Reduced dosage of *ERF* causes complex craniosynostosis in humans and mice, and links ERK1/2 signaling to regulation of osteogenesis

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Supplementary Movies 1-6

Animated morphs of faces of 14 *ERF* mutation-positive individuals from Families 1, 4, 6, 8, 10 and 11 are shown in Movies 1-6, respectively. The portrait views highlight varying degrees of hypertelorism throughout the cohort. Significant lateral/bi-temporal expansion is also seen in probands of Families 1 (IV-2), 6 (III-1) and 10 (II-1). Shortening and/or vertical displacement of the nose is evident in families 1, 4 and 10. The profile animations of both children in Family 1 (IV-1 and IV-2) and the proband in Family 4 (III-1) emphasize the co-ordination of this nasal displacement with posterior displacement of the lateral periorbit. The three generations of Family 1 demonstrate consistent alterations in orbit depth and shape, as do members of Families 11 (affected mother II-2 and maternal uncle II-3) and 6 (proband IV-2 and affected father III-2). Malar flattening occurs in families 6, 8 and 11, but not in all cases.

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Supplementary Table 1. Non-synonymous variants identified by exome sequencing of individual IV-1 in Family 1

Chr	Position	Gene	Variant	Coding	Gene_description
chr1	145587388	ENSG00000186364	G > A	T > I	nudix (nucleoside diphosphate linked moiety X)-type motif 17
chr1	150679247	ENSG00000143452	C > T	G > S	HORMA domain containing 1
chr1	151110510	ENSG00000143434	G > A	P > S	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6C
chr1	155408686	ENSG00000116539	T > A	S > C	ash1 (absent, small, or homeotic)-like (Drosophila
chr1	155903528	ENSG00000132680	C > T	A > T	KIAA0907
chr1	156642155	ENSG00000132688	C > G	K > N	nestin
chr1	161071849	ENSG00000143256	T > A	N > Y	prefoldin subunit 2
chr1	230907829	ENSG00000135773	G > A	G > R	calpain 9
chr1	234744889	ENSG00000168264	G > A	P > S	interferon regulatory factor 2 binding protein 2
chr2	38956836	ENSG00000143891	C > G	A > G	galactose mutarotase (aldose 1-epimerase)
chr2	216257711	ENSG00000115414	C > T	V > I	fibronectin 1
chr2	219878668	ENSG00000181378	T > C	D > G	coiled-coil domain containing 108
chr2	220353297	ENSG00000072195	C > T	R > W	SPEG complex locus
chr3	121416813	ENSG00000173230	G > A	L > F	golgin B1, golgi integral membrane protein
chr3	132405152	ENSG00000113971	C > T	R > Q	nephronophthisis 3 (adolescent)
chr3	132441046	ENSG00000113971	C > T	A > T	nephronophthisis 3 (adolescent)
chr4	106863842	ENSG00000168743	C > T	P > L	nephronectin
chr4	126412558	ENSG00000196159	A > G	M > V	FAT tumor suppressor homolog 4 (Drosophila)
chr4	140625183	ENSG00000085871	C > T	R > *	microsomal glutathione S-transferase 2
chr4	166416765	ENSG00000109472	C > T	A > V	carboxypeptidase E
chr4	177089839	ENSG00000150627	C > G	L > V	WD repeat domain 17
chr5	55466627	ENSG00000164512	A > C	W > G	ankyrin repeat domain 55
chr6	31960890	ENSG00000244731	A > G	T > A	Complement C4-A Precursor (Acidic complement C4)(C3 and PZP-like alpha-2-macroglobulin domain-containing protein 2) [Contains Complement C4 beta chain;Complement C4-A alpha chain;C4a anaphylatoxin;C4b-A;C4d-A;Complement C4 gamma chain]
chr6	31993628	ENSG00000224389	A > G	T > A	complement component 4B (Chido blood group)
chr6	32821022	ENSG00000168394	A > G	L > P	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
chr6	46803249	ENSG00000112818	G > A	G > S	meprin A, alpha (PABA peptide hydrolase)
chr6	137323081	ENSG00000016402	A > G	S > P	interleukin 20 receptor, alpha
chr6	160468304	ENSG00000197081	C > T	P > L	insulin-like growth factor 2 receptor
chr6	169621600	ENSG00000186340	G > A	P > L	thrombospondin 2
chr7	73119489	ENSG00000106089	T > C	K > E	syntaxin 1A (brain)
chr7	92098864	ENSG00000242950	G > A	R > C	endogenous retroviral family W, env(C7), member 1
chr7	92763720	ENSG00000177409	G > A	A > V	sterile alpha motif domain containing 9-like
chr7	102223282	ENSG00000105808	T > C	H > R	RAS p21 protein activator 4
chr7	103292112	ENSG00000189056	T > G	S > R	reelin
chr7	107800823	ENSG00000091129	T > A	K > M	neuronal cell adhesion molecule
chr7	127235688	ENSG00000106328	C > T	R > W	fascin homolog 3, actin-bundling protein,

					testicular (Strongylocentrotus purpuratus)
chr7	139756914	ENSG00000059378	A > C	S > A	poly (ADP-ribose) polymerase family, member 12
chr8	10464616	ENSG00000183638	G > A	T > M	retinitis pigmentosa 1-like 1
chr8	72977723	ENSG00000104321	G > A	A > V	transient receptor potential cation channel, subfamily A, member 1
chr8	73921195	ENSG00000147601	A > G	E > G	telomeric repeat binding factor (NIMA-interacting) 1
chr9	116187302	ENSG00000157653	C > G	P > A	Uncharacterized protein C9orf43
chr9	116191205	ENSG00000157653	A > C	N > T	Uncharacterized protein C9orf43
chr9	124526082	ENSG00000136848	G > A	A > T	DAB2 interacting protein
chr9	125512943	ENSG00000171459	C > T	R > W	olfactory receptor, family 1, subfamily L, member 6
chr9	130167270	ENSG00000136856	G > A	G > S	solute carrier family 2 (facilitated glucose transporter), member 8
chr9	130886050	ENSG00000148334	C > G	G > A	prostaglandin E synthase 2
chr10	23270355	ENSG00000165309	G > A	A > T	armadillo repeat containing 3
chr10	33017899	ENSG00000150076	T > C	I > T	Uncharacterized protein C10orf68
chr10	133784238	ENSG00000176171	G > A	T > M	BCL2/adenovirus E1B 19kDa interacting protein 3
chr10	135085954	ENSG00000151651	G > A	R > W	ADAM metallopeptidase domain 8
chr11	224194	ENSG00000142082	C > T	G > S	sirtuin (silent mating type information regulation 2 homolog) 3 (S. cerevisiae)
chr11	376331	ENSG00000182272	T > A	F > Y	beta-1,4-N-acetyl-galactosaminyl transferase 4
chr11	700653	ENSG00000177042	G > A	D > N	transmembrane protein 80
chr11	5878836	ENSG00000183269	T > A	I > F	olfactory receptor, family 52, subfamily E, member 8
chr11	6459615	ENSG00000110169	C > T	C > Y	hemopexin
chr11	22848804	ENSG00000198168	T > G	K > N	small VCP/p97-interacting protein
chr11	25098911	ENSG00000187398	C > T	P > S	leucine zipper protein 2
chr11	46917501	ENSG00000134569	G > A	R > W	low density lipoprotein receptor-related protein 4
chr11	48510505	ENSG00000237388	G > A	G > D	olfactory receptor, family 4, subfamily A, member 47
chr11	55032399	ENSG00000150244	A > C	Q > P	tripartite motif-containing 48
chr11	55927414	ENSG00000181752	C > T	C > Y	olfactory receptor, family 8, subfamily K, member 5
chr11	58206703	ENSG00000172362	C > T	A > T	olfactory receptor, family 5, subfamily B, member 12
chr11	58978486	ENSG00000197629	G > C	A > G	macrophage expressed 1
chr11	62444384	ENSG00000162191	C > G	E > Q	UBX domain protein 1
chr11	65631142	ENSG00000172732	A > G	H > R	MUS81 endonuclease homolog (S. cerevisiae)
chr11	66102593	ENSG00000174791	T > C	N > S	Ras and Rab interactor 1
chr11	66192465	ENSG00000174576	A > G	N > D	neuronal PAS domain protein 4
chr11	66243148	ENSG00000174516	C > T	A > V	pellino homolog 3 (Drosophila)
chr11	68483391	ENSG00000132749	C > A	A > S	metallothionein-like 5, testis-specific (tesmin)
chr11	71726122	ENSG00000137497	T > A	E > D	nuclear mitotic apparatus protein 1
chr11	73677267	ENSG00000187726	C > T	R > C	DnaJ (Hsp40) related, subfamily B, member 13
chr11	73850854	ENSG00000168014	G > A	P > L	C2 calcium-dependent domain containing 3
chr11	85396880	ENSG00000179071	A > C	N > K	coiled-coil domain containing 89
chr11	86111834	ENSG00000149201	C > A	T > N	coiled-coil domain containing 81 [Source:HGNC Symbol;Acc:26281]
chr11	118133306	ENSG00000149573	C > G	V > L	myelin protein zero-like 2

