Supporting Information for the article

High Incidence of Noonan Syndrome Features including short stature and Pulmonic Stenosis in patients carrying *NF1* Missense Mutations Affecting p.Arg1809: Genotype-Phenotype Correlation

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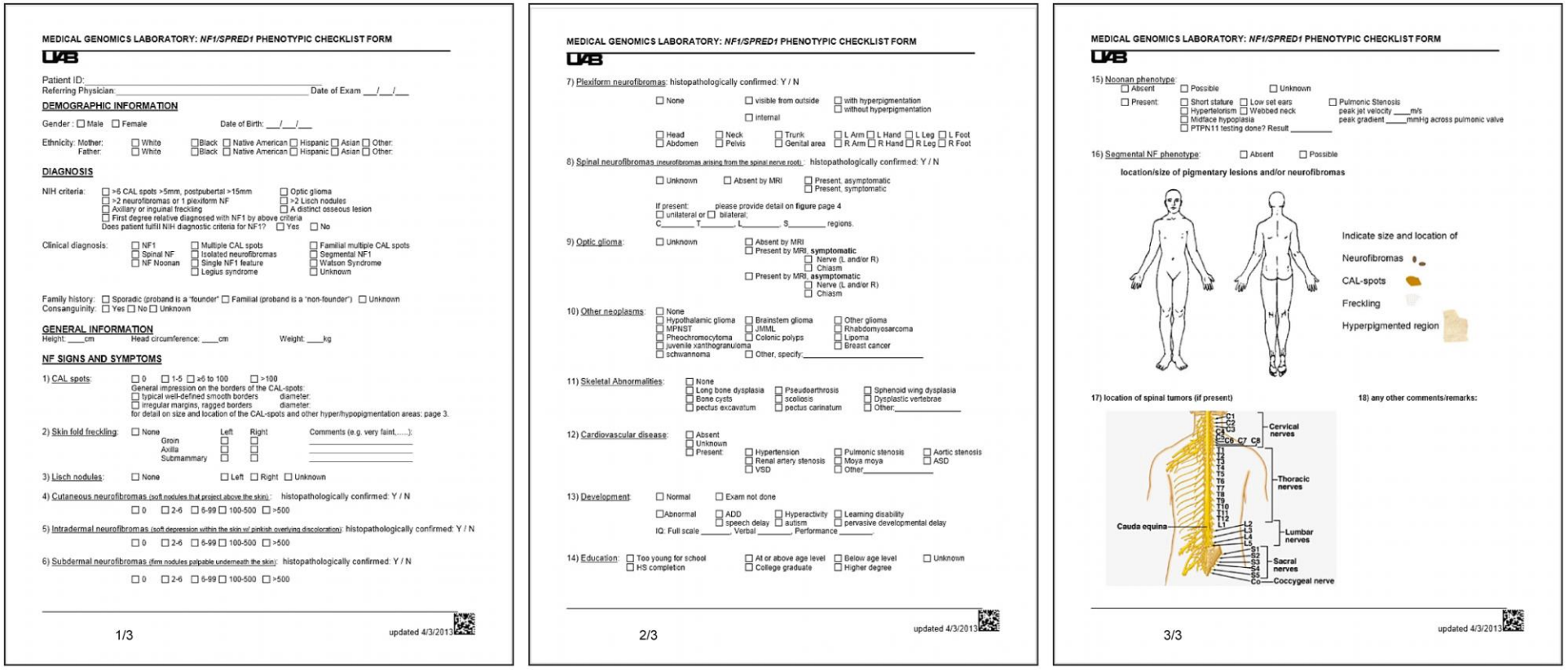


MEDICAL GENOMICS LABORATORY: *NF1/SPRED1* PHENOTYPIC CHECKLIST FORM

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

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

1/3 updated 4/3/2013

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 ☐ Abdomen ☐ Neck ☐ Pelvis ☐ Trunk ☐ Genital area ☐ L Arm ☐ L Hand ☐ L Leg ☐ L Foot ☐ 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 ☐ MPNST ☐ Pheochromocytoma ☐ juvenile xanthogranuloma ☐ schwannoma
☐ Brainstem glioma ☐ JMML ☐ Colonic polyps ☐ Other, specify:_____
☐ Other glioma ☐ Rhabdomyosarcoma ☐ Lipoma ☐ Breast cancer

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

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

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

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

2/3 updated 4/3/2013

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

UAB

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

16) Segmental NF phenotype: ☐ Absent ☐ Possible
location/size of pigmentary lesions and/or neurofibromas

