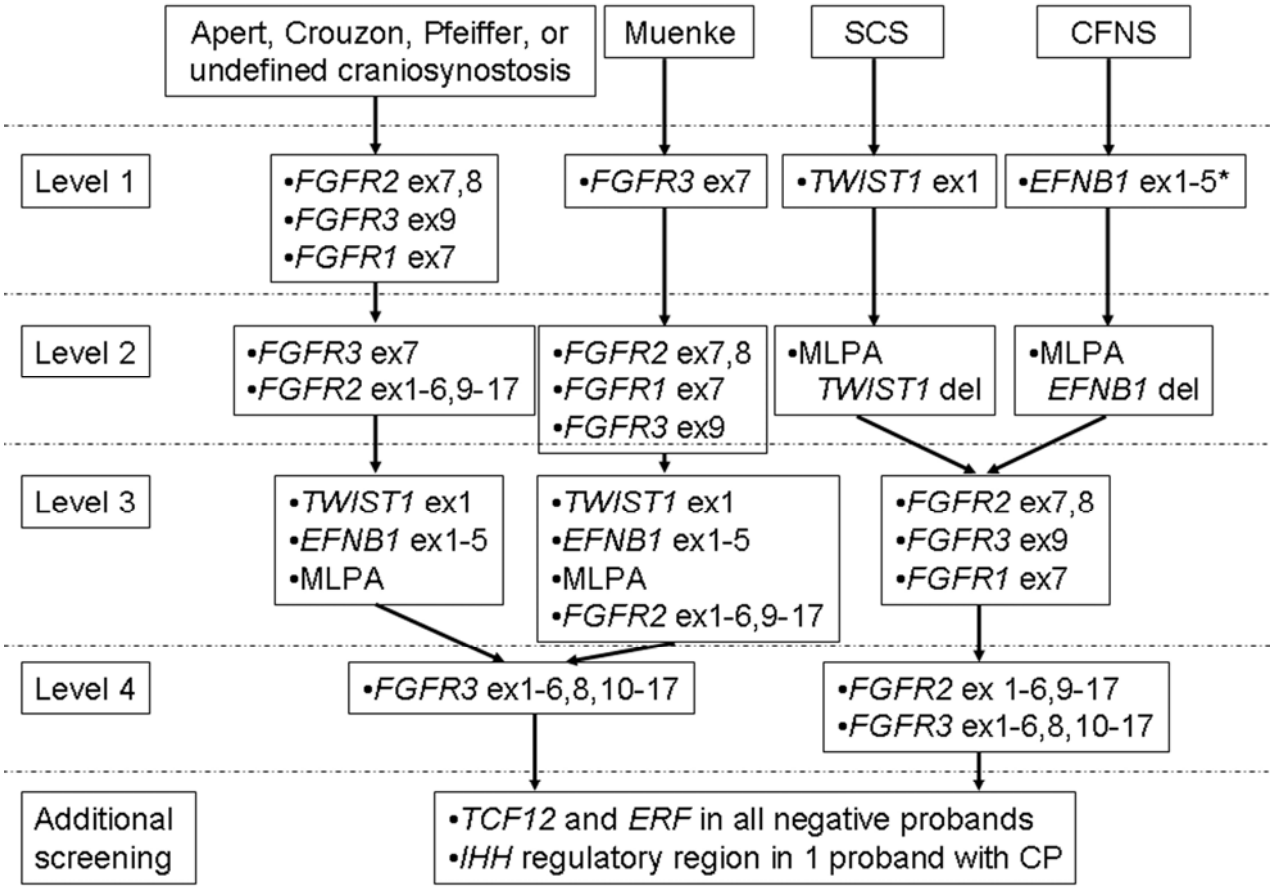
