no CRL-11268) were plated and transfected, using the polyethylenimine

method, with 0.1 ng of pFA-Elk1, 10 ng of pFR-Luc (carrying the GAL4 UAS fused luciferase gene) and 10 ng β-galactosidase gene (control), together with various Flag-tagged *SPRED1* constructs. After 24h, cells were treated with 50 ng/ml basic fibroblast growth factor (bFGF) (PeproTech) for 6h, and lysed with Reporter Lysis Buffer (Promega). Luciferase and β -galactosidase activities were analyzed using Luciferase Assay Systems (Promega) and o-nitrophenyl β -galactopilanoside (NacalaiTesque) as a substrate, respectively.[7] Overexpression of co-transfected wild type *SPRED1* constructs in this GAL4/Elk-1 reporter assay will efficiently inhibit the activation of the MAPK pathway after stimulation of the HEK293T cells with bFGF and only low luciferase activity will be measured. When *SPRED1* constructs with an inactivating mutation are used, stimulation with bFGF will result in MAPK pathway activation and a significant increase in luciferase activity is measured. Two different concentrations of each *SPRED1* construct were used for transfection of HEK293T cells (2 ng and 10 ng).

Homology modeling

Secondary structure prediction of mutant p.Thr102Arg and p.Cys74Phe SPRED1 and comparison with known protein structures were carried out using the HHpred server at the Max-Planck Institute for Developmental Biology (http://toolkit.tuebingen.mpg.de/hhpred). The program MODELER[8] was used to generate a single template model of the human EVH1 domain of SPRED1 using the *X. tropicalis* EVH1 domain (Protein Data Bank accession code 1XOD; www.rcsb.org/pdb/home/home.do)[9] as a template. Structures were superimposed using visualization software MacPyMol 2006, developed by DeLano, Open-Source PyMOL 0.99rc6.[10] SPRED1 missense mutations p.Cys74Phe and p.Thr102Arg were modeled using COOT.[11]

Results

Functional and in silico analysis of missense mutations

The functional effect of the missense mutations on the Ras-MAPK pathway was analyzed using two independent assays, differentiation of PC12 cells (eFigure 2A) and Elk-1 reporter assay (eFigure 2B). Differentiation of PC12 cells in vitro after stimulation with

NGF is dependent on MAPK activation. Wild-type SPRED1 can inhibit the RAS-MAPK pathway. Less than 30% of PC12 cells transfected with wild-type SPRED1 showed differentiation whereas 70% of PC12 cells transfected with an empty construct or with a construct containing a known inactivating *SPRED1* mutation showed differentiation. Results comparable with wild-type SPRED1 were observed with SPRED1 mutants p.Cys74Phe, p.Ser149Asn, p.Met188Ile, p.Thr196Ile, p.Thr313Met, p.Asp398Asn and p.Cys433Tyr. SPRED1 mutants p.Thr102Arg and p.Pro415Ala were associated with a high percentage of neurite outgrowth after transfection in PC12 cells, indicating that these mutants were unable to block MAPK activation in PC12 cells after stimulation with NGF (eFigure 2A).

One of the nuclear targets of MAPK is Elk-1; therefore FGF induced MAPK activation can be monitored by measuring Elk-1 dependent transcription. In the Elk-1 reporter assay only the p.Thr102Arg and the p.Pro415Ala SPRED1 mutants were unable to suppress FGF induced Elk-1 dependent transcription activation (eFigure 2B).

For the missense mutations located in the EVH1 domain (p.Cys74Phe, p.Thr102Arg) a theoretical three-dimensional protein model was generated based on the *X. tropicalis* Spred1 EVH1 domain crystal structure (eFigure 2C). EVH1 domains adopt a pleckstrin homology fold which binds and recognizes proline-rich peptide motifs. In Spred proteins, the EVH1 domain is thought to bind peptides in a groove formed by beta-strands β1, β2, β6 and β7. Our model of the human EVH1 domain shows a very similar structural arrangement as other EVH1 domains with a potential peptide-binding groove in the core of the domain. The p.Thr102Arg mutation is very likely to impair functionality of the EVH1 domain as it seems to block the access to the peptide-binding groove. Cys74 is located in an outer strand of the EVH1 beta-sandwich (β4) and the predicted effect of the p.Cys74Phe SPRED1 mutant is less dramatic compared to the p.Thr102Arg mutant. In addition to functional impairment, p.Pro415Ala and p.Thr102Arg were found in an affected first-degree relative (eTable 2), and thus both are classified as pathogenic mutations. p.Cys74Phe, p.Ser149Asn, p.Met188Ile, p.Thr196Ile, pThr313Met, p.Asp398Asn, and p.Cys433Tyr showed similar functional activity as wild-type SPRED1 toward the RAS-MAPK pathway in both functional tests (eFigure 2, A and B). p.Ser149Asn, p.Met188Ile, p.Thr196Ile, pThr313Met, p.Asp398Asn are not evolutionary conserved in all species and p.Cys74Phe, p.Ser149Asn,

p.Asp398Asn and p.Cys433Tyr were detected in an unaffected relative. Taking all data into account, p.Cys74Phe, p.Ser149Asn, p.Asp398Asn, and p.Cys433Tyr are rare benign variants. For mutations p.Met188Ile, p.Thr313Met and p.Thr196Ile no family segregation study could be performed and since effects outside the MAPK pathway cannot be excluded at this moment we classify these mutations as probably benign variants. Data supporting classification of missense mutations are summarized in eTable 3B.

References

1. Messiaen L, Wimmer K. NF1 mutational spectrum. In: Kaufmann D, ed. *Monographs Hum Genet Neurofibromatoses.* Basel, Switzerland: Karger; 2008:63-77.

