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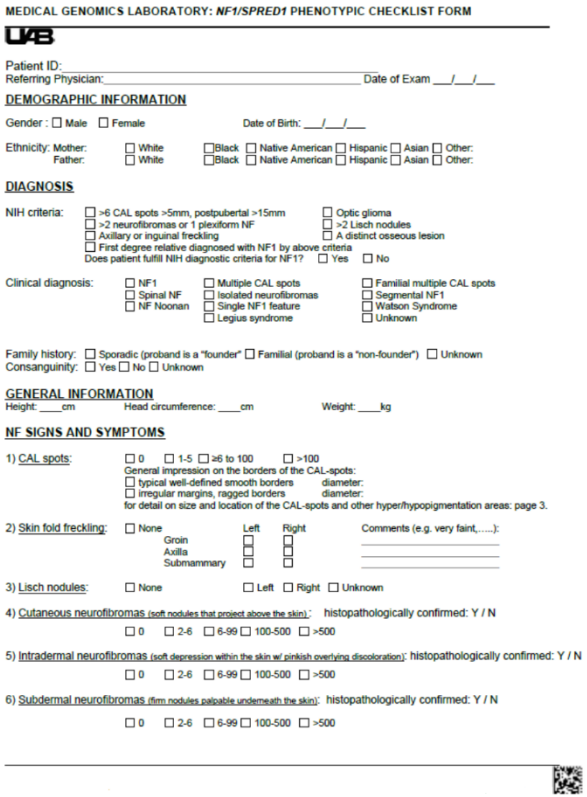
Supplemental Data

Genotype-Phenotype Correlation in NF1: Evidence for a More Severe Phenotype Associated with Missense Mutations Affecting *NF1* Codons 844–848

Magdalena Koczkowska, Yunjia Chen, Tom Callens, Alicia Gomes, Angela Sharp, Sherrell Johnson, Meng-Chang Hsiao, Zhenbin Chen, Meena Balasubramanian, Christopher P. Barnett, Troy A. Becker, Shay Ben-Shachar, Debora R. Bertola, Jaishri O. Blakeley, Emma M.M. Burkitt-Wright, Alison Callaway, Melissa Crenshaw, Karin S. Cunha, Mitch Cunningham, Maria D. D'Agostino, Karin Dahan, Alessandro De Luca, Anne Destrée, Radhika Dhamija, Marica Eoli, D. Gareth R. Evans, Patricia Galvin-Parton, Jaya K. George-Abraham, Karen W. Gripp, Jose Guevara-Campos, Neil A. Hanchard, Concepcion Hernández-Chico, LaDonna Immken, Sandra Janssens, Kristi J. Jones, Beth A. Keena, Aaina Kochhar, Jan Liebelt, Arelis Martir-Negron, Maurice J. Mahoney, Isabelle Maystadt, Carey McDougall, Meriel McEntagart, Nancy Mendelsohn, David T. Miller, Geert Mortier, Jenny Morton, John Pappas, Scott R. Plotkin, Dinel Pond, Kenneth Rosenbaum, Karol Rubin, Laura Russell, Lane S. Rutledge, Veronica Saletti, Rhonda Schonberg, Allison Schreiber, Meredith Seidel, Elizabeth Siqveland, David W. Stockton, Eva Trevisson, Nicole J. Ullrich, Meena Upadhyaya, Rick van Minkelen, Helene Verhelst, Margaret R. Wallace, Yoon-Sim Yap, Elaine Zackai, Jonathan Zonana, Vickie Zurcher, Kathleen Claes, Yolanda Martin, Bruce R. Korf, Eric Legius, and Ludwine M. Messiaen



MEDICAL GENOMICS LABORATORY: *NF1/SPRED1* PHENOTYPIC CHECKLIST FORM

UAB

Patient ID:_____
Referring Physician:_____ Date of Exam ___/___/___

DEMOGRAPHIC INFORMATION

Gender : ☐ Male ☐ Female Date of Birth: ___/___/___

Ethnicity: Mother: ☐ White ☐ Black ☐ Native American ☐ Hispanic ☐ Asian ☐ Other:
Father: ☐ White ☐ Black ☐ Native American ☐ Hispanic ☐ Asian ☐ Other:

DIAGNOSIS

NIH criteria: ☐ >6 CAL spots >5mm, postpubertal >15mm ☐ Optic glioma
☐ >2 neurofibromas or 1 plexiform NF ☐ >2 Lisch nodules
☐ Axillary or inguinal freckling ☐ A distinct osseous lesion
☐ First degree relative diagnosed with NF1 by above criteria
Does patient fulfill NIH diagnostic criteria for NF1? ☐ Yes ☐ No

Clinical diagnosis: ☐ NF1 ☐ Multiple CAL spots ☐ Familial multiple CAL spots
☐ Spinal NF ☐ Isolated neurofibromas ☐ Segmental NF1
☐ NF Noonan ☐ Single NF1 feature ☐ Watson Syndrome
☐ Legius syndrome ☐ Unknown

Family history: ☐ Sporadic (proband is a "founder" ☐ Familial (proband is a "non-founder") ☐ Unknown
Consanguinity: ☐ Yes ☐ No ☐ Unknown

GENERAL INFORMATION

Height: ____cm Head circumference: ____cm Weight: ____kg

NF SIGNS AND SYMPTOMS

1) CAL spots: ☐ 0 ☐ 1-5 ☐ ≥6 to 100 ☐ >100
General impression on the borders of the CAL-spots:
☐ typical well-defined smooth borders diameter:
☐ irregular margins, ragged borders diameter:
for detail on size and location of the CAL-spots and other hyper/hypopigmentation areas: page 3.

2) Skin fold freckling: ☐ None

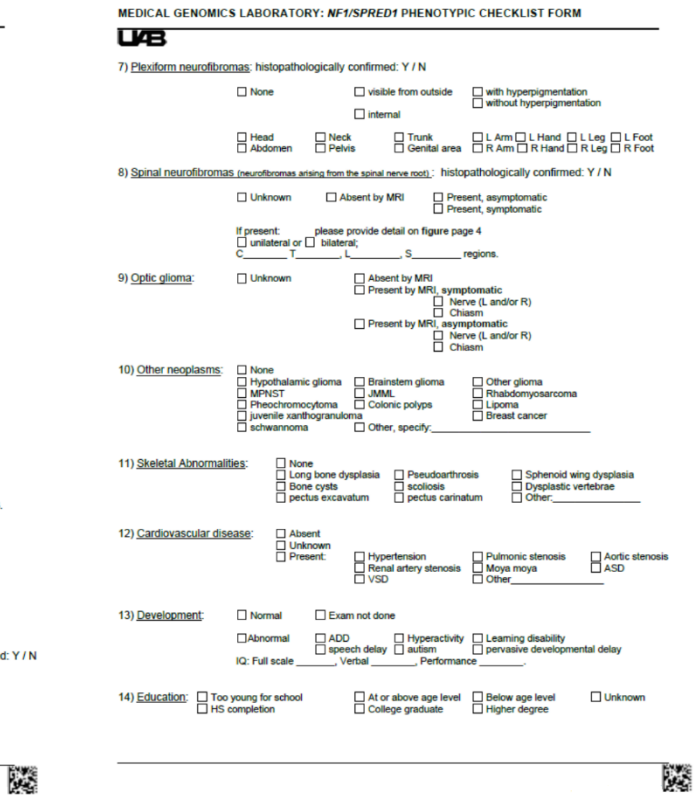
	Left	Right	Comments (e.g. very faint,....):
Groin	☐	☐	
Axilla	☐	☐	
Submammary	☐	☐	

3) Lisch nodules: ☐ None ☐ Left ☐ Right ☐ Unknown

4) Cutaneous neurofibromas (soft nodules that project above the skin): histopathologically confirmed: Y / N
☐ 0 ☐ 2-6 ☐ 6-99 ☐ 100-500 ☐ >500

5) Intradermal neurofibromas (soft depression within the skin w/ pinkish overlying discoloration): histopathologically confirmed: Y / N
☐ 0 ☐ 2-6 ☐ 6-99 ☐ 100-500 ☐ >500

6) Subdermal neurofibromas (firm nodules palpable underneath the skin): histopathologically confirmed: Y / N
☐ 0 ☐ 2-6 ☐ 6-99 ☐ 100-500 ☐ >500



MEDICAL GENOMICS LABORATORY: *NF1/SPRED1* PHENOTYPIC CHECKLIST FORM

UAB

7) Plexiform neurofibromas: histopathologically confirmed: Y / N
☐ None ☐ visible from outside ☐ with hyperpigmentation ☐ without hyperpigmentation ☐ internal
☐ Head ☐ Neck ☐ Trunk ☐ L Arm ☐ L Hand ☐ L Leg ☐ L Foot
☐ Abdomen ☐ Pelvis ☐ Genital area ☐ R Arm ☐ R Hand ☐ R Leg ☐ R Foot

8) Spinal neurofibromas (neurofibromas arising from the spinal nerve root): histopathologically confirmed: Y / N
☐ Unknown ☐ Absent by MRI ☐ Present, asymptomatic ☐ Present, symptomatic
If present: please provide detail on figure page 4
☐ unilateral or ☐ bilateral;
C_____ T_____, L_____, S_____ regions.

9) Optic glioma: ☐ Unknown ☐ Absent by MRI ☐ Present by MRI, symptomatic ☐ Nerve (L and/or R) ☐ Chiasm ☐ Present by MRI, asymptomatic ☐ Nerve (L and/or R) ☐ Chiasm

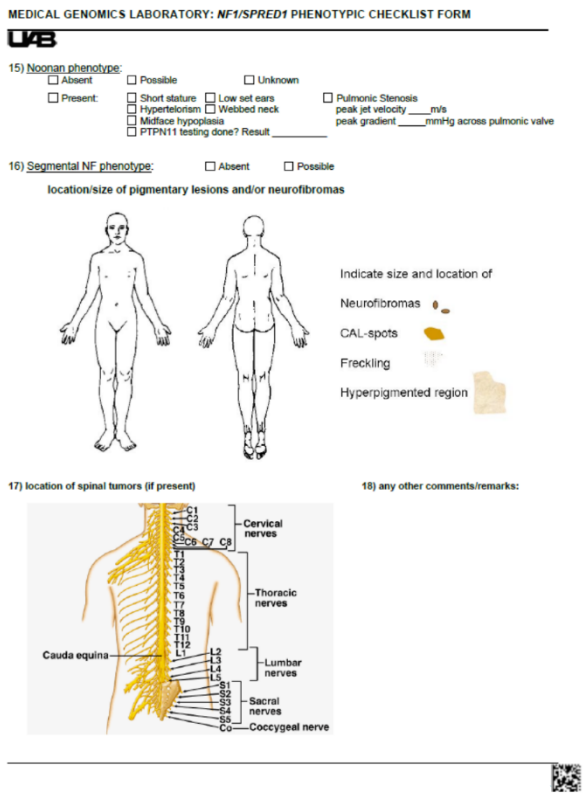
10) Other neoplasms: ☐ None
☐ Hypothalamic glioma ☐ Brainstem glioma ☐ Other glioma
☐ MPNST ☐ JMML ☐ Rhabdomyosarcoma
☐ Pheochromocytoma ☐ Colonic polyps ☐ Lipoma
☐ juvenile xanthogranuloma ☐ Breast cancer
☐ schwannoma ☐ Other, specify:_____

11) Skeletal Abnormalities: ☐ None
☐ Long bone dysplasia ☐ Pseudoarthrosis ☐ Sphenoid wing dysplasia
☐ Bone cysts ☐ scoliosis ☐ Dysplastic vertebrae
☐ pectus excavatum ☐ pectus carinatum ☐ Other:_____

12) Cardiovascular disease: ☐ Absent ☐ Unknown ☐ Present:
☐ Hypertension ☐ Pulmonic stenosis ☐ Aortic stenosis
☐ Renal artery stenosis ☐ Moya moya ☐ ASD
☐ VSD ☐ Other_____

13) Development: ☐ Normal ☐ Exam not done
☐ Abnormal ☐ ADD ☐ Hyperactivity ☐ Learning disability
☐ speech delay ☐ autism ☐ pervasive developmental delay
IQ: Full scale _____, Verbal _____, Performance _____.