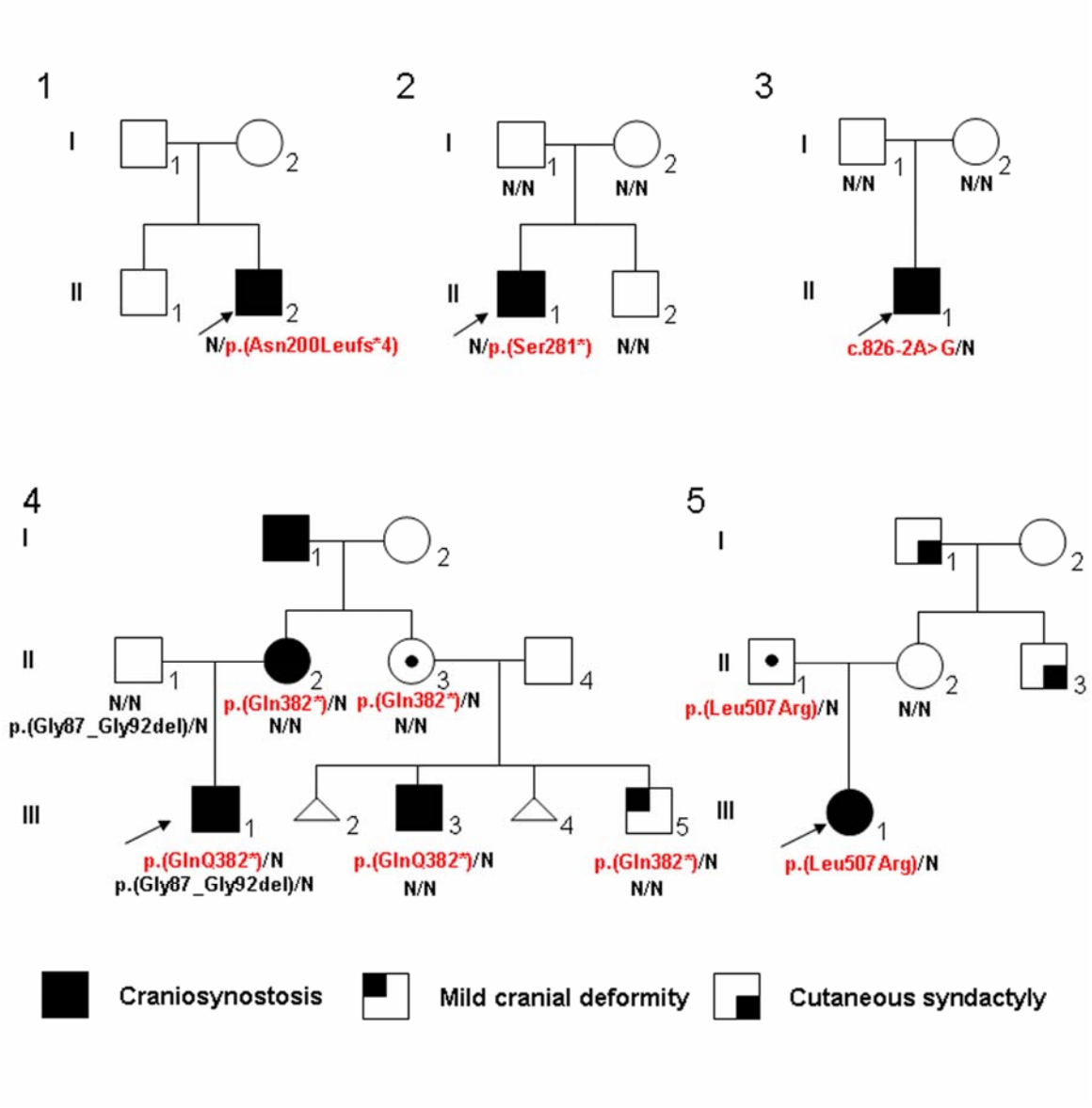
SUPPLEMENTARY DATA

