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Diagnoses of double heterozygous NF1 variants and dual RASopathy

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Abstract
Genetic testing in neurofibromatosis type 1 (NF1) occasionally reveals two heterozygous *NF1* variants in the same individual. Correct interpretation hinges on allelic phasing, elucidation of somatic second hits in lesions, and distinction from bona fide dual RASopathy diagnoses. We provide a focused, critical review of the primary literature on (i) *cis* doublets in *NF1*, (ii) purported germline *trans* configurations—particularly in spinal neurofibromatosis, (iii) biallelic somatic inactivation of *NF1* in café-au-lait macules, tumors, and juvenile myelomonocytic leukemia, and (iv) confirmed cases of dual RASopathy. We also include an index patient with two heterozygous *NF1* variants to illustrate clinical challenges. Phase-proven *cis* doublets are rare, often closely spaced, and generally accompany classic NF1 without reproducible phenotype escalation. Claims of germline *trans NF1* pathogenic variants weaken on reappraisal: second alleles reported in spinal neurofibromatosis typically do not meet current pathogenicity standards, while Nf1−/− mouse embryonic lethality underscores the biological implausibility of constitutive biallelic loss. In contrast, biallelic somatic *NF1* inactivation is pervasive across NF1 lesions, including café-au-lait macules, plexiform and cutaneous neurofibromas, and juvenile myelomonocytic leukemia, due to mitotic recombination (copy-neutral loss-of-heterozygosity), microdeletions, or a second variant. Confirmed dual RASopathy diagnoses (most often *NF1* plus *PTPN11*) are rare and produce blended systemic phenotypes. Interpretation of double *NF1* findings should prioritize independent American College of Medical Genetics and Genomics/Association for Molecular Pathology classification of each variant, rigorous phasing (segregation/RNA/allele-specific methods), and tumor-focused analysis where applicable. Our index case and synthesis support a practical workflow that clarifies counseling and helps avoid over-calling dual pathogenicity.

Introduction

Neurofibromatosis type 1 (NF1; MIM #162200, ORPHA:636) is a common RASopathy following an autosomal dominant inheritance pattern, near-complete penetrance, and striking variability of expression. Approximately half of the cases arise from de novo variants, and inter-individual courses span pigment-predominant phenotypes to heavy internal tumor burdens, with additional morbidity due to neurocognitive and behavioral difficulties. Despite decades of study, genotype–phenotype correlations remain limited at the individual level. Aside from a few established associations

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[e.g., 17q11.2 microdeletions and the recurrent alleles NM_000267.3(NF1):c.5425 C > T (p.Arg1809Cys) and NM_001042492.3(NF1):c.2970_2972del (p.Met992del)] the identity or class of the germline *NF1*variant does not reliably predict clinical outcomes such as the occurrence or burden of tumors, age at onset of optic pathway glioma, neurocognitive/behavioral difficulties, or vascular complications. This uncertainty complicates counseling and surveillance planning for families [1–3].

Molecularly, NF1 is a formidable diagnostic challenge. More than 3,000 distinct disease-causing germline variants have been described in the literature, predominantly single-nucleotide substitutions and small indels, with a significant minority of microdeletions and complex splice-altering changes [1]. Large public resources curate many thousands of additional sequence records. ClinVar lists > 16,000 variants mapping to *NF1* [single-nucleotide variants/indels and overlapping copy number variations (CNVs); access date November 2025], while a locus-specific *NF1* database (www.LOVD.nl/NF1) provides transcript-resolved entries (e.g. NM_000267.3; NM_001042492.3). The possibility of mosaicism further muddles interpretation.

In ~ 5–10% of individuals, NF1 results from recurrent 17q11.2 microdeletions that remove the entire NF1 gene plus flanking genes [4–6]. The most frequent, type-1 deletions (~ 1.4 Mb), arise by non-allelic homologous recombination between *NF1* and flanking repetitive sequences (termed REPs) and typically remove ~ 14 protein-coding genes including *SUZ12*. They are usually constitutional (germline) and account for ~ 70–80% of *NF1* microdeletions [7]. Type-2 deletions (~ 1.2 Mb) result from recombination between *SUZ12* and *SUZ12P1*and are often post-zygotic (mosaic) [8]. Type-3 (~ 1.0 Mb) and atypical deletions are less common with variable breakpoints [9]. On average, microdeletion genotypes associate with earlier and heavier tumor burden and additional dysmorphic/neurodevelopmental features. Awareness of these classes informs testing [e.g., multiplex ligation-dependent probe amplification (MLPA)/next-generation sequencing (NGS) CNV calling].

In response, comprehensive testing strategies that pair high coverage sequencing with copy-number analyses and, when indicated, dosage analysis and RNA studies for cryptic splice effects have become routine in many centers [10]. The 2021 international revision of diagnostic criteria explicitly integrated molecular testing and clarified the boundary with Legius syndrome (*SPRED1*-related; MIM #611431, ORPHA:137605), which is a key differential diagnosis in pigment-only presentations [11].

Contemporary multigene testing has highlighted that some individuals carry pathogenic or likely pathogenic (subsequently referred to as pathogenic) variants in more than one cancer-predisposition gene (variously termed multilocus inherited neoplasia or double heterozygosity). Although uncommon, such multilocus genotypes recur across cohorts and often influence surveillance and counseling—underscoring the need for careful variant interpretation in context [12, 13].

Against this backdrop, encountering two *NF1* variants in one individual raises the central question of allelic phase: do the variants reside on the same allele (*cis*; monoallelic) or on opposite alleles (*trans*; biallelic)? Establishing the phase is a foundational informational step and typically relies on segregation studies (parents), allele-specific polymerase chain reaction (PCR)/cloning, RNA/complementary DNA analysis for splice effects, or long-read/haplotype-based approaches when needed.