2. Messiaen LM, Callens T, Mortier G, et al. Exhaustive mutation analysis of the *NF1* gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects. *Hum Mutat*. 2000;15(6):541-555.</jrn>

3. Wimmer K, Yao S, Claes K, et al. Spectrum of single- and multiexon *NF1* copy number changes in a cohort of 1,100 unselected NF1 patients. *Genes Chromosomes Cancer*. 2006;45(3):265-276.</jrn>

4. Brems H, Chmara M, Sahbatou M, et al. Germline loss-of-function mutations in *SPRED1* cause a neurofibromatosis 1-like phenotype. *Nat Genet*. 2007;39(9):1120-1126.

5. Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*. 1995;80(2):179-185.

6. Wakioka T, Sasaki A, Kato R, et al. Spred is a Sprouty-related suppressor of Ras signalling. *Nature*. 2001;412(6847):647-651.

7. Nonami A, Kato R, Taniguchi K, et al. Spred-1 negatively regulates interleukin-3-mediated ERK/mitogen-activated protein (MAP) kinase activation in hematopoietic cells. *J Biol Chem*. 2004;279(50):52543-52551.

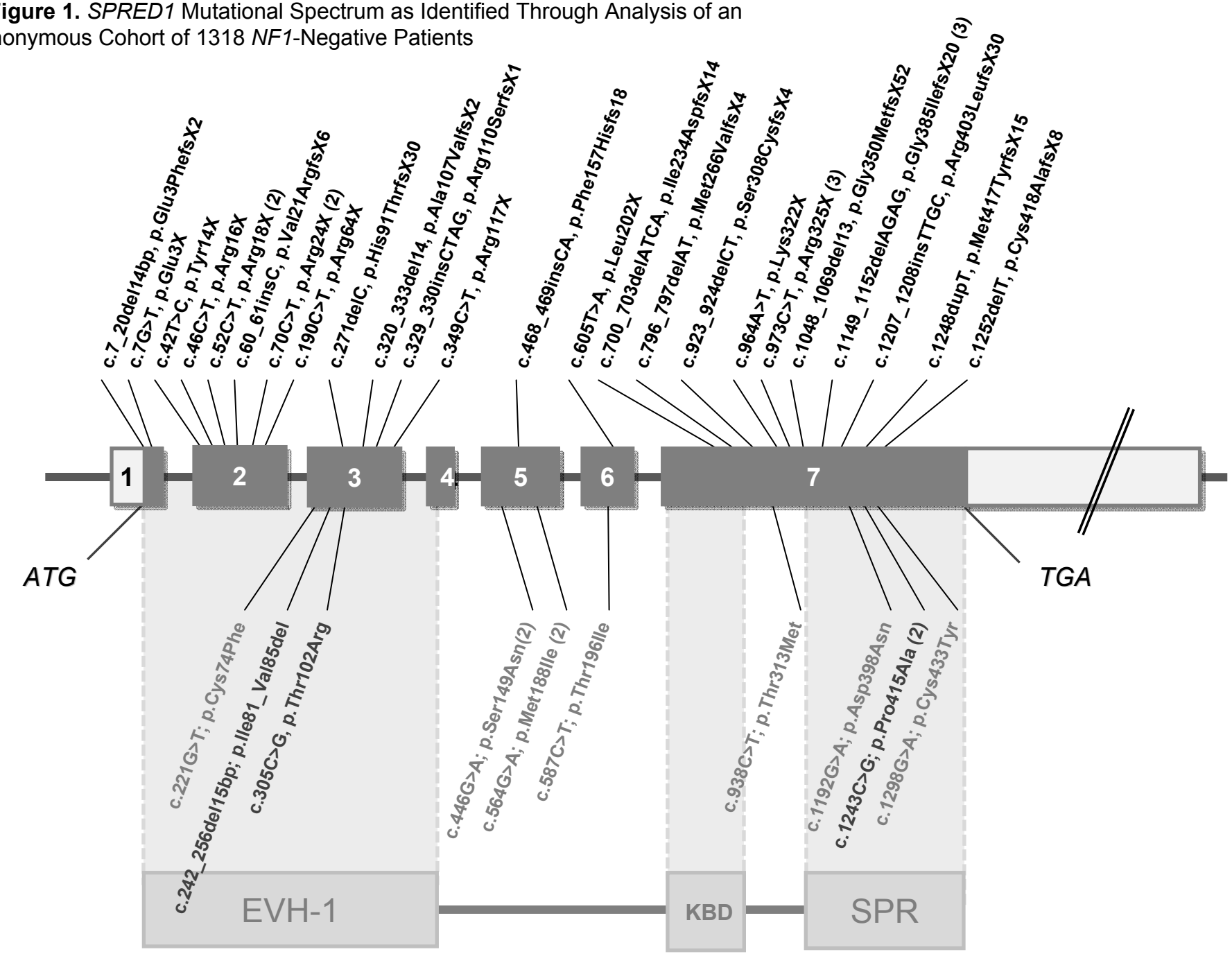
8. Sali A, Blundell TL. Comparative protein modeling by satisfaction of spatial restraints. *J Mol Biol*. 1993;234(3):779-815.

9. Harmer NJ, Sivak JM, Amaya E, Blundell TL. A crystal structure of the *X tropicalis* Spred1 EVH1 domain suggests a fourth distinct peptide-binding mechanism within the EVH1 family. *FEBS Lett*. 2005;579(5):1161-1166.

10. DeLano WL. PyMol Molecular Graphics System. 2006. http://www.pymol.org.

11. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr*. 2004;60(pt 12)(pt 1):2126-2132.