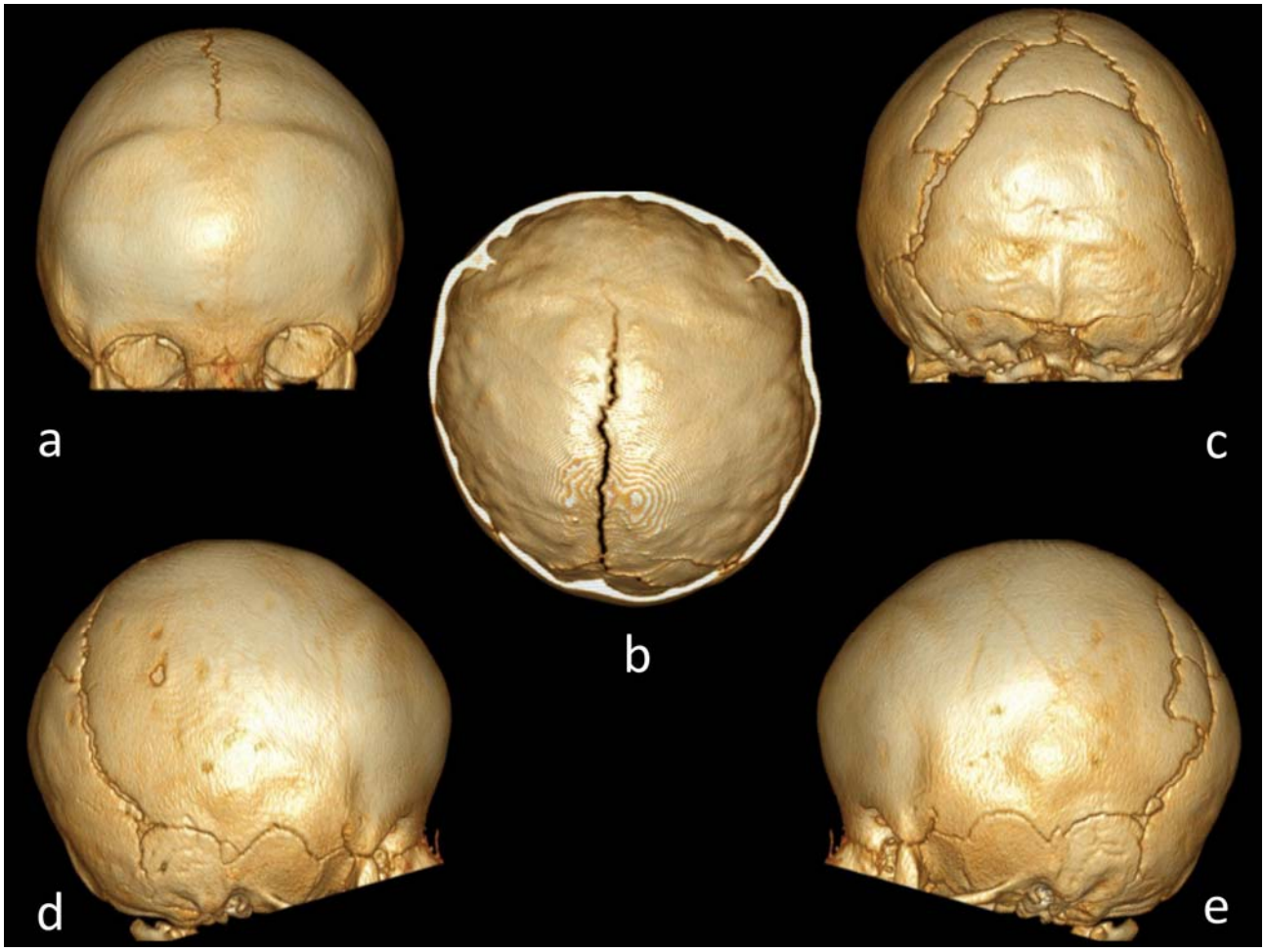
Supplementary Figure 1: Genetic screening cascade for craniosynostosis, including the analysis of *FGFR1, FGFR2, FGFR3, TWIST1,* and *EFNB1*. The exons analyzed for each gene at each level are indicated. *High incidence (19%) of mosaicism which should be taken into account when designing screening method[6]. *FGFR2* exons 7 and 9 are alternatively known as exons 8(IIIa) and exon 10 (IIIc). *FGFR3* exons 7 and 9 are alternatively known as exons 7 (IIIc) and 10 (TM)[161]. Additional screening including the analysis of *TCF12, ERF* and the regulatory regions of *IHH.*