The clinical importance of allelic status within a single gene is well illustrated in other hereditary conditions. In the DNA mismatch-repair genes, monoallelic pathogenic variants underlie Lynch syndrome (MIM #120435, ORPHA:144), whereas biallelic constitutional defects cause childhood-onset constitutional mismatch repair deficiency (CMMRD) with a distinct clinical spectrum (MIM #276300, ORPHA:252202) [14, 15]. In the homologous-recombination pathway, monoallelic variants in genes such as *BRCA2* or *PALB2* confer hereditary breast and ovarian cancer susceptibility (MIM #612555, ORPHA:145), while biallelic loss produces Fanconi anemia subtypes with early-onset hematologic and solid tumor risks (MIM #605724 and 610832, ORPHA:84) [16]. These single-gene examples demonstrate how the clinical and biological context may hinge on whether one or both alleles are affected, a principle that motivates careful attention to the *cis*/*trans* architecture, segregation, and potential multilocus genotypes in NF1.

By contrast, a dual RASopathy diagnosis denotes two independent germline conditions in different Ras/mitogen-activated protein kinase (MAPK) pathway genes (for example, *NF1* together with *PTPN11* or *KRAS*). This is a multilocus scenario, not a single-gene allelic-status issue, and it requires rigorous variant curation and segregation to avoid over-attribution [17].

Here, we present an index patient with two *NF1* variants and then synthesize primary literature on (i) *cis* doublets, (ii) germline *trans* configurations, (iii) biallelic somatic inactivation in café-au-lait macules (CALMs), tumors, and juvenile myelomonocytic leukemia (JMML), and (iv) bona fide dual RASopathy diagnoses, closing with a practical phasing/interpretation workflow.

Case report

A 10-year-old boy was referred for ongoing evaluation of NF1. Clinical features include multiple CALMs, bilateral Lisch nodules, plexiform neurofibromas, macrocephaly, focal areas of signal intensity on brain magnetic resonance imaging, and a low-grade glioma.

Neurodevelopmental/behavioral history is notable for attention-deficit disorder, motor developmental delay with muscular hypotonia, reading and writing disability, and balance difficulties. On this basis he fulfills the clinical diagnostic criteria for NF1 [11].

Germline testing was performed outside our institutions at an external accredited laboratory using Sanger sequencing and MLPA on peripheral-blood DNA and identified two heterozygous *NF1* variants [NM_001042492.3(NF1):c.3763 C > T (p.Gln1255*) and NM_001042492.3(NF1):c.3826 C > T (p.Arg1276*)], each classified as pathogenic. As expected for Sanger-based testing, quantitative variant allele fractions were not generated and read-backed phasing was not possible. Sensitivity for low-level mosaicism is limited and deep intronic or structural alterations beyond MLPA detection were not assessed in the work-up. A detailed three-generation history was non-diagnostic. On examination, both parents each had single CALM. Neither *NF1* variant identified in the proband was detected in parental samples by targeted Sanger sequencing of blood DNA. Allelic phase has not been established.

Given these constraints, two configurations were considered most plausible: (i) a *cis* doublet (both variants on the same allele), which is compatible with the child's classic NF1 phenotype, or (ii) a germline variant plus a post-zygotic hematopoietic (blood-restricted) variant, which can appear as a second hit on blood testing despite a monoallelic germline architecture. In that setting, the putative somatic change often shows a lower or discordant variant allele fraction and is absent in non-hematologic tissue. Comparison with an alternative specimen (e.g., cultured skin fibroblasts or hair roots) would clarify tissue distribution. A third possibility, reflecting the high *de novo* mutation rate in *NF1*, is that both variants arose as independent *de novo* germline events. In view of the biological implausibility of constitutional biallelic *NF1* loss, such double *de novo* changes would most plausibly reside in *cis*, consistent with clustered/multinucleotide events and with reports of distinct *NF1* pathogenic variants occurring in first-degree relatives [18]. In our patient, follow-up phase-resolved analysis on a new peripheral-blood sample established that the two *NF1* variants reside in *cis*. (Fig. 1) Observed allele fractions were compatible with heterozygosity, with no evidence for blood-restricted mosaicism. This result, together with the literature synthesis presented here, underpins the phasing/interpretation workflow included to guide similar cases in practice.

Monoallelic cis doublets in *NF1*

Reports of two pathogenic *NF1* variants on the same allele (*cis*; monoallelic) are rare but well documented. The most convincing demonstrations establish phase

