

The Origin of *EFNB1* Mutations in Craniofrontonasal Syndrome: Frequent Somatic Mosaicism and Explanation of the Paucity of Carrier Males

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Craniofrontonasal syndrome (CFNS) is an X-linked disorder that exhibits a paradoxical sex reversal in phenotypic severity: females characteristically have frontonasal dysplasia, craniosynostosis, and additional minor malformations, but males are usually mildly affected with hypertelorism only. Despite this, males appear underrepresented in CFNS pedigrees, with carrier males encountered infrequently compared with affected females. To investigate these unusual genetic features of CFNS, we exploited the recent discovery of causative mutations in the *EFNB1* gene, which encodes ephrin-B1, to survey the molecular alterations in 59 families (39 newly investigated and 20 published elsewhere). We identified the first complete deletions of *EFNB1*, catalogued 27 novel intragenic mutations, and used Pyrosequencing and analysis of nearby polymorphic alleles to quantify mosaic cases and to determine the parental origin of verified germline mutations. Somatic mosaicism was demonstrated in 6 of 53 informative families, and, of 17 germline mutations in individuals for whom the parental origin of mutation could be demonstrated, 15 arose from the father. We conclude that the major factor accounting for the relative scarcity of carrier males is the bias toward mutations in the paternal germline (which present as affected female offspring) combined with reduced reproductive fitness in affected females. Postzygotic mutations also contribute to the female preponderance, whereas true nonpenetrance in males who are hemizygous for an *EFNB1* mutation appears unusual. These results highlight the importance of considering possible origins of mutation in the counseling of families with CFNS and provide a generally applicable approach to the combined analysis of mosaic and germline mutations.

Craniofrontonasal syndrome (CFNS [MIM 304110]), first clearly delineated in 1979,^{1,2} is a distinctive disorder characterized by severe hypertelorism with a grooved nasal tip, coronal synostosis (unilateral or bilateral), fine frizzy hair, abnormal modeling of the clavicles, partial cutaneous syndactyly of the hands and feet, and longitudinal ridging of the nails. Cleft lip and/or palate, Sprengel shoulder, duplication of the thumbs or halluces, partial or complete agenesis of the corpus callosum, and learning difficulties occur in a proportion of cases.³ Over a 10-year period at the Oxford Craniofacial Unit, CFNS accounted for 6 of 287 prospectively ascertained cases that required an operation for craniosynostosis, which indicates a birth prevalence of ~1 in 120,000 (S.A.W. and A.O.M.W., unpublished data).

The genetic counseling of families with CFNS has been problematical because of the unusual inheritance pattern

of CFNS. The great majority of individuals who present with classic CFNS are females; only a small number of males with an apparently similar phenotype have been described.^{4,5} Usually, this sex bias in manifestation is caused by the segregation of an X-linked male-lethal mutation; indeed, this mechanism was proposed for CFNS.⁶ However, this explanation became untenable with the description of multiple pedigrees in which classically affected females were linked through an intermediate male relative.^{2,7-9} These obligate carrier males were always more mildly affected than were affected females, with a nonspecific phenotype comprising hypertelorism and occasional cleft lip. Nevertheless, the observation that the daughters of these male carriers invariably had CFNS supported the segregation of an X-linked mutation that, paradoxically, seemed to affect heterozygous females more severely than hemizygous males.⁸

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Table 1

Clinical Features and Molecular Analysis of the Newly Analyzed *EFNB1* Mutation

PROBAND (NO. AND SEX OF MUTATION-POSITIVE SUBJECTS ANALYZED)	CLINICAL FEATURES ^a						MUTATION ^b						
	Coronal Craniosynostosis	Cleft Lip and/or Palate	Duplex Thumb or Hallux	Agnesis of the Corpus Callosum	Other		DNA	Exon (Intron)	Protein	Familial or Sporadic	Confirmation	Mosaicism ^c	Parental Origin ^d
3205 (1 F)	–	–	–	–	...		<i>-4_Adel</i>	1	–	S	<i>BslI(+)</i>	–	U
3350 (1 F)	L	–	–	–	Developmental delay		<i>30C→T</i>	1	K11SfsX2 ^e	S	ASO	–	NI
3259 (1 F)	R and L	–	–	–	Sprengel deformity		<i>109T→G</i>	1	W37G	S	<i>BstXI(-)^f</i>	–	U
3429 (1 F)	R and L	–	–	–	Hypertrophic cardiomyopathy		<i>109T→G</i>	1	W37G	Fam (2 F)	<i>BstXI(-)^f</i>	NA	U
3605 (1 F and 1 M)	R	–	–	+	Duplex kidney, Bartholin cyst		<i>110G→A</i>	1	W37X	Fam (1 F and 1 M)	<i>BslI(-)</i>	+	PZ(M)
3533 (1 F)	–	–	LH	Partial	...		<i>123C→G</i>	1	N41K	S	<i>SmlI(+)</i>	–	Mat
3504 (1 M and 2 F)	L	–	–	–	Diaphragmatic hernia in son		<i>151_153delGTG</i>	2	V51del	Fam (1 M and 4 F)	<i>HpbI(-)</i>	NA	U
3217 (2 F and 1 M)	R	–	–	–	...		<i>161C→T</i>	2	P54L	Fam (1 F and 1 M)	<i>BslI(-)</i>	+	PZ(F)
3556 (1 F)	–	–	–	–	...		<i>170_171GA→TT</i>	2	G57V	S	<i>BslI(-)</i>	–	U
3412 (1 F)	–	–	–	U	...		<i>191G→A</i>	2	C64Y	Fam (2 F)	<i>MwoI(-)^f</i>	+	PZ(F)
3553 (2 F and 1 M)	L	–	–	U	...		<i>196delC</i>	2	R66EfsX93	Fam (1 F and 1 M)	<i>BglI(-)^f</i>	+	PZ(F)
3204 (1 F)	L	–	–	–	Intracranial dermoid and double uterus		<i>196C→T</i>	2	R66X	S	<i>AvaI(-)</i>	–	U
3518 (1 F)	R and L	CL, P	–	–	...		<i>196C→T</i>	2	R66X	S	<i>AvaI(-)</i>	–	Pat
3549 (1 F)	L	CL, P	RH and LH	Partial	Developmental delay		<i>196C→T</i>	2	R66X	S	<i>AvaI(-)</i>	–	U
3516 (1 F)	R and L	–	RH	–	...		<i>220G→T</i>	2	E74X	S	<i>HinI(-)^f</i>	–	U
3281 (3 F)	R and L	–	–	–	Sprengel deformity		<i>233T→C</i>	2	L78P	Fam (3 F)	<i>BceAI(+)</i>	–	U
3638 (1 F)	R	–	–	Partial	Duplex left kidney		<i>265T→C</i>	2	C89R	S	<i>SfcI(-)</i>	–	NI
3347 (1 F)	L	–	–	–	Ulnar clinodactyly, right middle finger		<i>266G→A</i>	2	C89Y	S	<i>Hpy8I(-)^f</i>	–	U
3262 (1 F)	R and L	–	–	–	...		<i>339G→C</i>	2	K113N	S	<i>Hpy8I(+)^f</i>	–	Pat
3608 (2 F and 1 M)	R and L	–	–	–	...		<i>355C→G</i>	2	P119A	Fam (3 F and 1 M)	<i>HbaI(+)</i>	NA	U
3575 (1 F)	R and L	–	–	–	Cerebellar dysplasia and vesico-ureteric reflux		<i>363C→A</i>	2	Y121X	S	<i>NlaIII(-)</i>	–	Pat
3258 (1 F and 1 M)	R and L	–	–	–	...		<i>368G→A</i>	2	G123D	Fam (1 F and 1 M)	<i>AvaII(+)</i>	– (M)	U
3213 (1 F)	–	–	LH	Partial	Developmental delay		<i>398delA</i>	2	Y133SfsX26	S	ASO	–	U
3494 (1 F)	R	–	–	–	...		<i>407-1G→A</i>	(2)	SP	S	<i>PstI(-)</i>	+	PZ(F)
3618 (1 F)	–	P (uvula)	RT	+	Developmental delay		<i>407C→T</i>	3	S136L	S	<i>TseI(-)</i>	–	Pat
3410 (1 M and 1 F)	+	CL and P	–	–	Diaphragmatic hernia and sacrococcygeal teratoma in son		<i>432delG</i>	3	L145WfsX14	Fam (1 M and 1 F)	<i>BglI(+)</i>	–	U
3563 (1 F)	R	P	–	–	...		<i>445G→T</i>	3	E149X	S	<i>NciI(-)</i>	–	U
3615 (2 F and 1 M)	U	U	U	U	...		<i>451G→A</i>	3	G151S	Fam (4 F and 1 M)	<i>AclI(-)</i>	NA	U
3576 (1 F)	U	–	–	+	Developmental delay		<i>452G→A</i>	3	G151D	S	<i>AclI(-)</i>	–	U
3467 (1 F)	R	–	–	–	...		<i>458G→C</i>	3	C153S	S	<i>MwoI(-)</i>	–	Pat
3265 (2 F and 1 M)	R and L	–	–	–	Developmental delay		<i>496C→T</i>	3	Q166X	Fam (2 F and 1 M)	<i>BglI(-)^f</i>	– (M)	U
3414 (2 F)	L	–	–	U	Developmental delay		<i>500-2A→G</i>	(3)	SP	Fam (4 F)	<i>BamHI(+)</i>	–	U
3269 (1 F)	–	–	–	U	...		<i>564_565insT</i>	4	V189CfsX10	S	<i>DrdI(-)</i>	–	Pat
3221 (1 F)	+	–	–	–	...		<i>587delC</i>	4	P196LfsX17	S	<i>XcmI(+)</i>	–	U
3473 (2 F)	L	–	–	–	Right diaphragmatic hernia		<i>635_636delTG</i>	5	V212EfsX19	Fam (2 F)	<i>Hpy8I(-)</i>	–	U
3319 (1 F)	R	–	–	–	Bilateral dysplastic hips		<i>993_994insCT</i>	5	Q332LfsX61	S	<i>Hpy188III(+)</i>	–	NI
3214 (1 F)	R and L	–	–	+	...		Deletion	1–5	...	S	Southern MLPA	–	Mat
3487 (2 F)	L	–	–	Partial	Mother has bicornuate uterus		Deletion	1–3	...	Fam (2 F)	Southern MLPA	–	Mat
3558 (1 F)	R	–	–	+	Sensorineural hearing loss		Deletion	1–5	...	S	MLPA	–	Pat