14) Education: ☐ Too young for school ☐ At or above age level ☐ Below age level ☐ Unknown
☐ HS completion ☐ College graduate ☐ Higher degree



MEDICAL GENOMICS LABORATORY: *NF1/SPRED1* PHENOTYPIC CHECKLIST FORM

UAB

15) Noonan phenotype:
☐ Absent ☐ Possible ☐ Unknown
☐ Present: ☐ Short stature ☐ Low set ears ☐ Pulmonic Stenosis
☐ Hypertelorism ☐ Webbed neck peak jet velocity ____m/s
☐ Midface hypoplasia peak gradient _____mmHg across pulmonic valve
☐ PTPN11 testing done? Result _____

16) Segmental NF phenotype: ☐ Absent ☐ Possible

location/size of pigmentary lesions and/or neurofibromas

17) location of spinal tumors (if present)

18) any other comments/remarks:

Figure S1. Standardized phenotypic checklist used to document the phenotype of the individuals included in this study.


HGMD
ClinVar
LOVD
UAB
Trp837Gly
Trp837Arg
Ile838Met
Thr862Ser
Pro869Leu
Arg873Gly
Arg873Cys
Val880Met
Asp888Gly
Met895Arg
Leu898Pro
Leu920Pro
SPLICE
Leu929Pro
Leu933Gln
Ala804Pro
Val813Ile
Arg816Gln
Gly823Glu
Ser825Cys
Asp832Gly
Asn857His
Asn857Ser
Ser858Cys
Ala861Pro
Met867Val
Arg873Cys
Gly875Val
Met877Val
Met881Val
Gly885Arg
Thr889Arg
Val891Leu
Pro890Ser
Arg897Pro
Arg897Trp
Arg914Gln
Asn916Ser
Val921Met
Gly922Ser
Met932Val
Asn935His
Thr940Ser
Ser947Cys
Gln950His
Ile826Leu
Glu836Lys
Met880Val
Leu933Pro
Ala804Thr
Lys814Arg
Ser829Phe
Thr841Pro
Phe894Ser
Gly910Arg
Arg914Trp
Val917Asp
Gly922Ser (EXONIC SPLICE)
Leu920Gln (EXONIC SPLICE)
Met932Val
Gln950His (EXONIC SPLICE)

Figure S2. The spectrum of missense mutations in the exon 21 [16] of *NF1* (codons 804-950): a comparison of variants deposited in the publicly available disease databases (LOVD, ClinVar and HGMD, as of 05/09/2017) and variants identified in ~8400 unrelated individuals from UAB cohort.

All individuals identified in UAB cohort had been analyzed using comprehensive *NF1* mutation analysis using a core RNA-based approach and MLPA as previously described.[1,2] The variants reported in UAB cohort were classified according to the ACMG recommendations.[3] The classifications of the variants deposited in the publicly available databases (LOVD, ClinVar and HGMD) are shown here as reported by the original author(s). Additionally, the evidence provided in the original publications for those variants deposited in the HGMD database were reviewed and re-classified, if needed, based on currently accepted criteria for pathogenicity.[3]

HGMD re-verification:

c.2514C>G (p.Ile838Met) - reported in 1/91 unrelated NF1 individuals fulfilling the NIH diagnostic criteria, also carrying the second *NF1* variant c.2531T>C (p.Leu844Pro); no phenotypic details available; no evidence for pathogenicity provided (Mattocks et al.[4]); **c.2509T>C (p.Trp837Arg), c.2585C>G (p.Thr862Ser), c.2606C>T (p.Pro869Leu), c.2798T>A (p.Leu933Gln)** - absence in normal controls, segregation studies, proven *de novo* with paternity confirmation, *in silico* analysis (van Minkelen et al.[5]); **c.2509T>G (p.Trp837Gly), c.2617C>G (p.Arg873Gly), c.2638G>A (p.Val880Met), c.2684T>G (p.Met895Arg)** - no evidence for pathogenicity provided by the original author(s) (Sabbagh et al.[6] and Pasmant et al.[7]); **c.2693T>C (p.Leu898Pro) -** reported once in sporadic case of 465 unrelated NF1 individuals; no phenotypic details available; no evidence for pathogenicity provided (Maynard et al.[8]**; c.2759T>C (p.Leu920Pro)** - reported once in sporadic case of 17 unrelated NF1 individuals; 2 CALMs and spinal neurofibromas; no evidence for pathogenicity provided (Kluwe et al.[9]); **c.2617C>T (p.Arg873Cys), c.2663A>G (p.Asp888Gly)** - both variants reported in the same Sicilian individual; the same person also carrying two *NF1* frameshift alterations: c.2480delA, c.2632delC and other missense and synonymous variants (Bongiorno et al.[10]); **c.2764G>A (p.Gly922Ser)** - RNA-based approach analysis demonstrated that variant p.Gly922Ser results in cryptic 5' splice site/ 2761del90 (Ars et al.[11]); **c.2786T>C (p.Leu929Pro)** - reported once, absent in >1000 normal chromosomes, no further evidence for pathogenicity provided (Ribeiro et al.[12]).

Each number in the circle corresponds with the total number of probands with a specific variant. The dotted black line indicates the region studied in this report (codons 844-848). For graph presenting the variants deposited in the HGMD database, right panel shows the variants' classifications originally reported by the author(s), while left panel indicates the classifications based on our re-verification and following the ACMG recommendations.[3] Different colors correspond with the pathogenicity score according to the legend presented below ("exonic splice variants" are not *true* missense mutations; these variants cause missplicing as observed by RNA-based analysis in blood). The phenotypes of individuals from UAB cohort carrying a particular alteration are presented below in the table. The figure was prepared using the ProteinPaint application.[13]

Variant	Phenotype	Classification
c.2410G>A (p.Ala804Thr)	30-yo Japanese individual with >5 CALMs, freckling, 6-99 cutaneous and 6-99 intradermal nf	**VUS**
c.2441A>G (p.Lys814Arg)	6-yo Asian individual with >5 CALMs and freckling, also carrying *NF1* c.1338delA (p.Leu447Phefs*26); p.Lys814Arg ALSO present in the unaffected mother	**Likely benign**
c.2486C>T (p.Ser829Phe)	16-yo Native American proband with right axillary freckling, abdominal internal nf, also carrying *NF1* c.2894T>G (p.Ile965Arg); p.Ser829Phe ALSO present in the unaffected mother, whereas p.Ile965Arg was *de novo*	**Likely benign**
c.2509T>C (p.Trp837Arg)	24-yo individual with >5 CALMs and freckling	**VUS**
c.2509T>G (p.Trp837Gly)	>5 CALMs and freckling (all individuals, from 3-yo to 20-yo); other complications: abnormal development and asymptomatic OPG (3-yo) and plexiform nf (20-yo)	**Pathogenic**
c.2521A>C (p.Thr841Pro)	>5 CALMs and freckling (3-yo); *de novo* but no identity test done	**VUS**
c.2570A>G (p.Asn857Ser)	2.5-yo individual with >5 CALMs; p.Asn857Ser ALSO present in the unaffected mother	**Likely benign**
c.2573C>G (p.Ser858Cys)	>5 CALMs and freckling, 2-6 cutaneous and 2-6 intradermal nf, long bone dysplasia (31-yo); presence of second nonsense variant c.4108C>T (p.Gln1370*)	**Likely benign**
c.2585C>G (p.Thr862Ser)	5-yo proband with 1-5 CALMs and pre B-cell acute lymphoblastic leukemia; p.Thr862Ser ALSO present in affected 33-yo mother with 4-5 CALMs, bilateral Lisch nodules and plexiform nf on the leg	**Pathogenic**
c.2643G>A (p.Met881Ile)	2-yo proband with 1-5 CALMs and freckling; variant ALSO found in 32-yo father with 3 CALMs	**VUS**
c.2681T>C (p.Phe894Ser)	10-yo proband with >5 CALMs and freckling; variant ALSO found in 12-yo sibling with >5 CALMs and freckling	**VUS**
c.2693T>C (p.Leu898Pro)	>5 CALMs and freckling (all individuals, from 1-yo to 38-yo); 2 individuals >26-yo have numerous cutaneous and subcutaneous nf and plexiform nf; proven *de novo* but no identity test done; p.Leu898Pro ALSO found in father and son of the one individual (son with OPG)	**Pathogenic**
c.2728G>C (p.Gly910Arg)	only 3-4 CALMs and abnormal development; the presence of second missense variant c.3632T>G (p.Leu1211Arg); familial history, but ONLY variant p.Leu1211Arg was identified in the individual's father and paternal grandfather with the NF1 features; p.Gly910Arg ALSO present in the unaffected mother	**Likely benign**
c.2740C>T (p.Arg914Trp)	both individuals with >5 CALMs; individual with abnormal development and pulmonic stenosis has the presence of the second variant (*NF1* deletion exons 2-3)	**Likely benign**
c.2747A>G (p.Asn916Ser)	10-mo individual with 1-5 CALMs, brainstem glioma and pectus excavatum (sporadic); 12-yo individual only with >5 CALMs and variant ALSO present in the individual's father with <6 CALMs and in 5-yo brother with >5 CALMS and freckling	**Likely pathogenic**
c.2750T>A (p.Val917Asp)	only >5 CALMs; the individual's mother has the NF1 features, but she had not been tested	**VUS**
c.2786T>C (p.Leu929Pro)	7-mo individual with >6 CALMs and freckles, proven *de novo*; for other individuals no data provided	**VUS**
c.2794A>G (p.Met932Val)	4-mo Asian individual with only >5 CALMs	**VUS**
c.2798T>C (p.Leu933Pro)	>5 CALMs and freckles; Lisch nodules (in 2/6 individuals), cutaneous (2/6) and intradermal (2/6) nf, plexiform (1/6) and spinal (1/6) nf, abnormal development (3/6); others: juvenile pilocytic astrocytoma, brainstem hamartoma and pseudarthrosis	**Pathogenic**

yo - **y**ears **o**ld; **mo** - **m**onths **o**ld; **nf** - **n**euro**f**ibromas; **OPG** - **o**ptic **p**athway **g**lioma; **VUS** - **v**ariant of **u**ncertain **s**ignificance