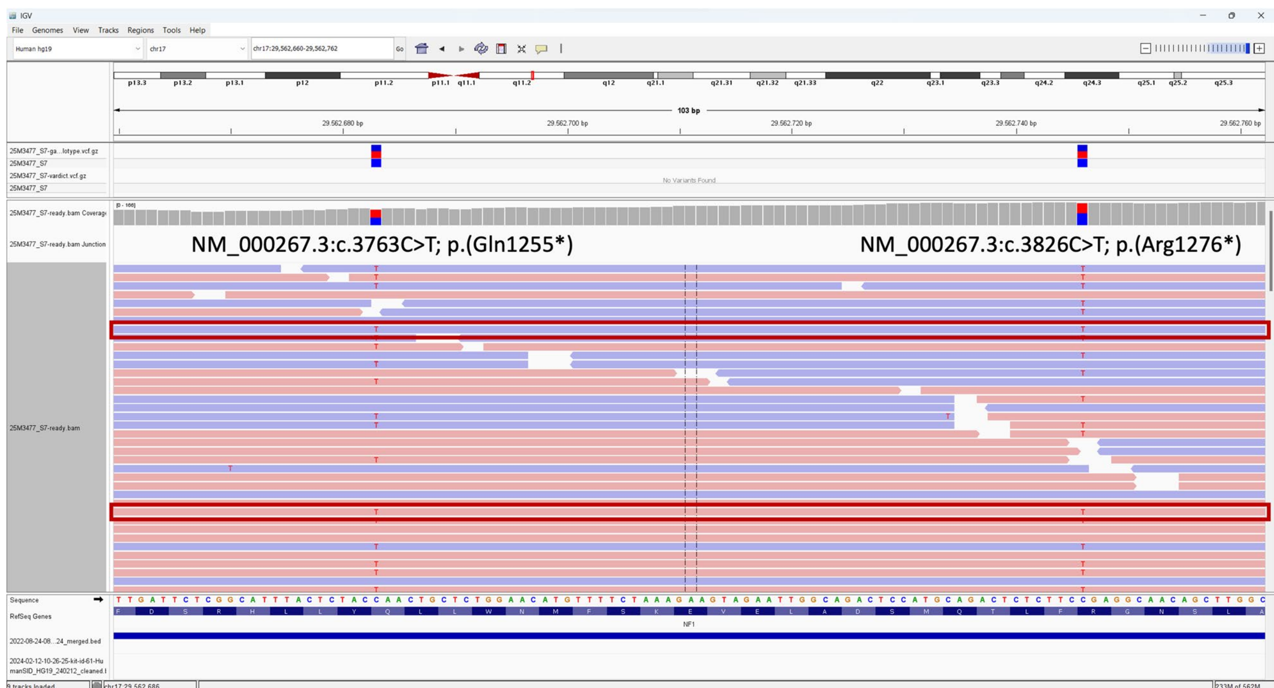

Fig. 1 Integrative Genomics Viewer (IGV) (https://igv.org/) visualization of forward (red) and reverse (blue) next generation sequence reads from NGS analysis. The two identified heterozygous variants NM_000267.3:c.3763 C > T; p.(Gln1255*) (altAF 0.4954, 54 of 109 reads) and c.3826 C > T; p.(Arg1276*) (altAF 0.4698, 70 of 149 reads) in exon 28 of the - *NF1* gene are located on the same allele (in *cis*). One forward and one reverse read were highlighted as examples (red boxes) to show the localization of both variants on the same reads in both directions

by allele-specific PCR/cloning and RNA/complementary DNA analysis. In two independent families, Stella et al. identified double *de novo*, closely spaced *NF1* variants on a single allele (for variant-level details and current interpretation please see Table 1). Both probands had classic NF1 without a reproducible increase in severity [19]. In a large pedigree, Hernández-Imaz et al. showed two truncating variants in *NF1* occurring in *cis* and segregating with NF1 [20]. Terzi et al. described a child with two pathogenic *NF1* variants and a mild phenotype [21]. While the frameshift variant c.5214dup (p.Thr1739fs) meets pathogenicity criteria, the missense variant c.2894T > A (p.Ile965Lys) remains a variant of uncertain significance (VUS)/likely pathogenic variant with conflicting ClinVar interpretation on current review. Together with the phenotype and subsequent literature, this constellation is most consistent with a *cis* configuration. More recently, Watanabe et al. reported two adjacent *de novo* missense variants in *NF1* [NM_001042492.3(NF1):c.3586 C > T (p.Leu1196Phe) and NM_001042492.3(NF1):c.3590 C > T (p.Ala1197Val)] proven in *cis*—an instructive example of closely spaced multiple mutations that most plausibly arose from a single, complex mutational event rather than two independent hits [22]. In this context, *cis* pairs are best interpreted on the haplotype-level allele rather than treated as two independent lesions. Closely spaced substitutions can constitute a multi-nucleotide variant, for which the joint codon-level consequence may differ from the sum of single-variant annotations. Haplotype-aware consequence calling and phase-aware pipelines help avoid such misannotation, especially for clustered de novo changes [23, 24]. Analogous germline phenomena have been attributed to transient hypermutability and double-strand-breaks-associated processes (including a maternal/oocyte-aging component), with population studies estimating that a measurable fraction of de novo single nucleotide variants occur in such clusters [25, 26].

Taken together, *cis* doublets appear to coincide with classic NF1 and do not consistently shift phenotype beyond the usual spectrum—though numbers are small and careful variant-level curation is still essential (e.g., rule out that one of the two is a VUS). Mechanistically, the following is expected: when *cis* changes render the transcript subject to nonsense-mediated decay or otherwise produce a null allele, a second pathogenic variant on the same allele cannot further reduce dosage beyond loss of that allele. Only in scenarios where both changes are missense (or otherwise expressed) on the same transcript, therefore producing a doubly mutated protein, is a theoretical additive effect conceivable; to date, convincing clinical aggravation from such *cis* missense doublets has not been demonstrated.

Rare germline *trans* configurations

True germline *trans* (compound-heterozygous) *NF1* configurations are uncommon and tend to weaken upon close reappraisal. In line with this, true germline biallelic loss-of-function (LoF; *trans*) is biologically implausible: *NF1* knockout mice die mid-gestation with cardiac/neural crest defects, supporting the notion that constitutive biallelic LoF is incompatible with prenatal survival [27]. When such configurations are discussed, it is typically in the context of spinal neurofibromatosis (SNF), a recognizable NF1 subset characterized by numerous bilateral spinal nerve-root neurofibromas and frequent familial accumulation [28].