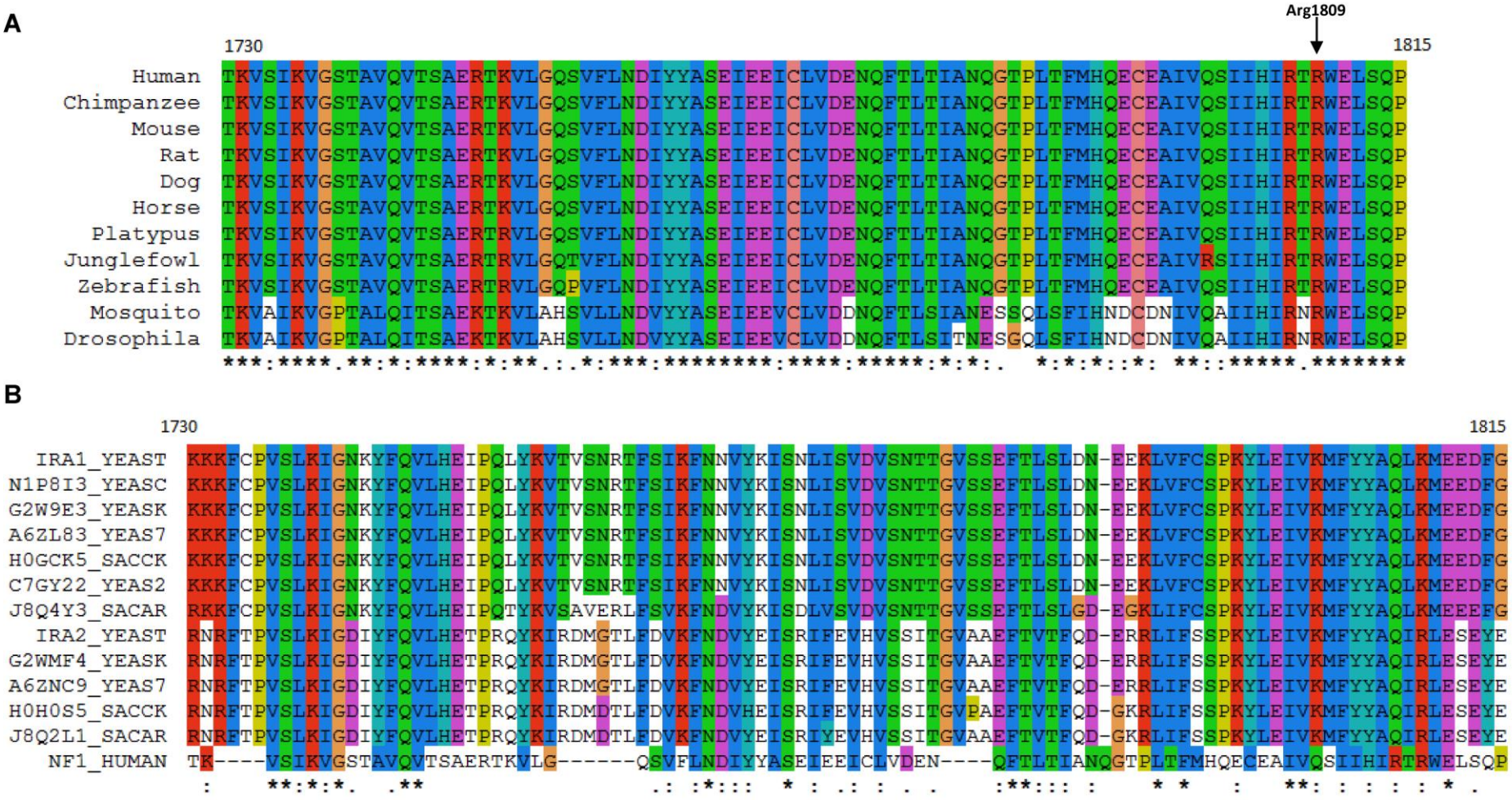
Indicate size and location of
Neurofibromas
CAL-spots
Freckling
Hyperpigmented region

17) location of spinal tumors (if present)