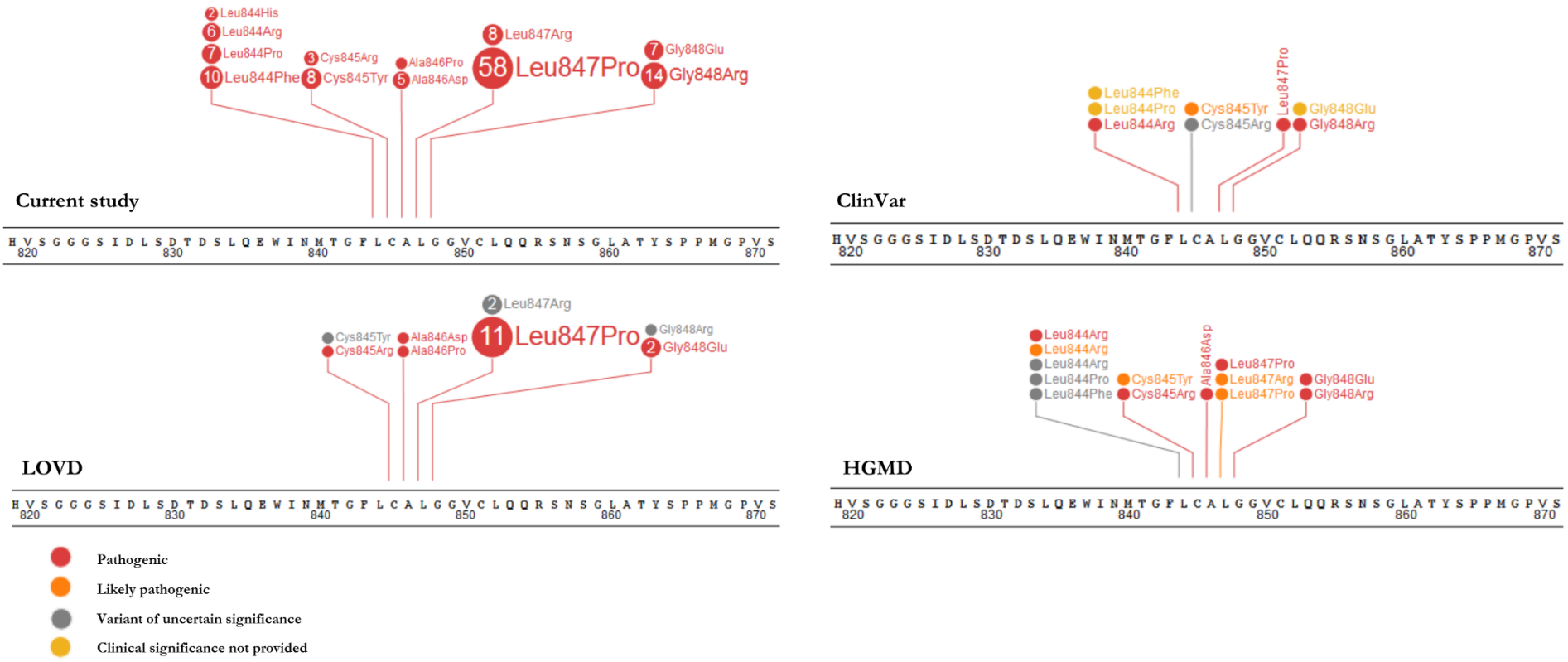


Figure S3. The classification of *NF1* missense mutations affecting codons 844-848: a comparison of the classification in the publicly available disease databases (LOVD, ClinVar and HGMD, as of 05/09/2017) with the current study, classifying variants according to the ACMG recommendations (details in Tables S2-S4).

The classifications of the variants deposited in the publicly available LOVD and ClinVar databases are shown here as reported by the original author(s). For variants deposited in the HGMD database, the originally reported variants' classifications were reviewed and if necessary re-classified according to the ACMG guidelines [3] based on the evidence for pathogenicity provided in the published papers by the authors (details in Table S3). Each number in the circle corresponds with the total number of probands with a specific variant. Different colors correspond with the pathogenicity score. The figure was prepared using the ProteinPaint application.[13]

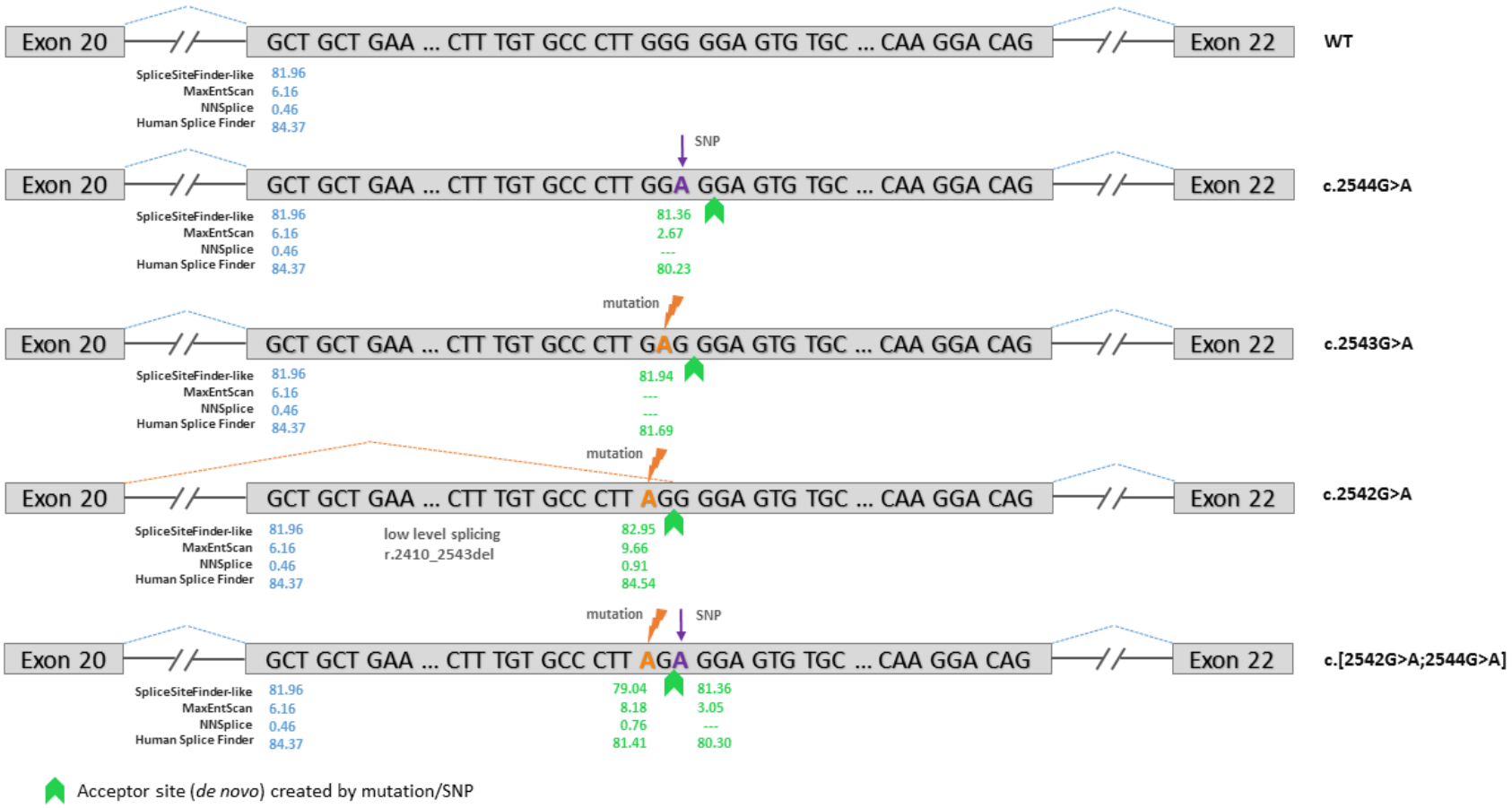


Figure S4. *In silico* prediction and *in vivo* analysis of the effect of the *NF1* c.2542G>A (p.Gly848Arg) and c.2543G>A (p.Gly848Glu) missense mutations as well as the single nucleotide polymorphism c.2544G>A (p.Gly848=) on splicing in short-term cultured blood lymphocytes.

Based on the results of *in silico* analysis (SpliceSiteFinder-like, MaxEntScan, NNSplice and Human Splice Finder embedded in Alamut visual software v.2.9.0), a novel splice acceptor site is created by the presence of *NF1* mutations (c.2542G>A and c.2543G>A) as well as by the single nucleotide polymorphism (SNP) at c.2544G>A (rs17883704). *In vivo* transcript analysis and sequencing revealed a minor effect on splicing ONLY for c.2542G>A, resulting in low level of r.2410_2543del transcript. No missplicing effect was observed in individuals carrying the *NF1* mutation c.2542G>A and SNP; both variants proven to reside in cis through next-generation sequencing.

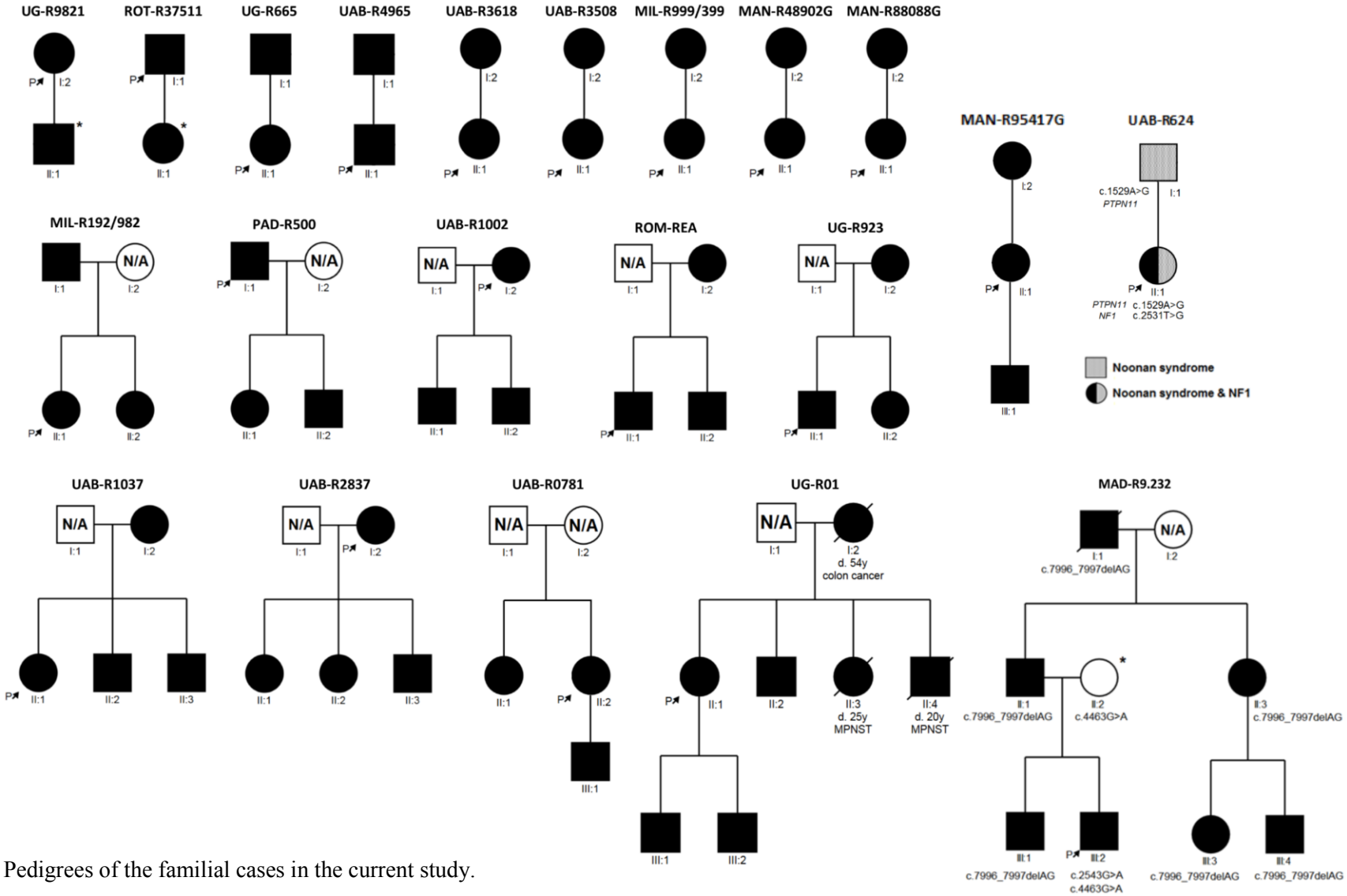


Figure S5. Pedigrees of the familial cases in the current study.

The filled black symbols represent the individuals who are positive for an *NF1* missense mutation affecting codons 844-848, while the open symbols represent relatives who are negative for the family specific mutations. The arrow indicates the proband of the family. For the first generation, father is always I:1 and mother is I:2.

Abbreviations: **N/A** - the relatives not available for genetic analysis; ***** - no clinical information available for these individuals.