In the largest SNF cohort, two unrelated probands were reported with two *NF1* variants in *trans* [29, 30]. In Family 1, the additional variant reported in *trans* to the family´s established variant NM_001042492.3(NF1):c.62T > A (p.Leu21His) (now curated as VUS) was *NF1* (NM_001042492.3):c.528T > A (p.Asp176Glu), inherited from a clinically unaffected parent. In Family 17, the established variant was NM_001042492.3(NF1):c.3314 + 2 T > C and the additional variant was NM_001042492.3(NF1):c.7595 C > T (p.Ala2532Val), inherited from a clinically not evaluated parent. Both additional alleles now have (likely) benign curations by multiple clinical laboratories, based on population frequency (gnomAD), ClinVar interpretations, and the criteria proposed by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) (Table 1). In addition, the suggestion that compound carriers were more severely affected rests on qualitative observations rather than standardized phenotypic assessment.

Fauth et al. additionally described an adult with sporadic SNF and malignant peripheral nerve sheath tumor (MPNST) who carried two variants in *trans*, c.3046T > C (p.Cys1016Arg) and a small in-frame deletion c.8194_8196del (p.Val2732del) [31]. Inheritance was not reported. Likewise, the small in-frame deletion lacks supportive functional/segregation data. Present-day submissions classify the variant as low-evidence/benign-leaning.

Taken together, across published cases the putative "second" *NF1* alleles do not constitute bona fide LoF. Most would now be classified as benign or, at most, of uncertain significance. Although subtle modifier effects of rare missense variation in *trans* cannot be excluded, there is no compelling evidence to reject the null hypothesis that these configurations reflect coincidental co-occurrence in a gene with substantial background missense variation. The apparent enrichment in SNF may likewise reflect ascertainment bias. Consistent with this, the literature provides no convincing examples of germline biallelic pathogenic *NF1* loss, whereas biallelic

Table 1 Germline variants in NF1 and other Ras-pathway genes with variant-level details and current interpretation

Reference	Gene	Variants (as reported)	Transcript reference	Curated[1]	Classification[2]	ClinVar Variation ID	gnomAD allele count[3]	Segregation	Interpretive note
NF1* variants in *cis									
Present report	*NF1*	c.3763C>T, p.Gln1255*	NM_001042492.3	c.3763C>T, p.Gln1255*	PATH	404504	Not observed	de novo	Proven in *cis* by single read analysis
		c.3826C>T,p. Arg1276*	NM_001042492.3	c.3826C>T, p.Arg1276*	PATH	237556	5:1613938	de novo	
Stella et al. 2022 Case 1	*NF1*	c.3198-1G>A, p.Asp1067Phefs*21	NM_000267.3	c.3198-1G>A	PATH	2901595	Not observed	Parents clinically unaffected, not available for testing	Proven in *cis* by segregation and cloning of PCR products Effect of splice site variant was verified on mRNA level as r.3198_3199delAG
		c.3295A>T, p.Lys1099*	NM_000267.3	c.3295A>T, p.Lys1099*	LPATH	Not available	Not observed		
Stella et al. 2022 Case 2	*NF1*	c.2546del, p.Gly849GlufsTer29	NM_000267.3	c.2546del, p.Gly849fs	PATH	996424	Not observed	de novo	Proven in *cis* by cloning of PCR products
		c.2548G>A, p.Val850Met	NM_000267.3	c.2548G>A, p.Val850Met	VUS	1318994	Not observed	de novo	
Hernandez-Imaz et al. 2013	*NF1*	c.6792C>A, p.Tyr2264*	NM_000267.2	c.6855C>A, p.Tyr2285*	PATH	185082	2:1613200	Co-segregation within family	Proven in *cis* by segregation within family
		c.6799C>T, p.Gln2267*	NM_000267.2	c.6862C>T, p.Gln2288*	LPATH /PATH	1755477	Not observed		
Terzi et al. 2012	*NF1*	c.2894T>A p. Ile965Lys	Not indicated	c.2894T>A p. Ile965Lys	Conflicting VUS / LPATH	947673	Not observed	Inherited from father	De novo variant assumed to have occurred on the paternal allele
		c.5213insT	Not indicated	c.5214dup, p.Thr1739fs	LPATH	Not available	Not observed	de novo	
Watanabe et al. 2023	*NF1*	c.3586C>T, p.Leu1196Phe	NM_001042492.3	c.3586C>T, p.Leu1196Phe	LPATH / PATH	1036158	1:1614048	de novo	NF1 variants proven in *cis*by single read analysis; patient in addition had a pathogenic *de novoGABBR1*missense variant which likely contributed to the phenotype
		c.3590C>T, p.Ala1197Val	NM_001042492.3	c.3590C>T, p.Ala1197Val	Conflicting VUS / LPATH	216401	Not observed	de novo	
NF1* variants in*trans									
Paterra et al. 2022 Fam. 1 Bettinaglio et al. 2024	*NF1*	c.62T>A ,p.Leu21His	NM_001042492.3	c.62T>A ,p.Leu21His	VUS	1523219	Not observed	Inherited from affected father	Proband carrying both variants reported to display a more severe phenotype than his affected father and brother who carried only the c.62T>A variant
		c.528T>A, p.Asp176Glu	NM_001042492.3	c.528T>A, p.Asp176Glu	Conflicting VUS / LBEN / BEN	41673	6645:1612888	Inherited from mother not meeting NF1 criteria	

Table 1 (continued)