NOTE.—Totals include mosaic cases. The 27 newly described intragenic mutations are shown in bold italics. U = unknown.

^a A plus sign (+) = present; a minus sign (–) = absent; R = right side affected; L = left side affected; CL = cleft lip; P = cleft palate; T = duplex thumb; H = duplex hallux.

^b SP = splice-site mutation; S = sporadic case; Fam = familial mutation; ASO = allele-specific oligonucleotide hybridization.

^c A plus sign (+) = proven mosaicism; a minus sign (–) = no mosaicism found; NA = not applicable. One somatic mosaicism was identified in a family published elsewhere.¹⁰

^d Pat = paternal origin; Mat = maternal origin; PZ = postzygotic origin (in F or M fetus); NI = not informative. Seven paternally originating germline mutations (344, 347, 372, 656, 723, 1219, and 2613) were identified in cases published elsewhere.¹⁰

^e RNA analysis shows that this apparently synonymous substitution creates a cryptic donor splice site.

^f Mutant oligonucleotide/digest.

Table 2

Primers Used for the Amplification of SNPs and CA Microsatellites

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Although generally accepted, this proposed inheritance pattern raised two major difficulties. The first was to explain how an X-linked mutation could result in a more severe phenotype in a heterozygous female than in a hemizygous male. Substantial insight into this issue was recently achieved with the identification of *EFNB1* (GenBank accession numbers AL136092 and NM_004429), which encodes the cell-surface signaling molecule ephrin-B1, as the major gene mutated in CFNS.^{10,11} This discovery confirmed that CFNS is X linked, because *EFNB1* is located at Xq13.1. Moreover, targeted inactivation of the mouse ortholog *Efnb1* results in a similar paradoxical pattern of phenotypic severity, with heterozygous females consistently more severely affected than hemizygous males.^{12,13} In the heterozygous female mice, abnormal sorting of cells into ephrin-B1-expressing and -nonexpressing patches was shown to correlate with the X-inactivation status of *Efnb1*.¹² As a result, ectopic tissue boundaries between these patches are generated, which lead to morphological abnormalities specifically in heterozygous females; these females are functional mosaics because of X inactivation. The term “cellular interference” has been proposed for this phenomenon (reviewed by Wieacker and Wieland¹⁴). In hemizygous males, by contrast, this process cannot occur. Since the diverse nature of *EFNB1* mutations—including missense, nonsense, frameshift, and partial gene deletions—implies loss of function in the protein, the generally mild male phenotype suggests that ephrin-B1 is redundant in most tissues in which it is expressed.

The confirmation that CFNS exhibits a paradoxical pattern of X-linked inheritance, as well as insight into its potential mechanism, highlights a second difficulty in explaining the genetics of this disorder. If the male phenotype is minor, why are there so few males in CFNS pedigrees? For example, the comprehensive review by Grutzner and Gorlin identified 58 affected females but only 8 affected/carrier males⁸; similarly, the nonoverlapping set of pedigrees analyzed in the present study includes 86 affected females but only 9 carrier males. The aggregate figures indicate a significant underrepresentation of males compared with the expected 2:1 female:male ratio for an X-linked mutation at equilibrium. One possible explanation is that many males with *EFNB1* mutations do not manifest any clinical abnormality. However, in our initial mutation analysis of 20 unrelated females with CFNS, we did not find a single instance in which the clinically unaffected father turned out to be a

carrier: either the mutation had arisen de novo or the clinically affected mother had transmitted the mutation.¹⁰

In an attempt to resolve this paradox, we set out to analyze the molecular origins of *EFNB1* mutations in a large patient cohort. One goal of this work was to determine the parental origin of new germline mutations, because paternally originating mutations can give rise only to affected females in the first generation.¹⁵ Given the importance of functional mosaicism (through X inactivation) in the CFNS phenotype, we also wanted to examine the possibility that somatic mosaicism of *EFNB1* leads to CFNS in either males or females. Collectively, our work identifies several factors that lead to the underrepresentation of males in CFNS pedigrees, the most important of which is a bias toward new mutations arising in the paternal germline. Our analysis also reveals a diversity of presentations of postzygotic *EFNB1* mutations that require careful consideration for genetic counseling.

Subjects and Methods

Clinical Ascertainment

The study was approved by the Oxfordshire Research Ethics Committee, and informed consent was obtained from all participants by the referring clinicians, in accordance with local guidelines. All families were ascertained through a clinically affected proband exhibiting the characteristic features of CFNS.³ This work analyzes 59 unrelated families in which a pathogenic mutation of *EFNB1* was identified. Of these families, 20 were reported elsewhere¹⁰ and 39 were recruited for this study; the latter are unpublished elsewhere except for subjects 3259, 3262, 3258, and 3265, who correspond to subjects 1, 2, 4/8, and 5/6/9, respectively, in the work of Kapusta et al.⁴ The clinical features and results of mutation analysis in these new families are summarized in table 1. We analyzed peripheral blood samples from all affected individuals and obtained additional buccal brushings and/or hair roots from three individuals with suspected cases of mosaicism. *EFNB1* mutations were not identified in a further 10 females with suspected CFNS referred by clinical geneticists, nor in 2 sporadic males with a CFNS-like phenotype.