chr12	6128388	ENSG00000110799	C > T	R > H	von Willebrand factor
chr12	6627110	ENSG00000010292	C > T	A > V	non-SMC condensin I complex, subunit D2
chr12	9875323	ENSG00000184293	T > C	T > A	C-type lectin-like 1
chr12	51693021	ENSG00000110934	C > T	E > K	bridging integrator 2
chr12	53452590	ENSG00000111077	C > G	H > D	tensin like C1 domain containing phosphatase (tensin 2)
chr12	55726161	ENSG00000205329	C > T	P > L	olfactory receptor, family 6, subfamily C, member 3
chr12	56397802	ENSG00000139531	C > T	P > L	sulfite oxidase
chr12	57398178	ENSG00000166860	G > A	P > L	zinc finger and BTB domain containing 39
chr12	57441459	ENSG00000166866	G > A	R > *	myosin IA
chr13	33703738	ENSG00000133121	C > T	R > H	StAR-related lipid transfer (START) domain containing 13
chr13	43918878	ENSG00000120658	T > C	K > E	ecto-NOX disulfide-thiol exchanger 1
chr13	57716532	ENSG00000204919	G > C	E > Q	proline rich 20A
chr13	114107571	ENSG00000153531	A > G	M > T	ADP-ribosylhydrolase like 1
chr14	76958012	ENSG00000119715	A > C	E > A	estrogen-related receptor beta
chr14	77845149	ENSG00000100583	G > A	G > E	SAM domain-containing protein C14orf174
chr14	102717282	ENSG00000080823	A > G	Y > H	renal tumor antigen
chr14	105609459	ENSG00000184916	G > C	A > G	jagged 2
chr15	50534647	ENSG00000140287	G > A	T > I	histidine decarboxylase
chr15	55664110	ENSG00000214882	G > C	A > G	cell cycle progression 1
chr16	23721844	ENSG00000134398	C > T	V > I	endoplasmic reticulum to nucleus signaling 2
chr16	30990985	ENSG00000099381	T > C	L > P	SET domain containing 1A
chr16	48226490	ENSG00000121270	T > A	I > F	ATP-binding cassette, sub-family C (CFTR/MRP), member 11
chr16	57601982	ENSG00000159618	G > C	V > L	G protein-coupled receptor 114
chr16	70289732	ENSG00000090861	G > A	R > W	alanyl-tRNA synthetase
chr16	71065672	ENSG00000157423	T > C	Y > C	hydrocephalus inducing homolog (mouse)
chr16	71094532	ENSG00000157423	C > T	G > S	hydrocephalus inducing homolog (mouse)
chr16	81209298	ENSG00000166473	C > G	G > A	polycystic kidney disease 1-like 2
chr17	5037281	ENSG00000129204	T > C	Y > H	ubiquitin specific peptidase 6 (Tre-2 oncogene)
chr17	8015486	ENSG00000179148	A > T	L > M	arachidonate lipoxygenase 3
chr17	15638936	ENSG00000214946	A > G	D > G	TBC1 domain family, member 26
chr17	15644436	ENSG00000214946	G > C	E > Q	TBC1 domain family, member 26
chr17	18544392	ENSG00000189375	T > C	D > G	TBC1 domain family, member 28
chr17	56690784	ENSG00000121101	G > A	R > *	testis expressed 14
chr17	58285542	ENSG00000170832	G > A	R > W	ubiquitin specific peptidase 32
chr19	37383068	ENSG00000185869	T > C	K > E	zinc finger protein 829
chr19	**42753717**	**ENSG00000105722**	**G > A**	**R > ***	**Ets2 repressor factor**
chr19	45912492	ENSG00000117877	G > C	K > N	CD3e molecule, epsilon associated protein
chr19	49167531	ENSG00000142233	A > G	I > T	netrin 5
chr19	52448007	ENSG00000176024	A > C	S > R	zinc finger protein 613
chr19	53208330	ENSG00000213020	C > T	V > M	zinc finger protein 611
chr19	54407979	ENSG00000126583	G > A	V > M	protein kinase C, gamma
chr19	54754973	ENSG00000105609	T > A	R > S	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 5
chr19	55263689	ENSG00000243772	A > G	E > G	killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2
chr19	55915741	ENSG00000108106	G > A	P > L	ubiquitin-conjugating enzyme E2S

chr19	57838058	ENSG00000178229	G > A	W > *	zinc finger protein 543
chr20	259928	ENSG00000196476	C > G	S > T	Uncharacterized protein C20orf96
chr20	2517926	ENSG00000149488	G > T	V > L	transmembrane channel-like 2
chr20	6060176	ENSG00000101311	C > A	R > S	fermitin family homolog 1 (Drosophila)
chr20	19674019	ENSG00000185052	G > T	A > S	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
chr20	23965995	ENSG00000149435	A > T	V > E	gamma-glutamyltransferase light chain 1
chr20	31598859	ENSG00000078898	C > T	R > W	bactericidal/permeability-increasing protein-like 1
chr20	62187957	ENSG00000125531	T > C	L > P	Uncharacterized protein C20orf195
chr21	44485379	ENSG00000160200	G > A	R > C	cystathionine-beta-synthase
chr21	45537819	ENSG00000241945	G > A	E > K	PWP2 periodic tryptophan protein homolog (yeast)
chr22	38039746	ENSG00000100092	C > T	T > M	SH3-domain binding protein 1
chr22	43275082	ENSG00000100266	C > T	D > N	protein kinase C and casein kinase substrate in neurons 2
chrX	1734138	ENSG00000196433	G > T	A > S	acetylserotonin O-methyltransferase
chrX	48121222	ENSG00000126752	T > G	F > C	synovial sarcoma, X breakpoint 1
chrX	50350757	ENSG00000158352	T > G	K > Q	shroom family member 4
chrX	155004014	ENSG00000168939	G > A	A > T	sprouty homolog 3 (Drosophila)

Supplementary Table 2. Clinical features of subjects heterozygous for *ERF* mutations

Family	ID	Sex	Mutation (protein)	Age at assessment (years)	Syndromic (S) or non-syndromic (N); C= Crouzon syndrome suggested	Craniosynostosis diagnosed (+ or -)	Diagnostic method (CT scan or SXR)	Sutures fused (specify S, Me, RC, LC, RL, LL or P, pansynostosis)	Chiari type I malformation	Raised ICP measured (Pre- or Post - first procedure) CB=copper-beaten appearance of skull	Age (years) at 1st craniofacial surgery	Number of major craniofacial surgical procedures	Craniofacial signs (hypertelorism (H), midface hypoplasia (M), exorbitism (E), macrocephaly >97th centile (MC))	Neurodevelopmental features	Developmental Quotient (Verbal/non-verbal)	Other features
1	II-2	**F**	R183*	62		na					na	0	MC			
1	III-3	F	R183*	38	S (C)	na					na	0	E, M			
1	IV-1	M	R183*	15	S	+	CT	P	Low cerebellar tonsils	Pre	10	1	H, E	Average verbal ability; low average perceptual organization, processing speed, attention and concentration		
1	IV-2	M	R183*	11	S	+	CT	Me,S, LC	-	Post	0.9	3	H	Pervasive developmental delay, severe speech and language delay, behavioural difficulties	80/77 aged 7.1 yr	Grommet R ear (middle ear effusion)
2	II-1	M	F504Lfs*27	4.0	S	+	na	na	-			na	H	Mild intellectual disability		Short digits
3	II-1	M	G299Rfs*9	10	S	+	SXR	P	-	CB	na	0	H, E, downslanting palpebral fissures	Global developmental delay, poor attention span and receptive language; behavioural difficulties		-
4	II-2	F	R86C	43		na					na	0				childhood epilepsy 5th finger clinodactyly
4	III-1	M	R86C	9.6	N	+	CT	RL	+ (surgery 7.4 yr)		1.9	1	MC	Speech and language delay	<50/88 aged 1.9 yr	middle ear effusions, tonsillectomy
5	II-1	M	R65Q	39	N	na					na	0	MC			
5	III-1	M	R65Q	9.9	N	+	CT	S (RC)	-		2.0	1			87/85 aged 1.6 yr	platelet disorder
6	II-1	M	M1I	69		na					na	0	H			Obstructive sleep apnoea
6	III-1	F	M1I	39		na					na	0	MC			bipolar disorder
6	III-2	M	M1I	41	S	na					na	0	H, M, E, MC			orthodontic brace upper teeth, broad halluces

6	IV-2	M	M1I	11	S (C)	+	CT	S, RC, LC, LL	-	Pre	5.4	1	H, M, E	Relative delay in expressive language and processing speed	116/142 aged 5.3 yr	adenotonsillectomy
7	II-2	F	G299Rfs*9	38	S	na					na	0	E			short digits
7	III-1	F	G299Rfs*9	18	S (C)	+	SXR	RC, LC, RL, LL		CB	na	0	H, M, E			
7	III-2	F	G299Rfs*9	7.3	S	+	SXR	RL, LL			na	0	H	Hyperactivity, poor concentration		
8	II-1	M	G299Rfs*9	9.4	N	+	CT	S, RL, LL	-	Pre	4.3	1		Specific language impairment	75/112 aged 4.3 yr	adenotonsillectomy, grommets
9	II-2	F	Q424*	46	S	na					na	0	H			Atrial or ventricular septal defect, von Willebrand disease
9	III-1	F	Q424*	10	S (C)	+	SXR	P	+ (surgery 9 yr)	CB	na	0	H, E	Mild early speech delay, poor coordination/ concentration; now academically ok		Thoracic and lumbar scoliosis, broad toes
9	III-2	M	Q424*	8	S	-			+		na	0	H, E	Poor concentration		Broad toes
10	II-1	M	R86C	5.6	S (C)	+	CT	S, RL, LL	+ (surgery 0.75 yr)	Pre	0.33	2	H, E	Speech articulation disorder with verbal dyspraxia; general motor developmental dyspraxia; Normal receptive and expressive language, cognitive abilities		Class 1 dental malocclusion Adenoidectomy Mild subglottic stenosis R inguinal hernia Broad thumbs and halluces
11	II-2	F	G8_F9ins147	23	S	na					na	0	MC			
11	II-3	M	G8_F9ins147	22		na					na	0	MC			
11	III-1	M	G8_F9ins147	1.7	S (C)	+	CT	S	-		1.4	1	H		103/100 aged 1.4 yr	
12	II-1	M	K401Efs*10	4	S	+	CT	P	-	Pre	2.4	1	H	Delayed development, poor attention span, problems with writing		Brachydactyly of hands and feet, broad halluces, dysplastic ears

Abbreviations not defined at top: F, female, M, male; CT, computed tomography, SXR, skull X-ray; +, present, -, absent, na, not applicable; Me, metopic, S, sagittal, RC right coronal, LC, left coronal, RL, right lambdoid, LL, left lambdoid.

Supplementary Table 3. Combinations of sutures fused in subjects assessed by CT scanning with 3-dimensional reconstruction

Family	Individual	Metopic	Sagittal	Right coronal	Left coronal	Right lambdoid	Left lambdoid
1	IV-1		+	+	+	+	+
1	IV-2	+	+		+		
4	III-1					+	
5	III-1		+	(+)			
6	IV-2		+	+	+		+
8	II-1		+			+	+
10	II-1		+			+	+
11	II-1		+				
Total/8		1	7	2(3)	3	4	4

(+) indicates fusion occurred post-operatively

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Supplementary Table 9. Summary statistics for exome sequencing			
			reads
1	Input	Raw data obtained from 51 bp paired-end sequencing	30,471,986 (x2)
2	MAP_SAM_OUTPUT: bowtie -a	Map to whole genome – all hits recorded	107,738,084
3	GET_HITS: get_mapped.pl	Remove unmapped sequences	85,397,636
4	GET_UNIQUE_PAIRS: parse_sam5.pl	Remove PCR artefacts (only paired-reads with highest total Phred score are retained)	81,546,798
5	FIND_BEST_HITS: find_best_hits.pl	Remove pseudogenes (if a read pair maps to more than 1 location only those which have the lowest number of mismatches over both ends and which map on or near an exome capture bait are retained)	32,061,328 (52.6% map on/near target)
Variant analysis			**variants**
Average read depth			43
Total number of variants			45,372
Number of exonic variants			20,332
dbSNP131/1000 genomes filtering			1,120
SNP array segregation analysis			653
Artefact exclusion (due to highly homologous sequences)			422
Number of synonymous changes remaining			185
Number of non-synonymous changes remaining			237
Excluding non-synonymous changes found in other 2 patients (likely artefacts)			135