Reference	Gene	Variants (as reported)	Transcript reference	Curated[1]	Classification[2]	ClinVar Variation ID	gnomAD allele count[3]	Segregation	Interpretive note
Paterra et al. 2022 Fam. 17	*NF1*	c.3314+2T>C	NM_001042492.3	c.3314+2T>C	PATH	1918167	Not observed	Inherited from affected mother	Proband carrying both variants reported to display a more severe phenotype compared to the relatives carrying one of the *NF1*variants
		c.7532C>T, p.Ala2511Val	NM_001042492.3	c.7595C>T, p.Ala2532Val	BEN / LBEN	41678	994:1613328	Inherited from father referred to as healthy	
Fauth et al. 2009	*NF1*	c.3046T>C, p.Cys1016Arg	Not indicated	c.3046T>C, p.Cys1016Arg	PATH	237543	Not observed	Not determined	Variants proven in *trans*by analysis of human/mouse hybrid cell lines containing the different chromosomes 17
		c.8131_8133delGTT, p.2711delVal	Not indicated	c.8194_8196del p.Val2732del	LBEN	1575712	Not observed	Not determined	
Dual RASopathy diagnoses									
Bertola et al. 2005	*NF1*	c.2531A>G, p.Leu844Arg	Not indicated	c.2531T>G, p.Leu844Arg	PATH	373	1:1611918	de novo	Neurofibromatosis-Noonan syndrome
	PTPN11	c.1909A>G, p.Gln510Arg	Not indicated	c.1529A>G, p.Gln510Arg	LPATH / PATH	13345	14:1614226	Inherited from father diagnosed with Noonan syndrome	
Thiel et al. 2009	*NF1*	c.4661+1G>C	Not indicated	c.4724+1G>C	LPATH / PATH	374	Not observed	Inherited from mother affected by NF1	The index patient's phenotype was considered to result from additive effects of her *NF1*and *PTPN11*variants
	PTPN11	c.5C>T, p.Thr2Ile	Not indicated	*de novo*	LPATH / PATH	13349	4:1528266	de novo	
Prada et al. 2011	*NF1*	c.4288A>G, p.Asn1430Asp	Not indicated	c.4351A>G, p.Asn1451Asp	LPATH / PATH	569136	Not observed	Inherited from mother	Index patient reported to exhibit severe clinical presentation of a RASopathy
	PTPN11	c.922A>G, p.Asn308Asp	Not indicated	c.922A>G, p.Asn308Asp	PATH	13326	12:1612192	Not determined	
Baquedano Lobera et al. 2019	*NF1*	c.7756G>T p.E2586X	Not indicated	c.7819T>G,p. Glu2607*	LPATH	Not available	Not observed	de novo	Dual RASopathy (NF1, Noonan syndrome)
	KRAS	c.40G>A,p.Val14Ile	Not indicated	c.40G>A,p. Val14Ile	PATH	12589	2:1612846	de novo	

[1]Standardized nomenclature for the respective MANE transcript: NF1: NM_001042492.3; KRAS: NM_004985.5; PTPN11: NM_002834.5

[2]Classification of pathogenicity according ClinVar entry (if available) or by evaluation on the basis of criteria proposed by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP)

[3]Global allele count in gnomAD 4.1.0 (January 2026)

inactivation in tumors/JMML arises via somatic second hits and is well established.

Biallelic somatic inactivation of *NF1* in café-au-lait macules, tumors, and juvenile myelomonocytic leukemia

Mechanism of the second hit

Loss of heterozygosity (LOH) or a second somatic mutation affecting the wild-type *NF1* allele is the canonical driver of NF1-associated neoplasia (Knudson´s two-hit model). Across NF1-associated lesions, including cutaneous and plexiform neurofibromas, MPNST, pilocytic astrocytoma, and myeloid disease, clonal cells typically harbor biallelic *NF1* inactivation, while the germline remains heterozygous [32]. Notably, even melanocytes in CALMs can carry a somatic second hit, underscoring that the two-hit paradigm extends to most or even all benign and malignant NF1-related focal lesions [33].

Multiple mechanisms of the second hit have been demonstrated in human NF1-related tumors: (i) Mitotic recombination leading to copy-neutral LOH (17q uniparental disomy, UPD): In neurofibromas, mitotic recombination that renders the Schwann-cell clone homozygous for the germline *NF1* variant is a recurrent mechanism. Copy-neutral LOH on 17q via segmental uniparental isodisomy is likewise common in hematologic disease [32, 34–38]. (ii) Interstitial deletions/microdeletions at 17q11.2: focal deletions removing the remaining wild-type *NF1* allele occur in a subset of tumors. In neurofibromas, distinct type-2 second-hit deletions have been catalogued [39]. (iii) Compound heterozygous inactivation by two distinct somatic variants: Tumors may acquire an independent pathogenic variant on the second allele in the absence of LOH [35]. (iv) Structural events: A single well-documented neurofibroma showed translocation-mediated disruption of both *NF1* copies [40]. Collectively, these data support biallelic somatic loss as the rule in NF1 tumorigenesis [32].

JMML: an NF1-driven subtype with recurrent 17q events

Children with clinical NF1 have a markedly elevated risk of JMML. Neurofibromin restrains RAS–MAPK in hematopoiesis. Haploinsufficiency confers Granulocyte/Macrophage Colony Stimulating Factor hypersensitivity and expands myelomonocytic progenitors, establishing a leukemogenic field that, in JMML, progresses via somatic second hits causing biallelic *NF1* loss. In NF1-associated JMML, leukemic cells almost invariably achieve biallelic *NF1* inactivation via 17q UPD leading to copy-neutral LOH, somatic *NF1* deletions encompassing the *NF1* locus on 17q11.2, or independent somatic *NF1* mutations on the second allele [41].

In the most comprehensive contemporary series, 25 JMML patients with NF1 were analyzed: ten had 17q UPD resulting in copy-neutral LOH at *NF1*, five had microdeletion of one allele with a pathogenic *NF1* variant on the other, and nine were compound-heterozygotes for *NF1* variants. The same study interrogated a comparison group of 16 JMML cases without clinical NF1 and lacking canonical Ras-pathway drivers (*PTPN11*/*KRAS*/*NRAS*/*CBL*): eight had *NF1* altered leukemias (three with microdeletions plus a hemizygous variant, three compound heterozygotes, and two simple heterozygotes) supporting routine *NF1* analysis in JMML even when NF1 is not clinically apparent [41].