Informativity for Analysis of Mutational Origin

Of the 59 families studied, 20 families had two or more affected individuals (samples were available from 42 individuals: 33 females and 9 males); the remaining 39 families had sporadically affected females. We distinguished among three categories of family sampling. In category 1 ($n = 6$), no sample was available from the first affected individual in the family, so that attempts to determine mutational origin were not ap-

Table 3

Primers Used for Parental-Origin Analysis by Allele-Specific PCR

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Table 4

Miscellaneous Primers

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plicable. In category 2 ($n = 27$), a sample was available from the first affected individual in the family but not from both clinically unaffected parents (only the maternal sample was required in the case of a first affected male). These samples were informative for mosaicism only. In category 3 ($n = 26$), availability of samples from both parents of a first affected child rendered the family potentially fully informative for both mosaicism and parental origin. This third category includes an additional subject (individual 373), for whom parental samples were obtained subsequent to the original publication.¹⁰

Detection of *EFNB1* Mutations

Intragenic mutations of *EFNB1* were detected by a combination of PCR, Wave denaturing high-performance liquid chromatography (DHPLC), and DNA sequencing, as described elsewhere.¹⁰ Additional PCR amplifications, performed with the primer pairs and conditions shown in tables 2–6 (online only), used the same reagents,¹⁰ except that the *Pwo* polymerase was omitted for non-Wave DHPLC applications. All mutations were confirmed independently, mostly by restriction enzyme digest; in cases where the mutation did not alter a restriction site, a primer containing a mismatch was designed to amplify the mutation, to introduce a suitable restriction site (preferably for the wild-type allele) (table 5). In two samples, allele-specific oligonucleotide hybridization was used for confirmation (table 6). To assess the likely effects of amino acid substitutions in ephrin-B1, residue contacts in the ephrin-B2/EphB2 structure¹⁷ were obtained with CSU software (see the LPC CSU Server).¹⁸

In samples that had normal results in the mutation screen described above, the possibility of a deletion in *EFNB1* was sought. Six polymorphic (CA)_n microsatellites (table 2 [online only]) surrounding *EFNB1* were amplified, and the products were separated by electrophoresis and were analyzed by blot hybridization with a ³²P-dCTP-labeled (CA)₁₀ probe. Apparent noninheritance of alleles at more than one locus indicated the existence of a deletion and demonstrated its parental origin. Deletions were confirmed using the multiplex ligation-dependent probe amplification (MLPA) P080 Craniofacial kit (MRC Holland). In patient 3214, after approximate localization of the breakpoints with Southern blot hybridization, a diagnostic breakpoint fragment was PCR amplified (table 4 [online only]) and sequenced.

Quantification of Mosaicism by Pyrosequencing

Single-stranded PCR products were obtained using a biotinylated primer (table 7) and immobilization to streptavidin beads, denatured with NaOH and annealed to the sequencing primer by heating to 80°C for 2 min. Pyrosequencing¹⁹ was performed on three independent PCRs from control and patient samples, with use of primers and dispensation orders designed to quantify each mutant (M) and corresponding nor-

mal control (NC) allele (table 7 and fig. 1), on a PSQ-HS96A system (Biotage). Peak heights were obtained using the Pyrosequencing software and were exported to an Excel spreadsheet. We extracted the heights of all independent pairs of dispensation peaks for the same nucleotide and specific to either the NC or the M alleles; where possible, these pairs represented the same nucleotide position extended at different dispensations (fig. 1). In two cases, it was necessary to compare a double peak for one allele with a single peak for the other allele; in those cases, we halved the height of the double peak before comparing the two measurements. We rejected dispensations when the average value of M/(M + NC) exceeded 0.07 in the normal control sample. We used the same calculation (corrected for background, estimated from a blank dispensation included in each assay) to estimate the proportion of mutant allele in the patient samples. One to four independent estimates of this proportion were obtained for each PCR (fig. 1); these estimates were averaged and then were compared among the three separate reactions.

Parental Origin of *EFNB1* Mutations

We used approaches described elsewhere^{16,20} to determine from which parent the mutation had arisen. Probands from fully informative families were assessed for heterozygosity at microsatellite polymorphisms or at SNPs located within 1.8 kb of the particular mutation. Informative polymorphisms were a (CA)_n microsatellite within intron 1 (CA c.129–485) (families 344, 372, 656, 2613, 3262, 3269, 3467, 3518, 3575, and 3618) and four SNPs, two newly identified in the present study, employed in the single families 3533 (dbSNP *rs421069*), 1219 (dbSNP *ss49854052*), 723 (dbSNP *rs626840*), and 347 (dbSNP *ss49854051*) (fig. 2). The methods used to deduce the phase of the mutation with respect to the polymorphism are detailed in tables 3 and 4 (online only). Correct relationships of DNA in sample trios were confirmed using a minimum of eight microsatellites, with a minimum heterozygosity of 67% (GDB Human Genome Database) and located on different chromosomes.

Results

In 49 consecutively referred, unrelated female patients with suspected CFNS, we identified 39 *EFNB1* mutations (table 1), of which 36 were intragenic and 3 were partial ($n = 1$) or complete ($n = 2$) gene deletions. The 36 intragenic mutations comprised 20 single-nucleotide and 1 double-nucleotide substitutions encoding missense ($n = 15$) or nonsense ($n = 6$) codons, frameshifting deletions ($n = 5$) or insertions ($n = 2$) of 1–2 nt, splicing mutations ($n = 3$), and initiation codon and in-frame deletion mutations (one each). Two mutations, W37G and R66X, were found recurrently. Of the intragenic mu-

Table 5

Primers Used for Genotyping of Mutations and SNPs

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 1 Quantification of *EFNB1* mutations by Pyrosequencing. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

tations, 27 are newly described (indicated in bold italics in table 1); the majority predict gross disruption of the protein. Of the 12 novel missense mutations, 5 locate at residues (W37, C89, P119, and G151) at which multiple independent substitutions have occurred in CFNS; 5 (N41K, C64Y, C89R, K113N, and S136L) were shown to have arisen de novo (see below). Of the substitutions not fulfilling either of these criteria (G57V, L78P, and G123D), two are familial and segregated with the phenotype (a total of five affected individuals); all are non-conservative and locate at residues highly conserved in vertebrate B-type ephrins.¹⁰ It is therefore likely that all the missense mutations that we identified are pathogenic.

We characterized the three submicroscopic gene deletions, using a combination of microsatellite analysis, MLPA, and Southern blotting (fig. 3). One complete deletion (in subject 3214, a de novo case of maternal origin) extended only for a short distance beyond the 5' end of the gene, excising 14,610 bp (fig. 3). Using primers flanking the deletion (table 4 and fig. 2), we amplified a diagnostic junction fragment in the proband but found no evidence of its presence in DNA extracted from the blood of the unaffected mother (not shown). The two other deletions of *EFNB1* (one complete and one partial) were partially characterized by microsatellite analysis and were shown to be of paternal origin. The complete deletion included at least part of the neighboring gene, *STARD8* (GenBank accession numbers AL732324.6 and AL360076.9), which encodes a RhoGAP protein (fig. 3).

A major goal of our work was to establish the parental origin of intragenic germline *EFNB1* mutations. To ensure correct interpretation of these data, it was essential to avoid contamination with cases of postzygotic origin,

Table 6

Oligonucleotides Used for Confirmation of Mutations

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

which might show no preference for arising on the paternal or maternal allele. We hypothesized that even low levels of somatic mosaicism might manifest in CFNS (in either females or males), since this could potentially lead to cellular interference by a mechanism analogous to that resulting from X inactivation. Therefore, our initial efforts were directed at identification of somatic mosaicism in the 53 cases that were potentially informative.

The six instances in which such mosaicism was established are shown in figure 4A. These presented in three distinct ways, but only one case had been suspected clinically. This was in family 3217 (fig. 4B, left), in which the female proband had classic CFNS and her brother had hypertelorism and a nasal pit, but their mother did not have any CFNS features apart from slight nasal asymmetry and longitudinal grooves in her nails. The *EFNB1* mutation 161C→T (P54L), described twice elsewhere,^{11,21} was present in DNA from blood of those three individuals but not in blood of the father or of the mother's parents. Quantification of this mutation, by Pyrosequencing of the mother's blood, showed a level indistinguishable from 50%; however, because we suspected that she was a mosaic, we analyzed hair roots and buccal brushings. On the diagnostic *Bs*I restriction digests, it was apparent that the mutant allele was underrepresented in these tissues (fig. 4B, right); this was quantified for the hair roots by Pyrosequencing (fig. 5).