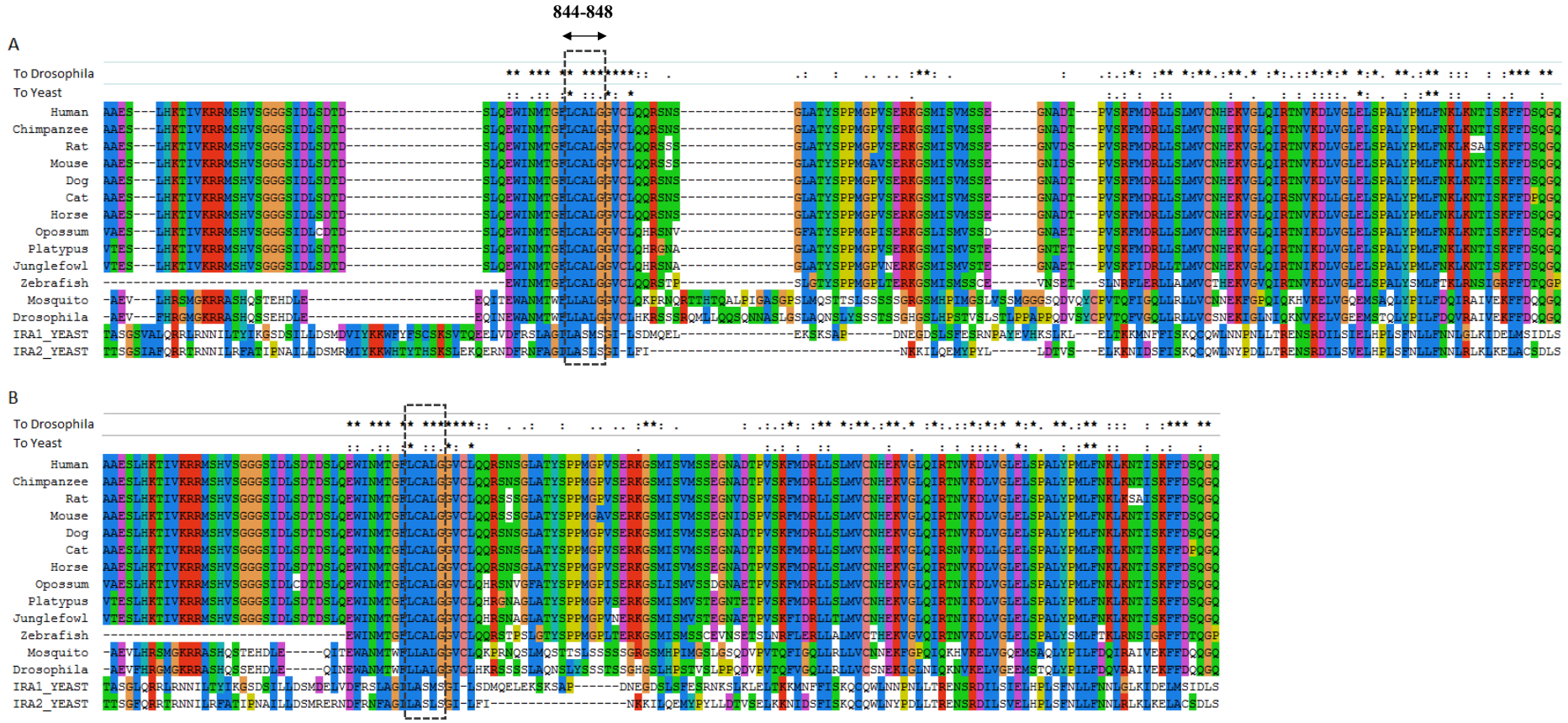


Figure S6. Multiple sequence alignment of the exon 21 [16] of *NF1* (A) and multiple sequence alignment of the same region adjusted to the human *NF1* without gaps (B).

Human (RefSeq: NP_000258.1 residues 804-950), Chimpanzee (TrEMBL: K7DMZ3 residues 804-950), Rat (SWISSPROT: P97526 residues 806-952), Mouse (SWISSPROT: Q04690-2 residues 806-952), Dog (RefSeq: XP_013971967.1 residues 799-945), Cat (RefSeq: XP_011287367.1 residues 807-953), Horse (TrEMBL: F6XRM7 residues 739-885), Opossum (TrEMBL: F6ZHU9 residues 564-710), Platypus (RefSeq: XP_007664859.1 residues 840-986), Junglefowl (RefSeq: XP_003642464.1 residues 799-945), Zebrafish (TrEMBL: E7FBD0 residues 751-865), Mosquito (TrEMBL: B0WYP5 residues 771-936), Drosophila (TrEMBL: O01397 residues 804-969), yeast IRA1 (SWISSPROT: IRA1_YEAST residues 1253-1419) and IRA2 (SWISSPROT: IRA2_YEAST residues 1263-1411) were included in the alignment. The multiple sequence alignment was performed using *Clustal 2.0.12* software with manually adjustment. The black dotted lines on the panels present the region 844-848. An asterisk (*), a colon (:) and a period (.) indicate the following positions: (*) a single fully conserved residue, (:) conservation between groups of strongly similar properties and (.) conservation between groups of weakly similar properties.

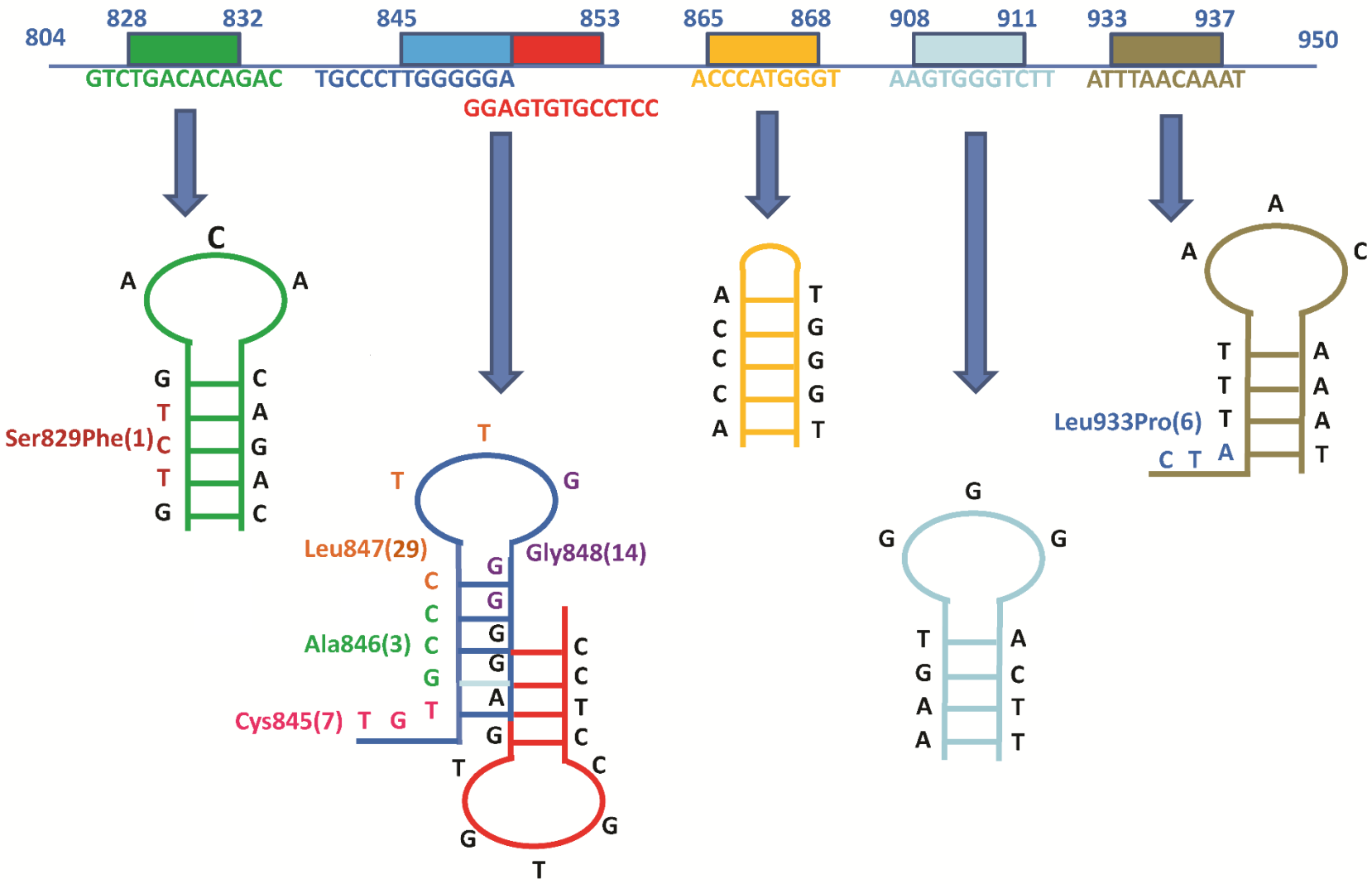


Figure S7. Schematic of five palindromic structures in the *NF1* codons 804-950.

Missense mutations with a total number of samples identified in the UAB cohort were annotated next to the palindromic structures with each color corresponding to the variant motif. Particularly, two palindromic sequences **TGCCCTTGGGGGA** (AA845-849) and **GGAGTGTGCCTCC** (AA849-853) may form a unique non-B DNA structure contributing recurrent missense mutations.

Table S1. Clinical details for 162 individuals with an *NF1* missense mutation affecting one of five codons 844-848 from 129 different families.

Table S1 is included as a separate Excel file.

[A] For individuals with one asterisk (*) the standardized phenotypic checklist forms were not updated by the referring physicians, the data are based on the originally submitted forms; individuals with two asterisks (**) had incomplete phenotypic checklist forms; individuals with three asterisks (***) were the newborn infants without the NF1 features at birth or without the clinical data provided.

[B] **UAB**: **U**niversity of **A**labama at **B**irmingham (67 probands and 11 relatives); **BRA**: Fluminense Federal University, Niterói, **Bra**zil (one proband); **CAR**: i/ Division of Cancer and Genetics, Cardiff University, **Car**diff, UK; ii/ Birmingham Women's and Children's NHS Foundation Trust, Birmingham, UK; iii/ Wessex Regional Genetics Laboratory, Salisbury NHS Foundation Trust, Salisbury, UK; iv/ St George's University Hospitals NHS Foundation Trust, London, UK; v/ Division of Medical Oncology, National Cancer Centre, Singapore (5 probands); **MAD**: Hospital Universitario Ramón y Cayal, Institute of Health Research (IRYCIS) and Center for Biomedical Research-Network for Rare Diseases (CIBERER), **Mad**rid, Spain (6 probands); **MAN**: University of **Man**chester, UK (4 probands and 4 relatives); **MIL**: Carlo Besta Neurological Institute, **Mil**an, Italy (6 probands and 3 relatives); **PAD**: University of **Pad**ova, Italy (5 probands and 2 relatives); **SGR**: Molecular Genetics Unit, Casa Sollievo della Sofferenza Hospital, IRCCS, **S**an **G**iovanni **R**otondo, Italy (4 probands and 2 relatives); **ROT**: Erasmus Medical Center, **Rot**terdam, the Netherlands (13 probands); **SHE**: Sheffield Chidlren's NHS Foundation Trust, **She**ffield, UK (one proband); **UL**: **U**niversity of **L**euven, Leuven, Belgium (one proband); **UF**: **U**niversity of **F**lorida College of Medicine, Gainesville, Florida, USA (one proband); **UG**: **U**niversity Hospital, **G**hent, Belgium (15 probands and 11 relatives).

[C] **F**: **f**amilial; **PrS**: **pr**oven **s**poradic; **RS**: **r**eportedly **s**poradic; **NS**: **n**ot **s**pecified

[D] Exact age was used to calculate Height and Head Circumferences, but provided as age-groups in Table S1: 0-24 months; 2-4 years; 5-8 years; 9-13 years; 14-18 years; 19-26 years; >26 years.