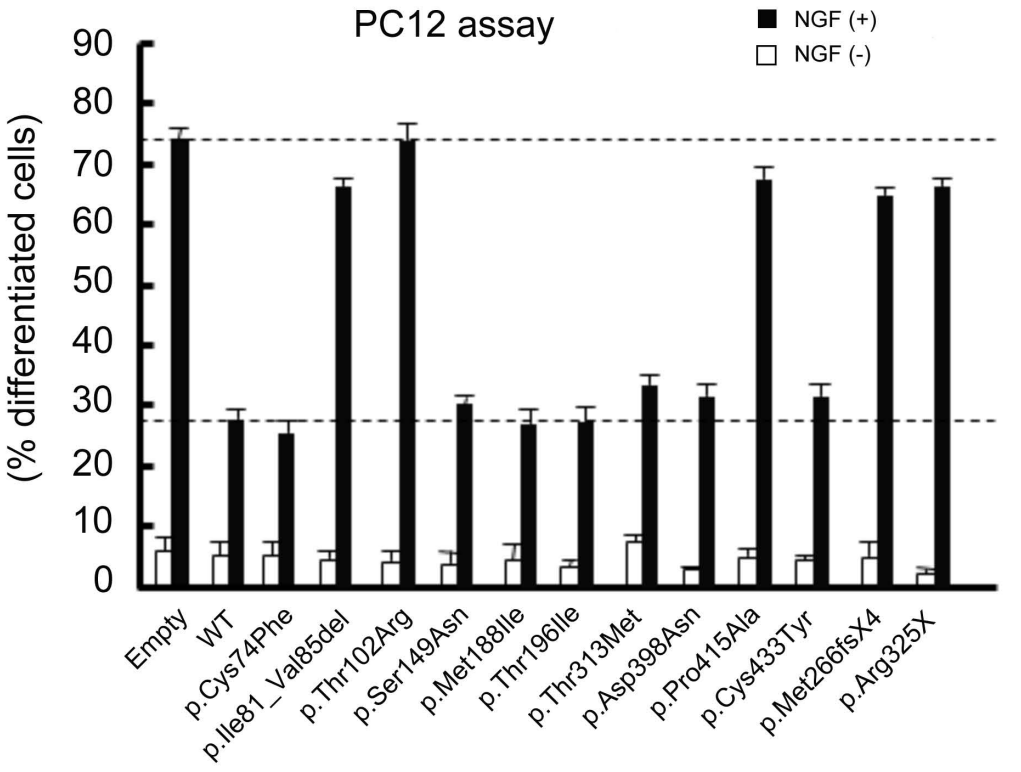
eFigure 1. *SPRED1* Mutational Spectrum as Identified Through Analysis of an Anonymous Cohort of 1318 *NF1*-Negative Patients

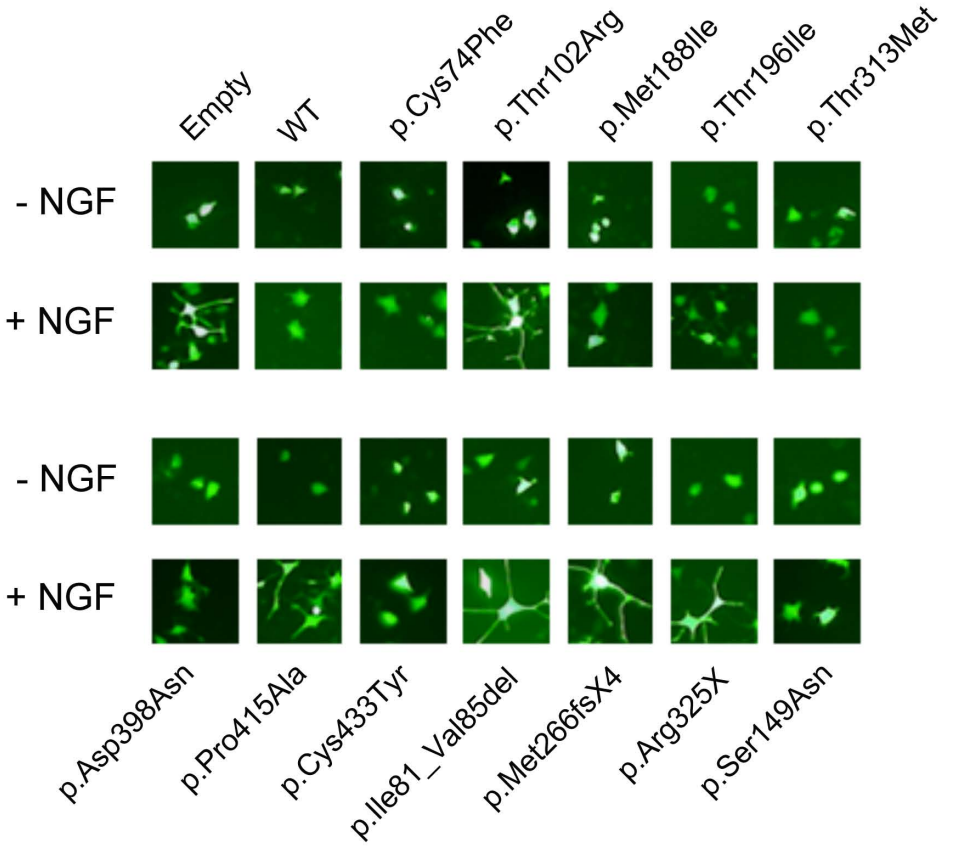


eFigure 1. *SPRED1* Mutational Spectrum as Identified Through Analysis of an Anonymous Cohort of 1318 *NF1*-Negative Patients (continued)

