SUPPORTING INFORMATION

Supp. Materials and Methods

Subjects

Study participants were recruited from multiple institutions collaborating within the International Craniosynostosis Consortium (https://genetics.ucdmc.ucdavis.edu/index.cfm). All probands were enrolled with a clinical diagnosis of non-syndromic craniosynostosis, based on physical examination by a clinical geneticist and review of medical documentation including available head CT and surgical reports. In a subset of six, involvement of an additional cranial suture was identified. Craniosynostosis syndromes were excluded by clinical assessment showing lack of extracranial malformations or developmental delays. In 117 cases mutations in the hot-spot areas for FGFR1, FGFR2, FGFR3 and the entire TWIST were excluded by direct sequencing [Boyadjiev et al., 2007]. This study was approved by the local institutional review boards and all subjects were consented prior to examination, review of records and sample collection.

PCR and sequencing

DNA was extracted from blood or saliva according to the manufacturer's protocol (PureGene 5 Prime™). PCR reactions were performed to amplify all exons of ALX4 with at least 100bp of intronic flanking sequences (primers and protocols available upon request) and the application fragments were visualized by agarose gel electrophoresis. Fragments were further purified with Shrimp Alkaline Phosphotase (SAP) and Exonuclease (EXO) (Affymetrix®) treatment. Final PCR products were sequenced with ABI 3730 DNA Analyzer. Electropherograms were analyzed with the VectorNTI™ ContigExpress software by two independent investigators and compared with ALX4 wild-type sequence (NM_021926.3). All polymorphic variants were confirmed via an independent PCR reaction and secondary sequencing using the reverse primer. Sequencing of the parental samples was performed to determine whether the polymorphisms are familial. Control samples were obtained from European Collection of Cell Cultures (ECACC), Sigma-Aldrich®.

Bioinformatic analysis

Mutations found in our sagittal NSC cohort were documented as suggested by the HGVS website (http://www.hgvs.org/mutnomen/). Nucleotide numbering corresponds with cDNA numbering, with "+1" being the "A" of the ATG start codon. Single nucleotide polymorphisms (SNPs) were considered novel if not present in the following databases: HapMap (Rel28, Aug 2010), NCBI BLAST, MapBack or 1000Genomes. SNPs found in any of those databases (previously reported) were then checked for availability of control allele frequency data in HapMap (Release 28, Phase II + III) and in 1000Genomes. The large size of these databases, particularly 1000Genomes, allowed us to use them for obtaining allele frequencies in the control population and circumvented the need to genotype a control population ourselves. If allele frequencies were available, deviation from Hardy-Weinberg equilibrium (HWE) was tested using FINETTI software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Possible harmful effect of the SNPs were determined by the SIFT and PPH prediction softwares (SIFT, Fred Hutchinson Cancer Research Center, Seattle, web software, http://blocks.fhcrc.org/sift/SIFT.html; PPH, Bork Group & Sunyaev Lab, Harvard, web software, http://genetics.bwh.harvard.edu/pph). SIFT scores of less than 0.05 are considered harmful, as are PolyPhen PSIC (Position-Specific Independent Counts [Sunyaev et al., 1999] of greater than 0.5. Grantham scores are calculated as a comparison of composition, polarity, and molecular volume [Grantham, 1974]. Grantham scores were grouped into conservative (0-50), moderately conservative (51-100), moderately radical (101-150), or radical (≥151)[Li et al., 1984], where scores over 100 are considered damaging.

Vector Construction

We used pCMV6-Entry to express all ALX4 variants and also used pGL3 as the reporter vector. The wild-type gene in the pCMV6-Entry vector was obtained from Origene® (PS100001) and mutant variants of ALX4 were introduced with Agilent® QuikChange Site-Directed Mutagenesis PCR. A variant known to segregate with parietal foramina (R272P) was used as a known loss-of-function control [Wuyts et al., 2000], The reporter vector was constructed by inserting the P3 sequence (the strongest binding site for ALX4 [Qu et al., 1999; Tucker and

Wisdom, 1999]) upstream of the promoter of luciferase in the pGL3 vector. The resulting vector is pGL3-P3-Luc. Control calvarial osteoblast cells were used as a host for expression analysis

Dual-luciferase Assay

Each variant of ALX4 was co-transfected with pGL3-P3-Luc and hRenilla vectors (pGL4-hRluc) into human fetal osteoblasts using Lipofectamine reagent (Invitrogen™). Promega™ Dual-Luciferase Reporter Assay System was used to provide luciferin substrate for luminescence production, which was measured with a Turner BioSystems™ 20/20 Luminometer. Transfection efficiency was normalized with the luminescence of the *Renilla* luciferase. The DNA ratio of ALX4 constructs to the *Renilla* vectors was set at 50 to 1 after a series of optimization trials. We used 0.5 ug of ALX4 construct DNA for each transfection. The reporter vector (pGL3-P3) contains the P3 element for ALX4 binding upstream of the promoter of firefly luciferase. The *Renilla* vector (pGL4-hRluc) serves as an internal measure for the efficiency of transfection. Untransfected cells produced negligible levels of both the firefly and *Renilla* luciferase activities (data not shown).

Statistical calculations

Six replicates were tested for each construct, each with a firefly and *Renilla* luminescence value. *Renilla* luminescence readings were then expressed as a fraction of the largest value and firefly luciferase was normalized by multiplying it with its corresponding *Renilla* fraction. The firefly values normalized for transfection efficiency were then averaged and expressed as the mean ± SEM (standard error of the mean, s.d./n where s.d. is standard deviation and n is the number of samples. Student's T-test was carried out on each construct compared to the wild-type to determine significance.

Supp. Table S1: Summary of SNPs found in a cohort of 203 probands with nonsyndromic craniosynostosis

SNP ID	Novel?	RS#	Nonsynonymous?	
c.19G>T V7F	Yes	-	Yes	◄
c.104G>C R35T	No	rs3824915	Yes	
c.304G>T P102S	No	rs12421995	Yes	
c.594C>A L198L	Yes	-	No	
c.631A>G K211E	Yes	-	Yes	◄
c.729G>A A243A	No	rs11037928	No	
c.879C>T L293L	No	rs12419361	No	
c.917C>T P306L	Yes	rs149897209	Yes	◄
c.918G>N P306P	Yes	-	No	
c.1074C>T H358H	No	rs3802805	No	
c.1464C>T	No	rs4755798	No	
c.1431C>A	Yes	-	No	
c.778-11G>A	Yes	-	No	
c.467-45C>T	No	rs11037929	No	
c.777+70A>G	No	rs11037927	No	
c.906+133G>A	Yes	-	No	

SNPs were categorized by novelty (absence from known SNP databases) and base pair change that resulted in an amino acid substitution. The three SNPs tested by dual-luciferase assay are marked with arrowheads. P306L is given an rs identification number because it was reported in a database after our identification.

Supp. References

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