These findings extend earlier work showing that, in NF1-associated JMML, copy-neutral LOH at *NF1* due to mitotic recombination on 17q is frequent. Among the remaining cases, biallelic inactivation typically arises through compound heterozygosity for *NF1* variants or interstitial deletions, with no consistent genotype–phenotype differences across mechanisms [35]. They also align with broader hematologic data establishing copy-neutral LOH as a recurrent mechanism in myeloid malignancies and with the World Health Organization/International Consensus Classification 2022 classifications, which recognize JMML, NF1-associated, as a distinct molecularly defined category [42, 43]. Of note, secondary cooperating lesions (e.g., *ASXL1* and/or *SETBP1* mutations) are common in both NF1-JMML and NF1-negative/JMML-negative subsets, underscoring the value of a comprehensive genomic work-up [41].

Solid tumors and hyperproliferative lesions

Across NF1-associated lesions, a second somatic inactivation event in *NF1* is a prerequisite for clonal outgrowth, but it is not sufficient for malignant transformation. Benign neurofibromas, including plexiform neurofibromas, typically show biallelic *NF1* inactivation in the Schwann-cell lineage. Progression toward malignancy requires additional, cooperating genetic and epigenetic alterations beyond the *NF1* second hit [44, 45].

The evolution typically proceeds from plexiform neurofibroma to atypical neurofibromatous neoplasm of uncertain biologic potential (ANNUBP) and ultimately to MPNST. Along this continuum, recurrent cooperating alterations accumulate, most prominently *CDKN2A/B* copy-number loss at 9p21 and inactivation of the Polycomb Repressive Complex 2 via *SUZ12* or *EED* LoF, with consequent loss of trimethylation of lysine 27 on histone H3. Additional events (e.g., *TP53* mutation, broad copy-number remodeling) are frequent in high-grade disease. These cooperating events are rarely, if ever, present in ordinary neurofibromas, and underscore the stepwise nature of transformation [46–49].

Constituional *NF1* microdeletions illustrate this logic clinically. Individuals with germline type-1 17q11.2 microdeletions have more numerous neurofibromas, earlier tumor onset, and a markedly elevated lifetime

MPNST risk (≈16–26%) compared with patients harboring intragenic *NF1* variants. Concomitant *SUZ12* haploinsufficiency, due to co-deletion within the type-1 interval, has been implicated as a key modifier, consistent with the central role of *PRC2* LoF in malignant progression [3, 4, 50, 51].

Within benign lesions, second-hit mechanisms include mitotic recombination leading to copy-neutral LOH (17q UPD) that renders the germline *NF1* variant homozygous, segmental 17q11.2 deletions removing the wild-type allele, and, less commonly, compound heterozygous somatic inactivation by two distinct somatic variants in *trans* [39].

Recent integrative and single-cell studies reinforce this framework: *PRC2* loss is recurrent in NF1-MPNST and closely linked to malignant progression. Pan-genomic surveys catalog *NF1*, *CDKN2A*, and *PRC2* alterations as core lesions across stages, and consensus pathology recognizes ANNUBP as a bona fide precursor with molecular features intermediate between neurofibroma and MPNST. Together, these data support a multi-hit model in which *NF1* biallelic *NF1* inactivation is necessary but never sufficient for malignancy [52–54].

Bona fide dual RASopathy diagnoses

Double diagnoses involving pathogenic variants in *NF1* plus another RASopathy gene have been documented in a small number of individuals who show a blended phenotype, Noonan-spectrum traits (Noonan syndrome, MIM #163950, ORPHA:648) together with characteristic NF1 lesions. The most common situation pairs *NF1* with *PTPN11*. In the index report, a girl with neurofibromatosis–Noonan features carried two independent pathogenic variants—*de novo NF1* c.2531T > G (p.Leu844Arg) and a paternally inherited *PTPN11*c.1529 A > G (p.Gln510Arg)—providing the first molecular proof of co-occurrence of both disorders in one person [55]. In a subsequent family, an 18-month-old girl with typical Noonan features, multiple CALMs, and bilateral optic pathway gliomas harbored a *de novo PTPN11* variant together with a maternally inherited *NF1* splice variant in *NF1* c.4724 + 1G > C. Relatives who carried only the *NF1* variant had few CALMs as their sole NF1 sign consistent with an additive, blended presentation [56].

A third case described a male neonate with severe congenital heart disease, arrhythmia, hypertrophic cardiomyopathy, airway compression, and early death in whom pathogenic variants in both *NF1* c.4351 A > G (p.Asn1451Asp) and *PTPN11* c.922 A > G (p.Asn308Asp) were identified [57]. Although Ras/MAPK hypersignaling was proposed as a unifying mechanism, the fatal course may also be plausibly explained by Noonan-associated cardiac and lymphovascular complications.

Mechanistically, such dual Ras/MAPK pathway variants are not equivalent to biallelic *NF1* loss. In tissues that have not acquired a somatic second hit, one *NF1* allele remains intact. A germline Noonan-spectrum gain-of-function variant (e.g., *PTPN11*) would be expected to modestly raise baseline Ras/MAPK tone rather than recapitulate complete *NF1* loss. Conversely, once a tumor clone undergoes biallelic *NF1* inactivation, pathway signaling is already deregulated. A concurrent germline Noonan-spectrum variant may increase phenotypic severity but is therefore not expected to be a major additional oncogenic driver, and there is currently no empirical evidence that tumor behavior is systematically intensified by the presence of a second RASopathy gene variant [58].