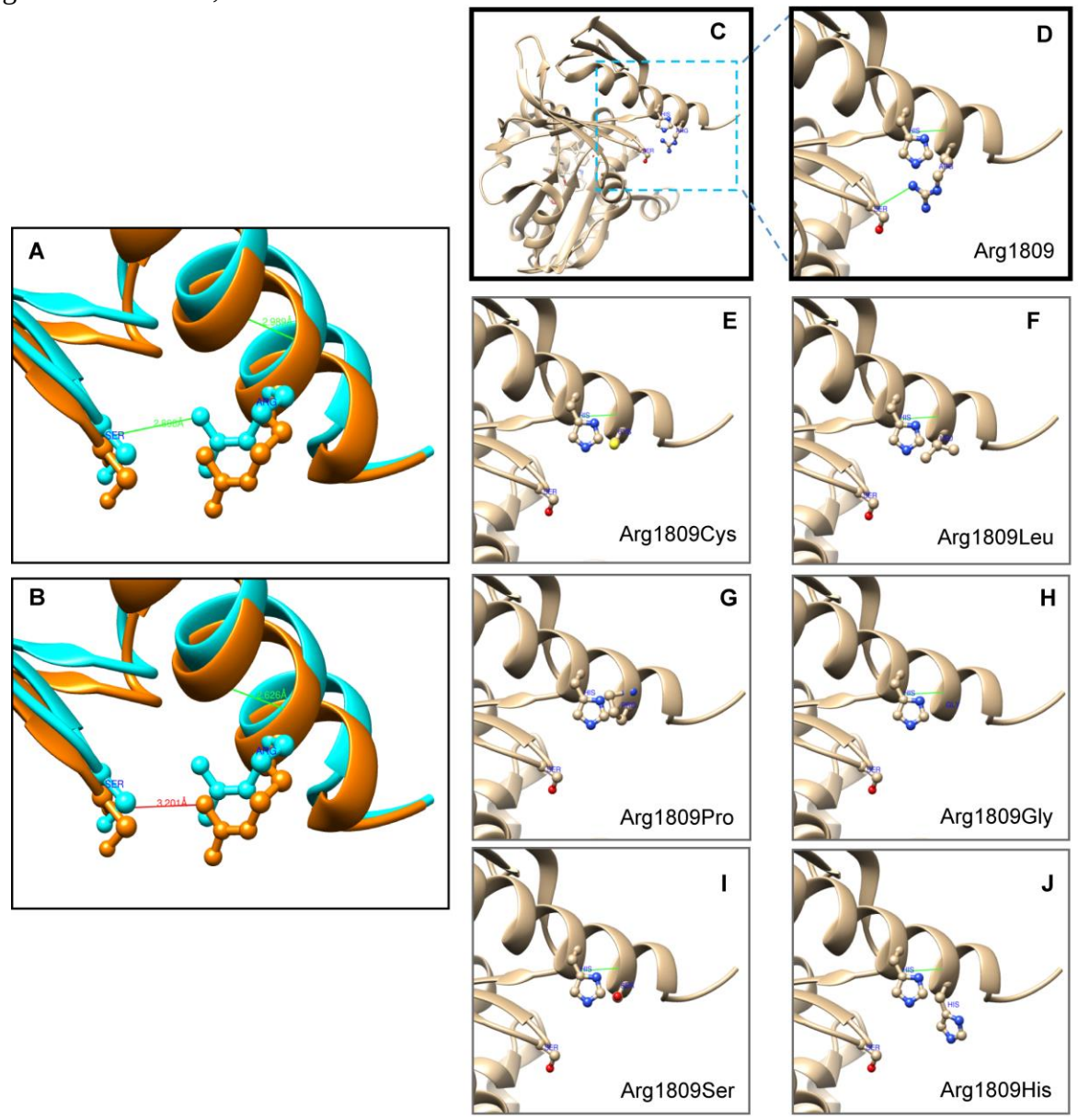
18) any other comments/remarks:

3/3 updated 4/3/2013

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



Supp. Figure S2. **A.** Multiple sequence alignment of a region of NF1. NF1 from 11 different species were included in the alignment: Human, RefSeq: NP_000258.1 residues 1730-1815; Chimpanzee, TrEMBL: K7DMZ3 residues 1730-1815; Mouse, SWISSPROT: Q04690-2 residues 1732-1817; Rat, SWISSPROT: P97526 residues 1732-1817; Dog, RefSeq: XP_537738.3 residues 1736-1821; Horse TrEMBL: F6XRM7 residues 1665-1750; Platypus, RefSeq: XP_007664859.1 residues 1787-1872; Junglefowl, RefSeq: XP_003642464.1 residues 1725-1810; Zebrafish, TrEMBL: E7FBD0 residues 1720-1805; Mosquito, TrEMBL: B0WYP5 residues 1739-1824; and Drosophila, TrEMBL: O01397 residues 1782-1867. The multiple sequence alignment was performed with ClustalW2 program. **B.** Multiple sequence alignment of Human NF1 residue 1730-1815 (isoform2), and corresponding regions of yeast IRA1 and IRA2. Human NF1 (NF1_HUMAN, i.e. RefSeq NP_000258.1), 7 yeast IRA1 (IRA1_YEAST, N1P8I3_YEASC, G2W9E3_YEASK, A6ZL83_YEAS7, H0GCK5_SACCK , C7GY22_YEAS2, J8Q4Y3_SACAR) and 5 yeast IRA2 (IRA2_YEAST, G2WMF4_YEASK, A6ZNC9_YEAS7, H0H0S5_SACCK, J8Q2L1_SACAR) sequences were included in the alignment. The multiple sequence alignment was performed with ClustalW2 program.



K

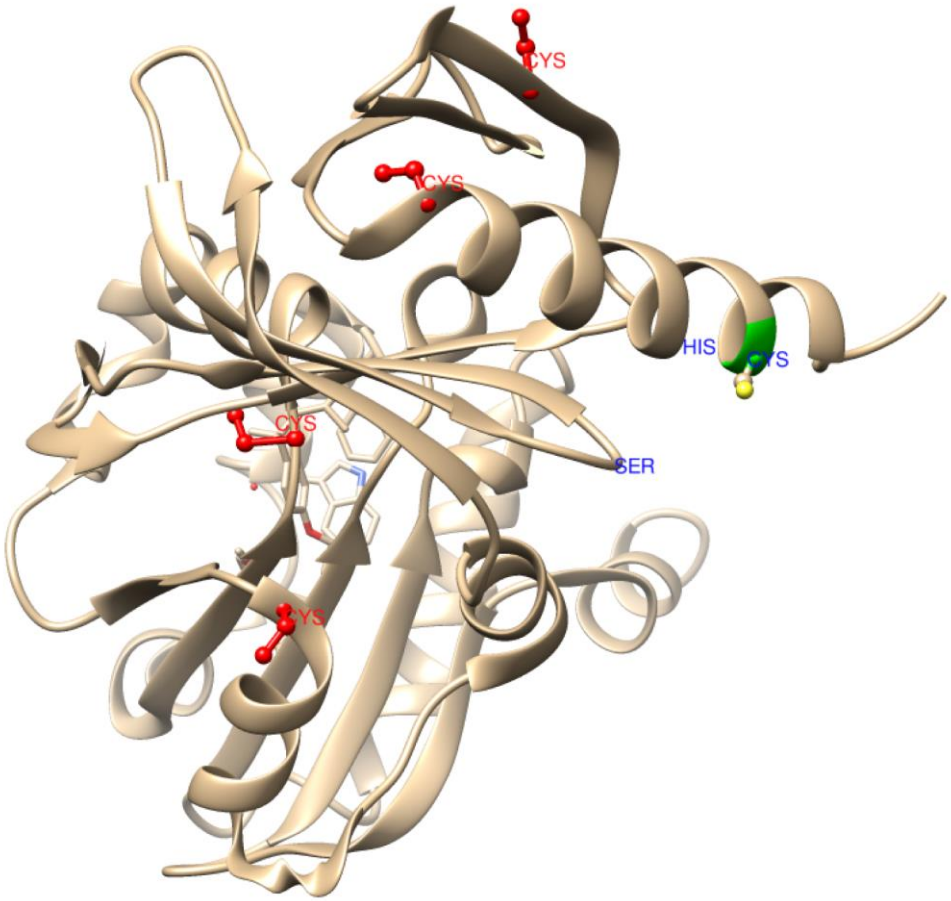
Variant	Hydrogen bond with Ser1738	Hydrogen bond with His1805
p.Arg1809	Present (2.898 Å)	Present (2.989 Å)
p.Arg1809Cys	Lost	Present (2.989 Å)
p.Arg1809Leu	Lost	Present (2.989 Å)
p.Arg1809Pro	Lost	Loss
p.Arg1809Gly	Lost	Present (2.989 Å)
p.Arg1809Ser	Lost	Present (2.989 Å)
p.Arg1809His	Lost	Present (2.989 Å)

Supp. Figure S3. A and B: Comparison of H-bonds involving the residue Arg1809 in two crystal structures of human NF1, PDB-Accession code: 2D4Q and 2E2X. Crystal structures of human NF1 (2D4Q in cyan and 2E2X in orange) were overlaid and close-up views of Arg1809 and its H-bonds with Ser1738 the His1805 were shown: **(A)** 2D4Q and **(B)** 2E2X. Two structures show a slight difference: for **2D4Q**, the donor-acceptor distances between Arg1809-His1805 is 2.989 Å and Arg1809-Ser1738 is 2.898 Å; for **2E2X**, the distances are 2.626 Å and 3.201 Å, respectively.