In three other families (1818, 3412, and 3494), by contrast, mosaicism was established because the *EFNB1* mutation was underrepresented in the blood of a classically affected female. In one of these (patient 3494), the *EFNB1* mutation 407-1G→A (described by Twigg et al.¹⁰) was not identifiable on sequencing of DNA from blood, but the mutation was revealed because our mutation screening included Wave-DHPLC, which is more

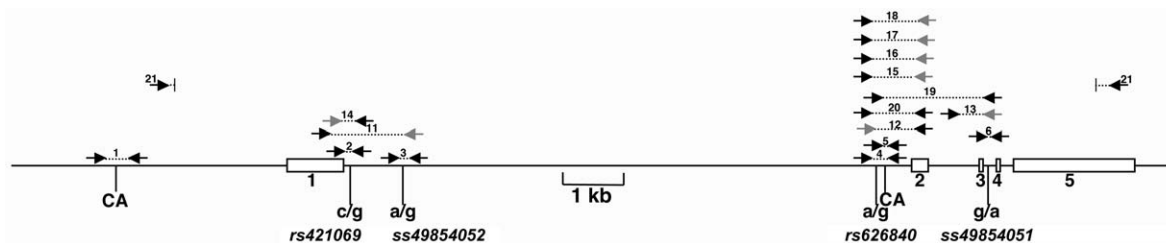


Figure 2 Scale map of the *EFNB1* gene showing key polymorphic sites and primers used in the analysis. Positions of (CA)_n repeats and SNPs are shown beneath the *EFNB1* exons, which are boxed and numbered 1–5. Above, Numbered dotted lines connecting pairs of arrows correspond to PCR products and primer pairs featured in tables 2–4 (online only). Allele-specific primers are indicated in gray.

Table 7**PCR and Pyrosequencing Oligonucleotides**

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

sensitive than DNA sequencing for detection of trace amounts of mutation (fig. 4C, left). After cloning the PCR product that exhibited the abnormal waveform, the G→A mutation was revealed in 1 of 16 clones sequenced (fig. 4C, middle). The diagnostic *Pst*I restriction digest confirmed that the mutant allele was underrepresented (fig. 4C, right), and this was quantified as 13% by Pyrosequencing (fig. 5). In contrast, the other two cases had higher levels of the mosaic mutation in blood, but these levels were quantifiably <50%. We initially suspected these cases either because the mutant allele appeared underrepresented on the DNA sequencing chromatogram (fig. 4D, left) or by comparison of the intensities of the normal control and the mutant alleles in the confirmatory restriction digests (see the “Discussion” section; for examples, see fig. 4B and 4C). We confirmed the mosaicism by quantification of the allelic proportions, using Pyrosequencing (fig. 4D, right, and fig. 5).

In the third group (families 3553 and 3605), the asymptomatic parent of a sporadic affected individual was found to be a mosaic (fig. 4A). Only 24 families (those in category 3, except for subjects 3487 and 3558 with the larger de novo deletions) could be tested for this type of mosaicism. In family 3553, the only clinical manifestation in the mosaic grandmother was marked longitudinal nail ridges (noted retrospectively). The other case, the father in family 3605, is the first male mosaic to be identified with an *EFNB1* mutation. His facial features appeared mildly dysmorphic with nasal asymmetry, but he did not have significant telecanthus (inner canthal distance 35 mm; +1.6 SD). In both of these cases, the level of mosaicism was quantified at ≤13% (figs. 4A and 5), but the mutation was detected on diagnostic restriction digests of multiple tissues.

No other cases of mosaicism were found, either by examination of the relative heights of normal control and mutant peaks on DNA sequence chromatograms or of relative fragment intensity on restriction digests (data not shown). However, since we recognized that both these methods are subjective, we quantified the relative amounts of normal and mutant alleles by Pyrosequencing (fig. 1). We selected four classes for analysis: (1) suspected cases of mosaicism (described above), (2) familial mutations in which a first-generation affected individual (potential mosaic) could be compared with a second-generation offspring (germline mutation), (3) mutations in which multiple independent cases were available for comparison, and (4) all cases in which we determined the parental origin of mutation (see below). The

results are illustrated in figure 5 and show that, apart from class 1, there were no confirmed cases of mosaicism in the analysis of blood ($n = 24$).

In all apparently nonmosaic, nondeletion de novo cases ($n = 20$), we attempted to determine the parental origin of the mutation by establishing the phase of the mutant allele with respect to a neighboring polymorphic site. Most useful for this purpose was a highly polymorphic (CA)_n microsatellite located within intron 1 (c.129–485) that has a heterozygosity of ~90%. Patients with mutations in the downstream exon 2 could be analyzed directly by allele-specific PCR amplification, whereas, for patients with mutations in exon 3, a two-stage process of long-range PCR followed by cloning was required. Four other less polymorphic SNPs were used in analysis

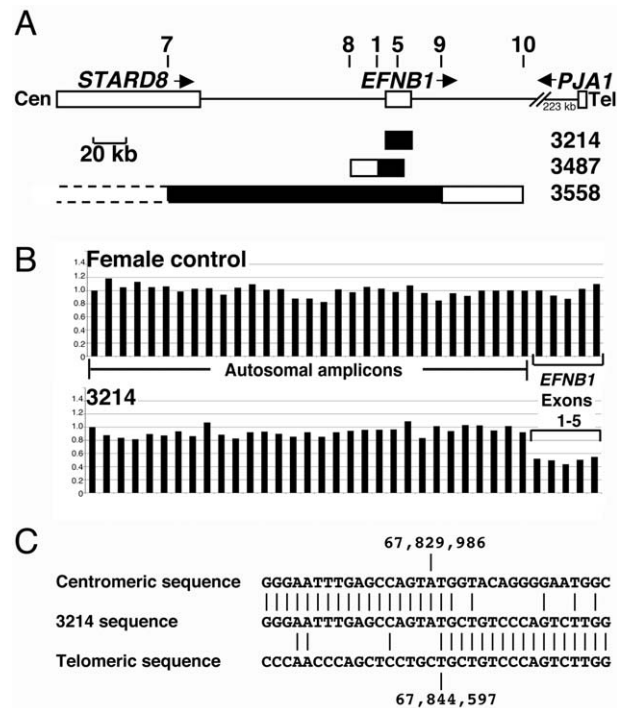


Figure 3 Complete and partial deletions of *EFNB1*. A, Scale diagram showing position of *EFNB1* relative to its flanking genes *STARD8* and *PJA1* (GenBank accession number AL157699.13). The (CA)_n primer pairs (1, 5, and 7–10) used to detect hemizygosity or heterozygosity at different positions correspond to numbered PCR primer pairs featured in table 2 (online only). The extent of confirmed deletions in three cases is indicated by blackened horizontal bars, with unblackened boxes that delineate the range of possible deletion break-points. The centromeric (Cen) extent of deletion in 3558 has not been determined (dashed lines). Tel = telomeric. B, MLPA analysis of subject 3214 (below) compared with a normal female control (above). The estimated dose of *EFNB1* exons 1–5 in 3214 is approximately half in comparison with 31 different autosomal amplicons. C, DNA sequence across *EFNB1* deletion in case 3214, compared with the normal sequences at the centromeric and telomeric ends of the break-point. There is homology of only 2 nt (TG) at the site where the deleted sequences are joined.

of individual families. Representative examples of these analyses are shown in figure 6. We established the parental origin of 14 of the 20 cases with de novo intragenic mutations: 13 originated from the father and 1 from the mother (table 1). With inclusion of the 3 de novo deletions, there were 15 mutations of paternal origin and 2 of maternal origin, which indicates a significant paternal excess (95% CI for proportion of paternal mutations 0.64–0.99). For the 15 paternally originating mutations, the mean (\pm SE) of the paternal age at the time of the child's birth was 32.1 ± 1.25 years. This does not indicate a strong paternal age effect, a conclusion that is supported by matching the nine subjects born in England and Wales with the mean paternal age for the year of birth: the mean of the paternal age excess was 0.95 ± 1.61 years. Inclusion of the five uninformative cases gave similar findings.