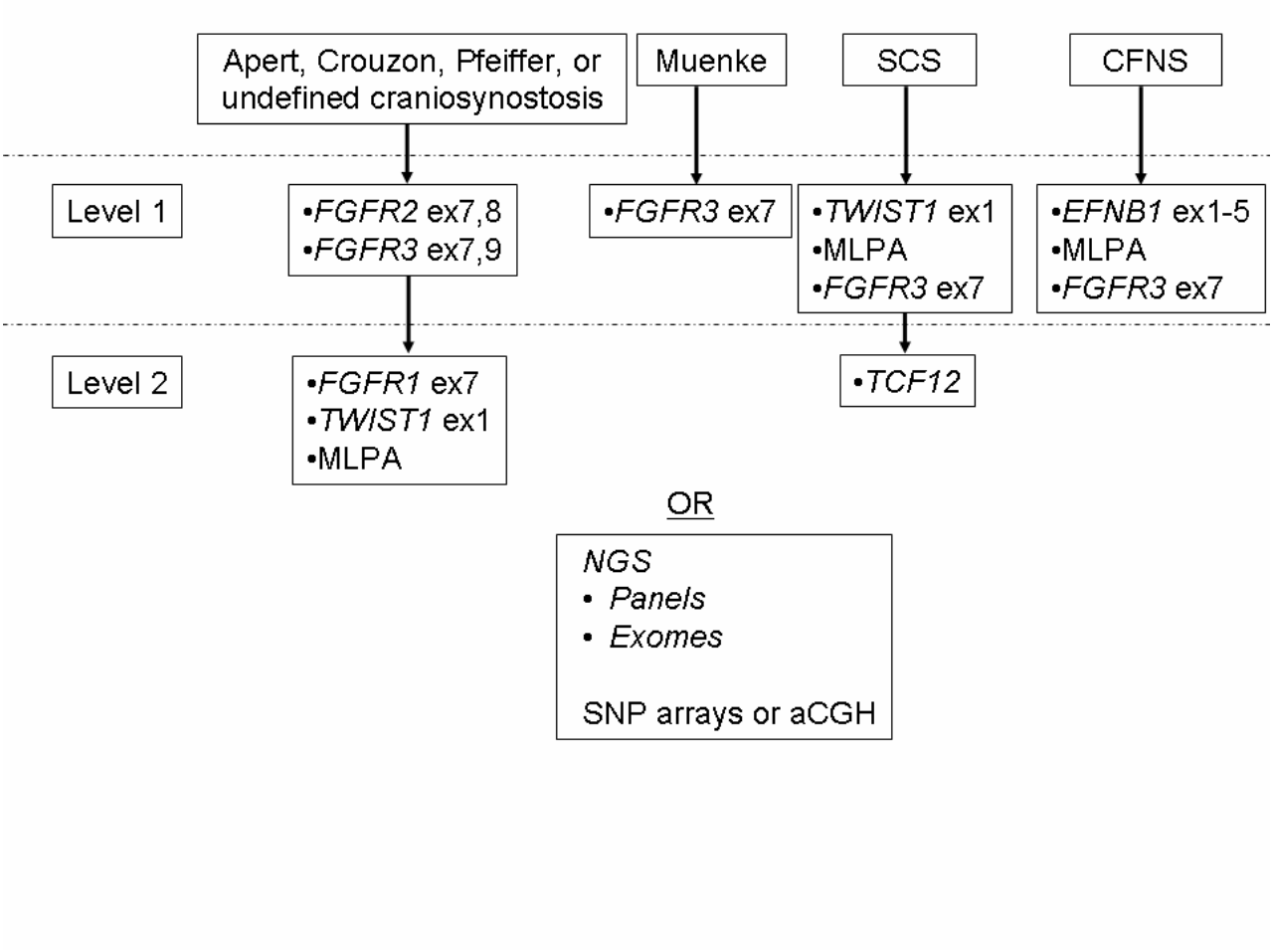
Supplementary Figure 2. Pedigrees of the five families with *TCF12* mutations. Family 1 with the c.596dupC (p.Asn200Lysfs*4) mutation in exon 9. Family 2 with the c.842G>C (p.(Ser281*)) mutation in exon 11. The mutation had either arisen *de novo* or was due to germ line mosaicism. Family 3 with the exon 11 mutation, c.826-2A>G. The mutation had either arisen *de novo* or was due to germ line mosaicism. Family 4 - It's notable that the proband from this family, not only has the *TCF12* p.(Gln382*) mutation, but also an 18 bp deletion in *TWIST1*, p.(Gly87_Gly92del). Cosegregation analysis showed that the proband and unaffected father carried the *TWIST1* deletion. This deletion has also been observed in normal controls, but its pathogenicity is unclear[17]. Thus, it appears that the *TCF12* mutation is the pathogenic mutation in our family and that the *TWIST1* deletion is a non-pathogenic variant. The *TCF12* mutation showed incomplete penetrance whilst the *TWIST1* copy number variant was paternally inherited and did not segregate with the craniosynostosis, thus showing that it is a non-pathogenic CNV. Family 5 with the c.1520T>G (p.(Leu507Arg)) mutation. Two family members reported bilateral cutaneous syndactyly of the second and third toes. The mutation was inherited from the asymptomatic father.