These considerations help delineate Neurofibromatosis–Noonan syndrome (NFNS; MIM #601321, ORPHA:638) from bona fide two-gene diagnoses. Most individuals labeled NFNS carry *NF1* variants alone, and recognized genotype–phenotype enrichments account for Noonan-like features with a relative paucity of cutaneous/plexiform neurofibromas [59–61]. In clinical practice, a patient with *NF1* and *PTPN11* variants will often still be diagnosed as NFNS on phenotypic grounds. To distinguish NF1-only NFNS from true digenic cases molecular testing is necessary. However, a proportion of individuals labeled NF1-only NFNS may harbor undetected variants in Noonan-spectrum genes or regulatory/noncoding changes not captured by panel or exome tests. Where phenotype suggests a blended Ras/MAPK presentation but targeted testing is uninformative, genome sequencing (to access intronic/regulatory and structural variants), RNA sequencing from an informative tissue (to detect aberrant splicing/expression), and long-read sequencing (to resolve phasing and complex rearrangements) merit consideration [62]. Because gene–disease knowledge and curation standards evolve, periodic reanalysis of existing exome/genome sequencing data increases diagnostic yield and should be incorporated into counseling and follow-up plans [63, 64].

Beyond *PTPN11*, dual diagnoses have been reported with *KRAS*. A child harboring a truncating *NF1* variant c.7819T > G (p.Glu2607*) together with *KRAS* c.40G > A (p.Val14Ile) displayed features within the upper severity range expected for this Noonan-associated *KRAS* allele, rather than a clearly novel or uniformly aggravated phenotype due to the combination [65]. Finally, co-occurrence of *NF1* and *SPRED1* pathogenic variants within the same individual appears exceptional. Many putative examples resolve to family-level co-segregation (different relatives) or to a *SPRED1* VUS alongside a clearly pathogenic *NF1* variant, underscoring the risk of over-calling dual diagnoses without robust pathogenicity and segregation data [66, 67]. As a differential diagnosis, however,

SPRED1 remains important in pigment-predominant, tumor-negative presentations. Roughly ~ 1–2% of NF1-like clinic cohorts ultimately have Legius syndrome, and about ~ 8% of children with ≥ 6 café-au-lait macules and no other NF1 criteria harbor *SPRED1* variants [68].

In summary, bona fide dual RASopathy diagnoses involving *NF1* and a second gene are rare and best interpreted as additive/blended systemic phenotypes. Lethality is not the rule. Current data do not demonstrate that adding a germline Noonan-spectrum variant increases the oncogenic consequences beyond those driven by biallelic *NF1* loss within lesions. For suspected dual cases, we advocate comprehensive RASopathy-panel or exome testing with ACMG/AMP-level curation of each variant and, where possible, segregation/trio data—while resisting VUS-driven over-interpretation.

Outside RASopathies specifically, large exome cohorts find ~ 2–5% of solved cases carry multiple molecular diagnoses, indicating that dual diagnoses do occur but are uncommon, and careful curation is essential before concluding two syndromes [17, 69, 70].

Diagnostic implications

Interpretation of reports listing two *NF1* variants is shaped by several recurrent sources of error. Many individuals with Noonan-like features and NF1 harbor a single missense or in-frame *NF1* variant (i.e., NFNS), rather than two pathogenic variants in both genes. Apparent biallelic *NF1* in tumors or copy-neutral LOH of 17q in JMML reflects somatic evolution, not a germline *trans* state. Co-variation of *NF1* and *SPRED1* within families frequently involves a VUS in one of the two genes, arguing against inferring a dual diagnosis until independent pathogenicity is established. Mosaicism of *NF1* adds further complexity, as low variant allele fractions in blood may indicate post-zygotic changes rather than a second germline allele. Finally, although many *NF1* variants arise de novo and often on the paternal allele, evidence for a paternal-age effect remains limited and is best communicated cautiously [11, 71–73].

For individuals with clinical suspicion of NF1, we consider an NGS-based *NF1* assay that captures both sequence variants and exon- or gene-level copy-number changes (via validated NGS-based CNV calling and/or MLPA) an appropriate first-line diagnostic approach, consistent with current molecular testing practice for NF1 [1]. In pigment-predominant presentations without detectable tumor manifestations, commonly encountered in children, and in the absence of an affected parent, we recommend upfront inclusion of *SPRED1* to address the key differential diagnosis of Legius syndrome. Inclusion of *LZTR1* and *PTPN11* should be considered, ideally within a panel-based testing framework, to cover LZTR1-related multiple café-au-lait spots and Noonan syndrome with multiple lentigines. When the phenotype is broad or atypical for NF1, an extended panel or exome/genome-based analysis should encompass a wider range of differential diagnoses, including other RASopathies, CMMRD, and other conditions. Trio-based testing can be applied when it provides added value for assessment of de novo status or variant phasing. Targeted Sanger sequencing is best reserved for familial testing and/or orthogonal confirmation. Finally, we note practical assay considerations for mosaicism detection (read depth and variant allele fractions, and, when indicated, alternative tissues), RNA-based analyses for suspected splice effects, and a selected role for long-read sequencing in unresolved phasing or structural-variant cases. For unresolved NF1-only NFNS presentations or suspected dual RASopathy without a confirmed second locus, reanalysis of prior exome/genome sequencing at defined intervals (e.g., 12–24 months) is recommended, given consistent incremental yields with updated pipelines, annotations, and gene lists [74].

Where two pathogenic variants in different Ras/MAPK genes are confirmed (e.g., *NF1* plus a Noonan-spectrum gene), clinical management typically integrates surveillance frameworks from both conditions (NF1-directed tumor surveillance together with Noonan-directed cardiology and growth/endocrine evaluation).