Discussion

The paradoxically greater phenotypic severity of CFNS in females than in males and the apparent paucity of carrier males has puzzled clinical geneticists for decades. The identification of causative mutations in *EFNB1* makes it possible to explore the molecular bases of these phenomena. In the present study, we extended our previous analysis of 20 *EFNB1* mutations¹⁰ to include a further 39 independent mutations, 27 of which are newly described intragenic changes (table 1). The mutations are diverse, which suggests partial or complete loss of function in ephrin-B1. No genotype-phenotype correlation is apparent, in agreement with previous studies.^{10,21} Most of the newly identified missense mutations occur at completely or partly buried residues and are predicted to disrupt protein folding,²² but three substitutions (at N41, K113, and G123) locate at sites of interaction between ephrin-B2 and EphB2.¹⁷ The mutation 993_994insCT predicts a frameshift in the protein 15 aa from the C terminus. This segment includes a PDZ-binding domain, in the last 4 aa, that is required for binding of intracellular targets,²³ which suggests that disruption of the reverse signaling activity of ephrin-B1 contributes to the CFNS phenotype.²¹ This is consistent with the observation that removal of the terminal amino acid in murine ephrin-B1 affects embryonic development.¹³

In addition to the intragenic mutations, we identified three heterozygous deletions in individuals with typical CFNS. Using a combination of microsatellite analysis and breakpoint isolation, we showed that two deletions do not involve any other gene but that the third disrupts at least the centromeric gene *STARD8*. These patients showed no consistent phenotypic differences compared with those harboring intragenic mutations.

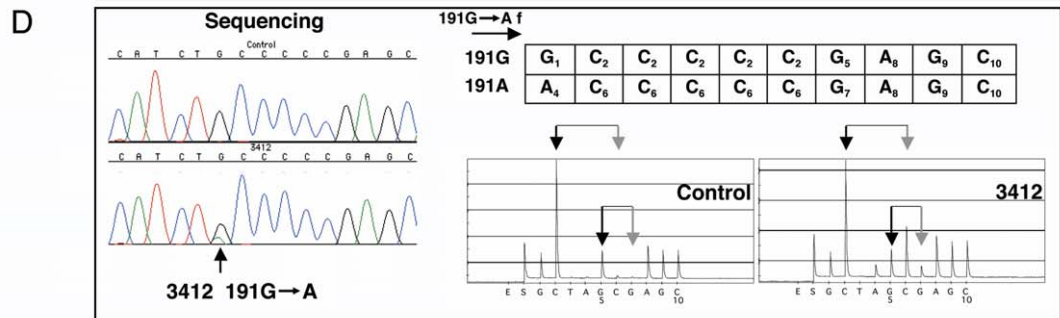
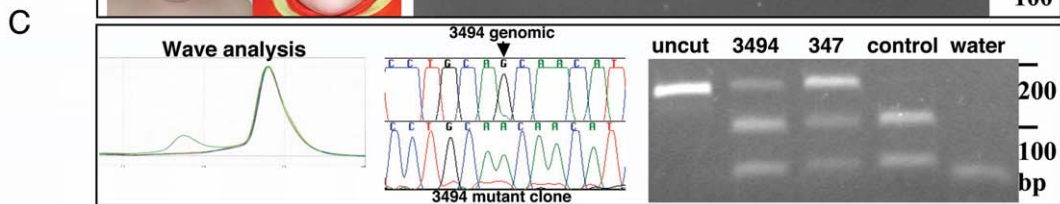
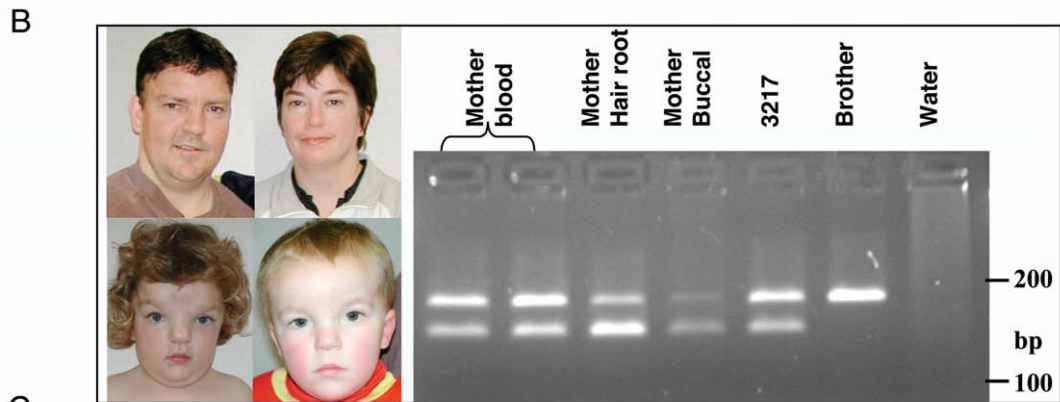
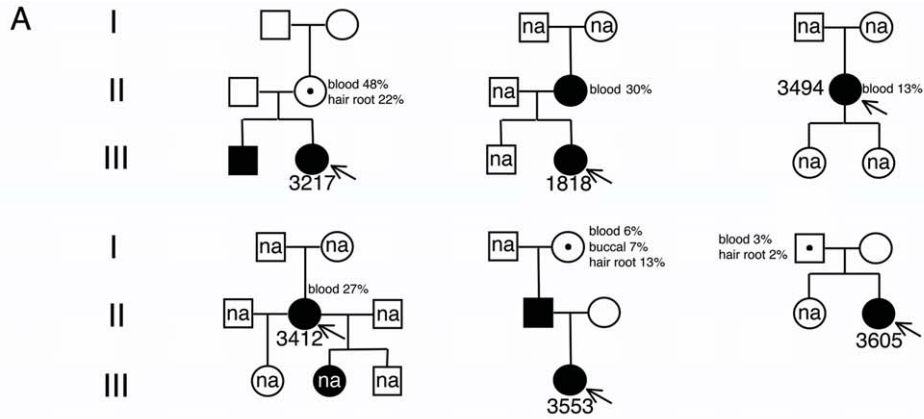
In an effort to understand the underrepresentation of carrier males, we aimed to define the parental origin of

new germline mutations. A significant advantage of the present study, compared with those of X-linked recessive disorders, was that the relative proportions of germline mutations arising on the paternal and maternal X could be estimated directly, which avoided the need to correct for ascertainment bias.²⁴ However, we encountered another potential confounder: the occurrence of mosaicism, which indicated postzygotic mutation. Mutability of the X chromosomes of maternal and paternal origin in the early embryo might be similar, because imprinting is erased at this stage²⁵; thus, contamination with mosaic cases would cause misinterpretation of parental origin data for true germline mutations. Therefore, we first sought evidence of mosaicism, using Wave-DHPLC, restriction digests, and Pyrosequencing. Of note, at least one case would not have been detected had we used DNA sequencing as our only mutation detection method (fig. 4C; see also Jones et al.²⁶).

Once a mutation had been identified, we found that use of restriction digests was an effective way to screen for mosaicism. The digests were designed so that the mutant allele abolished a restriction site; because of incomplete extensions in the PCR at higher cycle numbers, the final product contains a significant proportion of heteroduplex molecules that are resistant to digestion.²⁷ In high-level mosaics, the relative intensity of the non-digested and digested fragments is reversed (fig. 4B), whereas low-level mosaics can be detected because a majority of mutant molecules is present in heteroduplexes, nearly doubling the amount of PCR product resistant to digestion (fig. 4C). We could readily identify, by Pyrosequencing estimates, mutations that were present at the <5% level (fig. 5).