[E] **F**: **f**emale; **M**: **m**ale

[F] **W**: **W**hite; **His**: **His**panic; **AA**: **A**frican **A**merican; **As**: **As**ian; **NaA**: **N**ative **A**merican; **Pa**: **Pa**kistani; **Fil**: **Fil**ipino; **A**: **A**frican; **Jew**: **Jew**ish

[G] An individual was classified as having „NF-Noonan" if at least two of the following features were present: short stature (SS), low set ears (LSE), hypertelorism (HTL), midface hypoplasia (MH), webbed neck (WN) or pulmonic stenosis (PS).

[H] Height percentiles for Hispanic and Asian individuals were provided in square brackets to indicate that they were excluded from the data analysis on frequency of short or normal stature due to the lack of ethnic-specific growth charts.

[I] Individuals with ADD/ADHD, but normal development were still classified as normal.

Abbreviations:

NS: **n**ot **s**pecified; **bil**: **bil**ateral; **gr**: **gr**oin; **ax**: **ax**illary; **OPG**: **o**ptic **p**athway **g**lioma; **N, UN**: **n**o symptomatic OPG or symptomatic spinal neurofibroma, **un**known if any asymptomatic OPG or asymptomatic spinal neurofibromas are present; **N, MRI**: **n**o symptomatic and asymptomatic OPG or spinal neurofibroma detected by **MRI**; **Y, MRI**: **y**es, presence of OPG or spinal neurofibroma confirmed by **MRI**; **ASD**: **a**trial **s**eptal **d**efect; **LD**: **l**earning **d**isability; **HTL**: **h**yper**t**e**l**orism; **MH**: **m**idface **h**ypoplasia; **SS**: **s**hort **s**tature; **PT**: **pt**osis; **PS**: **p**ulmonic **s**tenosis; **DPF**: **d**ownslanting **p**alpebral **f**issures; **LSE**: **l**ow **s**et **e**ars; **ADD**: **a**ttention **d**eficit **d**isorder; **ADHD**: **a**ttention **d**eficit **h**yperactivity **d**isorder; **SD**: **s**peech **d**elay; **F**: **f**ather: **M**: **m**other; **S**: **s**ibling; **C**: **c**hild.

Table S2. In *silico* prediction of pathogenicity and *in vivo* splicing analysis of *NF1* missense mutations affecting codons 844-848 in the studied group.

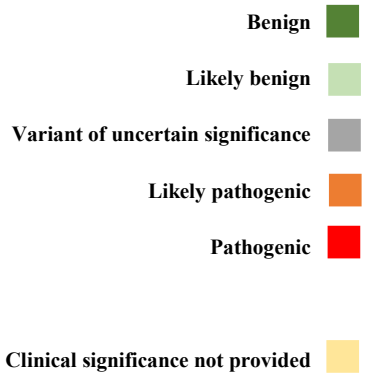
Variant	Protein	844-848 positive probands	844-848 positive UAB probands	gnomAD (123,136)	EVS (6,503)	1000 G	HGMD [A]	LOVD [A] (3,384)	ClinVar [A]	Grantham distance	SIFT	PolyPhen	CADD	SSF/ MaxEntScan/ NNSplice/HSF	RNA observed	Other evidence [B]
c.2530C>T	p.Leu844Phe	10/129	5/67	0	0	0	Present (DM)	0	Present (NP)	22	Deleterious (0)	Probably damaging (0.997)	27.2	No change	Normal	Proven *de novo* (UAB-R2425; UAB-R8475; UAB-R0903); segregation in family (MAN-R48902G)
c.2531T>A	p.Leu844His	2/129	1/67	0	0	0	0	0	0	99	Deleterious (0)	Probably damaging (0.999)	26.6	No change	Normal	Proven *de novo* (UAB-R4444)
c.2531T>C	p.Leu844Pro	7/129	5/67	0	0	0	Present (DM)	0	Present (NP)	98	Deleterious (0)	Probably damaging (0.997)	25.9	No change	Normal	Proven *de novo* (UAB-R3714; UAB-R2087; UAB-R8047; UAB-R0075)
c.2531T>G	p.Leu844Arg	6/129	3/67	0.00041%	0	0	Present (DM)	0	Present (P)	102	Deleterious (0)	Probably damaging (0.997)	26.8	No change	Normal	Proven *de novo* (UAB-R624)
c.2533T>C	p.Cys845Arg	3/129	1/67	0	0	0	Present (DM)	1 (P)	Present (VUS)	180	Deleterious (0.010	Possibly damaging (0.571)	22.6	No change	Normal	Proven *de novo* (UAB-R3112); segregation in family (MAN-R88088G)
c.2534G>A	p.Cys845Tyr	8/129	6/67	0	0	0	Present (DM?)	1 (VUS)	Present (LP)	194	Deleterious (0.01)	Possibly damaging (0.891)	23.2	No change	Normal	Proven *de novo* (UAB-R9135; UAB-R7836; UAB-R2696)
c.2536G>C	p.Ala846Pro	1/129	0/67	0	0	0	0	1 (P)	0	27	Deleterious (0.03)	Probably damaging (0.999)	27	No change	Normal	Segregation in family (UG-R0781)
c.2537C>A	p.Ala846Asp	5/129	3/67	0	0	0	Present (DM)	1 (P)	0	126	Deleterious (0.01)	Probably damaging (0.999)	31	No change	Normal	Proven *de novo* **(ROT-R02233)**; segregation in family (UAB-R3618; UG-R665)
c.2540T>C	p.Leu847Pro	58/129	27/67	0	0	0	Present (DM)	11 (P)	Present (P)	98	Deleterious (0.01)	Probably damaging (0.997)	26.1	No change	Normal	Proven *de novo* (UAB-R9676; UAB-R2266; **ROT-R22853; ROT-R17435**; UG-R5831); segregation in multiple families (UAB-R4965; UAB-R3508; UG-R01; PAD-R500; MIL-R192/982)
c.2540T>G	p.Leu847Arg	8/129	2/67	0	0	0	Present (DM?)	2 (VUS)	0	102	Deleterious (0)	Probably damaging (0.997)	27	No change	Normal	Proven *de novo* (UAB-R6913, UG-R399)
c.2542G>C	p.Gly848Arg	8/129	5/67	0	0	0	Present (DM)	1 (VUS)	Present (P)	125	Deleterious (0.02)	Probably damaging (1)	27.2	No change	Normal	Proven *de novo* (UAB-R45401FN.201); segregation in multiple families (UAB-R1002; UAB-R1037; UAB-R2837, UG-R923; MIL-R999/399)
c.2542G>A	p.Gly848Arg	6/129	5/67	0	0	0	0	0	0	125	Deleterious (0.02)	Probably damaging (1)	28.2	Creates a novel splice acceptor sequence in the exon that is used in a small fraction of transcripts: from WT cc/cc/cc to MUT - 83.0 / 9.7 / 0.9 / 84.5 (Fig S4)	Low level of r.2410_2543del	Proven *de novo* (UAB-R4476; UAB-R3513)
c.2543G>A	p.Gly848Glu	7/129	4/67	0	0	0	Present (DM)	2 (P)	Present (NP)	98	Deleterious (0.03)	Probably damaging (1)	27.2	Creates a novel splice acceptor sequence in the exon: from WT cc/cc/cc/ to MUT - 81.9 / - / - / 81.7 (Fig S4)	No missplicing observed	Proven *de novo* (UAB-R1333, MAD-R9.232); segregation in family (SGR-REA; MAN-R95417G)

Abbreviations - gnomAD: the **G**e**nom**e **A**ggregation **D**atabase; **EVS**: **E**xome **V**ariant **S**erver; **1000 G**: **1000 G**enomes Project; **HGMD**: **H**uman **G**ene **M**utation **D**atabase; **LOVD**: **L**eiden **O**pen **V**ariation **D**atabase; **CADD**: **C**ombined **A**nnotation **D**ependent **D**epletion; **SSF**: **S**plice**S**ite**F**inder-like; **HSF**: **H**uman **S**plicing **F**inder; **VUS**: **v**ariant of **u**ncertain **s**ignificance; **P**: **p**athogenic; **LP**: **l**ikely **p**athogenic; **DM**: disease causing; **DM?**: likely disease causing; **NP**: clinical significance **n**ot **p**rovided.

[A] The variants' classifications presented in round brackets have been exactly the same as reported by the original author(s). **[B]** Individuals proven *de novo* with the confirmed biological relationships are shown in bold.

Table S3. The classification of *NF1* missense mutations affecting codons 844-848 reported in the studied group: a comparison of the publicly available disease databases (LOVD, ClinVar and HGMD, as of 05/09/2017) with the current study.

Variant	Protein	LOVD [A]	ClinVar [A]	HGMD [B]	This study [C]
c.2530C>T	p.Leu844Phe			a,b	
c.2531T>A	p.Leu844His				
c.2531T>C	p.Leu844Pro			b	
c.2531T>G	p.Leu844Arg			c-e	
c.2533T>C	p.Cys845Arg			f	
c.2534G>A	p.Cys845Tyr			f	
c.2536G>C	p.Ala846Pro				
c.2537C>A	p.Ala846Asp			f	
c.2540T>C	p.Leu847Pro			g,h	
c.2540T>G	p.Leu847Arg			f	
c.2542G>A	p.Gly848Arg				
c.2542G>C	p.Gly848Arg			i	
c.2543G>A	p.Gly848Glu			h	



[A] The variants' classification is provided as reported by the original author(s); [B] the originally reported variants' classifications have been reviewed and if necessary re-classified based on evidence for pathogenicity provided by the authors in the published papers and according to the ACMG recommendations; [C] the variants' classification according to the ACMG recommendations (details in Table S4).

HGMD: [a] reported in 1/93 unrelated NF1 individuals fulfilling the NIH diagnostic criteria; no phenotypic details available; no evidence for pathogenicity provided (Girodon-Boulandet et al.[19]); [b] reported in 2/91 unrelated NF1 individuals fulfilling the NIH diagnostic criteria; no phenotypic details available; no evidence for pathogenicity provided (Mattocks et al.[4]); [c] reported once in sporadic case of 465 unrelated NF1 individuals; no phenotypic details available; no evidence for pathogenicity provided (Maynard et al.[8]); [d] proven *de novo*, no other evidence for pathogenicity provided (Wang et al.[20]); [e] proven *de novo,* absence in normal controls, phenotype highly specific for a disease (Bertola et al.[14]); [f] absence in normal controls, segregation studies, proven *de novo* with paternity confirmation, *in silico* analysis (van Minkelen et al.[5]); [g] reported in 1/521 unrelated NF1 individuals, *in silico* analysis, no other evidence for pathogenicity provided (Fahsold et al.[21]); [h] absence in normal controls, disease phenotype segregated with the mutation in familial cases, *in silico* analysis (De Luca et al.[22]; De Luca et al.[23]); [i] disease phenotype segregated with the mutation in familial cases, phenotype highly specific for a disease (Pascual-Castroviejo et al.[17])

Table S4. The classification of *NF1* missense mutations affecting codons 844-848 reported in the studied group according to the ACMG recommendations.[3]