Supplementary Table 10. Primers and amplification conditions

Screening

	Primer sequence 5′→3′ (M13 tags in lowercase)		Product size (bp)	Amplification conditions[a]
Amplicon	Forward	Reverse		
Ex1 F/R	gtaaaacgacggccagtGGAGGGGGCGGGCGCAGTGTCTCCATGG	agcggataacaatttcacacaggaGGTCCCCCAGAACTGGGGATCACTC	386	66°C + DMSO
Ex2 F/R	gtaaaacgacggccagtCCCATGTTGAAGGCAGGGCAGAGGTGG	agcggataacaatttcacacaggaCCCAAGGTCACACAGCTAGGATTTGG	463	66°C
Ex3 F/R	gtaaaacgacggccagtGTGTTAAGGTGTGGAGTCTAGACCTGGG	agcggataacaatttcacacaggaGAAGAGGGAAGATGAAGATGAAGAGC	421	66°C
Ex4 F1/R1	gtaaaacgacggccagtGTGCCGTCGGGTGGTAGCCACTTCCG	agcggataacaatttcacacaggaGTCCTCAGGGCTGAAGGAGAAGTGGG	525	66°C
Ex4 F2/R2	gtaaaacgacggccagtGCTGAGCCCGATGTACCCCAGTGGTGG	agcggataacaatttcacacaggaCTGATGTCAGTCACCTCTACCTCCTC	487	66°C
Ex4 F3/R3	gtaaaacgacggccagtGATCAAGGTGGAGCCCATCTCGGAAGG	agcggataacaatttcacacaggaGTGGGGAGGGAAAAGGGAGGAGGCAGGG	506	66°C

Mutation confirmation

	Primer sequence 5′→3′		Product size (bp)	Amplification conditions[a]	Digest
Mutation	Forward	Reverse			
547C>T	GTACCAGGGATGGTTGGTGTGGAGAGGG	GACTCGGAAGACACCAGGGTCATGGGG	414	66°C	HaeIII(-)
1512delT	GATCAAGGTGGAGCCCATCTCGGAAGG	GTGGGGAGGGAAAAGGGAGGAGGCAGGG	506	66°C	SEQ[b]
891_892delAG	GCTGAGCCCGATGTACCCCAGTGGTGG	CTGATGTCAGTCACCTCTACCTCCTC	487	66°C	AvaI(+)
256C>T	CCCATGTTGAAGGCAGGGCAGAGGTGG	CCCAAGGTCACACAGCTAGGATTTGG	463	66°C	AciI(-)
194G>A	CCCATGTTGAAGGCAGGGCAGAGGTGG	CCCAAGGTCACACAGCTAGGATTTGG	463	66°C	HpaII(-)
3G>A	GCGGCCCGGAATCGGGGCGCTTCG	GGTCCCCCAGAACTGGGGATCACTC	181	+ DMSO	NlaIII(-)
1270C>T	TTCCTGCACTACCCTGGGCTGGTGGTGCCC	ACTCGCCTTCCGAGATGGGCTCCACC**AG**GATCT[c]	313	66°C	AlwNI(-)
21A>T	GGAGGGGGCGGGCGCAGTGTCTCCATGG	GGTCCCCCAGAACTGGGGATCACTC	386	66°C	TspRI(+)

Primers and amplification conditions for cDNA analysis of 21A>T (Family 11)

cDNA	Primer sequence 5′→3′		Product size (bp)	Amplification conditions[a]	Primers
Fragment	Forward	Reverse			
WT	GCGGCCCGGAATCGGGGCGCTTCG	CACTCTGGGGCACTGCACCCCCAGCCAACC	432	+ DMSO	1F1, ex3/4R
Mutant	GCGGCCCGGAATCGGGGCGCTTCG	CGTGAAGGTCACCAGCAGGACCGCGCA	482	+ DMSO	1F1, I1R
Mutant	CCCCTCAAGCACTGGAGACCTGTTGCTTCTC	CACTCTGGGGCACTGCACCCCCAGCCAACC	694	+ DMSO	I1F, ex3/4

Primers used for parental origin analysis in Family 8

	Primer sequence 5′→3′		Product size (bp)	Amplification conditions[a]	Digest
Allele	Forward	Reverse			
C	GGAGGAACCGCTGGGAGAGGATCCCCG**C**G	GTCCTCAGGGCTGAAGGAGAAGTGGG	340	63°C	AvaI(+)
G	GGAGGAACCGCTGGGAGAGGATCCCCG**G**G	GTCCTCAGGGCTGAAGGAGAAGTGGG	340	63°C	AvaI(+)

Multiplex ligation-dependent probe amplification (MLPA)[d]

	Primer sequence 5′→3′		Product size (bp)
Probe	Forward	Reverse	
1 (Intron 1)	gggttccctaagggttggagCGTCCACCGGGCCCGCTATCCCT	GGTTCTGCGCTGCGCGGTCCTGCTGGTGACCTTCatctagattggatcttgctggcac	101
2 (exon 2)	gggttccctaagggttggaGGGCGTCATTGCCTGGCAGGGGGACTACGGGGAATTCGTCATCA	AAGACCCTGATGAGGTGGCCCGGCTGTGGGGCGTTCGCAAGTGCAAgtctagattggatcttgctggcac	133
3 (exon 4-5′)	gggttccctaagggttggaGGGCCGAGGCTCAGTCAGTGACTGTA	GTGATGGCACGTCAGAGCTGGAGGAACCGCTGGGAGAGGAtctagattggatcttgctggcac	108
4 (exon 4-3′)	gggttccctaagggttggaGACTGACATCAGTGATGAGGATGAGGAAGACGGGGAGGTGTTCA	AGACGCCCCGTGCCCCACCTGCACCCCCTAAGCCTGAGCCctctagattggatcttgctggcac	127

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Double-strand oligonucleotides encompassing the core Ets/Runx binding site - 5'-AGAGGATGTGGTTT-3', 5'-AAACCACATCCTCT-3'		
***Erf* targeting**		
StopF2/5578R	ACCGAGATTCCTGAGAGCTAT	AGAGACTAAAGAGAGCTGTCC
Intr1-2/5578R	ATCATACATGTTTCTGAGGGG	AGAGACTAAAGAGAGCTGTCC

Primers used for quantitative mRNA analysis of E16 calvaria[e]

	Primer sequence 5′→3′		Intron span
Gene	5'	3'	
Ibsp (BSP)	GAAACGGTTTCCAGTCCAG	CTGAAACCCGTTCAGAAGG	Yes
Col1a2	TGAAGTGGGTCTTCCAGGTC	GACCAGGCTCACCAACAAGT	Yes
Fos	ATGGGCTCTCCTGTCAACAC	ACGGAGGAGACCAGAGTGG	Yes
Fosb	CCTTCAACCAGCACAACCAC	CTTCAAGCTGATCAGTTTCCG	Yes
Jun	GAAAAGTAGCCCCCAACCTC	AATCAGACAGGGGACACAGC	No
Junb	GCAGCTACTTTTCGGGTCAG	AAAAGTACTGTCCCGGAGGC	No
Sp7	TCTGCTTGAGGAAGAAGCTC	TCCATTGGTGCTTGAGAAGG	Yes
Tgfb1	GGAGAGCCCTGGATACCAAC	AAGTTGGCATGGTAGCCCTT	Yes
Serinc5	ACGTGATGATGACTCTGACC	CACCAGTTGCTGCTTAAACC	Yes
Prkg2	CAACCACCCGAACCTATGAC	CTGGGGATCCAATCTCTTCA	Yes
Capg	CTGGACAGATGAATCTGACC	CATTCGAGAGATGAAGCCATC	Yes
Anxa1	CCGGGAAGACAAGCAAATAC	CCCAGGACCACCTTTGTATG	Yes
Hpgd	CAGCAACCTGTTTATTGTGC	TAATCCATTGGCAATGGTTG	Yes
Apcdd1	ACTCAGACCCTGTCTGCAAG	CACTGAAGACATTGAGGAGG	Yes
Runx2	GAACCAAGAAGGCACAGACA	AACTGCCTGGGGTCTGAAAA	Yes
Bglap-rs1/Bglap2	CTCTGTCTCTCTGACCTCACAG	CAGGTCCTAAATAGTGATACCG	Yes
Alpl (AP)	AATGCCCTGAAACTCCAAAAGC	CCTCTGGTGGCATCTCGTTATC	Yes
Fgf2	TTCTTCCTGCGCATCCATCC	CTGGAGTATTTCCGTGACCG	Yes
Erf	TGTGGCACTTTATCCTGGAG	CTTGTAGGTGAACCGTTTCC	Yes
Gapdh	GACTCCACGACATACTCAGC	CCAGTATGACTCCACTCACG	No
***Erf* RNA *in situ* probe**			
mErfF2/mErfR1	GCTGGAGAGAAGGCTCTAGGAGGCACTG	GGTTAAGGCAGCAAAAGCTCAGGGAGTGG	

Note.—[a]DNA was obtained from whole blood samples by phenol-chloroform extraction and was amplified in a total volume of 20 µl containing 15 mM TrisHCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM each dNTP, 0.4 µM primers, and 0.5 units of Amplitaq Gold polymerase (Applied Biosystems) with or without 10% DMSO. Cycling conditions consisted of an 8 min denaturation step at 94°C, followed by 35 cycles of 94°C for 30 s, 63°C or 66°C for 30 s and 72°C for 30s, with a final extension at 72°C for 10 min. Mutation confirmation was carried out by PCR using the above conditions and indicated primers, followed by restriction digest of 8 µl of PCR product.