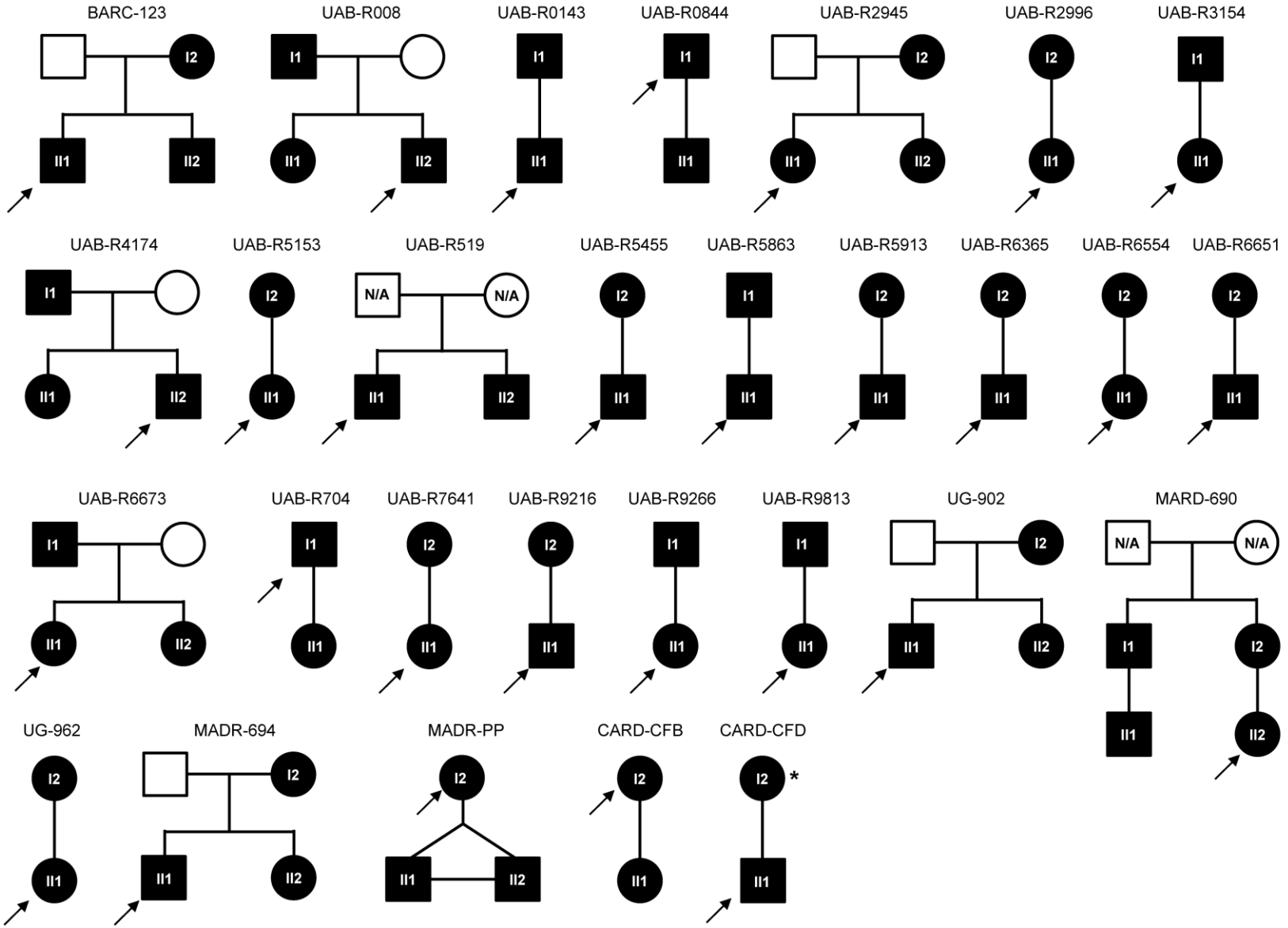
Most H-bonds in proteins are in the moderate category with donor-acceptor distances at 2.5 Å ~3.2 Å. In the figures, moderate H-bonds were marked with green lines and weak ones were marked with red lines (donor-acceptor distances at 3.2 Å ~4.0 Å).