Supplementary Figure 3: Magnetic resonance images (MRI) reconstruction of the cranial bones of proband 4. Metopic and bicoronal synostosis (a, b, d, e) and ossification defects in the parietal bones (c, d) are shown for proband 4, with the TCF12 variant p.(Gln382*).



Supplementary Figure 4: Proposed genetic screening method for classical mutation screening or for NGS bioinformatics analysis. *FGFR2* exons 7 and 9 are alternatively known as exons 8(IIIa) and exon 10 (IIIc). *FGFR3* exons 7 and 9 are alternatively known as exons 7 (IIIc) and 10 (TM)[16].



Supplementary Table 1: Oligonucleotide sequences and PCR conditions for *FGFR1, FGFR2, FGFR3, TWIST1* and *EFNB1*.

Gene	Exon	Sense oligonucleotide (5´-3´)	Antisense oligonucleotide (5´-3´)	Size (bp)	Annealing temperature (°C)
FGFR1	7	CAACCCATCACTGGGAAAGCCAAG	TGTGTGCCTGAAGCGTGAGGAATGATC	420	65[1]
FGFR2	2	CTGACTCGCCAATCTCTTTC	GGCAAAGGACCTTCTCTCA	459	60
FGFR2	3	CCTCCACTGACCTTTGTTG	AAACTATGAAGCTGTATGCCT	470	59[1]
FGFR2	4	TTTGCGAGGGTTCCTGGGT	ATCGGAGCCGGGCAGTTACT	217	59
FGFR2	5	AAGCTGTCCATCAGTATA	TGATGTTCTGAAAGCTTA	239	56
FGFR2	6	ATTCTGTGCTAGGATTGTTA	AGGACTTAACGTTCATGCTT	255	55
FGFR2	7	GGTCTCTCATTCTCCCATCCC	CCAACAGGAAATCAAAGAACC	325	59
FGFR2	8	CCTCCACAATCATTCCTGTGTC	CGAAGCTCCAACCCCTAGACCC	256	55
FGFR2	9	GGTTGTGCTATGATGCGTCA	TCGCACATGGAAGCTCACAG	395	60
FGFR2	10	AACCCATTCCTTTCTAAGA	GGCCAAGAGAAGTACTCAC	277	56
FGFR2	11	ATGCTATGTGCTAATCCC	ACTTTCTTGATAAGACTC	215	54
FGFR2	12	TAACAGTAGCTGCCCATGAG	GGGCGAATGCAGTTT	204	53
FGFR2	13	TCCTGGCCTCATGTGA	CCAGCCAAGTAGAATGTGAA	421	59
FGFR2	14	CGGCCACACTGTATTTC	TGTTACTGCCATCGACTTAC	293	56
FGFR2	15	CCCTATTGAGCCTGCTAA	GATAACCGCTTTGTAGTTGC	431	57
FGFR2	16	GGGCAGGAAAGAGCACATAG	CCAGAGAGCTTCAGCCATT	431	63
FGFR2	17	TTCGGAGGAACTGGCAGG	TCCAACCAACAGCCAACAGG	383	56
FGFR2	18	TGTTATCCTGACCCAAGA	AGTCCACTGCTCCAGAAA	432	55[3]
FGFR3	2	CCGGGCCGTGGGGGGCAGCAT	GCGTTGGGGACGGGAACCGGC	245	69[2]
FGFR3	3	TCCACTGCTGTCTCTGTAAAGGG	CCCTCCTCTCCACCAATGACC	454	62[2]