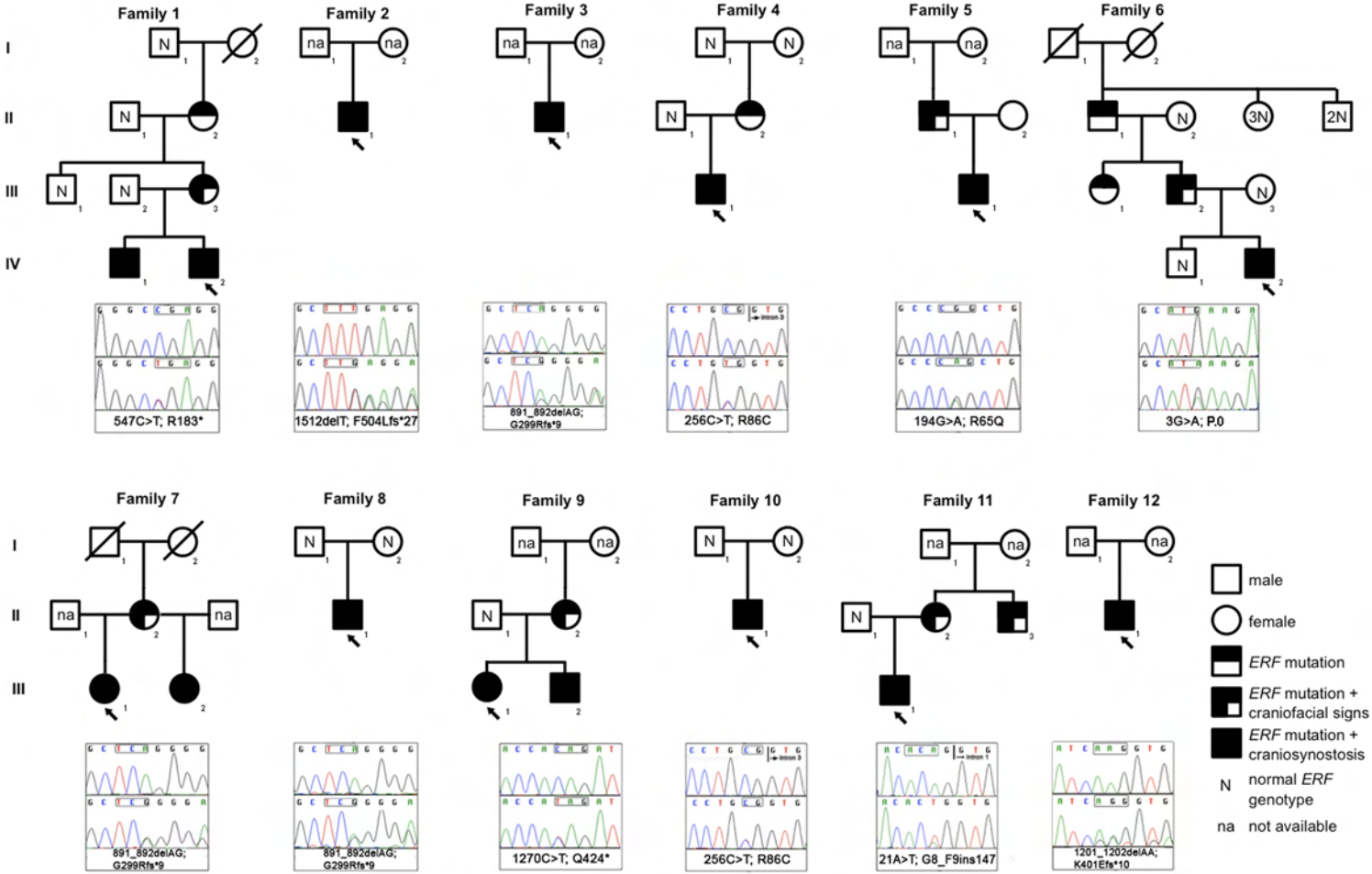
[b]Confirmation carried out by dideoxy-sequencing alone.

[c]The reverse oligonucleotide was mutated (bases in bold) to allow discrimination of the mutation with AlwNI.

[d]Multiplex-ligation-dependent probe amplification was performed using synthetic oligonucleotide probes designed to *ERF* according to protocols available from MRC-Holland: http://www.mrc-holland.com/pages/indexpag.html. Fragments were analyzed by capillary electrophoresis using an ABI 3130 containing POP-7 polymer. Peaks were visualized using Gene Mapper v3.7 (Applied Biosystems). Common PCR primer annealing sequences are shown in lower case, hybridizing sequences are shown in upper case and the 3' probe sequence is 5' phosphorylated.

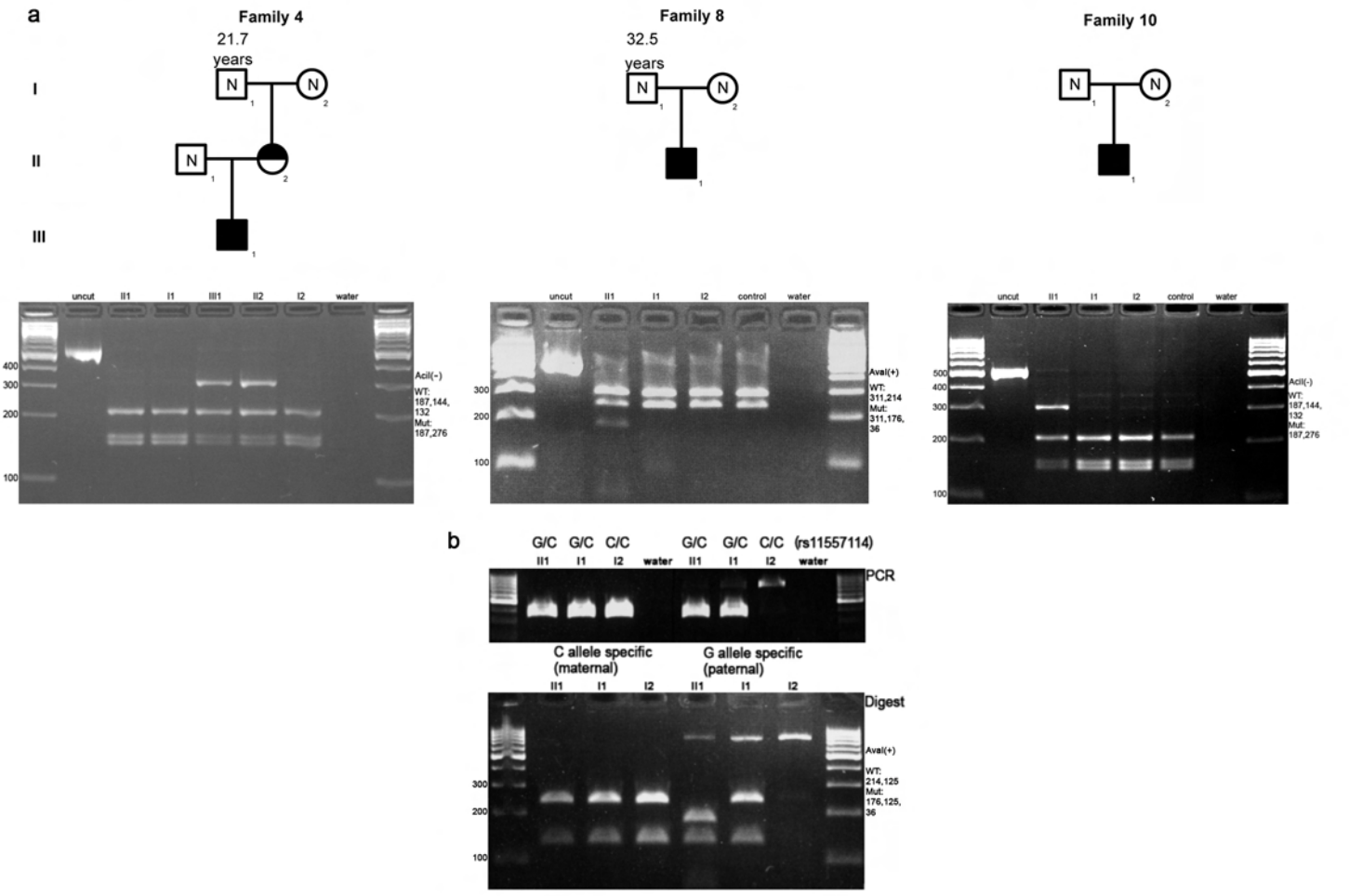
[e]cDNA was prepared from RNA extracted from E16.5 calvariae dissected free of skin and brain and stored in Trizol, using the Stratagene AffinityScript Multi Temperature cDNA synthesis kit according to the manufacturer's instructions. Quantitative PCR was performed on a Stratagene Mx3005P instrument with Brilliant III SYBR® Green reaction reagent. Cycling conditions consisted of a 10 min denaturation step at 95°C, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s.

Supplementary Figure 1. Pedigrees of 12 families with mutations in *ERF* and results of dideoxy-sequencing



For each family the individuals analyzed in the pedigree (see key to symbols) are shown together with a representative sequence chromatogram for a normal control (above) and affected individual (below).

Supplementary Figure 2. Analysis of *de novo ERF* mutations and independent origins of identical mutations



a-b, Restriction digest analysis to demonstrate *de novo* mutations. For families 4, 8 and 10, diagnostic digests demonstrate absence of the mutation in either the parental (Families 8, 10) or grandparental (Family 4) generation (upper panel). The lower panel, **b**, illustrates allele-specific PCR analysis of rs11557114 C/G SNP, demonstrating the paternal origin of mutation in Family 8 (in Family 4, microsatellite analysis was employed, see section **d**). Ages of unaffected fathers at birth of their affected child are indicated. **c-e**, Analysis of chromosome segregation around *ERF* using 4 microsatellite markers. **c**, In Family 6, the mutation is shown to have arisen *de novo* in individual II-1 because 3 of his mutation-negative siblings (II-3, II-4, II-7) inherited the same chromosome that bears his *ERF* mutation. **d**, In Family 4, the mutation is shown to have arisen from the unaffected grandfather. **e**, Families 7 and 3 with the same mutation have differing microsatellite genotypes at the *CA2* locus. Disease haplotypes are boxed with black surrounding lines in individuals bearing the *ERF* mutation and gray surrounding lines in *ERF* mutation-negative subjects. The position of *ERF* in relation to the microsatellites (separation in kb) is shown in the key.