Variant	Protein	PS1	PS2 [A]	PS3	PS4	PM1 [B]	PM2	PM3	PM4	PM5 [C]	PM6 [D]	PP1 [E]	PP2	PP3	PP4 [F]	PP5 [G]	Variant classification [H]
c.2530C>T	p.Leu844Phe	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	**YES**	**YES**	N/A	?	**YES**	**YES**	NO	**VUS** → **Pathogenic**
c.2531T>G	p.Leu844Arg	N/A	N/A	?	**YES**	?	NO	N/A	N/A	N/A	**YES**	**YES**	?	**YES**	**YES**	**YES** [2,3]	**Pathogenic** → **Pathogenic**
c.2531T>C	p.Leu844Pro	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	**YES**	**YES**	N/A	?	**YES**	**YES**	NO	**VUS** → **Pathogenic**
c.2531T>A	p.Leu844His	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	**YES**	**YES**	N/A	?	**YES**	**YES**	N/A	**?** → **Pathogenic**
c.2533T>C	p.Cys845Arg	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	N/A	**YES**	N/A	?	**YES**	**YES**	**YES** [1,3]	**Pathogenic** → **Pathogenic**
c.2534G>A	p.Cys845Tyr	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	**YES**	**YES**	N/A	?	**YES**	**YES**	NO	**VUS** → **Pathogenic**
c.2536G>C	p.Ala846Pro	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	N/A	N/A	**YES**	?	**YES**	**YES**	**YES** [1]	**Pathogenic** → **Pathogenic**
c.2537C>A	p.Ala846Asp	N/A	**YES**	?	**YES**	?	**YES**	N/A	N/A	N/A	N/A	N/A	?	**YES**	**YES**	**YES** [1,3]	**Pathogenic** → **Pathogenic**
c.2540T>C	p.Leu847Pro	N/A	**YES**	?	**YES**	?	**YES**	N/A	N/A	N/A	N/A	**YES**	?	**YES**	**YES**	**YES** [1-3]	**Pathogenic** → **Pathogenic**
c.2540T>G	p.Leu847Arg	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	**YES**	**YES**	N/A	?	**YES**	**YES**	NO	**VUS** → **Pathogenic**
c.2542G>A	p.Gly848Arg	**YES**	N/A	?	**YES**	?	**YES**	N/A	N/A	**YES**	**YES**	N/A	?	**YES**	**YES**	N/A	**?** → **Pathogenic**
c.2542G>C	p.Gly848Arg	N/A	N/A	NO [I]	**YES**	?	**YES**	N/A	N/A	N/A	**YES**	**YES**	?	**YES**	**YES**	**YES** [2,3]	**Pathogenic** → **Pathogenic**
c.2543G>A	p.Gly848Glu	N/A	N/A	?	**YES**	?	**YES**	N/A	N/A	N/A	**YES**	**YES**	?	**YES**	**YES**	**YES** [1,3]	**Pathogenic** → **Pathogenic**

PS1: same amino acid change as a previously established pathogenic variant regardless of nucleotide change; **PS2**: proven *de novo* (both maternity and paternity confirmed); **PS3**: well-established functional studies; **PS4:** the prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls; **PM1**: located in a mutational hot spot and/or in critical functional domain; **PM2**: absent from controls; **PM3**: for recessive disorders, detected in *trans* with a pathogenic variant; **PM4**: protein length changes due to in-frame or stop-loss variants; **PM5**: novel missense change at amino acid residue where a different pathogenic missense change has been seen before; **PM6**: assumed *de novo*, but without confirmation of paternity and maternity; **PP1**: co-segregation with disease in multiple affected family members; **PP2**: missense variant in a gene that has a low rate of benign missense variation; **PP3**: multiple lines of computational evidence support a deleterious effect on the gene or gene product; **PP4**: individual's phenotype or family history is highly specific for a disease; **PP5**: reputable source reports variant as pathogenic; **N/A**: not applicable; **YES**: the criterion has been fulfilled; **NO**: the criterion has not been fulfilled; **?**: due to the lack of evidence, it is difficult to establish whether the criterion has been fulfilled or not.

[A] based on the data from LOVD database (only individuals confirmed as *de novo* and identified in the Netherlands where biological relationships were confirmed as part of the *de novo* assessment have been taken into account)**; [B]** CSRD domain has not been proved to be critical to protein function (as function is largely unknown still), thus this evidence could not be considered as moderate evidence of pathogenicity; **[C]** variants classified as *pathogenic* in the publicly available disease databases (LOVD, ClinVar, HGMD) have been taken into account; **[D]** only individuals confirmed as *de novo* at UAB have been taken into account, except for variants fulfilling the PS2 criterion (p.Ala846Asp and p.Leu847Pro); **[E]** segregation with at least three affected family members needed to fulfill this criterion; **[F]** at least one individual with "classic" NF1 phenotype, including neurofibroma(s) and/or Lisch nodules was considered required to fulfill this criterion; **[G]** based on the variant's classification as pathogenic reported in LOVD (1), ClinVar (2) and/or HGMD (3) databases (details in Table S3); **[H]** prior variants classification as reported in the publicly available disease databases (LOVD, ClinVar, HGMD) and *a posteriori* classification based on our study and following the ACMG recommendations; **[I]** based on functional studies by Li et al.[24] and Toonen et al.[25]

To classify variant as pathogenic the following criteria need to be fulfilled: ≥2 strong (PS1-PS4) OR 1 strong (PS1-PS4) and ≥3 moderate (PM1-PM6) OR 1 strong (PS1-PS4) and 2 moderate (PM1-PM6) and ≥2 supporting (PP1-PP5) OR 1 strong (PS1-PS4) and 1 moderate (PM1-PM6) and ≥4 supporting (PP1-PP5).

Table S5. The age of the individuals who did present with CALMs-only (either >5 or < 6), but carried a missense mutation affecting one of five *NF1* codons 844-848 in this study.

Family ID	cDNA level	Protein level	Age group
>5 CALMs			
UAB-R8475	c.2530C>T	p.Leu844Phe	14-18 years
UAB-R8047	c.2531T>C	p.Leu844Pro	0-24 months
ROT-R13734	c.2534G>A	p.Cys845Tyr	0-24 months
UG-R391	c.2534G>A	p.Cys845Tyr	2-4 years
ROT-R36375	c.2540T>G	p.Leu847Arg	0-24 months
UAB-R6913	c.2540T>G	p.Leu847Arg	5-8 years
UAB-R09001FN.101	c.2540T>C	p.Leu847Pro	0-24 months
UAB-R6106	c.2540T>C	p.Leu847Pro	0-24 months
UAB-R3537	c.2540T>C	p.Leu847Pro	0-24 months
ROT-R2CMUL	c.2540T>C	p.Leu847Pro	0-24 months
CAR-R655763	c.2540T>C	p.Leu847Pro	0-24 months
UG-R746	c.2540T>C	p.Leu847Pro	0-24 months
UAB-R9676	c.2540T>C	p.Leu847Pro	2-4 years
ROT-R49013	c.2540T>C	p.Leu847Pro	>26 years
UAB-R45401FN.201	c.2542G>C	p.Gly848Arg	9-13 years
UAB-R1333	c.2543G>A	p.Gly848Glu	0-24 months

Family ID	cDNA level	Protein level	Age group
<6 CALMs			
UAB-R2696 [A]	c.2534G>A	p.Cys845Tyr	14-18 years
* UAB-R9135 [B]	c.2534G>A	p.Cys845Tyr	19-26 years
* UG-R5831 [C]	c.2540T>C	p.Leu847Pro	5-8 years †
UAB-R9493	c.2542G>A	p.Gly848Arg	5-8 years
UAB-R1037-S2	c.2542G>C	p.Gly848Arg	0-24 months
UAB-R2837-C1	c.2542G>C	p.Gly848Arg	2-4 years
UAB-R2837-C3	c.2542G>C	p.Gly848Arg	2-4 years
UAB-R2837-C2	c.2542G>C	p.Gly848Arg	5-8 years
UAB-R1037-M	c.2542G>C	p.Gly848Arg	>26 years
MAD-R9.232	c.[2543G>A;4463G>A]	p.[Gly848Glu;Arg1488His]	5-8 years

[A] Individual had also two superficial plexiform neurofibromas on trunk and back and symptomatic spinal neurofibroma; [B] Individual had one superficial plexiform neurofibroma on trunk and no CALMs; [C] Individual had very severe phenotype, presenting shortly after birth; three plexiform neurofibromas, including right orbital tumor, one located in right middle ear and one on neck, visible from outside; protrusion of the right eye with hemangiomas on the right cheek confirmed by MRI; severe epilepsy with attacks occurring mostly in sleep or during feeding, causing vomiting; individual was not able to eat (tube feeding); deceased at age of ~8 years.

* None CALMs had been observed in these individuals.

Table S6. Spinal neurofibromas in individuals carrying a missense mutation affecting one of five codons 844-848 in this study.

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
SYMPTOMATIC SPINAL NEUROFIBROMAS									
UAB-R2696	c.2534G>A	p.Cys845Tyr	PrS	14-18 years	<6	-	plexiform neurofibromas on trunk and back (visible from outside)	mild scoliosis, hypertension, Noonan phenotype features	MRI: innumerable spinal, intercostal, brachial and lumbosacral plexiform neurofibromas; large right-sided paraspinal neurofibroma in the lumbosacral region (12 cm in length)
UG-R399	c.2540T>G	p.Leu847Arg	PrS	5-8 years	>5	NS	plexiform neurofibroma on trunk (visible from outside and internal, without hyperpigmentation)	scoliosis, Noonan-phenotype features, abnormal development, gross motor delays, LD, ADHD, SD	extensive spinal neurofibromas on the costal nerves T8-11 right side; visible externally but much larger on MRI
UAB-R3508	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	plexiform neurofibromas on neck, trunk and left arm (visible from outside, with hyperpigmentation)	symptomatic OPG, abnormal development, LD, ADD, ADHD	none
UAB-R01701FN.103	c.2540T>C	p.Leu847Pro	F	19-26 years	>5	+	plexiform neurofibromas on neck, left leg, left foot and abdomen (visible from outside); multiple cutaneous and subcutaneous neurofibromas on all parts of the body (#unknown)	Lisch nodules, LD, ADD	spinal neurofibromas on T5-T6 and L4-L5 regions
UF-R1	c.2540T>C	p.Leu847Pro	F	19-26 years	>5	-	large nodular neurofibroma on right thigh, surgically removed during the teenage years; some cutaneous (<10) and subcutaneous neurofibromas (#unknown)	scoliosis, chronic arthritis multiple joints, possible OPG (small lesion on optic nerve by MRI scan), Noonan phenotype features (PT, LSE, DPF), macrocephaly, non-ossifying fibroma on femur/knee, bilateral ankle clonus, knee hyperreflexia and esotropia	individual has suffered from proximal weakness in lower extremities that can be associated with the nerve root enlargements, significant difficulty walking, weakness of iliopsoas and quadriceps muscles; in addition, the individual has depigmented spots on back and left chest (vitiligo) and piebaldism (presence of white forelock); genetic analysis of *KIT* and *SNAI2* revealed presence of the *KIT* missense alteration c.1195G>A (p.Val399Ile), likely contributing to the piebaldism phenotype in this case
UAB-R0008	c.2542G>A	p.Gly848Arg	RS	14-18 years	>5	+	-	scoliosis, LD (dyslexia associated with working memory and phonological awareness issues), history of recurrent migraine headaches	p.Gly848Arg causing low level missplicing r.2410_2543del; father was diagnosed with a bilateral distal sensory neuropathy, his spinal MRI was normal
ROT-R95424	c.2542G>C	p.Gly848Arg	F	>26 years	<6	+	2-6 cutaneous and 2-6 subcutaneous neurofibromas	normal development, concentration problems, macrocephaly	individual's mother has many small CALMs spots and some neurofibromas
UAB-R1002	c.2542G>C	p.Gly848Arg	F	19-26 years	>5	+	mediastinal mass on chest (internal) and large plexiform neurofibroma bilateral on both sides of neck; 1 cutaneous neurofibroma in the right ear filling the entire canal	scoliosis, hypertension, renal artery stenosis, conductive hearing loss in the right ear, likely secondary to right ear being filled with a neurofibroma	individual's brother, father and paternal uncle have NF1
UG-R923	c.2542G>C	p.Gly848Arg	F	19-26 years	0	-	plexiform neurofibromas on both sides of the neck	moderate scoliosis, LD, ADHD	clumsiness with impaired walking, moderate atrophy of the distal leg muscles; generalized nerve sheath tumors, presence of symmetric neurofibromas in all spinal nerves of the body following the peripheral course of the nerves; case reported by Pascual-Castroviejo et al.[17]
UAB-R2492	c.2543G>A	p.Gly848Glu	F	>26 years	>5	-	plexiform neurofibroma on right leg (visible from outside); 100-500 cutaneous/subcutaneous neurofibromas	macrocephaly	none