FGFR3	4	GGGGGTCTCTCTGGTCATTGGTG	CCCAGCCCCTCCTGTATCCTG	366	63[1]
FGFR3	5	CACAGGACGGGAAACTGAGG	GGTGAGTGAGCGGAGGCA	350	64[2]
FGFR3	6	CCGCCAACACCGTCCGCTTCC	TGAGCGTCATCTGCCCCCACA	410	67[1]
FGFR3	7	GTGGAGAACAAGTTTGGCAGCATC	CTGCCCCCGAAGCTCCAACCCCTA	452	63[1]
FGFR3	8	CCTGCCGTGTGGACTCTGTG	GTGTCCCGAGCCAGCGTC	280	69[1]
FGFR3	9	GCCCATGTCTTTGCAGCCGAGGA	GCTCACACAGCCCAGGACCAGCGTG	300	62[1]
FGFR3	10	ACCCCCGGCTGTACCTCC	CAGTCCCTGCCATACACCCGTCC	383	62
FGFR3	11/12	GGCTGAGAGTGGGCGAGTTT	CGGGCTCCTCAGACGGG	439	65
FGFR3	13	AGGGCGGTAGGTGCGGTAG	CCCGCTGCTCCCAGCATCTC	343	63
FGFR3	14-15	GGTGGAGAGGCTTCAGCCCT	AGGGCTGCGCTGCTGCC	459	65
FGFR3	16	GGGGGCAGCAGCGCAGCCCT	AGGGCTGCGCTGCTGCCCCC	419	68
FGFR3	17	CCAACTGCACACACGACC	TCTTCGCACAGCCACCTCTG	409	65

FGFR3	18	GGTGGCTGTGCGAAGAGGG	GGGTGGGCTGCTAGGGAC	252	68
TWIST1	1 (A2)	GGGACTGGAAAGCGGAAACT	CTGGCTCTTCCTCGCTGTTG	523	61[2]
TWIST1	1 (B2)	CGTGTCCAGCTCGCCAGTCT	GGGCAGCGTGGGGATGATCT	406	66[2]
TWIST1	1 (C2)	CAGCGGGTCATGGCCAACG	GCCTGCCGTCTGCCACCT	379	2-step PCR (76/68)[1]
EFNB1	1	CCCCGTCGCGCCTCGTG	GTGTTTTCCTGCGGGCGGGC	557	66
EFNB1	2	GTCCGCTTCCCTGGTTCTGG	ATTCTCGCCCCAACATTTA	542	57
EFNB1	3/4	TTGGCTGAAGCAGAATGGGAGT	GACTGTTCCTTCCCCTTTCC	659	58[2]
EFNB1	5	GGGCTCCCTGGGTGACTCTGAT	CGGACCTCTCCTTTCGCC	661	61[2]

FGFR1 exon 7 (NM_015850.3 NG_007729.1), *FGFR2* exons 2-18 (NM_000141.4, NG_012449.1), *FGFR3* exons 2-18 (NM_000142.4, NG_0126321), *TWIST1* exon 1 (NM_000474.3, NG_008114.1) and *EFNB1* exons 1-5 (NM_004429.4, NG_008887.1)
The standard HRM PCR cycling conditions was 94 ^{o}C 5mins, 40 cycles at 94^{o}C 40s, annealing temp 40s, 72 ^{o}C 1min and a final extension of 8mins at 72 ^{o}C. TD- Touchdown PCR, 0.5^{o}C decrease/cycle for 16 cycles starting at the first indicated temperature, then 24 cycles at the second indicated temperature. Two step PCR of a combined annealing and extension cycle. [1]5% DMSO or [2]10% DMSO added. [3]44 cycles. *TWIST1* exon 1 was divided into three fragments (A2, B2 and C2).