To supplement this qualitative approach with a quantitative one, we used Pyrosequencing to show that presumptive high-level mosaic cases indeed deviated from 50:50 in the ratio of mutant:normal alleles (fig. 4D). To confirm mosaicism, we assayed both strands independently, compared the presumptive mosaic with a known germline mutation, or sampled additional tissues (buccal brushings and hair roots) (figs. 4 and 5). Artfactual explanations, such as inadvertent contamination of the sample with normal genomic DNA or PCR product, or unequal allelic amplification because of a polymorphism underlying the PCR primer sequences were avoided by repeat assays, DNA sequencing of primer binding regions, and replication of results with use of different combinations of primers.

Using these approaches, we confirmed occult parental mosaicism in 3 of 24 fully informative families and confirmed mosaicism of the first affected female in 3 of 50 remaining eligible cases. An unbiased estimate of the prevalence of mosaicism in new cases of *EFNB1* mutation is therefore 18.5%. These mosaic cases encompassed a wide variety of clinical presentations. In fe-



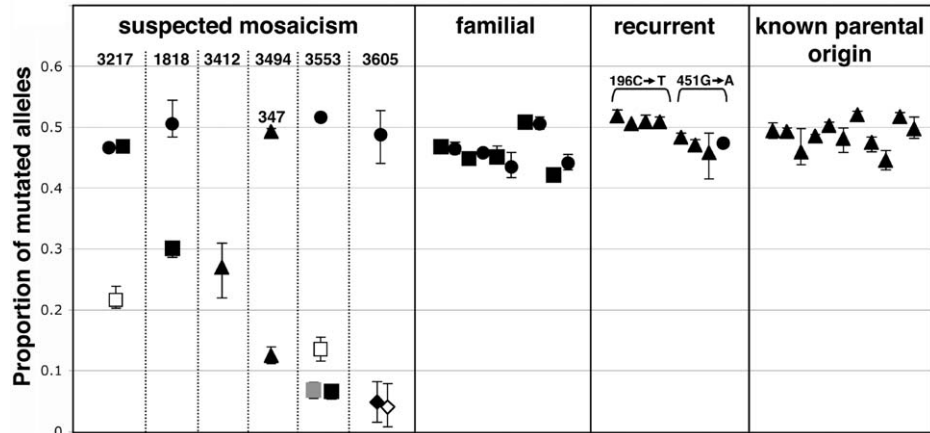


Figure 5 Quantification of proportion of mutated *EFNB1* alleles in different patients and tissues. Samples from blood, buccal brushings, and hair roots are indicated with blackened, gray, and unblackened symbols, respectively, and are plotted as the mean and range of measurements from triplicate PCR products. For related individuals, mothers, fathers, and daughters are shown as squares, diamonds, and circles, respectively. All other data are from unrelated, first affected cases and are indicated as triangles. For clarity, the results are divided into four classes. From left to right, these are (1) subjects in whom mosaicism was suspected and subsequently confirmed (proportion of mutated *EFNB1* allele <0.5), (2) mother-daughter pairs with the same mutation, (3) unrelated subjects with the recurrent mutations 196C→T and 451G→A, and (4) 11 subjects for whom the parental origin was established (the other 3 cases are plotted in previous classes). Family numbers for the individual points are shown in fig. 1B.

males, the most extreme contrasts are provided by the mother in family 3217, who had minimal clinical manifestations but appeared to have a constitutional (~50%) level of mutation in blood, although she had lower mutation levels in hair root and buccal brushings (fig. 4B), and subject 3494, who despite having classic CFNS, had a level of mutation in blood of only 13% (fig. 4C). The poor correlation between the measured level of mosaicism and clinical features presumably reflects variation

in mutation levels—and possibly X inactivation—in tissues that we were unable to sample.

Several other genetic disorders have been described in which somatic mosaicism for diverse intragenic mutations accounts for >10% of new cases; for example, retinoblastoma,²⁸ tuberous sclerosis complex,²⁹ hemophilia B,²⁴ X-linked lissencephaly/double cortex syndrome,³⁰ hemophilia A,³¹ and neurofibromatosis 2.³² However, low levels of somatic mosaicism for *EFNB1* mutations may

Figure 4 Subjects with CFNS who are mosaic for *EFNB1* mutations. *A*, Pedigrees of six confirmed mosaics (squares represent males; circles represent females; blackened symbols represent clinically affected individuals; a dot within the symbol represents a mosaic carrier with mild clinical symptoms; na indicates that no DNA was available for analysis). For the mosaic individual in each pedigree, the percentage of *EFNB1* mutation in each tissue sampled is indicated. *B*, High-level gonosomal mosaicism in the unaffected mother of subject 3217. Photographs show facial appearance of subject 3217 with clinical CFNS (below left), her mildly affected brother (below right), and her clinically unaffected parents (top). Right, Diagnostic restriction digest of exon 2 PCR product. The 161C→T mutation abolishes a *Bs*II site, which indicates that subject 3217 is heterozygous and her brother is hemizygous for the mutation. In the mother, the ratio of mutant:normal alleles appears identical to that of subject 3217 in DNA samples from blood but is reduced in the hair root and buccal samples. *C*, Low-level somatic mosaicism in the blood of subject 3494 with CFNS. Left, Wave-DHPLC analysis of exon 3 PCR fragments from five different individuals. Superimposition of the waveforms reveals abnormal migration of the fragment from subject 3494. Center, DNA sequencing of this fragment appeared normal (upper trace, arrowhead indicates position of mutation). On sequencing individual clones of this fragment, 1 of 16 contained the mutation 407-1G→A (lower trace). Right, Diagnostic restriction digest of exon 3 PCR product. The mutation abolishes a *Pst*I site: only a small amount of *Pst*I-resistant product is present in the sample from subject 3494, compared with subject 347, who is constitutionally heterozygous for the same mutation.¹⁰ Quantification by Pyrosequencing showed that the mutation level was 13% (panel A). *D*, High-level somatic mosaicism in the blood of subject 3412. Left, DNA sequencing of exon 2 PCR product showing apparent underrepresentation of the mutant 191G→A allele in the patient (lower trace). Right, Quantification by Pyrosequencing. The dispensation program for the forward Pyrosequencing primer is shown at the top right. The ratio of the 191A:191G alleles was estimated by comparing peak heights at dispensations C₄:C₂ (which quantify five consecutive C residues) and G₂:G₅. In the pyrograms (bottom right), peaks diagnostic of the 191A (mutant) and 191G (normal) alleles are indicated with gray and black arrows, respectively. In the control sample, mutant peak heights are at background levels and fulfill quality-control criteria. In the patient sample, note consistently lower heights for 191A peaks matched with 191G peaks. Quantification of pyrograms from triplicate PCR products, combined with data for the reverse Pyrosequencing primer, indicates that the 191G→A mutation was present at a 27% level in the blood (panel A).