SYMPTOMATIC SPINAL NEUROFIBROMAS									
UAB-R3237	c.2543G>A	p.Gly848Glu	RS	>26 years	<6	+	plexiform neurofibromas on head, neck, left and right leg (visible from outside, without hyperpigmentation); 1 cutaneous neurofibroma	asymptomatic OPG, Lisch nodules	none
MAN-R95417G	c.2543G>A	p.Gly848Glu	F	>26 years	0	+	multiple cutaneous and subcutaneous neurofibromas developed from her teenage years	SS, macrocephaly, normal development, possible Noonan phenotype (specific facial features, but no details available)	MRI: tumors present on nerve roots throughout the neuroaxis, particularly around the cauda equina, and a large retroperitoneal mass; repeated few years later MRI showed a very extensive burden of nerve sheath tumors, involving the large majority of spinal nerve roots; case reported by Burkitt-Wright et al.[18]
MAN-R95417G-C	c.2543G>A	p.Gly848Glu	F	>26 years	0	-	-	Lisch nodules, pectus carinatum, normal development, possible Noonan phenotype (specific facial features, but no details available)	MRI: a very extensive burden of nerve sheath tumors, including a particularly large lobulated lesion in the occipital/posterior neck region; case reported by Burkitt-Wright et al.[18]
ASYMPTOMATIC SPINAL NEUROFIBROMAS									
UAB-R0147	c.2534G>A	p.Cys845Tyr	RS	19-26 years	>5	+	innumerable solitary and plexiform neurofibromas on left arm, neck, trunk, left leg, right leg, abdomen (visible from outside, with hyperpigmentation); left arm extensive neurofibroma confirmed histopathologically as a focally cellular neurofibroma with degenerative atypia; 6-99 cutaneous and 6-99 subcutaneous neurofibromas	abnormal development, LD	numerous subcentimeter subcutaneous scalp solitary T2 hyperintense nodules: likely small peripheral nerve sheath tumors?
ROT-R21382	c.2540T>C	p.Leu847Pro	F	>26 years †	>5	+	left nervus ischiadicus, surgically removed; >500 cutaenous and 6-99 subcutaneous neurofibromas	pericarditis carcinomatosa, metastasized MPNST, superior vena cava syndrome (SVCS), dyspnea on effort	individual died of MPNST at age of 30 years; family history: affected mother and sister
UG-R01-C1	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	plexiform neurofibroma on trunk (visible from outside); 2-6 cutaneous neurofibromas	scoliosis, short stature, odontogenic fibroma lower jaw (removed at age of 11)	individual attends vocational training; muscular build; paravertebral plexiform neurofibroma at the site of scoliosis surgery
MIL-R999/399	c.2542G>C	p.Gly848Arg	F	19-26 years	<6	-	2-6 cutaneous and 2-6 subcutaneous neurofibromas	scoliosis, mitral insufficiency, Lisch nodules	none
UG-R923-S	c.2542G>C	p.Gly848Arg	F	14-18 years	<6	-	several cutaneous neurofibromas, #unknown	symptomatic OPG, scoliosis, short stature, cerebellar cystic tumors, borderline mental level, LD	brain MRI: glial cystic tumor of low degree of malignancy in the left cerebellar hemipshere; symmetrical spinal neurofibromas that affected all the spinal nerves; case reported by Pascual-Castroviejo et al.[17]
UG-R923-M	c.2542G>C	p.Gly848Arg	F	>26 years	<6	-	several cutaneous-subcutaneous neurofibromas	-	individual showed asymmetrical spinal neurofibromas in all the spinal nerves; her father died at 40 years of age of multiple hepatic neurofibromas, but reportedly had no cutaneous manifestations of NF1; case reported by Pascual-Castroviejo et al.[17]
SGR-REA	c.2543G>A	p.Gly848Glu	F	>26 years	>5	-	-	mild pulmonic insufficiency, Lisch nodules	none

PrS: **p**roven **s**poradic; **F**: **f**amilial; **RS**: **r**eportedly **s**poradic; **NS**: **n**ot **s**pecified; **LD**: **l**earning **d**ifficulties; **SD**: **s**peech **d**elay; **ADD**: **a**ttention **d**eficit **d**isorder; **ADHD**: **a**ttention **d**eficit **h**yperactivity **d**isorder; **OPG**: **o**ptic **p**athway **g**lioma; **SVCS**: **s**uperior **v**ena **c**ava **s**yndrome**; MPNST**: **m**alignant **p**eripheral **n**erve **s**heath **t**umor; **PT**: **pt**osis; **LSE**: **l**ow **s**et **e**ars; **DPF**: **d**ownslanting **p**alpebral **f**issures

Table S7. Optic pathway gliomas in individuals carrying a missense mutation affecting one of five codons 844-848 in this study.

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
SYMPTOMATIC OPTIC PATHWAY GLIOMAS									
UAB-R624	c.2531T>G	p.Leu844Arg	PrS	14-18 years	>5	+	plexiform neurofibroma on right cheek; cutaneous neurofibromas (#unknown)	bowed long bones, pectus carinatum, pulmonic stenosis, aortic coarctation, Noonan-phenotype features (and presence of pathogenic *PTPN11* mutation), short stature, pilocytic astrocytoma, Lisch nodules	MRI: bulky optic nerves, especially on the right, and a solid expansive lesion in the suprasellar region involving the optic chiasm and deforming the third ventricle; at 7 years of age, the individual complained of visual loss and cranial MRI showed progression with signs of hydrocephalus; the tumor, a pilocytic astrocytoma, was partially resected at age of 8 years; no further therapy was administered and the individual experienced progressive visual loss; case reported by Bertola et al.[14]
PAD-R300	c.2531T>C	p.Leu844Pro	RS	2-4 years	>5	+	-	hypothalamic glioma, abnormal development, SD	OPG involved optic nerves and chiasm
ROT-R02233	c.2537C>A	p.Ala846Asp	PrS	14-18 years	>5	+	plexiform neurofibroma on head (visible from outside, without hyperpigmentation); 2-6 cutaneous neurofibromas	normal development, executive function problems	none
UG-R665	c.2537C>A	p.Ala846Asp	F	5-8 years	>5	+	-	LD, ADHD, hemangioma left (2-3 cm)	OPG involved optic nerves, especially on the left, and chiasm
UL-R83988968	c.2540T>G	p.Leu847Arg	RS	5-8 years	>5	-	-	hypertension, Noonan-like features, macrocephaly, short stature, juvenile xanthogranulomas, LD, Lisch nodules	OPG involved optic nerves; individual needed treatment for **pubertas precox**; hyper nasal speech after adenoidectomy and tonsillectomy
UAB-R3508	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	plexiform neurofibromas on neck, trunk and left arm (visible from outside, with hyperpigmentation); symptomatic spinal neurofibromas (unilateral C1-C3)	short stature, abnormal development, LD, ADD, ADHD	OPG involved optic nerves; individual treated with bevacizumab and chemotherapy for 9-10 months; OPG is no longer symptomatic
CAR-R8012M6	c.2540T>C	p.Leu847Pro	NS	5-8 years	>5	NS	NS	individual attends special school (LD?)	none
MAD-R3.793	c.2540T>C	p.Leu847Pro	RS	19-26 years	>5	+	plexiform neurofibroma on left side of the face (visible from outside) and orbital neurofibroma resected	scoliosis, sphenoid wing dysplasia, short stature, macrocephaly, LD, Lisch nodules	OPG involved left optic nerves and chiasm; hemimegalencephaly with parenchymal atrophy and complex partial seizures
UG-R01-S1	c.2540T>C	p.Leu847Pro	F	>26 years	>5	+	-	short stature, focal epilepsy, psychiatric problems	-
UAB-R1474	c.2542G>A	p.Gly848Arg	RS	5-8 years	>5	+	2-6 intradermal neurofibromas	pectus carinatum, macrocephaly, embryonal rhabdomyosarcoma, abnormal development, gross motor delays, LD, ADHD, Lisch nodules	OPG involved optic nerves and chiasm; p.Gly848Arg causing low level missplicing r.2410_2543del
UG-R923-S	c.2542G>C	p.Gly848Arg	F	14-18 years	<6	-	several cutaneous neurofibromas (#unknown); asymptomatic spinal neurofibromas affected all the spinal nerves	scoliosis, short stature, cerebellar cystic tumors, LD	OPG involved left optic nerve and chiasm; MRI: glial cystic tumor with low degree of malignancy in the left cerebellar hemisphere
SGR-REA-S	c.2543G>A	p.Gly848Glu	F	19-26 years †	>5	-	plexiform neurofibroma on neck (visible from outside, without hyperpigmentation)	short stature, MPNST	OPG involved optic chiasm

PrS: proven **s**poradic**; F**: **f**amilial; **RS**: **r**eportedly **s**poradic; **LD**: **l**earning **d**ifficulties; **SD: s**peech **d**elay; **ADD**: **a**ttention **d**eficit **d**isorder; **ADHD**: **a**ttention **d**eficit **h**yperactivity **d**isorder; **MPNST**: **m**alignant **p**eripheral **n**erve **s**heath **t**umor; **OPG: o**ptic **p**athway **g**lioma**; NS**: **n**ot **s**pecified

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
ASYMPTOMATIC OPTIC PATHWAY GLIOMAS									
UAB-R0903	c.2530C>T	p.Leu844Phe	PrS	9-13 years	>5	+	absent	LD, special education	none
UAB-R0713	c.2531T>G	p.Leu844Arg	RS	5-8 years	>5	+	2-6 cutaneous neurofibromas	macrocephaly, abnormal development	OPG involved optic nerves
MIL-R168	c.2531T>G	p.Leu844Arg	RS	>26 years	>5	-	2-6 cutaneous and 2-6 subcutaneous neurofibromas	scoliosis, possible brainstem glioma, Lisch nodules	OPG involved optic nerves
SGR-RSO	c.2531T>G	p.Leu844Arg	RS	19-26 years	>5	+	absent	scoliosis	none
UAB-R3714	c.2531T>C	p.Leu844Pro	PrS	0-24 months	>5	+	absent	Lisch nodules	none
UAB-R0252	c.2540T>C	p.Leu847Pro	RS	19-26 years	>5	+	plexiform neurofibroma on trunk (with hyperpigmentation); 2-6 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	-	none
UAB-R2934	c.2540T>C	p.Leu847Pro	F	19-26 years	>5	+	2-6 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	Lisch nodules	bilateral OPG, no OPG treatment
UAB-R4965	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	a few subcutaneous neurofibromas on lower back	Lisch nodules, bone cysts, LD, ADD, Asperger syndrome, anger problems, mild hearing loss, migraines, non-ossifying fibroma of the right knee and anger/impulse control issues	OPG involved optic nerves and chiasm; individual had a tethered cord repair and had complaints of chronic pain in various areas
UAB-R2546	c.2540T>C	p.Leu847Pro	RS	14-18 years	>5	+	a few subcutaneous neurofibromas	ADD, migraine headaches (very strong family history of migraines); unusual bone infarctions at the site of some probable non-ossifying fibromas	OPG involved left chiasm and has been present for many years; it has remained asymptomatic and unchanged in size
UAB-R1928	c.2540T>C	p.Leu847Pro	RS	19-26 years	>5	+	6-99 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	dural ectasia, 4th lumbar vertebrae fragmentation, mild pervasive developmental delay, LD	OPG involved optic nerves
MAD-R3.7001	c.2540T>C	p.Leu847Pro	RS	5-8 years	>5	+	plexiform neurofibroma on head (visible from outside); 2-6 cutaneous neurofibromas	Lisch nodules, macrocephaly, LD	OPG involved optic nerves
MAD-R3.409	c.2540T>C	p.Leu847Pro	RS	9-13 years	>5	+	absent	LD	MRI: anomalous posterior fossa venous drainage; OPG involved left optic nerve
MIL-R361/018	c.2540T>C	p.Leu847Pro	F	14-18 years	>5	+	absent	abnormal development	OPG involved optic nerves, especially on the left; MRI: unidentified bright objects (UBOs)
ROT-R89874	c.2540T>C	p.Leu847Pro	F	9-13 years	>5	+	absent	abnormal development	none
UG-R004	c.2540T>C	p.Leu847Pro	F	9-13 years	>5	+	2-6 subcutaneous neurofibromas	ADD, ADHD, special education (LD?)	OPG involved left optic nerve and chiasm; brother was diagnosed with ADHD, but no NF1
UG-R01	c.2540T>C	p.Leu847Pro	F	>26 years	>5	+	2-6 cutaneous and 2-6 intradermal neurofibromas (symptomatic internal neurofibroma in left knee)	Lisch nodules, bone cysts in femur	OPG has remained asymptomatic for 16 years
UAB-R3513	c.2542G>A	p.Gly848Arg	F	2-4 years	>5	+	2-6 cutaneous neurofibromas	-	individual's brother has developmental delay, but he does not have any NF pigmentary signs
UAB-R3237	c.2543G>A	p.Gly848Glu	RS	>26 years	<6	+	plexiform neurofibromas on head, neck, left and right leg (visible from outside, without hyperpigmentation); 1 cutaneous neurofibroma, symptomatic spinal neurofibroma	-	OPG involved optic nerves