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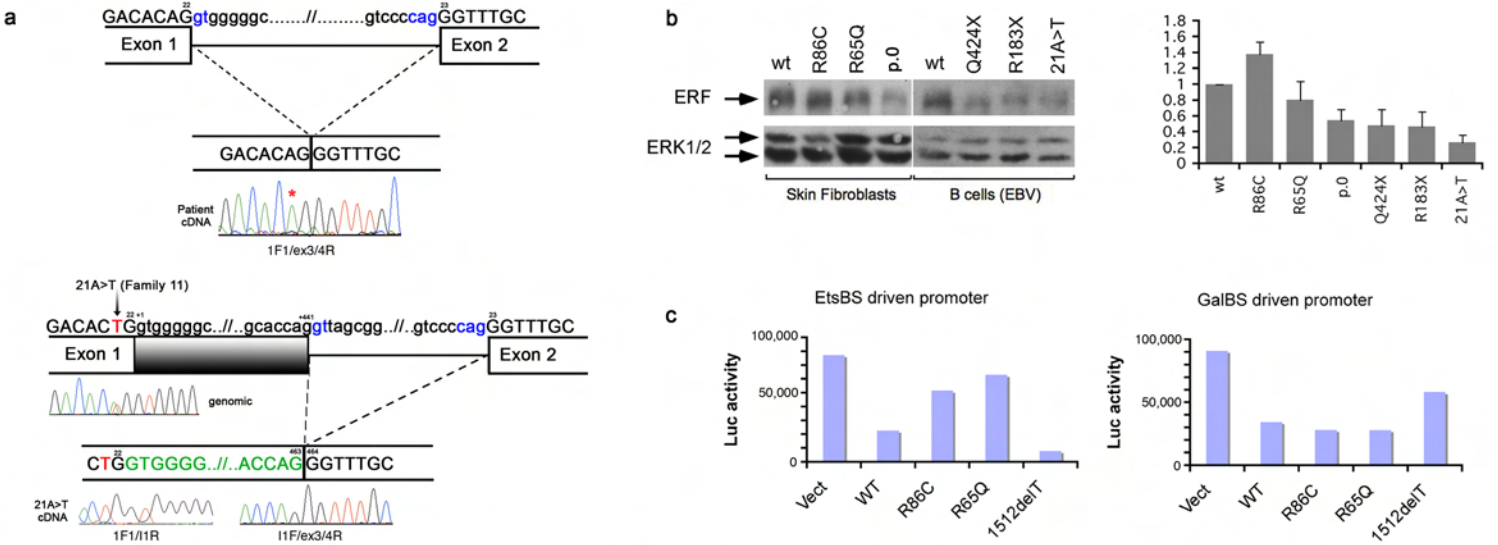
Supplementary Figure 3. RNA and protein studies on selected ERF mutations



a, Sequence analysis of lymphoblastoid cell line cDNA from individual III-1 (Family 11, **Supplementary Figure 1**, heterozygous for 21A>T mutation). The upper panel shows the result of RT-PCR using primers located in exon 1 and exon 3/4 (1F1 and ex3/4R; see **Supplementary Table 8**): only the normal A allele (red asterisk) is visualized, indicating complete loss of the mutant T allele in the normal mRNA. In the lower panel, the intron 1 sequence contains a potential cryptic donor splice site starting at nucleotide 442. RT-PCR using primers in intron1 (reverse primer I1R combined with 1F1, and forward primer I1F combined with ex3/4R), demonstrate that the use of this cryptic splice site occurs exclusively on the allele harbouring the 21A>T mutation. **b**, Western blot analysis of selected ERF mutations in fibroblasts or lymphoblastoid cell lines. Reduced amounts of ERF are produced in cells expressing disruptive mutations (p.0, Q242X, R183X, 21A>T). The quantification in the right hand panel shows the mean ±SD compared to wild type. ERK1/2 antibody reaction was used to control for variation in sample loading. **c**, DNA-binding domain mutants (R65Q, R86C) fail to repress Ets-binding site (EtsBS)-containing promoters (left) but function when tethered to the gal4-DNA binding domain on gal4 binding site (GalBS)-containing reporters (right). Conversely, the deletion/frame-shift mutation (1512delT) failed on gal4 but worked on the EtsBS promoter; this is expected as the ets-DNA binding domain, when overexpressed, is sufficient to out compete endogenous factors.

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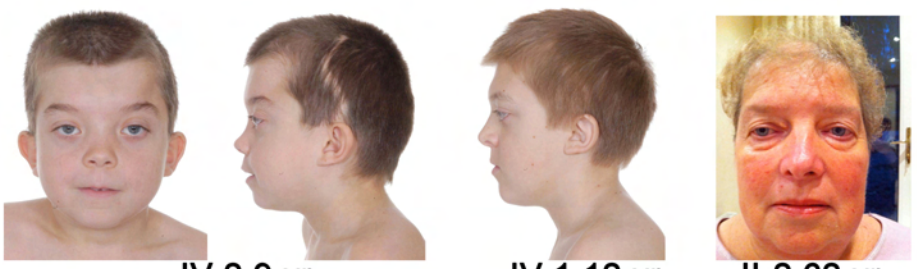
Supplementary Figure 4. Survey of facial features in subjects with *ERF* mutations

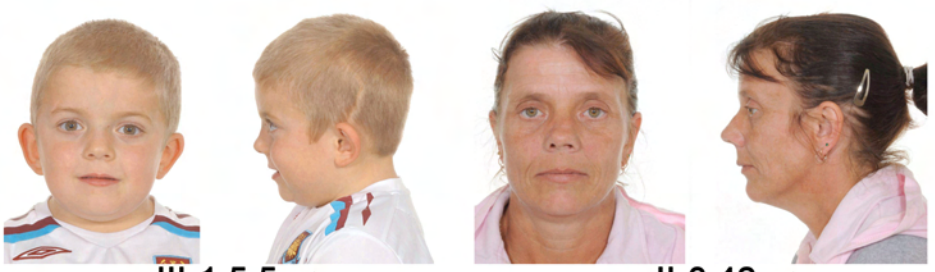
a



IV-2 9 yr IV-1 12 yr II-2 62 yr

Family 1

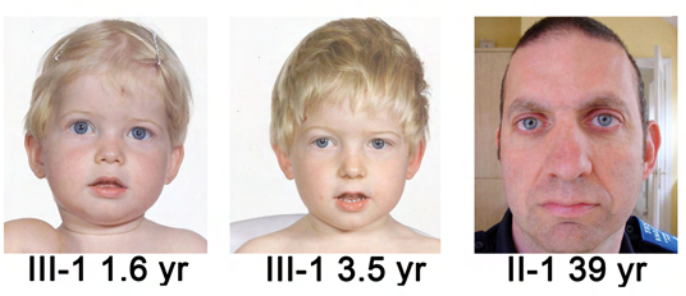
b



III-1 5.5 yr II-3 42 yr

Family 4

c



III-1 1.6 yr III-1 3.5 yr II-1 39 yr

Family 5

d

IV-2 5.3 yr III-2 39 yr

III-1 38 yr II-1 63 yr

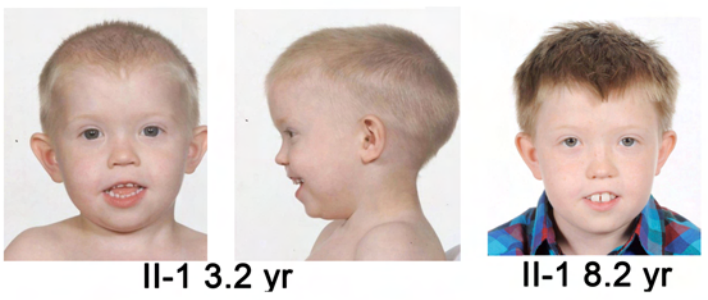
Family 6

e

III-2 6.7 yr III-1 18 yr II-2 38 yr

Family 7

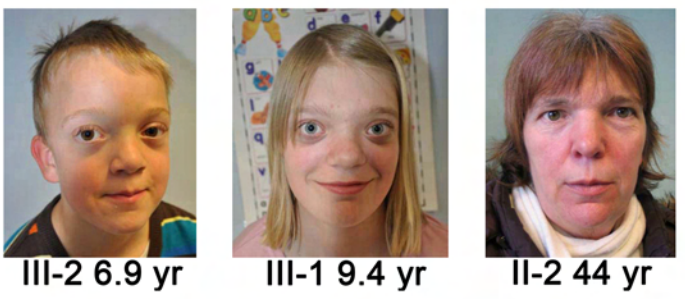
f



II-1 3.2 yr II-1 8.2 yr

Family 8

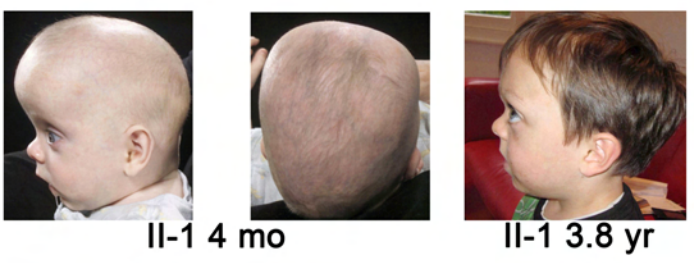
g



III-2 6.9 yr III-1 9.4 yr II-2 44 yr

Family 9

h



II-1 4 mo II-1 3.8 yr

Family 10

i

III-1 1.4 yr II-2 23 yr II-3 22 yr

Family 11

a-i, *ERF* mutation-positive individuals from 9 families. Written consent was provided for the publication of all photograps.