C to K: Tertiary structure prediction of human NF1 p.Arg1809 mutations. **(C)** Crystal structure of human NF1 (PDB-Accession code: 2D4Q, detergent bound form), in which three residues Arg1809, Ser1738 and His1805 were highlighted. **(D)** Zoomed view of the three residues (Arg1809, Ser1738 and His1805) and H-bonds (shown as green line) involving the residue Arg1809. **(E-J)** Zoomed view of NF1 p.Arg1809 mutations: Arg1809Cys **(E)**, Arg1809Leu **(F)**, Arg1809Pro **(G)**, Arg1809Gly **(H)**, Arg1809Ser **(I)**, and Arg1809His **(J)**. The H-bonds involving p.Arg1809 mutations (Cys, Leu, Pro, Gly, Ser and His) were shown as green lines and a summary of the H-bond alterations based on 2D4Q 3D model was listed in the table (Panel K).

2E2X was also used to analyze p.Arg1809 mutations (results not shown) and the results showed no difference compared to 2D4Q, except for the mutation Arg1809Pro. In 2D4Q, the mutation Arg1809Pro leads to loss of both H-bonds with Ser1738 and His1805; however, in 2E2X, only the weak H-bond with Ser1738 is lost, the H-bond with His1805 retains, just like all other p.Arg1809 mutations.