Exon-intron structure of *SPRED1* showing non-coding sequences as open boxes, protein-coding sequences as dark-grey boxes. Exons are numbered from 1 to 7. Grey boxes represent SPRED1 structural domains: EVH1: Ena/vasodilatator-stimulated phosphoprotein homology domain; KBD: KIT-binding domain; SPR: SPROUTY-related domain. Numbers between parenthesis indicate the number of unrelated patients identified with a given mutation. Mutations displayed in black are pathogenic Loss-of-Function; mutations displayed in gray are likely benign rare missense variants. Top: 24 different truncating Loss-of-Function mutations (nonsense mutations, and out-of-frame insertions/deletions). Bottom: 3 different non-truncating Loss-of-Function mutations (1 in-frame deletion, 2 missense mutations, shown in black) and 7 different likely non-pathogenic missense mutations (shown in grey). Seven out of the 43 patients with a pathogenic or likely non-pathogenic *SPRED1* mutation belonged to the anonymous UAB cohort previously reported in Brems et al.[4]

eFigure 2. Effect of SPRED1 Constructs on Differentiation of PC12 Cells

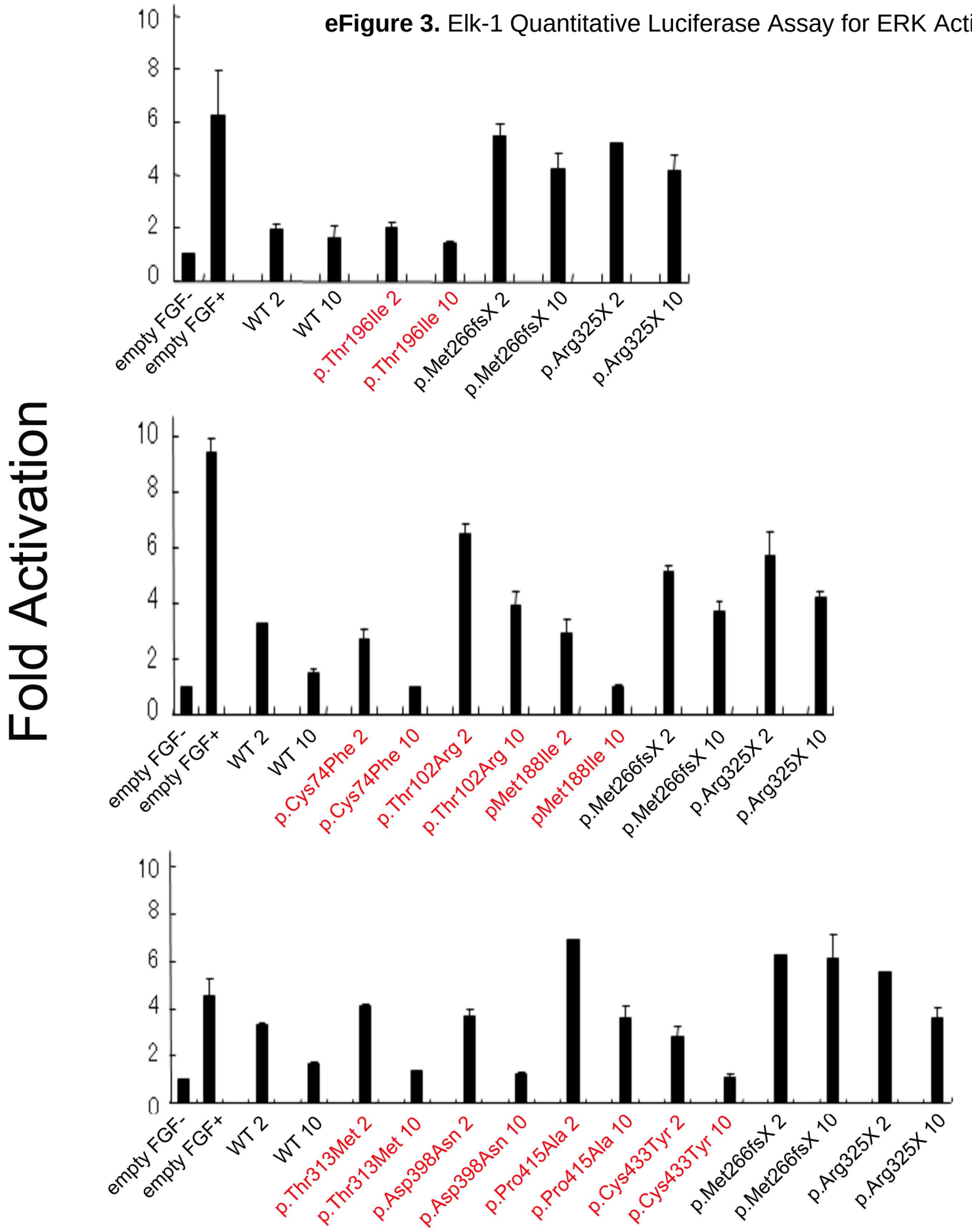




eFigure 2. Effect of SPRED1 Constructs on Differentiation of PC12 Cells (continued)