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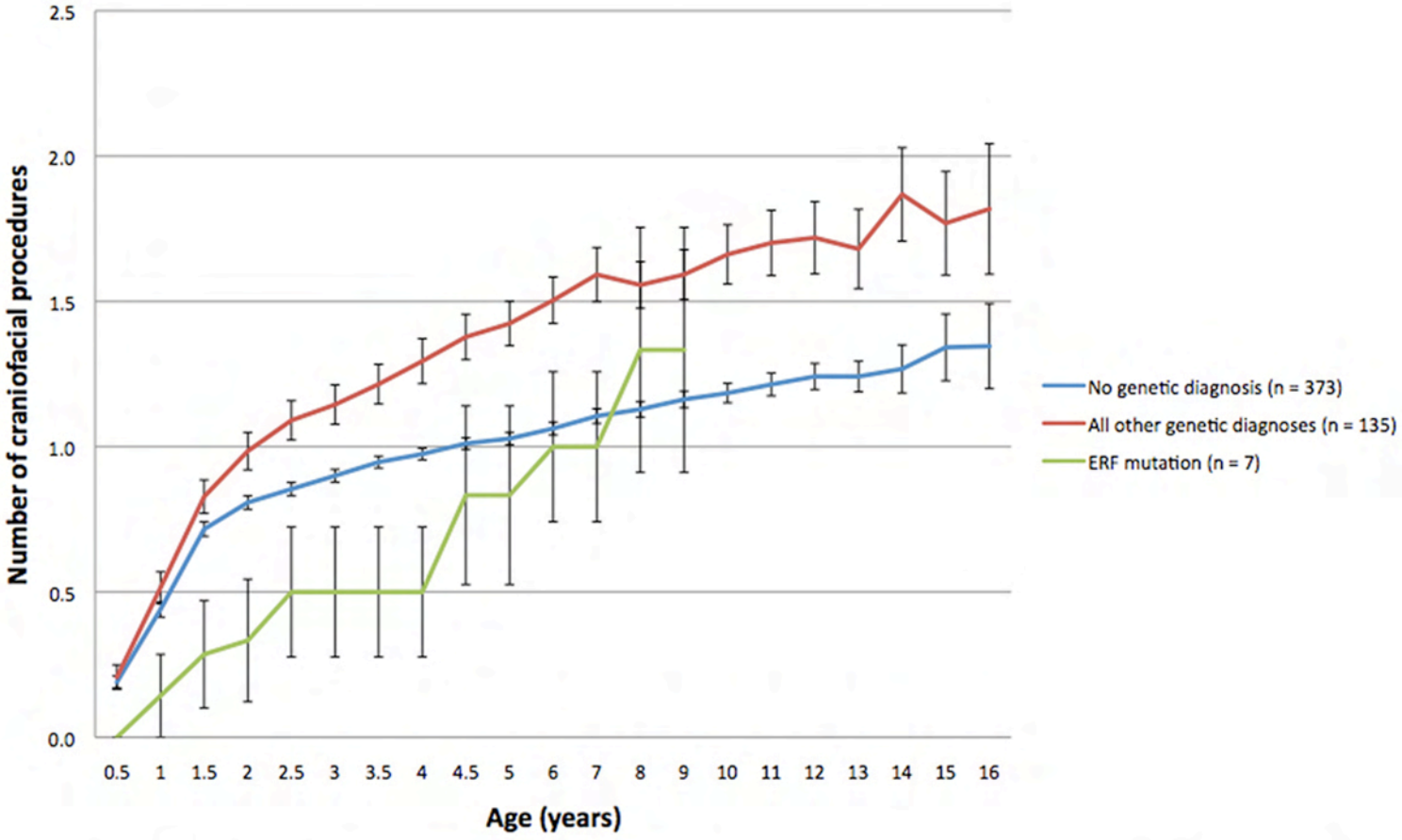
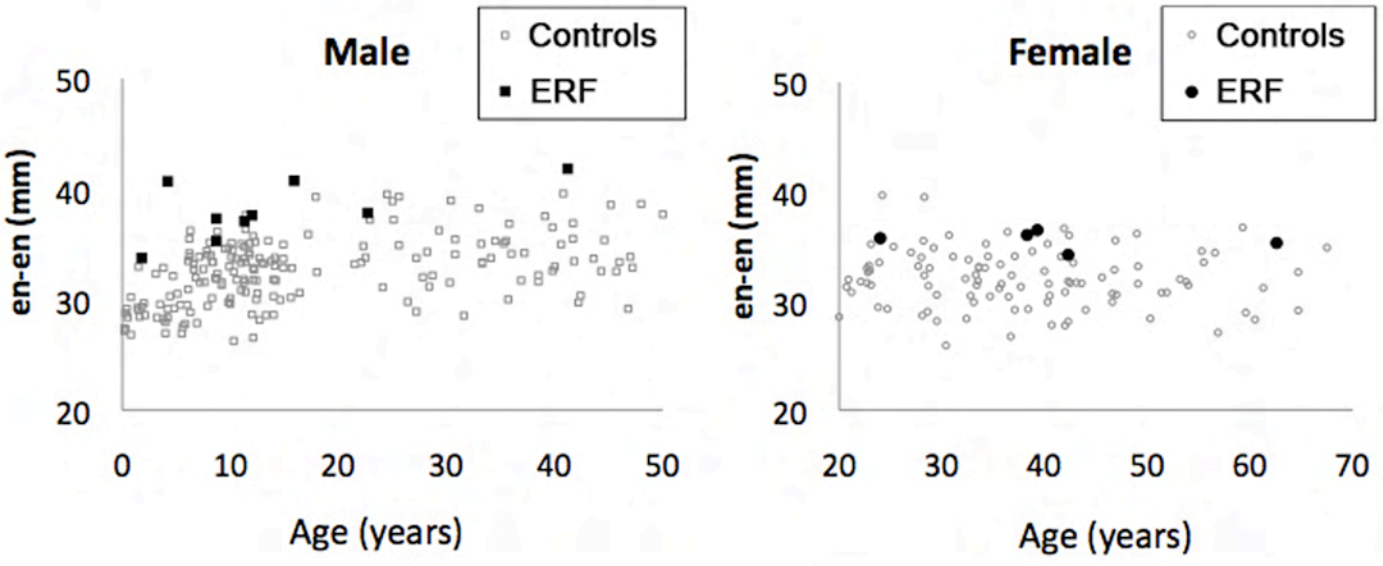
Supplementary Figure 5. Timing of major craniofacial surgery in *ERF* mutation-positive individuals with craniosynostosis



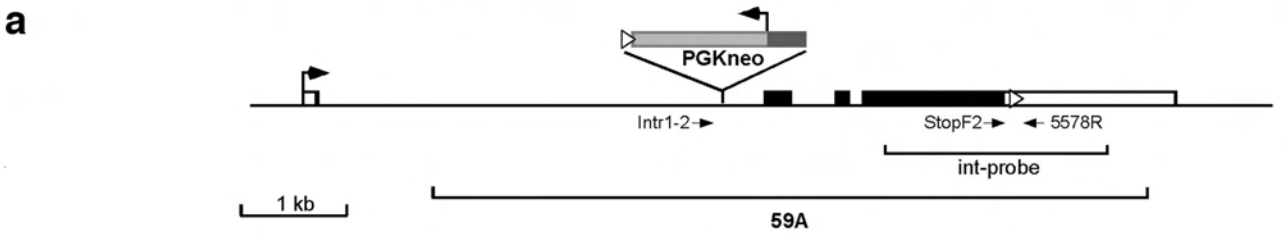
Points show the average number of major craniofacial procedures undertaken at a given age ±standard error of the mean. Comparative data with cases either having a different genetic diagnosis, or no genetic diagnosis made, are for Oxford patients born between 1993 and 2006 (updated from ref. 13). Data for ERF are not plotted beyond age 9 years because insufficient subjects were available (n < 6).

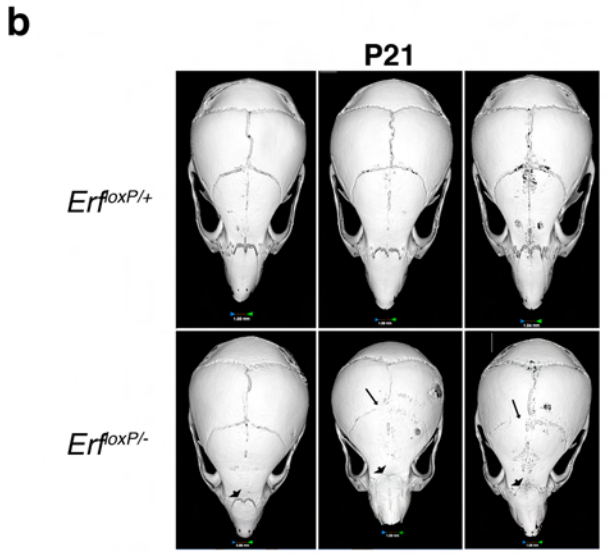
Supplementary Figure 6. Inner canthal separation

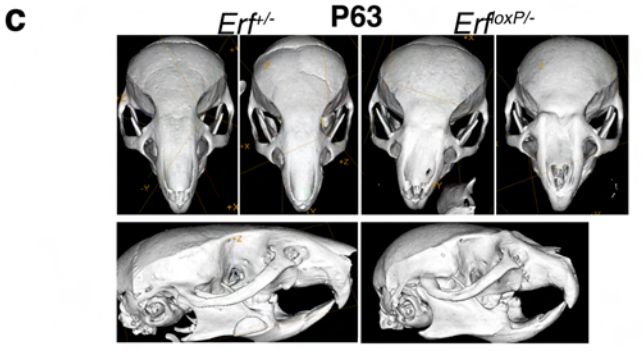


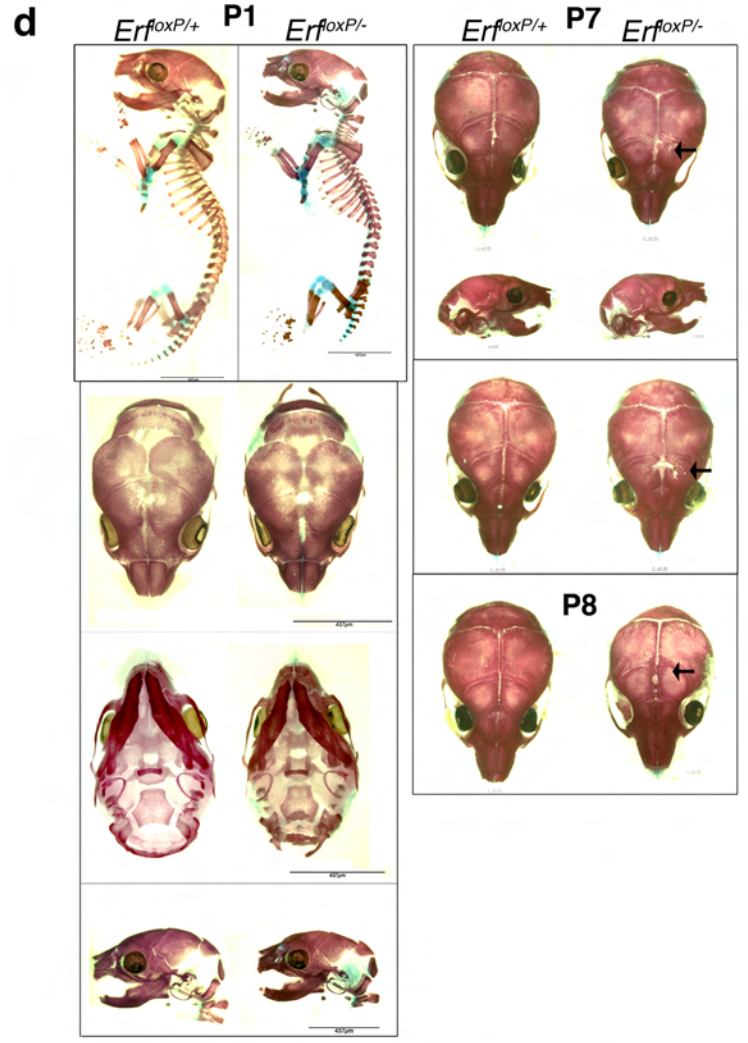
Inner canthal separation (en-en) determined from facial imaging of 14 ERF mutation-positive individuals and 384 controls. Hypertelorism is highly significant in both affected females ($P < 0.00005$) and affected males ($P < 0.0005$).