PrS: proven **s**poradic**; F**: **f**amilial; **RS**: **r**eportedly **s**poradic; **LD**: **l**earning **d**ifficulties; **ADD**: **a**ttention **d**eficit **d**isorder; **ADHD**: **a**ttention **d**eficit **h**yperactivity **d**isorder; **OPG: o**ptic **p**athway **g**lioma

Table S8. Malignant neoplasms in individuals carrying a missense mutation affecting one of five codons 844-848 in this study.

Family ID	cDNA level	Protein level	Family history	Age	CALMs	Freckles	Neurofibromas	Others	Comments
RHABDOMYOSARCOMA									
UAB-R1227	c.2540T>C	p.Leu847Pro	RS	2-4 years	>5	+	plexiform neurofibroma on back (visible from outside, with hyperpigmentation)	normal development	MRI: mass in left lobe of liver with possible involvement of the right atrium (diagnosis: embryonal rhabdomyosarcoma)
ROT-R49013	c.2540T>C	p.Leu847Pro	RS	>26 years	>5	NS	-	scoliosis, macrocephaly	**embryonal rhabdomyosarcoma** of left testis at 2 years old and **astrocytoma grade II** at 15 years old
UAB-R1474	c.2542G>A	p.Gly848Arg	RS	5-8 years	>5	+	2-6 intradermal neurofibromas	Lisch nodules, symptomatic OPG, pectus carinatum, macrocephaly, abnormal development, gross motor delays, LD, ADHD	diagnosis: embryonal rhabdomyosarcoma; p.Gly848Arg causing low level missplicing r.2410_2543del
MPNST									
MAN-R48902G	c.2530C>T	p.Leu844Phe	F	>26 years	>5	+	6-99 cutaneous, 2-6 subcutaneous and 2-6 intradermal neurofibromas	hypertension	low grade MPNST in right distal femur
CAR-R18010M61	c.2540T>C	p.Leu847Pro	F	>26 years	>5	+	6-99 cutaneous neurofibromas	-	individual has two sisters with multiple neurofibromas and brother who had colorectal cancer in his 40s
CAR-RNIS	c.2540T>C	p.Leu847Pro	F	>26 years	<6	-	plexiform neurofibroma in pelvis; 100-500 cutaneous neurofibromas	-	**MPNST** and **breast cancer**; genetic *BRCA1/2* testing was negative by whole exome sequencing (WES); WES analysis revealed the presence of p.Leu847Pro in all the three tumors and independent somatic "second hits" in *NF1* as well as multiple copy number changes in the breast cancer and MPNST, but not in the plexiform neurofibroma; case reported by McPherson et al.[16]
ROT-R21382	c.2540T>C	p.Leu847Pro	F	>26 years †	>5	+	left nervus ischiadicus, surgically removed; >500 cutaneous and 6-99 subcutaneous neurofibromas, asymptomatic spinal neurofibromas (from T11 and up)	pericarditis carcinomatosa, superior vena cava syndrome (SVCS) and dyspnea on effort	metastasized MPNST; **individual died of MPNST at age of 30 years**
UG-R01-S2	c.2540T>C	p.Leu847Pro	F	19-26 years †	>5	+	cutaneous and subcutaneous neurofibromas (#unknown); possible internal tumors	-	malignant MPNST of mediastinum and abdomen; **individual died of MPNST at age of 25 years**
UG-R01-S3	c.2540T>C	p.Leu847Pro	F	19-26 years †	NS	NS	NS	NS	**individual died of MPNST at age of 20 years**; individual was diagnosed with NF1, no further information available
SGR-REA-S	c.2543G>A	p.Gly848Glu	F	19-26 years †	>5	-	plexiform neurofibroma on neck (visible from outside, without hyperpigmentation)	symptomatic OPG, short stature	**individual died of MPNST at age of 26 years**
OTHERS									
UG-R01-M	c.2540T>C	p.Leu847Pro	F	>26 years †	>5	+	cutaneous neurfoibromas (#unknown); possible internal neurofibroma	Lisch nodules	**individual died of metastasized colon cancer (adenocarcinoma) at age of 54 years**
MIL-R192/982-F	c.2540T>C	p.Leu847Pro	F	>26 years	<6	+	plexiform neurofibroma on palate (without hyperpigmentation); 6-99 cutaneous and 2-6 subcutaneous neurofibromas	Lisch nodules, scoliosis, severe mood depression	**medullary thyroid carcinoma**
UAB-R9493	c.2542G>A	p.Gly848Arg	F	5-8 years	<6	-	-	-	**NF1-associated JMML** (second hit identified: c.1246C>T, p.Arg416*)

F: **f**amilial; **RS**: **r**eportedly **s**poradic; **NS**: **n**ot **s**pecified; **LD**: **l**earning **d**ifficulties; **ADHD**: **a**ttention **d**eficit **h**yperactivity **d**isorder; **OPG**: **o**ptic **p**athway **g**lioma; **JMML**: **j**uvenile **m**yelo**m**onocytic **l**eukemia

Table S9. Comparison of clinical features (malignant neoplasms, >5 CALMs, skinfold freckles, cutaneous and symptomatic spinal neurofibromas) in cohorts of individuals carrying different missense mutations affecting codons 844-848 - additional statistical calculations.

NF1 clinical features	Number of individuals (%)		p value (2-tailed Fisher's exact test)
	p.Leu847Pro	**Other mutations in AA 844-848**	
Malignant neoplasms ≥14 years	8/38 (21.1)	2/40 (5)	**0.0448**
	p.Gly848	**Other mutations in AA 844-848**	
Symptomatic spinal neurofibromas ≥9 years	8/20 (40)	4/59 (6.8)	**0.0012**
>5 CALMs	15/34 (44.1)	115/123 (93.5)	**<0.0001**
Skinfold freckles ≥9 years	11/23 (47.8)	65/72 (90.3)	**<0.0001**
Cutaneous neurofibromas ≥19 years	5/18 (27.8)	42/51 (82.4)	**<0.0001**

Table S10. List of all adjusted p-values after applying the Benjamini-Hochberg correction for multiple testing with false discovery rates at 0.05 and 0.01.

p-value	index	Statistical significance at p<0.05		Statistical significance at p<0.01	
		B-H critical [A]	value [B]	B-H critical [A]	value [B]
0.8794	1	0.0250		0.0050	
0.5830	2	0.0246		0.0049	
0.5727	3	0.0242		0.0048	
0.4489	4	0.0237		0.0047	
0.4296	5	0.0233		0.0047	
0.3590	6	0.0229		0.0046	
0.2968	7	0.0225		0.0045	
0.2916	8	0.0220		0.0044	
0.1349	9	0.0216		0.0043	
0.1323	10	0.0212		0.0042	
0.1160	11	0.0208		0.0042	
0.1029	12	0.0203		0.0041	
0.0693	13	0.0199		0.0040	
0.0619	14	0.0195		0.0039	
0.0451	15	0.0191		0.0038	
0.0409	16	0.0186		0.0037	
0.0404	17	0.0182		0.0036	
0.0384	18	0.0178		0.0036	
0.0341	19	0.0174		0.0035	
0.0276	20	0.0169		0.0034	
0.0263	21	0.0165		0.0033	
0.0241	22	0.0161		0.0032	
0.0186	23	0.0157		0.0031	
0.0174	24	0.0153		0.0031	
0.0164	25	0.0148		0.0030	
0.0125	26	0.0144	*	0.0029	
0.0080	27	0.0140	*	0.0028	
0.0076	28	0.0136	*	0.0027	
0.0067	29	0.0131	*	0.0026	
0.0061	30	0.0127	*	0.0025	
0.0060	31	0.0123	*	0.0025	
0.0043	32	0.0119	*	0.0024	
0.0042	33	0.0114	*	0.0023	
0.0028	34	0.0110	*	0.0022	
0.0025	35	0.0106	*	0.0021	
0.0023	36	0.0102	*	0.0020	
0.0022	37	0.0097	*	0.0019	
0.0020	38	0.0093	*	0.0019	
0.0012	39	0.0089	*	0.0018	*
0.0007	40	0.0085	*	0.0017	*
0.0005	41	0.0081	*	0.0016	*
0.0004	42	0.0076	*	0.0015	*
0.0002	43	0.0072	*	0.0014	*
0.0002	44	0.0068	*	0.0014	*
0.0001	45	0.0064	*	0.0013	*
0.0001	46	0.0059	*	0.0012	*
0.0001	47	0.0055	*	0.0011	*
0.0001	48	0.0051	*	0.0010	*
0.0001	49	0.0047	*	0.0009	*
0.0001	50	0.0042	*	0.0008	*
0.0001	51	0.0038	*	0.0008	*
0.0001	52	0.0034	*	0.0007	*
0.0001	53	0.0030	*	0.0006	*
0.0001	54	0.0025	*	0.0005	*
0.0001	55	0.0021	*	0.0004	*
0.0001	56	0.0017	*	0.0003	*
0.0001	57	0.0013	*	0.0003	*
0.0001	58	0.0008	*	0.0002	*
0.0001	59	0.0004	*	0.0001	*

[A] according to Thissen et al.[26]; [B] an asterisk (*) indicates the statistically significant p-value after B-H correction

Table S11. A mosaic neurofibromatosis type-1 case (13.5-year-old girl presenting with >5 CALMs and skinfold freckling) with the *NF1* c.2540T>C (p.Leu847Pro) mutation found as the "second hit" in the cultured melanocytes from one CALM.

Family ID	**Biopsy**	**Mutation**	
		"First hit" [A]	**"Second hit"**
UAB-R921FNES	1st CALM biopsy		**c.2540T>C (p.Leu847Pro)**
* analysis performed on successfully cultured	2nd CALM biopsy	c.5547-1G>A	absent
melanocytes from three biopsies of CALMs	3rd CALM biopsy		c.1432A>G (p.Lys478Glu)

A mutation leads to out-of-frame skipping of exon 38 [29] during *NF1* mRNA splicing and out-of-frame skipping exons 38 and 39 [29 and 30] in a proportion of *NF1* mRNA transcripts; the c.5547-1G>A mutation was not found in the blood lymphocytes by next-generation sequencing (0/600 reads, 3 different amplicons)

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