Supplementary Table 2: Oligonucleotide sequences and PCR conditions for *TCF12*.

Exon	Sense oligonucleotide (5´-3´)	Antisense oligonucleotide (5´-3´)	Size (bp)	Annealing Temp (^{o}C)
2	GCGTAATCTTCCCCAGTACC	CAGATGGAGTTCACGCTGCC	308	TD 66/58
3	TAAATGATCGGGTCTTC	AGATTTCTGAGGGAACTGTCA	475	59[1]
4a	GATTGATACTTAGCTCTTCCAC	AAAAATGGCTAAATACATATGGAG	494	53
4b	AAGGGGAAAAAGGTATACAT	TCTGGCTTTATTTAAACCTAACTT	309	50
5	GAGTGTCATTATGCTCAATTAGCG	CAAGAAAAGCCCTAGATGC	314	54
6	TACCATGAATAGTCTAGCAGTTTG	AATGGAGGTGGGTTATGAA	362	53
7	ATGGGTGCGTTCTAAGTT	AAGCATAGCCAGAAGTACAG	339	55
8	TTTTTCACTGGGACTAGTTA	ATCGAATGTACCCTTGTAG	311	50
9	TAAGCCCCTTACAGAATATAGA	TGCCTAAATAAGGCCTGAATA	302	50
10	CCCTTGAATTTTTATAAGCA	TTTTAGAAAGGCATGGTAAA	369	51
11	TTTACCATGCCTTTCTAAAA	ATAAAGATATCGGGGTCACT	365	51
12	GATGATAGAGAACACCCTT	ATCAATACAAACCGAGAATA	307	52
13	ATGATGAAACAGTCTAAAACTTG	GGCTCTCTTTACACACTTTCTTATAG	371	53
14	ATCTTTACCCTTTCCTTCACAAC	CCCTCATAACACAATTTTAACATCA	389	56
15	AGCCTTTTCATATCTTAATAAAATAG	AAAAGCACATGCCAGTAGAGC	435	53
16	CACTTGCTATCTTCCACATATCACAT	CCTTCCTTTCAGAAAGATCCTC	407	56
17	AAAAAGTTGCTGAAATCAGATGAGT	GGCTCTATTCATCAATAAGTATCTGT	443	53
18	AGGATATTCACCAGCTAGAAA	TATATAGTCCCTCAAATGGTATAGTA	493	51
19	TTGTGCACAATCAGCATATCTTAC	TGATACAGTAGAAGAAGTACTGGG	426	53
20	TAGTATGGGAATGAAGTTACACAA	GGCCACTGCTCACTAGAGA	358	54

TCF12 transcript NM_207037.1. Exon 4 was divided into two overlapping fragments (a, b). [1]Final $MgCl_2$ concentration 2.5mM and 44 cycles. TD- Touchdown PCR, 0.5^{o}C decrease/cycle for 16 cycles starting at the first indicated temperature, then 24 cycles at the second indicated temperature.

Supplementary Table 3: Oligonucleotide sequences and PCR conditions for *ERF*.