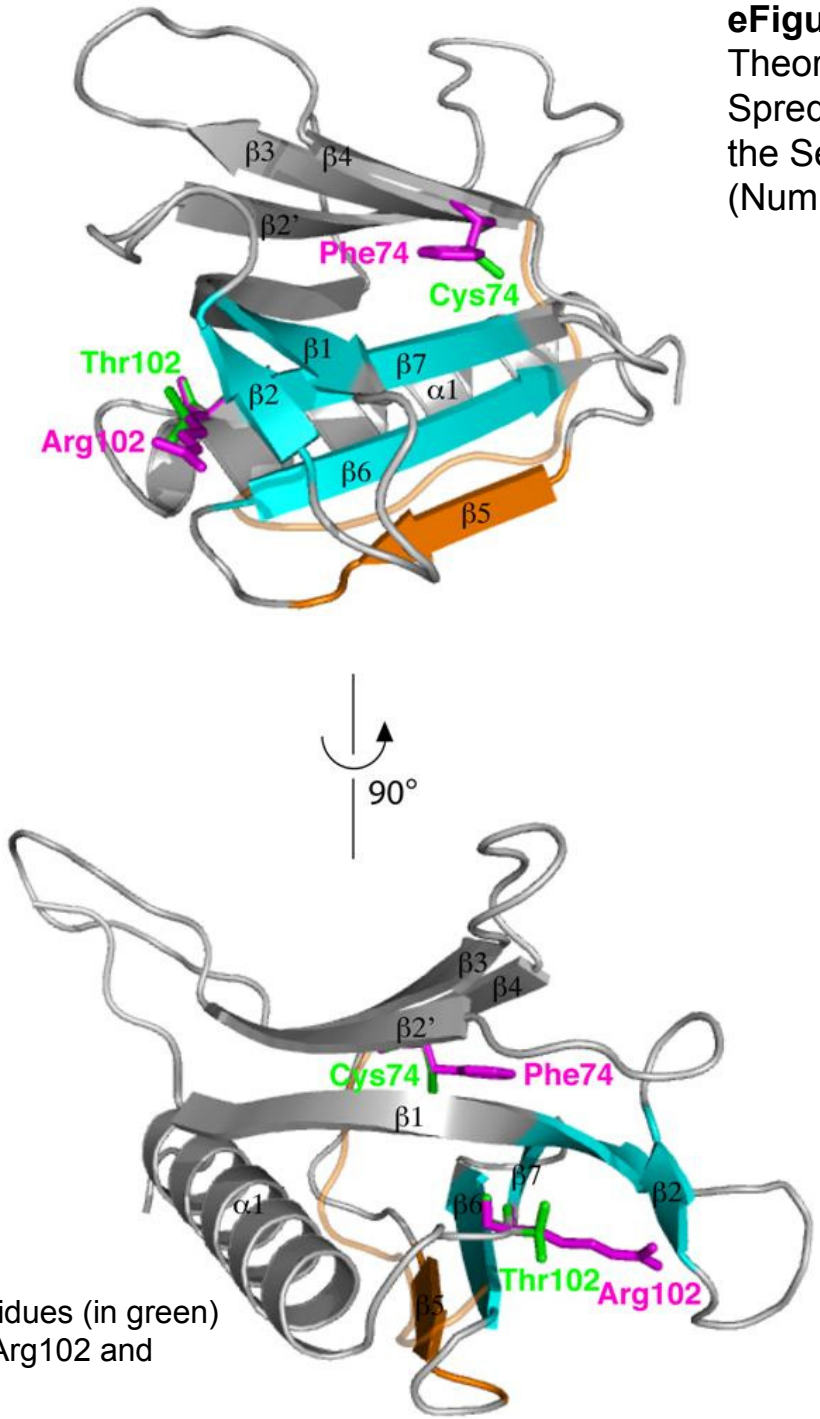
PC12 cells were co-transfected with a pEGFP expression vector, empty vector or a Flag-tagged wild-type (WT) or mutant SPRED1 containing expression vector and stimulated with Nerve Growth Factor (NGF). Top panel: The percentage of differentiated cells is shown. Error bars represent standard deviation. Constructs containing known inactivating mutations p.Met266fsX4 and p.Arg325X and the WT SPRED1 are in each experiment used as controls. Cells transfected with mutations p.Ile81_Val85del, p.Thr102Arg, and p.Pro415Ala showed the same percentage of differentiated cells as cells transfected with empty vector or as cells transfected with known inactivating mutations p.Met266fsX4 and p.Arg325X. Expression vectors containing other missense mutations inhibit differentiation of PC12 cells similar to cells transfected with the wild type SPRED1 construct. Lower panel: Representative fluorescent microscopy pictures of PC12 cells co-transfected with a pEGFP expression vector, empty vector or a Flag-tagged wild-type (WT) or mutant SPRED1 containing expression vector +/− stimulation with Nerve Growth Factor (NGF). Cells with processes longer than 1.5 times the diameter of the cell body were considered as neurite outgrowth (differentiated cells).

eFigure 3. Elk-1 Quantitative Luciferase Assay for ERK Activation



eFigure 3. *Elk-1* Quantitative Luciferase Assay for ERK Activation (continued)

Empty vector or vectors carrying wild-type (as a control) or mutant SPRED1 (2 ng or 10 ng of plasmid, indicated as 2 and 10) were transfected into HEK293T cells along with Elk-1 reporter plasmids. Known inactivating mutations p.Arg325X, p.Met266fsX4 were used as positive controls. Elk-1 activation is measured as an increase in luciferase activity after stimulation with bFGF. Elk-1 activation in the cells containing *SPRED1* missense mutations are indicated in red. The bars represent the average increase in luciferase activity of 3 replicates and error bars represent the standard deviation. Wild-type SPRED1 vector suppresses efficiently bFGF induced Elk-1 activation in comparison to empty vector. On the contrary known inactivating *SPRED1* mutations (p.Arg325X, p.Met266fsX4) and *SPRED1* missense mutations p.Pro415Ala and p.Thr102Arg were not able to down-regulate the increase in luciferase activity after bFGF stimulation. The other missense mutations suppress Elk-1 activation similar to wild-type *SPRED1*. The results of the different missense mutations are shown together with the results of empty vector, vector containing wild type SPRED1 and known inactivating mutations (p.Arg325X, p.Met266fsX4) used in the same set of experiments (3 sets of experiments to cover all missense mutations).



eFigure 4. Ribbon Diagram of the Theoretical Model of the Human Spred1 EVH1 Domain With Labels for the Secondary Structure Elements (Numbered as in Harmer et al[9])

Superimposed onto the wild-type residues (in green) are the relevant modeled mutations Arg102 and Phe74 (in magenta).