When neoplasia is present, particularly JMML or enlarging/atypical plexiform neurofibromas, germline studies are complemented by tumor–normal analyses. Copy-neutral LOH/UPD (e.g., on single-nucleotide polymorphism arrays), CNVs (MLPA/next-generation sequencing), and targeted *NF1* sequencing frequently document biallelic somatic inactivation. These findings clarify that the germline remains heterozygous. Recurrence risk therefore follows the germline variant rather than the tumor genotype. In JMML, identification of biallelic *NF1* loss supports classification as NF1-associated JMML even when clinical NF1 features are subtle. In pigment-only lesions (e.g., CALMs), demonstration of a second hit reinforces the two-hit model without implying additional germline risk. In cases initially reported with two *NF1* variants, analysis of tumor tissue helps prevent misattribution of somatic architecture to a germline *trans* configuration.

Taken together, consistent application of ACMG/AMP classification to each variant, rigorous phasing, and, where tissue is available, tumor-focused investigation provide a coherent framework for interpretation (Fig. 2). On present evidence, lesion biology in NF1 is best explained by single-allele germline NF1 with somatic second hits as the proximate driver, whereas true germline *trans* pathogenic configurations, particularly in SNF, remain unsubstantiated.

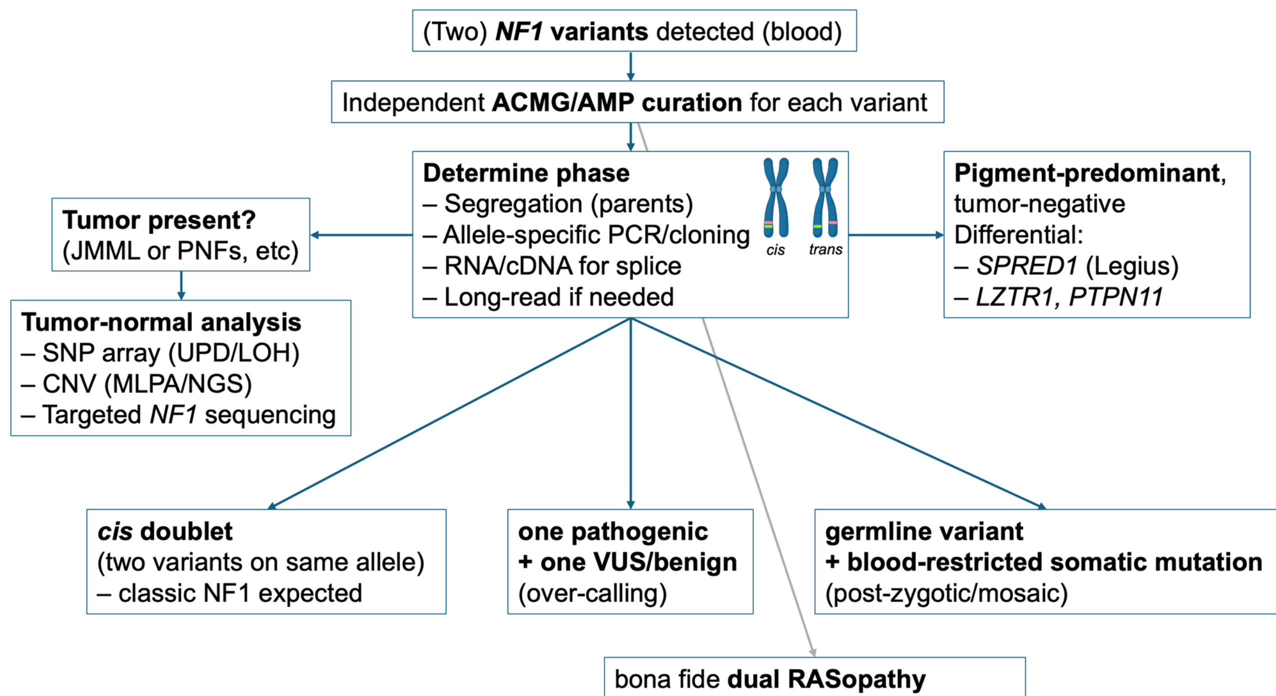

Fig. 2 Interpreting (two) *NF1* variants detected in blood: phasing and differential workflow *Note*: Haplotype-aware consequence calling is recommended when two cis variants fall within the same codon/region

Concluding remarks

Monoallelic *cis* doublets, including closely spaced, de novo pairs, are rare but well documented and generally tracked with classic NF1, with no reproducible phenotype escalation. Germline *trans* configurations reported in SNF weaken on reappraisal: the "second" alleles typically do not satisfy current pathogenicity standards, the perceived severity relies on qualitative impressions, and follow-up functional data are equivocal. By contrast, somatic second hits producing biallelic *NF1* loss are ubiquitous in lesions from CALMs to JMML and MPNST. Bona fide dual RASopathy diagnoses exist, most convincingly *NF1* plus *PTPN11*, and yield *blended* systemic phenotypes. However, there is no empirical evidence that a concurrent Noonan-spectrum variant further intensifies tumor behavior once a clone has lost both *NF1* alleles. The practical corollary is simple: in putative "double" cases, prove phase, prove pathogenicity, and look to tumor tissue to avoid mistaking somatic architecture for germline biology.

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Authors' contributions

DAT and MK had the idea for the article. DAT, MZ, and MK performed the literature search, data analysis, and data curation. DS performed sequencing and phase analysis. MK drafted the manuscript. DAT, DS, PV, MCF, TR, and MZ critically revised the work for important content.

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Data availability

Not applicable.

Declarations

Ethics approval and consent to participate

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Competing interests

The authors declare no competing interests.

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