Exon	Sense oligonucleotide (5´-3´)	Antisense oligonucleotide (5´-3´)	Size (bp)	Annealing Temp (°C)
1	GGGGCGGGCGCAGTGTCT	CCCCCAAAGTTTCTCCGTTCG	333	65[1]
2	TTGGCTGAGGTGAGACAGATTTC	CCCAAGGTCACACAGCTAGGATT	486	64
3	ACCCTCTGGGCACTTGATTTGTC	AGGGAAGCGGAAGTGGCTAC	410	61.7
4a	AAACTGGTGCTGGTCAATTACCC	CCTCCAGCTCTGACGTGCCATC	353	63[2]
4b	TCTTCCCTCTTCTCGGCTGTGGT	GGGGGCTGAGGTGGTAGTTGTA	478	63[1]
4c	CCCACGCTGAGCCCGATGTA	CCGAGATGGGCTCCACCTTGATCTG	454	64[1]
4d	CCGTAGCCGGTGCTGACAAG	CAAGAGAGCTGCCCTCACCTCC	559	67[1]

ERF transcript (NM_004429.2). Exon 4 was divided into four overlapping fragments (a, b, c, and d). [1]10% DMSO or [2]5% DMSO added.

Supplementary Table 4: Oligonucleotides for the construction of the *TCF12* minigene vectors.

Mutant cDNA[1]	Exon /Intron	Sense oligonucleotide (5´-3´)	Antisense oligonucleotide (5´-3´)	Size (bp)	Annealing Temp (^{o}C)
c.-1 A>G	Int 1	GGAGCTCGAGGCGTAATCTTCCCCAGTACC	GGAGGGATCCCAGATGGAGTTCACGCTGCC	382	62
c.826-2 G>A	Int 10	GGGGGAATTCCTTCAAGGCTTACCGCGTTTA	GGGGCTCGAGATCGGGGTCACTGAAAGTCCA	394	60

[1]*TCF12* transcript NM_207037.1.

Supplementary Table 5: Non-synonymous variants or possibly pathogenic variants identified in *TCF12* in the study, which were subsequently shown to be non-pathogenic.

cDNA	Exon	Amino acid	Pathogenicity exclusion criteria and Frequency in control populations
c.-1G>A[1]	2	N/A	Patient presented with craniosynostosis and other clinical features. A minigene assay did not reveal that splicing was altered. Subsequently the patient was clinically diagnosed with Mulibrey Nanism (MIM 253250) and a homozygous *TRIM37* mutation was identified, thus the patient was withdrawn from cohort. The variant was absent from the public databases: EVS and 1000 genomes, and 200 Spanish healthy controls.
c.454C>T[1]	7	p.(Pro152Ser)	Failed to segregate with familial craniosynostosis. Found in low frequency in EVS: European Americans 6/8584; African Americans 0/4384.
c.898G>A	11	p.(Gly300Ser)	EVS: European Americans 285/8584; African Americans 49/4384. Common variant in control population.

[1]The c-1G>A and c.454C>T variants have been submitted to the LOVD gene variant database at www.LOVD.nl/CAV3 (patient IDs #00017836 and #00017837, respectively).

Supplementary Table 6: Details of the craniosynostosis probands (n=20) where the genetic diagnosis differed from the referral diagnosis.

Referral diagnosis	**Mutation details**	**Genetic diagnosis**	**Comments**
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Muenke	*FGFR2* c.1010G>C (p.Ala337Pro)	Crouzon	
Pfeiffer	*FGFR2* c.1124A>G (p.Tyr375Cys)	BSS	Prenatal case
Pfeiffer	*TWIST1* Deletion Ex1-2	SCS	
Pfeiffer	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Pfeiffer	*FGFR2* c.1040C>G (p.Ser347Cys)	Crouzon	
Pfeiffer	*FGFR3* c.1172C>A (p.Ala391Glu)	CSAN	5 month old on referral
Pfeiffer	*FGFR3* c.749G>C (p.Pro250Arg)	Muenke	
Crouzon/Apert/Pfeiffer	*FGFR2* c.1124A>G (p.Tyr375Cys)	BSS	Prenatal case
Crouzon/Pfeiffer	*TWIST1* Deletion Ex1-2	SCS	
Crouzon/Pfeiffer	*FGFR3* c.1172C>A (p.Ala391Glu)	CSAN	3 month old on referral

BSS: Beare Stevenson syndrome; SCS: Saethre-Chotzen syndrome; CSAN: Crouzon syndrome with acanthosis nigricans.

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