eTable 1. Primer Sequences for Amplification *SPRED1* Exons and RT-PCR Exons 1-7

Exon	Forward primer	Reverse primer
Previous "exon 1"	5′-*TTCGCCTCCTTCTCTCGC-3′	5′-#GCAAGCTTTCCGGCACC-3′
Exon 1	5′-*CGTTCTGGGTGAGGCAT-3′	5′-#GCAAGCTTTCCGGCACC-3′
Exon 2	5′-*CATGTTAACTAATGTGTTCTTTGGTTTC-3′	5′-#TCACAACAGTATACTAATCATAACTTTAAACAGA-3′
Exon 3	5′-*CTCAGTTTGTATTTATGAGCTACATTAGAT-3′	5′-#CTTTAAGAAAGATAACCTCTACTATATATAACCGAT-3′
Exon 4	5′-*AATTAGTCATTAATACTGGACTCTAAGACAA-3′	5′-#TTTATGTTTACAAATTCCTTCCAAGCC-3′
Exon 5	5′-*CATAGCGATGGTAGCGATATATACT-3′	5′-#ACACATATAAACAACGCGCC-3′
Exon 6	5′-*CTGCTAATAAATTATGAGGTTTTGGAACA-3′	5′-#AGTTAAATTCCATATCACAAGATAAACTAACA-3′
Exon 7.1	5′-*CATAGCCCTCATTGCAGTT-3′	5′-#GTCTGGAGCATCCTGACATT-3′
Exon 7.2	5′-*CTGCGTATACTGCCAGGAAA-3′	5′-#GTGTGGGCTCAATGATACC-3′
Exon 1-7 (cDNA)	5′-GCCTGCTGTTGCTCCTCCAT-3′	5′-GCTTCCTCCATGTGTGGGCTC-3′

5′-*: at the 5′-end M13-specific forward universal sequencing nucleotides were attached; 5′-#: at the 5′-end M13-specific reverse universal sequencing nucleotides were attached.

eTable 2. Clinical Features of 42 Individuals With a Loss of Function *SPRED1* Mutation From 23 Families (Clinical Cohort)

Patient	F/S	Mutation (exon)	Type of mutation	Age (y)/ Gender	CALM	Freckling	NIH#	HC[d] (percentile)	Length[d] (percentile)	Development	Other findings
S13-II1	F	c.7G>T; p.Glu3X (1)	NS	6/M	>6	None	-	50th	25th	hyperactive	
S13-I1	F	c.7G>T; p.Glu3X (1)	NS	37/M	4	None	-	U	U	Normal	mild hearing loss
S11-II1	F	c.7_20del14 bp; p.Glu3PhefsX2 (1)	FS	3.5/Fe	>6	A&I	+	10-25th	50th	Normal	clinodactyly 5th finger
S11-I2	F	c.7_20del14 bp; p.Glu3PhefsX2 (1)	FS	27/Fe	6	U	+	U	U	Normal	clinodactyly 5th finger; congenital mild PS, MVP
S3-II1	F	c.42T>C; p.Tyr14X (2)	NS	2/M	4[a]	1 (outside LA)	-	>97th	50-75th	difficulties with pronunciation	2 CALM in 1-yo sib, S3-II2; WT *SPRED1*
S3-I1	F	c.42T>C; p.Tyr14X (2)	NS	35/M	<6	None	-	>97th	50-75th		
S21	S	c.52C>T; p.Arg18X (2)	NS	9.5/M	22	thoracic	-	75-90th	>97th	speech delay; normal intelligence	de novo, no CALM in parents; meatal stenosis, hypertelorism, mild hearing loss L ear
S16	U	c.60_61insC; p.Val21ArgfsX6 (2)	FS	2/Fe	14	1 CALM LA	-	50-75th	25-50th	hyperactive	mild pectus excavatum; mild retrognatia, simple ears. Mother: no signs, WT *SPRED1*; father NA.

eTable 2 (continued)

S8	S	c.70C>T; p.Arg24X (2)	NS	4/Fe	14	None	-	3rd	10th	hyperactive	de novo; no signs in parents and in 2-mo sib
S18-II1	F	c.70C>T; p.Arg24X (2)	NS	9/Fe	>6	BA	+	50th	10th	Normal	
S18-II2	F	c.70C>T; p.Arg24X (2)	NS	$7^{5/12}$/M	>6[c]	None	+	50th	10-25th	Normal	
S18-II3	F	c.70C>T; p.Arg24X (2)	NS	2/Fe	0	None	-	80th	25th	Normal	
S12 (UAB31)	S	c.242_256del15bp; p.Ile81_Val85del (3)	IFD	4/M	>15	1 in LA/RI Pigmentation in RA	+	60th	50th	Normal; normal brain MRI	*de novo,* no signs in parents and 15-mo and 9-yo sibs; pectus excavatum (Figure 3a)
S24-II1	F	c.305C>G; p.Thr102Arg (3)	LOF M	17/M	>6	BI; 2-3 in LA	+	50-75th	<5th	normal; completed high school	Large R temporal venous anomaly in brain; progressive dystonia of unexplained etiology
S24-I2	F	c.305C>G; p.Thr102Arg (3)	LOF M	47/Fe	>6	BA&I	+	25-50th	50th	Normal; Higher degree	vascular anomaly left lower leg
S4-II2	F	c.320_333del14bp; p.Ala107ValfsX2 (3)	FS	$6^{5/12}$/M	>6	BA &I/R Neck; faint	+	>97th	50-75th	Normal	3 CALs in 8-yo sib, S4-II1, WT *SPRED1*

eTable 2 (continued)