Supplementary Figure 7. Construction and characterization of the murine *Erf*^{loxP} allele









Generation of *Erf*^{loxP} allele and skeletal analysis of mutant mice. **a**, Design of targeting vector. Exons and untranslated regions are shown as filled and unfilled rectangles, respectively. *LoxP* sites are denoted by triangles and positions of the *PGKneo-loxP* cassette, diagnostic probes and PCR primers are indicated. **b**, micro-CT scans of postnatal day (P)21 skulls from *Erf*$^{loxP/+}$ (top) and *Erf*$^{loxP/-}$ (bottom) littermates. Note that coronal craniosynostosis is beginning to be apparent in 2 of 3 *Erf*$^{loxP/-}$ animals (arrows). The nasal bones (arrowheads) are already abnormal in all three *Erf*$^{loxP/-}$ animals, associated with simplified morphology of the frontonasal suture. **c**, micro-CT scans of P63 skulls from 2 *Erf*$^{+/-}$ and 2 *Erf*$^{loxP/-}$ littermates (3 of these mice are also illustrated with different views in **Fig. 3b-f**). Top, frontal views of all four mice (*Erf*$^{+/-}$ on left not shown in **Fig 3**). Note twisted broad snouts and telorbitism of *Erf*$^{loxP/-}$ mutants. Bottom, side views of 2 mice not shown in **Fig 3e, f**. **d**, Representative Alizarin red/Alcian blue-stained preparations at P1 (left) and P7/8 (right). At P1 note smaller but proportionate skeleton of *Erf*$^{loxP/-}$ animal compared with *Erf*$^{loxP/+}$ littermate (top; whole skeletons are composite images). No craniosynostosis is evident at this stage; 5 *Erf*$^{loxP/-}$ and 3*Erf*$^{loxP/+}$ were examined. By P7, skulls of *Erf*$^{loxP/-}$ animals are noticeably shorter and domed but craniosynostosis is not evident; in addition most *Erf*$^{loxP/-}$ mutants showed an irregular margin of the medial part of frontal bone with reduced mineralization (arrows). Eight *Erf*$^{loxP/-}$ and 11 *Erf*$^{loxP/+}$ were examined.

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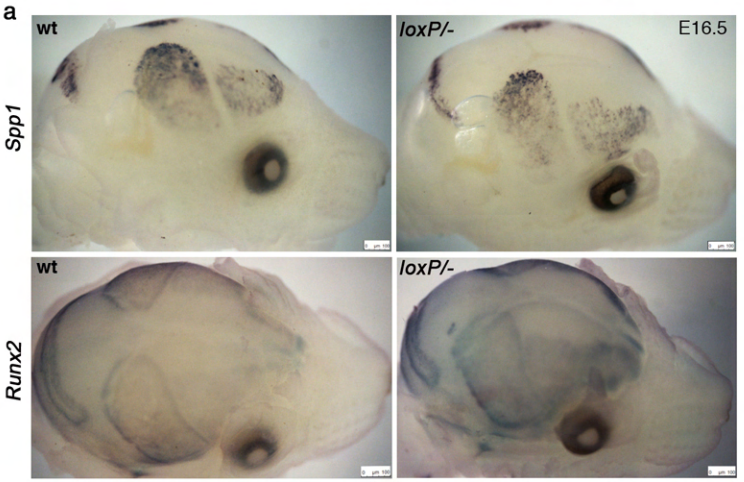
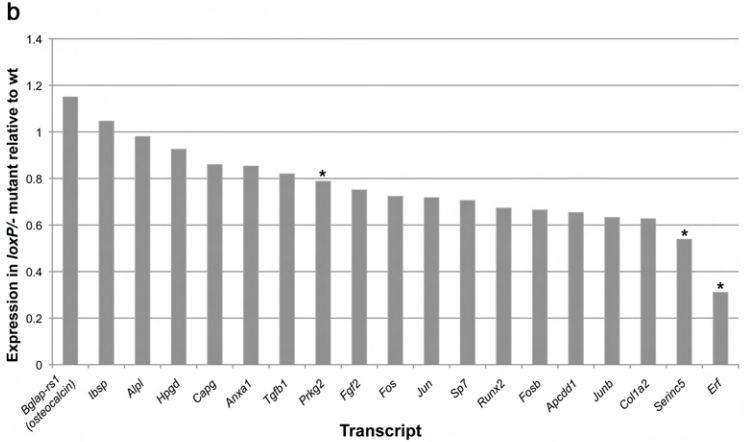
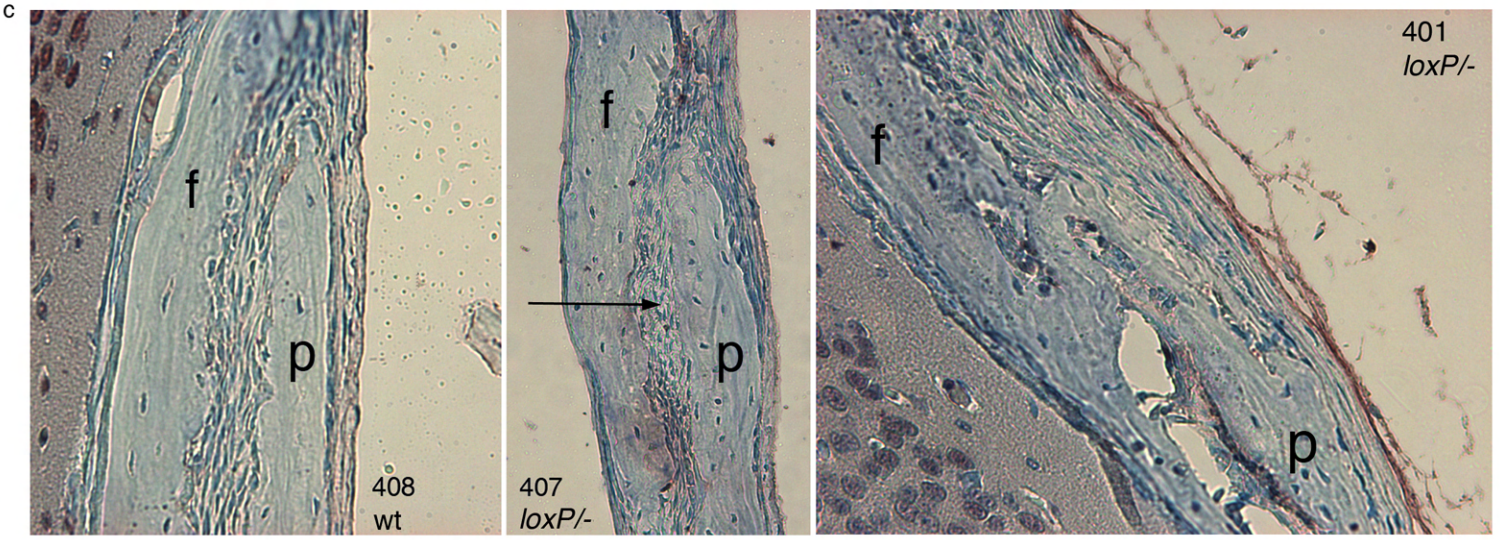
Supplementary Figure 8. Analysis of embryonic transcripts and P14 coronal sutures







a, Whole mount RNA *in situ* hybridization showing expression of *osteopontin* (*Spp1*) and *Runx2* in calvaria of representative E16.5 wildtype (wt) and $Erf^{loxP/-}$ embryos. No consistent differences in expression for a given gene were detected between wt and mutant embryos. **b**, Real time RT-PCR analysis of *Erf* and 18 osteogenic marker transcripts in dissected E16.5 calvaria. Data are based on comparison of wild type ($n = 5$) and $Erf^{loxP/-}$ ($n = 4$) mice from two litters. The average ratio of expression in the mutants to the wild types is shown, asterisks indicate differences significant at $P<0.05$ (*t*-test). **c**, Representative sagittal sections through the coronal suture of P14 littermates 408 (wt) 407 and 401 (both $Erf^{loxP/-}$), stained with a RUNX2 antibody and counterstained with Haematoxylin. The coronal suture is patent in $Erf^{loxP/-}$ mouse 407 (arrow) but fused in $Erf^{loxP/-}$ 401. f, frontal bone; p, parietal bone.

Supplementary Figure 9. Analysis of ChIP-Seq data

a

TSS

Name	Motif logo	Pattern	Nmembers	Freq	Ratio	Info	Score	FDR	Palindrome
C001		YGDGRACTACAWTTCCCAGMAKRS	56	3206	3.6226	16.0016	79.4190	0.0000	0
C002		RCSSCACTTCCGSYYSCGG	73	6910	2.9442	10.1024	50.4810	0.0000	0
C003		SSMSYGCGCABGCGCRSBS	22	1610	3.2724	10.4452	76.2150	0.0000	1
C004		GSGCCCCGCCCCCCY	18	1916	2.8218	8.1877	41.4220	0.0000	0
C005		CCCAAGATGCGCGCSG	10	633	3.0000	9.6134	26.1300	0.0000	0
C006		VYCGGARSYGGARGY	4	395	3.4052	7.7586	31.3910	0.0000	0
C007		VKGGCCCCGCCCCSC	5	574	2.5286	7.7654	14.3790	0.0000	0
C008		BTCCCASMATGCMCYGCGM	2	114	3.8000	11.7432	18.2140	0.0000	0
C009		VTGTGACGTCAB	3	133	3.5000	8.2476	14.7100	0.0000	0
C010		SRGCCCSGCCCC	3	349	2.3267	6.3430	13.0970	0.0000	0

non-TSS

Name	Motif logo	Pattern	Nmembers	Freq	Ratio	Info	Score	FDR	Palindrome
C001		KSTSDVVTGASTCABHSBBBC	104	10735	2.9387	8.9711	93.1940	0.0000	1
C002		GKNCCCCWCCCCCGS	14	1525	3.1971	7.3305	35.5660	0.0000	0
C003		WSNYTGTGGTTTSWDG	27	2940	2.4338	8.1564	25.2180	0.0000	0
C004		GAVYCAYTTCCTBTBNK	31	3971	2.2110	9.6474	12.9100	0.0000	0
C005		YDCCBBCCCCBHCCGV	3	320	2.9358	5.8427	26.2080	0.0000	0
C006		YRCACACACACACACRC	6	788	2.1768	9.3115	14.5860	0.0000	0
C007		DDRAAADMAARMAAANM	3	260	2.4074	6.6339	18.6840	0.0000	0
C008		BNCCCHNGCCCCMHK	5	459	2.2282	6.6712	9.8050	0.0000	0
C009		BBTCCCWGGRGAMS	3	230	2.2115	8.3799	11.0580	0.0000	1
C010		DVKGASTMACWSAS	3	176	2.8852	6.7458	10.5630	0.0000	0

>3

Name	Motif logo	Pattern	Nmembers	Freq	Ratio	Info	Score	FDR	Palindrome
C001		KBCBMRSTCTCGCGAGABYTSRVA	133	12100	3.7931	12.2526	272.1460	0.0000	1
C002		DNRRACTACAAHTCCCAGRA	44	2590	3.6895	13.4008	65.4360	0.0000	0
C003		SSSVGYGCGCABGCGCRSBG	37	2851	3.0427	11.1442	63.5670	0.0000	0
C004		SRGGCCCCGCCCCSBG	36	5410	2.5210	8.6521	51.2210	0.0000	0
C005		RVSSCCCCSSCCCCGS	14	1697	2.3868	7.6386	29.6900	0.0000	0
C006		SSSCAAGATGGCGGCGBY	9	648	3.4652	11.4372	26.1530	0.0000	0
C007		CBCAGCCAATCAGMNY	11	581	3.5864	9.8639	23.6490	0.0000	0
C008		CCACTTCCGGBYG	6	530	2.7749	8.8443	31.4440	0.0000	0
C009		RGGCCSSSSCCCSSCCCCSS	8	938	2.2548	8.4238	19.7180	0.0000	0
C010		SCCCGSCCCCYCSSGS	2	224	2.7654	8.7812	19.2620	0.0000	0