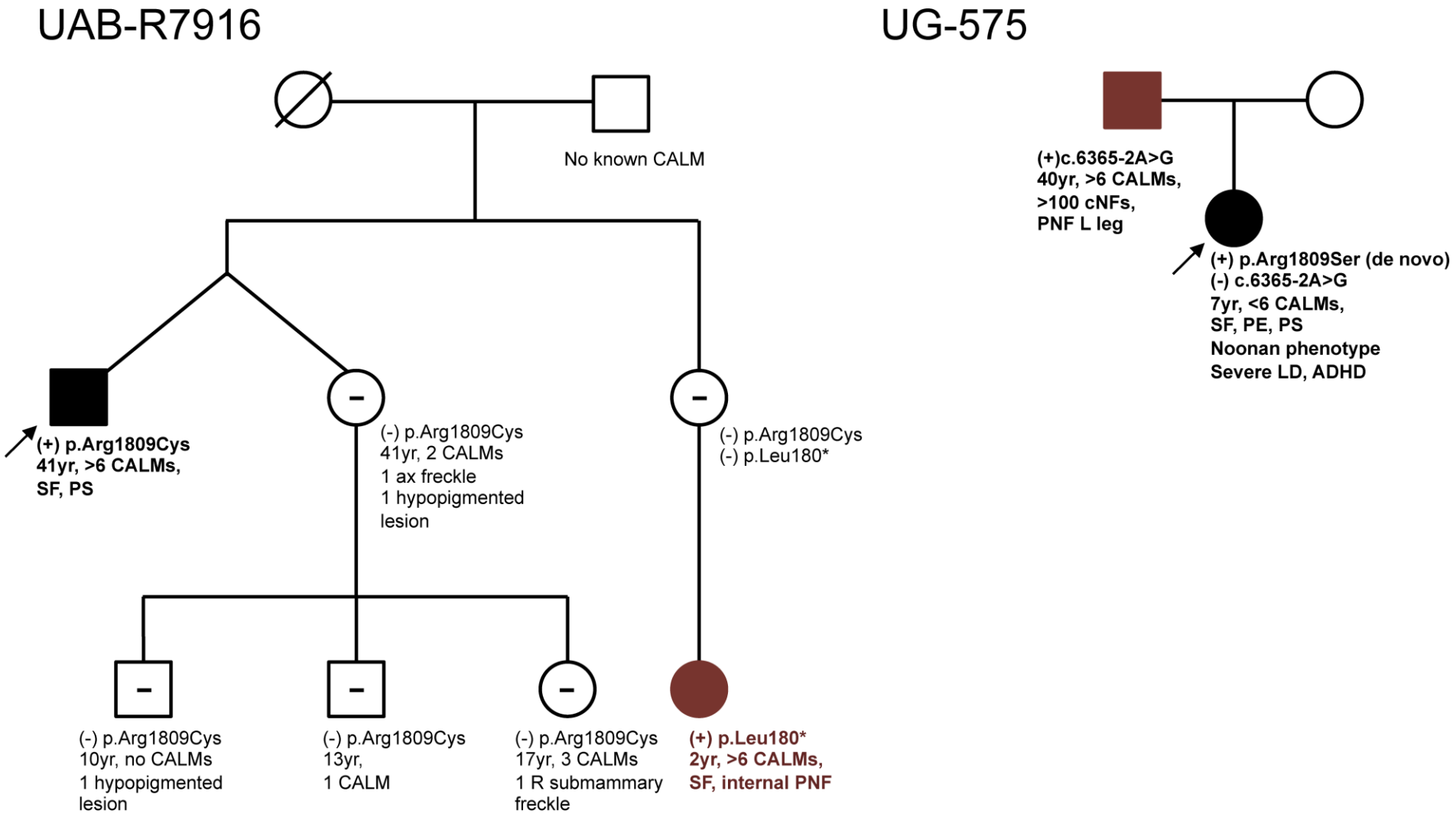


Supp. Figure S4. 3D structure of the 2D4Q detergent-bound form. Localization of all Cys side chains (red) within the Sec14-PH domain, with Arg1809Cys depicted in green. None of these Cysteines are located at a distance that would allow p.Arg1809Cys to engage in a disulfide bond.



Supp. Figure S5. Pedigrees of the familial cases in this study. The filled symbols represent the patients who are positive for Arg1809 mutations, and the open symbols are relatives who are negative for the family specific mutations. Note that "N/A" indicates the relatives were not available for genetic analysis. The arrow annotates the proband of the family. For the first generation, father is always I1 and mother is I2.

*: Limited clinical information was provided for CARD-CFD-I2 ("multiple CALs and mutation positive"), therefore this individual is only mentioned in the comments section in the Supp. Table S2.



Supp. Figure S6. Pedigrees of two families with a missense mutation at p.Arg1809 in the proband: p.Arg1809Cys in family UAB-R7916 and p.Arg1809Ser in family UG-575. A different truncating mutation was identified in both families in a 2nd-degree (UAB-R7916) or 1st-degree relative (UG-575) presenting with classic NF1 (depicted in dark brown).

Abbreviations: yr, years; CALM, café-au-lait macule; SF, skinfold freckling; PS, pulmonic stenosis; ax, axillary; R, right; L, left; PNF, plexiform neurofibroma; (-), mutation absent; cNF, cutaneous neurofibroma; PE, pectus excavatum; LD, learning disability; ADHD, attention deficit hyperactivity disorder.

Supp. Table S1. Clinical details for 136 individuals from 98 different families carrying a constitutional *NF1* missense mutation affecting p.Arg1809

Supp. Table S1 is included as a separate Excel file under the Supporting Information for this article.

[a]. two distinct pathogenic *NF1* mutations were identified in the family (see also Supp. Figure S6)

[b]. exact ages were used to calculate Height and Head Circumferences, but data are provided as "age-groups" in this Table: 0-24 months; 2-4 years; 5-8 years; 9-13 years; 14-18 years; 19-26 years; >26 years

[c]. fulfilling NIH criteria when taking family history into account and assuming the patient indeed has few small neurofibromas (none however histopathologically proven, nor biopsied)

[d]. height evaluated using the WHO or CDC charts results in value PC≤3 but given the Hispanic or Asian ethnicity this patient is not considered to have true Short Stature due to the lack of ethnic-specific growth charts. [ ] height percentiles for Hispanic and Asian individuals are provided between square brackets to indicate that they were not included for the data analysis on frequency of short stature.

[e]. The patients UAB-R2376, MARD-690-II2 and MARD-690-II1 were NOT considered having cognitive impairment/LD, despite presence of ADHD/ADD, as the referring physician said "normal development and IQ" on the phenotypic checklist.

Abbreviations: Ethnicity: W: white; His: hispanic; AA: African American; As: Asian; NaA: Native American; Pa: Pakistani; Ar: Arabic; In: Indian; A, African; F, Familial; PrS, proven sporadic; RS, reportedly sporadic; UN, unknow; PC, Percentile; NS, Not specified, i.e. no value provided on the phenotypic checklist; N, UN, no symptomatic optic glioma (OG), unknown if an asymptomatic OG is present as no MRI data available; LD, learning disability; HTL, hypertelorism; MH, midface hypoplasia; SS, short stature; WN, webbed neck; DPF, downslanting palpebral fissures; LSE, low Set ears; PC, pectus carinatum; PE, pectus excavatum; PS, pulmonic stenosis; Pt, ptosis; SN, short neck; EF, epicantus folds; LPH, low posterior hairline; BBE, bright blue eyes; PRE, posteriorly rotated ears; DD, developmental delay; SD, speech delay; PNF, plexiform neurofibromas.