S4-II3	F	c.320_333del14bp; p.Ala107ValfsX2 (3)	FS	4/M	>6	BA, few, faint	+	50-75th	25-50th	Normal	
S4-I2	F	c.320_333del14bp; p.Ala107ValfsX2 (3)	FS	42/M	>6	some neck	+	U	U	Normal	
S19-II1	S	c.605T>A; p.Leu202X (6)	NS	$7^{4/12}$/Fe	>6	None	-	50th	25-50th	Normal, bright	de novo; 1 CALM in mother S19-I2; no signs in father
S6	S	c.700_703del4bp; p.Ile234AspfsX14 (7)	FS	$1^{3/12}$/M	12-15, mostly <5mm	None	-	>95th	>95th	Normal	de novo, no signs in parents
S2-II1	F	c.923_924delCT; p.Ser308CysfsX4 (7)	FS	4/Fe	10	None	-	25-50th	25th	Normal	9-yo sib, WT *SPRED1*, no signs
S2-I1	F	c.923_924delCT; p.Ser308CysfsX4 (7)	FS	50/M	1	None	-	97th	10-25th		
S10-III1* (UAB48)	F	c.964A>T; p.Lys322X (7)	NS	$3^{5/12}$/Fe	9[b]	None	-	>97th	75th	mild speech delay, difficulties with pronunciation	bright blue eyes, downslanting palpebral fissures, short neck, Noonan-like, distal pectus excavatum. PTPN11 and PTEN testing negative
S10-II2	F	c.964A>T; p.Lys322X (7)	NS	33/Fe	8[b]	None	-	97th	25-50th	Normal	

eTable 2 (continued)

S10-I2 *	F	c.964A>T; p.Lys322X (7)	NS	68/Fe	2	None	-	90-97th	5th	Normal	2 lipomas
S17-II1	F	c.973C>T; p.Arg325X (7)	NS	8.5/Fe	10	2 I	+	10th	25th	Abnormal, special education; attention deficit	Adopted
S17-II2	F	c.973C>T; p.Arg325X (7)	NS	5/M	10	None	+	25-50th	10th	language delay; speech therapy	Adopted
S20	F?	c.973C>T; p.Arg325X (7)	NS	40/Fe	7	L A	+	50th	25-50th	Normal	dermoid tumor L ovary; 12-yo daughter with >6 CALM: NA
S9-I2	F	c.1149_1152delAGAG; p.Ser385IlefsX20 (7)	FS	35/Fe	>6	BA&I	+	U	U	Normal	L forearm: angiolipoma; L index finger: tenosynovial giant cell tumor localized type
S9-II1	F	c.1149_1152delAGAG; p.Ser385IlefsX20 (7)	FS	4/M	8	None	+	90-97th	25th	Normal	M sib $1^{6/12}$-yo, WT *SPRED1*, no signs

eTable 2 (continued)

S23-III3	F	c.1149_1152delAGAG; p.Ser385IlefsX20 (7)	FS	$0^{11/12}$/Fe	16	None	-	50th	50-75th		postaxial polydactyly hands and feet, anal stenosis (proband S23-III3 submitted due to classic NF1 in paternal cousin, S23-III1, mutation c.2755delG); 2.5-yo sibling of S23-III3, no signs, WT *SPRED1*
S23-II3	F	c.1149_1152delAGAG; p.Ser385IlefsX20 (7)	FS	33/M	6	None	-	U	U	Normal	30-yo M sibling, S23-II2 (father of NF1 patient S23-III1): irregular, spotty, faint hyperpigmented region, WT *SPRED1*, WT *NF1*
S23-I2	F	c.1149_1152delAGAG; p.Ser385IlefsX20 (7)	FS	55/Fe	6	None	-	U	U	Normal	

eTable 2 (continued)

S22-III4	S	c.1207_1208insTTGC; p.Arg403LeufsX30 (7)	FS	7/Fe	6[a]	None	-	90th	75th	Normal; at or above age level	de novo; five 1st, 2nd and 3rd degree relatives of Irish/Scottish ancestry with 2-4 typical and atypical CALMs but no mutation in NF1 or *SPRED1*
S14	F?	c.1243C>G; p.Pro415Ala (7)	LOF M	$3^{10/12}$/M	12	Axillary pigmentation: 7 mm diameter CALM	-	20th	25th	language delay, fine motor delay	father multiple CALM: NA child in foster care
S 15-II1	F	c.1243C>G; p.Pro415Ala (7)	LOF M	12/Fe	>6	none	+	U	U	U	
S15-12	F	c.1243C>G; p.Pro415Ala (7)	LOF M	36/Fe	>6	A	+	U	U	U	
S1-II1	F	c.1248dupT; p.Met417TyrfsX15 (7)	FS	21mo/Fe	>6	none	-	75th	10-25th		
S1-I2	F	c.1248dupT; p.Met417TyrfsX15 (7)	FS	32/Fe	>6	none	-	50th	U	Normal; hx of seizures	
S7-II1	F	c.1252delT; p.Cys418AlafsX6 (7)	FS	$4^{9/12}$/M	10	LI/few	+	>>97th	75-90th	language delay, PDD, hypotonia, Lack of coordination, behavior and attention problems, seizures	

eTable 2 (continued)

S7-II2*	F	c.1252delT; p.Cys418AlafsX6 (7)	FS	2 $^{4/12}$/Fe	>6	1L & 1RA;1 RI	+	90-95th	10-25th	no DD	Unequal gluteal folds
S7-I2	F	c.1252delT; p.Cys418AlafsX6 (7)	FS	33y/M	4; 2< 5 mm	1 in L A	+	U	U	Normal	2 brothers with CALM

Bold: proband. # "-"denotes that NIH criteria are not fulfilled; "+" denotes that NIH criteria are fulfilled. Scoring of CALM according to NIH criteria: >5mm in pre-pubertal and >15mm in post-pubertal individuals. NS: nonsense mutation, FS: frame shift mutation, IFD: in frame deletion, LOF M: loss-of-function missense mutation, S: sporadic; F: familial; I: inguinal; A: axillary; L: left; R: right; B: bilateral, Fe: female; M: male; NA: not available; U: unknown; WT: wild type. PS: pulmonic stenosis; MVP: mitral valve prolapsed; Hx: history; PDD: pervasive developmental delay.