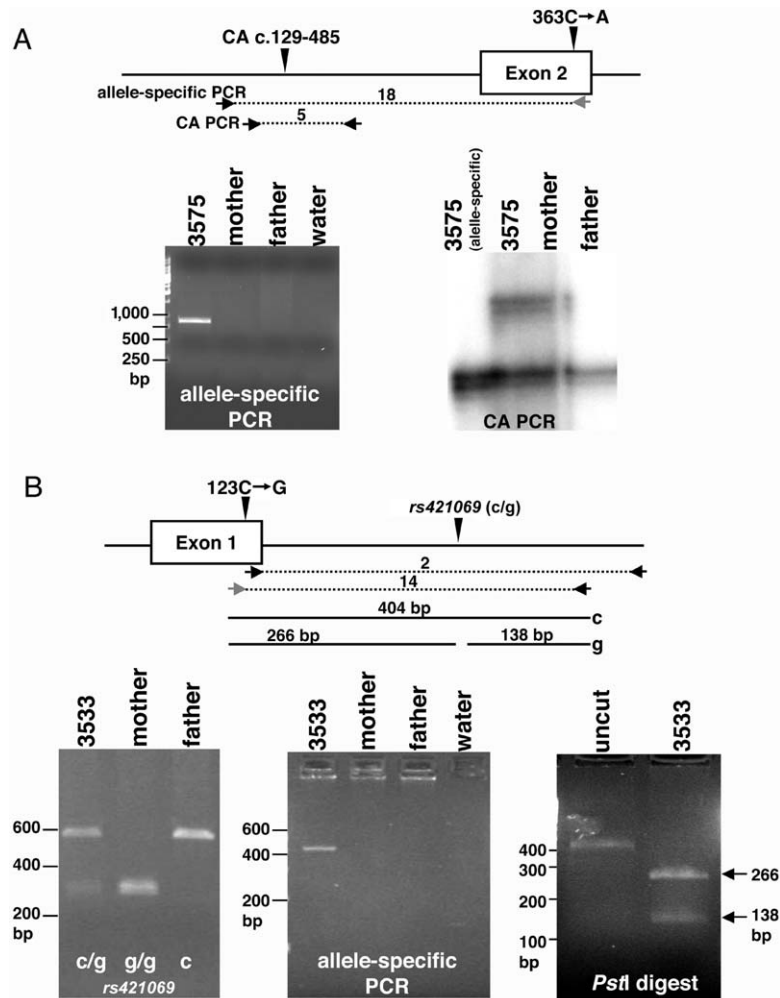


Figure 6 Analysis of the parental origin of *EFNB1* mutations. Representative results from two patients are shown, each below a diagram of the PCR strategy and product numbers (see tables 2 and 3 online only). **A**, Patient 3575 (363C→A in exon 2) informative for CA c.129–485. *Left*, Allele-specific PCR by use of mutation-specific primer (product 18), which confirms presence of the mutation in the patient’s blood but not in either parent. *Right*, (CA)_n genotyping of the family (product 5) by blot hybridization. The mutation-specific PCR product contains the paternal (CA)_n allele, which indicates that the mutation arose from the father. **B**, Patient 3533 (123C→G in exon 1) informative for the SNP 128+235c/g. The g allele of the SNP creates a *PstI* restriction site. *Left*, *PstI* genotyping (product 2) showing that patient 3533 is heterozygous for the SNP. *Center*, Allele-specific PCR by use of mutation-specific primer (product 14), which confirms the presence of the mutation in blood from the patient but not in either parent. *Right*, *PstI* genotyping of the allele-specific PCR product from patient 3533 showing that it contains the g allele of the SNP, which indicates that the mutation arose from the mother.

be particularly likely to manifest, because the proposed mechanism of the greater phenotypic severity in females involves functional mosaicism (i.e., X inactivation). We speculated that rare male cases with apparent phenocopies of CFNS might also be mosaics for an *EFNB1* mutation, but we did not identify a mutation in two such males, including a patient reported elsewhere.⁴ We did find mosaicism in one male, the father of a classically affected female (family 3605); however, the mutation was present at very low proportions in both his blood (3%) and his hair roots (2%). This man had previously fathered an unaffected girl, which indicates that he is

also mosaic in his testes; he manifested mild features of the male-carrier state rather than a more severe CFNS-like phenotype.

After this analysis, we were left with 20 families in which both parents were negative for the apparent germline intragenic mutation that was present in the child. Of the 14 trios with a suitable polymorphism near the mutation, 13 mutations originated from the father. After incorporating data from the three deletions (two paternal and one maternal in origin) and correcting for the relative proportions of intragenic mutations and deletions, we estimated that ~92% of de novo germline *EFNB1*

mutations have a paternal origin. This pattern of predominant but not exclusive paternal mutations without marked elevation in paternal age has been observed in mutations of several other genes associated with loss of function; for example, *NF1*,³³ *RB1*,³⁴ and *MECP2*.³⁵ This suggests that age-independent factors, such as DNA methylation, might be at least as important as replication error in contributing to the male excess of mutations in these genes.³⁶ The situation for *EFNB1* is clearly distinct from craniosynostosis-associated mutations in *FGFR2*, in which an exclusive paternal origin of mutations and significant paternal-age effect²⁰ have been attributed to positive selection of gain-of-function mutations in spermatogonial cells.^{37,38}

The identification of *EFNB1* mutations enables us to analyze why males are apparently underrepresented in CFNS pedigrees. Our series includes eight hemizygous males (table 1): six had typical mild features with hypertelorism,⁴ and the remaining two had major manifestations that required surgical procedures shortly after birth.³⁹ We have not yet encountered a case in which a father was thought to be unaffected, as assessed by a dysmorphologist, but turned out to be hemizygous for the mutation. This indicates that nonpenetrance is not the major explanation for the paucity of carrier males. Instead, we propose that three other factors contribute: (1) the predominant paternal origin of de novo germline *EFNB1* mutations, since these can affect only females in the first generation; (2) the lower genetic fitness of heterozygous females compared with hemizygous males, since carrier males for X-linked disorders can inherit a mutation from their mother only; and (3) the occurrence of postzygotic mutations, which are expected to occur twice as frequently in female embryos and may also be more likely to manifest clinically because of X inactivation. For illustration, if we adopt a simple model of mutation-selection equilibrium in which we ignore mosaicism, use a ratio of male:female germline *EFNB1* mutations of 9:1, and assume that reproductive fitness for *EFNB1*-mutant hemizygous males and heterozygous females is 1.0 and 0.4, respectively, we calculate that 80% of individuals harboring *EFNB1* mutations would be female, which accords with clinical observations (see the introduction above).

Apart from theoretical interest, our findings are important for genetic counseling of patients with CFNS. The specific information given to those who seek counseling will depend on how confidently mosaicism has been excluded in the unaffected parental and first affected generations; in particular, the possibility of occult germinal mosaicism in one of the parents of a child with a germline mutation should be considered. Germinal mosaicism can be confidently excluded only if it is shown that both (1) the mutation has arisen from the father and (2) it is not present at significant levels in the sperm.

It would be instructive to quantify the *EFNB1* mutation levels in the father's sperm in cases of known paternal origin. On the other hand, when it can be convincingly demonstrated—as it was in several cases in the present study—that the mutation is present at <50% levels in at least one tissue, then it is likely to have arisen postzygotically. In that situation, the recurrence risk for the parents of this affected individual is likely to be low and risk for the offspring of the affected individual might be <50%, but female offspring inheriting the mutation would do so in a nonmosaic state and might be more severely affected. However, careful exclusion of artifactual explanations of the apparent mosaicism would be essential before counseling along those lines.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/> (for *rs626840*, *rs421069*, *ss49854051*, and *ss49854052*)
GDB Human Genome Database, <http://gdbwww.gdb.org/> (for allele frequencies of reference microsatellites)
GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for human *EFNB1* [accession number AL136092], *EFNB1* cDNA [accession number NM_004429], *STARD8* [accession numbers AL732324.6 and AL360076.9], and *PJA1* [accession number AL157699.13])
LPC CSU Server, <http://bip.weizmann.ac.il/oca-bin/lpcsu> (for CSU software used for analysis of PDB entry 1KGY)
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for CFNS)

References

1. Cohen MM Jr (1979) Craniofrontonasal dysplasia. Birth Defects Orig Artic Ser 15:85–89
2. Slover R, Sujansky E (1979) Frontonasal dysplasia with coronal