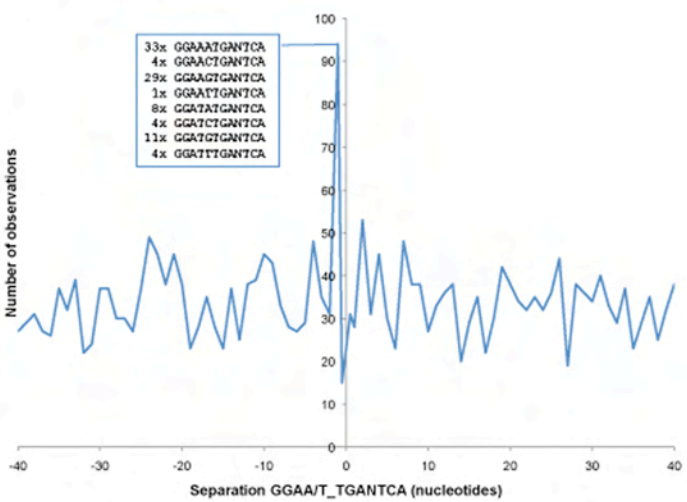
non-TSS

Name	Motif logo	Pattern	Nmembers	Freq	Ratio	Info	Score	FDR	Palindrome
C001		GYACATRYACACACAYRYACAYAYW	25	839	3.1423	13.1871	18.8190	0.0000	0
C002		DBTCCCCWGGGAMS	6	252	3.6522	8.9813	30.0760	0.0000	1
C003		CMSTCCCKGGGGAMW	1	39	4.9420	13.4500	21.7980	0.0001	0
C004		STCTCGCGAGAA	2	49	4.0833	8.9043	13.8080	0.0000	1
C005		HWCACAVACAYACWK	2	106	3.1176	6.8791	13.2240	0.0000	0
C006		DMACAYWGWHWCACAKA	2	84	3.1111	8.0144	10.5350	0.0000	0
C007		DCCCYWCCYCMG	1	37	3.4380	9.0728	13.0190	0.0180	0
C008		CRCATGBRHVCATGYR	1	20	3.7650	9.3674	11.0570	1.0000	1
C009		WSYCGCGAGAVAG	2	43	2.8667	9.7213	7.8110	0.0000	0
C010		YSYCMCTCCNCCBR	1	28	3.4300	8.4200	10.7860	0.4100	0

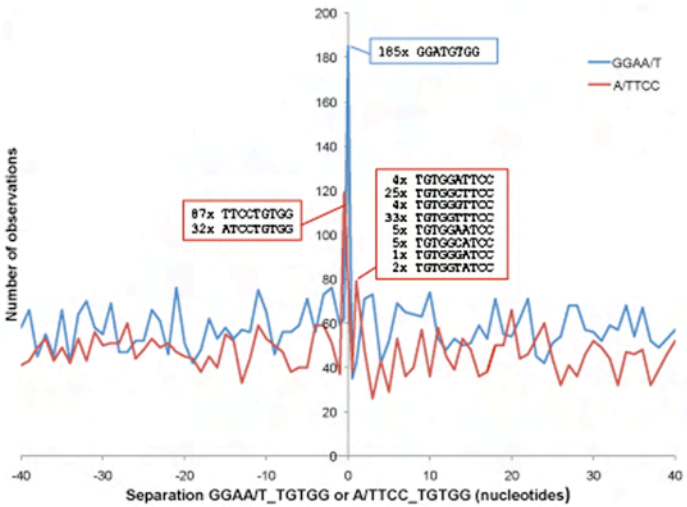
<3

Note: Frequency of clusters of motifs = sum of frequencies of members

b



c



a, MEME analysis[27] of sequence under ChIP-Seq peaks within 1 kb of transcription start sites (TSS) or not (non-TSS), and with a –FCS/+FCS ratio of greater or less than 3. **b,c**, plots showing number of sequences observed, at particular separations, of closely spaced ETS and palindromic AP1 binding sites (**b**) and ETS and RUNX sites (**c**), in peaks defined as non-TSS and FCS-/FCS+ >3. The *x*-axis shows the number of nucleotides separating the two motifs, except that in both plots, abutting motifs are plotted at -0.5 and +0.5, and in plot **c**, the specific overlapping motif GGATGTGG is plotted at 0. Sequence compositions of peaks representing closely adjacent motifs are shown individually. Owing to the palindromic nature of the AP1 site only a single plot is shown, whereas for the ETS-RUNX pairing the two possible orientations are shown separately.

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Supplementary Figure 10. Metacore analysis of –FCS/+FCS ratio >3, non-TSS ChIP-Seq targets

a

Process Networks
Statistically significant networks

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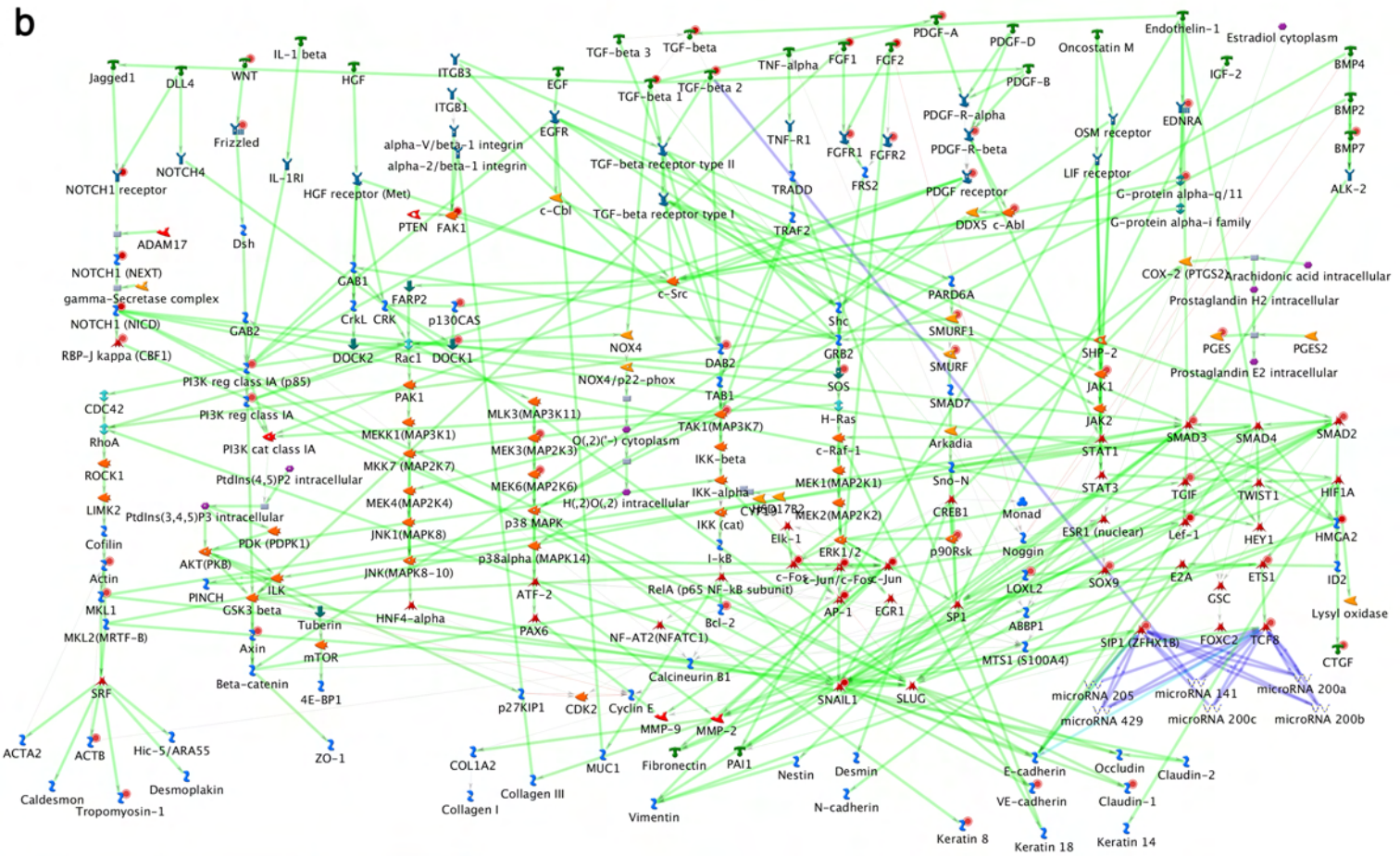
#	Networks	-log(pValue)	pValue	Ratio
1	Development_EMT_Regulation of epithelial-to-mesenchymal transition		4.086e-12	60/232
2	Development_Neurogenesis_Axonal guidance		9.091e-11	57/230
3	Cell adhesion_Synaptic contact		4.706e-9	46/184
4	GLI3 = GLI-3R, GLI-3R, c-Src, Hamartin, RelA (p65 NF-kB subunit)		6.594e-9	106/605
5	Cell adhesion_Attractive and repulsive receptors		8.512e-9	44/175
6	Development_Neurogenesis_Synaptogenesis		1.831e-7	42/180
7	Cell adhesion_Integrin-mediated cell-matrix adhesion		2.375e-7	47/214
8	Cell cycle_G1-S Growth factor regulation		2.593e-7	44/195
9	Apoptosis_Anti-Apoptosis mediated by external signals via MAPK and JAK/STAT		4.371e-7	41/179
10	Apoptosis_Anti-Apoptosis mediated by external signals via PI3K/AKT		5.281e-7	49/233
11	Signal Transduction_TGF-beta, GDF and Activin signaling		9.502e-7	36/152
12	Development_Blood vessel morphogenesis		1.647e-6	47/228
13	Signal transduction_Neuropeptide signaling pathways		4.329e-6	35/155
14	Signal transduction_ESR1-nuclear pathway		4.931e-6	44/216
15	Development_Regulation of angiogenesis		5.002e-6	45/223

b



GeneGo Metacore pathway analysis (http://www.genego.com/metacore.php) of the 2033 Erf ChIP-Seq targets selected as -FCS/+FCS >3 and non-TSS **(Supplementary Table 4a)**. **a**, List of networks ranked by statistical significance. **b**, Cartoon of the EMT regulation network (#1). Erf is not in the Metacore database, but would be positioned downstream of ERK1/2 alongside the transcription factors c-Fos, c-Jun and AP-1. Genes from the Supplementary Table 4a non-TSS input list are denoted by a circled red spot (darker shades of red reflect higher frequency of Erf ChIP-Seq peaks within that gene). Activation is indicated by green lines, and inhibition by blue.

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