Supp. Table S2. *In silico* prediction of pathogenicity of all possible missense mutations affecting Arg1809, and effect on splicing as observed *in vivo* (RNA from lymphocytes)

Mutation	Protein	Mutation Frequency			Grantham distance	SIFT	MutationTaster	PolyPhen	CADD v1.0	MutPred Splice prediction	RNA observed	Other evidence
		Arg1809-positive probands	*NF1*- positive probands UAB cohort	ExAC control population								
c.5425C>G	p.Arg1809Gly	1/98	0/7000	0/61,486	125	deleterious (0.02)	disease causing (p=1)	probably damaging 0.991 (sens 0.71; spec 0.97)	21.9	0.43 - Splice Neutral Variant	normal	segregation in family MADR-690
c.5425C>T	p.Arg1809Cys	75/98	67/7000	0/61,486	180	deleterious (0.00)	disease causing (p=1)	probably damaging 1.000 (sens 0.00; spec 1.00)	23.5	0.42 - Splice Neutral Variant	normal	segregation multiple families (BARC-123; UAB-R008; UAB-R0143; UAB-R2945; UAB-R3154; UAB-R4174; UAB-R5153; UAB-R519; UAB-R5455; UAB-R5863; UAB-R5913; UAB-6554; UAB-R6651; UAB-R6673; UAB-R704; UAB-R7464; UAB-R7641; UAB-R9216; UAB-R9266; UG-902; UG-962); proven de novo multiple probands (UAB-R1583; UAB-R2261; UAB-R2635; UAB-R3175; UAB-R3412; UAB-R3885; UAB-R3926; UAB-R3933; UAB-R4816; UAB-R4844; UAB-R6935; UAB-R7626; UAB-R8141; UAB-R9044; UAB-R9851); somatic mutation in melanocytes of segmental patient UAB-RSeNF23
c.5425C>A	p.Arg1809Ser	3/98	1/7000	0/61,486	110	deleterious (0.02)	disease causing (p=1)	probably damaging 0.991 (sens 0.71; spec 0.97)	24.3	0.41 - Splice Neutral Variant	normal	proven de novo (UG-575; UAB-R4906)
c.5426G>T	p.Arg1809Leu	15/98	13/7000	0/61,486	102	deleterious (0.02)	disease causing (p=1)	probably damaging 0.979 (sens 0.76; spec 0.96)	34	0.69 - Splice Affecting Variant - Strong	skipping of exon 2 (OOF) and exon 29+30 (OOF) in a small fraction of transcripts	segregation in multiple families (UAB-R0844; UAB-R6365; UAB-R9813; CARD-CFD); proven de novo (UAB-R7171; UAB-R7545; UAB-R8423; CARD-CFE)
c.5426G>C	p.Arg1809Pro	4/98	4/7000	0/61,486	103	deleterious (0.02)	disease causing (p=1)	probably damaging 0.998 (sens 0.27; spec 0.99)	29.3	0.57 - Splice Neutral Variant	skipping of exon 2 (OOF) and exon 29+30 (OOF) in a small fraction of transcripts	proven de novo (UAB-R3133; UAB-R7442)
c.5426G>A	p.Arg1809His	0/98	0/7000	0/61,486	29	deleterious (0.02)	disease causing (p=1)	probably damaging 0.997 (sens 0.41; spec 0.98)	34	0.46 - Splice Neutral Variant	ND	not observed

Abbreviations: sens: Sensitivity; spec: Specificity

Supp. Table S3. Cutaneous, dermal and/or subdermal neurofibromas in individuals ≥19 years old carrying a missense mutations at Arg1809 in this study and previous studies

Age group	**This study**	**Nyström el al, Ekvall et al, Pinna et al, Santoro et al**	Total
19-26yrs	0/10	0/3	0/13
27-35yrs	2/8 (1 with possible subdermal NF, 1 with 1-2 possible subdermal NF)	0/3	2/11*
36-50yrs	2/16 (1 with possible small NF on R forearm and 1 with possible subdermal NF)	0/8	2/24*
>50yrs	1/5 (1 with possible small subdermal NF)	0/6	1/11*

* Clear-cut multiple cutaneous, dermal and/or subdermal neurofibromas were not seen in any individual. Few cases had 1-2 possible neurofibromas, but none were biopsied for histopathology.