- craniosynostosis in three sibs. *Birth Defects Orig Artic Ser* 15:75–83
3. Cohen MM Jr (2000) Craniofrontonasal syndrome. In: Cohen MM Jr, MacLean RE (eds) *Craniosynostosis: diagnosis, evaluation and management*. Oxford University Press, Oxford, United Kingdom, pp 380–384
 4. Kapusta L, Brunner HG, Hamel BCJ (1992) Craniofrontonasal dysplasia. *Eur J Pediatr* 151:837–841
 5. Natarajan U, Baraitser M, Nicolaidis K, Gosden C (1993) Craniofrontonasal dysplasia in two male sibs. *Clin Dysmorphol* 2:360–364
 6. Young ID, Moore JR (1984) Craniofrontonasal dysplasia—a distinct entity with lethality in the male? *Clin Genet* 25:473–475
 7. Pruzansky S, Costaras M, Rollnick BR (1982) Radiocephalometric findings in a family with craniofrontonasal dysplasia. *Birth Defects* 18:121–138
 8. Grutzner E, Gorlin RJ (1988) Craniofrontonasal dysplasia: phenotypic expression in females and males and genetic considerations. *Oral Surg Oral Med Oral Pathol* 65:436–444
 9. Kere J, Ritvanen A, Marttinen E, Kaitila I (1990) Craniofrontonasal dysostosis: variable expression in a three-generation family. *Clin Genet* 38:441–446
 10. Twigg SRF, Kan R, Babbs C, Bochukova EG, Robertson SP, Wall SA, Morriss-Kay GM, Wilkie AOM (2004) Mutations of ephrin-B1 (*EFNB1*), a marker of tissue boundary formation, cause craniofrontonasal syndrome. *Proc Natl Acad Sci USA* 101:8652–8657
 11. Wieland I, Jakubiczka S, Muschke P, Cohen M, Thiele H, Gerlach KL, Adams RH, Wieacker P (2004) Mutations of the ephrin-B1 gene cause craniofrontonasal syndrome. *Am J Hum Genet* 74:1209–1215
 12. Compagni A, Logan M, Klein R, Adams RH (2003) Control of skeletal patterning by ephrinB1-EphB interactions. *Dev Cell* 5:217–230
 13. Davy A, Aubin J, Soriano P (2004) Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev* 18:572–583
 14. Wieacker P, Wieland I (2005) Clinical and genetic aspects of craniofrontonasal syndrome: towards resolving a genetic paradox. *Mol Genet Metab* 86:110–116
 15. Thomas GH (1996) High male:female ratio of germ-line mutations: an alternative explanation for postulated gestational lethality in males in X-linked dominant disorders. *Am J Hum Genet* 58:1364–1368
 16. Rannan-Eliya SV, Taylor IB, de Heer IM, van den Ouweland AMW, Wall SA, Wilkie AOM (2004) Paternal origin of *FGFR3* mutations in Muenke-type craniosynostosis. *Hum Genet* 115:200–207
 17. Himanen J-P, Rajashankar KR, Lackmann M, Cowan CA, Henkemeyer M, Nikolov DB (2001) Crystal structure of an Eph receptor-ephrin complex. *Nature* 414:933–938
 18. Sobolev V, Sorokine A, Prilusky J, Abola EE, Edelman M (1999) Automated analysis of interatomic contacts in proteins. *Bioinformatics* 15:327–332
 19. Langaee T, Ronaghi M (2005) Genetic variation analyses by pyrosequencing. *Mutat Res* 573:96–102
 20. Moloney DM, Slaney SF, Oldridge M, Wall SA, Sahlin P, Stenman G, Wilkie AOM (1996) Exclusive paternal origin of new mutations in Apert syndrome. *Nat Genet* 13:48–53
 21. Wieland I, Reardon W, Jakubiczka S, Franco B, Kress W, Vincent-Delorme C, Thierry P, Edwards M, König R, Rusu C, Schweiger S, Thompson E, Tinschert S, Stewart F, Wieacker P (2005) Twenty-six novel *EFNB1* mutations in familial and sporadic craniofrontonasal syndrome (CFNS). *Hum Mutat* 26:1–6
 22. Nikolov DB, Li C, Barton WA, Himanen J-P (2005) Crystal structure of the ephrin-B1 ectodomain: implications for receptor recognition and signaling. *Biochemistry* 44:10947–10953
 23. Lin D, Gish GD, Songyang Z, Pawson T (1999) The carboxyl terminus of B class ephrins constitutes a PDZ domain binding motif. *J Biol Chem* 274:3726–3733
 24. Ketterling RP, Vielhaber E, Li X, Drost J, Schaid DJ, Kasper CK, Phillips JA III, Koerper MA, Kim H, Sexauer C, Gruppo R, Ambriz R, Paredes R, Sommer SS (1999) Germline origins in the human *F9* gene: frequent G:C→A:T mosaicism and increased mutations with advanced maternal age. *Hum Genet* 105:629–640
 25. Sado T, Ferguson-Smith AC (2005) Imprinted X inactivation and reprogramming in the preimplantation mouse embryo. *Hum Mol Genet* 14:R59–R64
 26. Jones AC, Sampson JR, Cheadle JP (2001) Low level mosaicism detectable by DHPLC but not by direct sequencing. *Hum Mutat* 17:233–234
 27. Cohn DH, Starman BJ, Blumberg B, Byers PH (1990) Recurrence of lethal osteogenesis imperfecta due to parental mosaicism for a dominant mutation in a human type I collagen gene (*COL1A1*). *Am J Hum Genet* 46:591–601
 28. Sippel KC, Fraioli RE, Smith GD, Schalkoff ME, Sutherland J, Gallie BL, Dryja TP (1998) Frequency of somatic and germ-line mosaicism in retinoblastoma: implications for genetic counseling. *Am J Hum Genet* 62:610–619
 29. Verhoef S, Bakker L, Tempelaars AMP, Hesselting-Janssen ALW, Mazurczak T, Jozwiak S, Fois A, Bartalini G, Zonnenberg BA, van Essen AJ, Lindhout D, Halley DJJ, van den Ouweland AMW (1999) High rate of mosaicism in tuberous sclerosis complex. *Am J Hum Genet* 64:1632–1637
 30. Gleeson JG, Minnerath S, Kuzniecky RI, Dobyns WB, Young ID, Ross ME, Walsh CA (2000) Somatic and germline mosaic mutations in the *doublecortin* gene are associated with variable phenotypes. *Am J Hum Genet* 67:574–581
 31. Leuer M, Oldenburg J, Laverge J-M, Ludwig M, Fregin A, Eigel A, Ljung R, Goodeve A, Peake I, Olek K (2001) Somatic mosaicism in hemophilia A: a fairly common event. *Am J Hum Genet* 69:75–87
 32. Kluwe L, Mautner V, Heinrich B, Dezube R, Jacoby LB, Friedrich RE, MacCollin M (2003) Molecular study of frequency of mosaicism in neurofibromatosis 2 patients with bilateral vestibular schwannomas. *J Med Genet* 40:109–114
 33. Jadayel D, Fain P, Upadhyaya M, Ponder MA, Huson SM, Carey J, Fryer A, Mathew CGP, Barker DF, Ponder BAJ (1990) Paternal origin of new mutations in von Recklinghausen neurofibromatosis. *Nature* 343:558–559
 34. Dryja TP, Morrow JF, Rapaport JM (1997) Quantification of the paternal allele bias for new germline mutations in the retinoblastoma gene. *Hum Genet* 100:446–449
 35. Trappe R, Laccone F, Cobilanschi J, Meins M, Huppke P, Hanefeld F, Engel W (2001) *MECP2* mutations in sporadic cases of Rett syndrome are almost exclusively of paternal origin. *Am J Hum Genet* 68:1093–1101
 36. Hurst LD (2003) Mutation rate: sex biases. In: Cooper DN (ed) *Nature encyclopaedia of the human genome*. Vol 4. Nature Publishing Group, London, pp 218–222
 37. Goriely A, McVean GAT, van Pelt AMM, O'Rourke AW, Wall SA, de Rooij DG, Wilkie AOM (2005) Gain-of-function amino acid substitutions drive positive selection of *FGFR2* mutations in human spermatogonia. *Proc Natl Acad Sci USA* 102:6051–6056
 38. Wilkie AOM (2005) Bad bones, absent smell, selfish testes: the pleiotropic consequences of human FGF receptor mutations. *Cytokine Growth Factor Rev* 16:187–203
 39. Vasudevan PC, Twigg SRF, Mulliken JB, Cook JA, Quarrell OWJ, Wilkie AOM. Expanding the phenotype of craniofrontonasal syndrome: two unrelated boys with *EFNB1* mutations and congenital diaphragmatic hernia. *Eur J Hum Genet* (in press)