[a]And multiple smaller hyperpigmented spots.

[b]With diffuse hyperpigmentation.

[c]With hypopigmented lesions.

[d]Percentiles according to growth charts of CDC United States (http://www.cdc.gov/nchs/data/ad/ad314.pdf).

*relative macrocephaly.

eTable 3. Clinical Features in 9 Individuals With a Likely Nonpathogenic *SPRED1* Missense Mutation

Patient	Mutation	Age/Gender	# CALM	Freckling	Comments
S5/SB4	c.221G>T; p.Cys74Phe	30/F	>6	Only in hyperpigmented quadrant	Segmental pigmentary NF phenotype; no *NF1* mutation in blood, *SPRED1* p.Cys74Phe also present in mother with no pigmentary or any other signs, Caucasian, clinical testing
S26/SB1-II1 (UAB43)	c.446G>A; p.Ser149Asn	5/F	5 (including 3 with ragged borders)	none	Same mutation in father, Jewish ancestry, with no clinical signs, clinical testing
AC-1	c.446G>A; p.Ser149Asn	2/M	<6 CALM with ragged borders	none	No other data
AC-2	c.564>A; p.Met188Ile	7/F	0	0	Caucasian girl with an isolated "swelling" on the trunk; no other data
AC-3	c.564>A; p.Met188Ile	2/F	>6	L inguinal	Asian girl; no other data
AC-4	c.587C>T; p.Thr196Ile	10/M	>6 smooth and ragged borders	0	Abnormal development (IQ: 52); no other data
AC-5	c.938C>T; p.Thr313Met	2/M	<6 CALs	none	Cortical cysts; no other data
S25/SB5-IV2	c. 1192G>A; p.Asp398Asn	6.5/M	3	none	Hispanic boy with macrosomia, pulmonic stenosis, hypertelorism, short narrow palpebral fissures, low posterior hairline, posteriorly rotated ears, hyperactivity and short neck. Sequence analysis for *PTPN11*, *KRAS*, *RAF1 and SOS1* was negative for a pathogenic mutation (clinical testing reported by a commercial US laboratory). *SPRED1* p.Asp398Asn was present in his 20-yo half-sister and 45-yo mother both having only 1 CALM and no other signs. The 20-yo half-brother with classic NF1 (c.99A>G; p.Lys33Lys; r.100_204del105) did also carry *SPRED1* p.Asp398Asn.
S42/SB3	c.1298G>A; p.Cys433Tyr	32/F	0	none	Mother (Hispanic) of a child with severe NF1. The NF1 patient carries the *NF1* mutation c.4911dupT but not the *SPRED1* missense identified in his mother

F: female; M: male.

eTable 4. Evaluation of Missense Mutations Using Functional Assays, Evolutionary Conservation, *In Silico* Prediction (by SIFT and Polyphen) and Family Studies

Name Mutation	Elk-reporter assay	PC12-assay	Evolutionary conservation	SIFT	PolyPhen	Family studies	Conclusion
p.Cys74Phe	WT	WT	Conserved	Not Tolerated	Probably damaging	Not supportive	Rare benign variant
p.Thr102Arg	Defect	Defect	Conserved	Not Tolerated	Probably damaging	Supportive	LOF pathogenic
p.Ser149Asn	WT	WT	Not conserved	Tolerated	Benign	Not supportive	Rare benign variant
p.Met188Ile	WT	WT	Not Conserved	Tolerated	Benign	NA	Likely rare benign variant
p.Thr196Ile	WT	WT	Not Conserved	Tolerated	Benign	NA	Likely rare benign variant
p.Thr313Met	WT	WT	Not Conserved	Tolerated	Possibly damaging	NA	Likely rare benign variant
p.Asp398Asn	WT	WT	Not Conserved	Tolerated	Benign	Not supportive	Rare benign variant
p.Pro415Ala	Defect	Defect	Conserved	Not Tolerated	Probably damaging	Supportive	LOF pathogenic
p.Cys433Tyr	WT	WT	Conserved	Not Tolerated	Benign	Not supportive	Rare benign variant

WT: Wild-type; NA: not available; LOF: loss-of-function. Family studies: Not supportive: indicates that the missense mutation also was found in at least 1 non-affected adult relative; Supportive: indicates that the missense mutation segregated with the phenotype within a family.

eTable 5. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) to Detect a *SPRED1* Mutation in Cohorts of *NF1*-Negative Patients With Specific Combinations of Clinical Features (Between Parentheses: 95% Confidence Interval)

Clinical features	**No *NF1***		**Sensitivity**	**Specificity**	**PPV**	**NPV**
	No *SPRED1*	***SPRED1***				
ALL	1284	34	1 (.915-1.0)	0 (0.0-.002)	.026 (.019-.034)	-
>5 CALM	606	31	.912 (.763-.981)	.528 (.512-.558)	.049 (.035-.065)	.996 (.987-.999)
>5 CALM +/− freckling + fam.	16	18	.529 (.376-.678)	.988 (.981-.992)	.529 (.376-.678)	.988 (.981-.992)
>5 CALM +/− freckling + fam. + no other crit.	7	18	.529 (.376-.678)	.995 (.990-.997)	.720 (.538-.860)	.988 (.981-.992)
>5 CALM +/− freckling + spor. + no other crit.	530	13	.382 (.243-.538)	.587 (.564-.610)	.024 (.014-.038)	.973 (.961-.982)
Any CALM +/− freckling + no other crit.	954	34	1 (.915-1.0)	.257 (.237-.278)	.034 (.025-.045)	1 (.990-1.0)
>5 CALM +/− freckling + no other crit.	537	31	.912 (.763-.981)	.582 (.559-.605)	.055 (.040-.073)	.996 (.990-.999)