Human ZIC1 (zinc finger protein of cerebellum 1), one of five homologs of the Drosophila pair-rule gene odd-paired, encodes a transcription factor previously implicated in vertebrate brain development. Heterozygous deletions of ZIC1 and its nearby paralog ZIC4 on chromosome 3q25.1 are associated with Dandy-Walker malformation of the cerebellum, and loss of the orthologous Zic1 gene in the mouse causes cerebellar hypoplasia and vertebral defects. We describe individuals from five families with heterozygous mutations located in the final (third) exon of ZIC1 (encoding four nonsense and one missense change) who have a distinct phenotype in which severe craniosynostosis, specifically involving the coronal sutures, and variable learning disability are the most characteristic features. The location of the nonsense mutations predicts escape of mutant ZIC1 transcripts from nonsense-mediated decay, which was confirmed in a cell line from an affected individual. Both nonsense and missense mutations are associated with altered and/or enhanced expression of a target gene, engrailed-2, in a Xenopus embryo assay. Analysis of mouse embryos revealed a localized domain of Zic1 expression at embryonic days 11.5–12.5 in a region overlapping the supraorbital regulatory center, which patterns the coronal suture. We conclude that the human mutations uncover a previously unsuspected role for Zic1 in early cranial suture development, potentially by regulating engrailed 1, which was previously shown to be critical for positioning of the murine coronal suture. The diagnosis of a ZIC1 mutation has significant implications for prognosis and we recommend genetic testing when common causes of coronal synostosis have been excluded.

**Introduction**

Among the varied causes of craniosynostosis (premature fusion of one or more sutures of the skull vault), a monogenic etiology is most commonly identified in individuals with fusion of the coronal sutures, the major pair of transverse sutures crossing the vertex of the skull. Coronal synostosis, which can be present bilaterally (bicoronal) or unilaterally (unicoronal), affects approximately 1 in 10,000 children and is the type most commonly associated with an identifiable syndrome. Common monogenic disorders that characteristically present with coronal synostosis are Muenke (MIM: 602849) and Apert (MIM: 101200) syndromes, caused by localized gain-of-function mutations encoded by FGFR3 (MIM: 134934) and FGFR2 (MIM: 176943), respectively; Saethre-Chotzen syndrome (MIM: 101400) (TWIST1 [MIM: 601622] haploinsufficiency); TCF12-related craniosynostosis (MIM: 600480 and 615314) (also a haploinsufficiency); and craniofrontonasal syndrome (MIM: 304110) (cellular interference involving variants in the X-linked EFNB1 gene [MIM: 300035]).

Even in the absence of an obvious syndromic diagnosis, a specific mutation can be identified in about 60% of individuals with bicoronal and 30% with unicoronal synostosis. The high monogenic load in coronal synostosis can be accounted for by the specific developmental origin of the coronal suture, which lies at an embryonic tissue boundary between neural-crest-derived frontal bone and mesoderm-derived parietal bone. Based on analysis of mouse models, coronal synostosis is frequently caused by disruption in the maintenance of the population of stem cells within the suture during early development (typically, embryonic days [E]12.5–14.5), caused, for example, by abnormalities in migration of neural crest cells or abnormal paracrine signaling through fibroblast growth factor receptors.

An alternative possibility is that coronal synostosis could be caused by a primary failure of the suture to develop. Lineage tracing demonstrates that the cells of the future

1**Clinical Genetics Group, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK; 2Department of Cell Biology and Neuroscience, 513 Leon Johnson Hall, Montana State University, Bozeman, MT 59717, USA; 3Department of Plastic Surgery, Erasmus MC, University Medical Center Rotterdam, PO Box 2040, 3000 CA Rotterdam, the Netherlands; 4Developmental Biology and Cancer Programme, UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK; 5Department of Clinical Genetics, Erasmus MC, University Medical Center Rotterdam, PO Box 2040, 3000 CA Rotterdam, the Netherlands; 6Department of Bioinformatics, Erasmus MC, University Medical Center Rotterdam, PO Box 2040, 3000 CA Rotterdam, the Netherlands; 7Department of Pediatric Radiology, Erasmus MC, University Medical Center Rotterdam, PO Box 2040, 3000 CA Rotterdam, the Netherlands; 8Computational Biology Research Group, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK; 9Craniofacial Unit, Department of Plastic and Reconstructive Surgery, Oxford University Hospitals NHS Trust, John Radcliffe Hospital, Oxford OX3 9DU, UK

10These authors contributed equally to this work

11Deceased

*Correspondence: andrew.wilkie@imm.ox.ac.uk
http://dx.doi.org/10.1016/j.ajhg.2015.07.007. ©2015 The Authors
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
coronal suture originate from paraxial cephalic mesoderm at E7.5 and migrate laterally to locate above the developing eye.\textsuperscript{11} This region constitutes the supraorbital regulatory center and during E11.5–E13.5, cells from this zone migrate apically to form and populate the coronal suture.\textsuperscript{6,11,12} One of the genes characteristically expressed by these cells is \textit{engrailed 1} (\textit{En1}), a homolog of the \textit{Drosophila engrailed} segment polarity gene. Mice with homozygous loss of \textit{En1} function have generalized calvarial bone hypoplasia and persistent widening of the sutural gaps, which is associated with a posterior shift in the boundary between cells of neural crest and mesodermal origin.\textsuperscript{14,15} An orthologous mutation has not yet been described in humans.

Here, we report an additional genetic etiology for coronal synostosis, caused by heterozygous variants in the final exon of \textit{ZIC1} (zinc finger protein of cerebellum 1 [MIM: 600470]), identified in four simplex case subjects and a three-generation pedigree. \textit{ZIC1}, located on chromosome 3q25.1, belongs to a family of five genes encoding Zn-finger transcription factors, which are arranged as one unpaired and two paired paralogs in the human and mouse genomes;\textsuperscript{14} \textit{ZIC} genes are homologous to the \textit{Drosophila} pair-rule gene \textit{odd-paired}, which is required for activation of embryonic \textit{engrailed} expression.\textsuperscript{15} Vertebrate \textit{ZIC}s have important roles in multiple developmental processes, including neurogenesis, left-right axis formation, myogenesis, and skeletal patterning.\textsuperscript{16,17} Heterozygous complete deletions of \textit{ZIC1} were previously associated with Dandy-Walker malformation (DWM); hypoplasia and upward rotation of the cerebellar vermis and cystic dilatation of the fourth ventricle [MIM: 220200]),\textsuperscript{18} we now show that mutations affecting the highly conserved C terminus of the protein, which are likely to be associated with a gain of function, lead to a distinct phenotype of coronal suture fusion and learning disability. In addition to its previously established importance for neurogenesis,\textsuperscript{19,20} this work shows that \textit{ZIC1} is required for normal coronal suture development. We find that murine \textit{Zic1} is expressed in the supraorbital regulatory center, suggesting that this gene acts at a very early stage of coronal suture development,\textsuperscript{7} potentially (reflecting a similar epistatic relationship to that in \textit{Drosophila}) by regulating \textit{En1}.

Subjects and Methods

Subjects

The clinical studies were approved by Oxfordshire Research Ethics Committee B (reference CO2.143), London Riverside Research Ethics Committee (reference 09/H0706/20), and the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam (MEC-2012-140 and MEC-2013-547). Written informed consent to obtain samples for genetics research was obtained from each child’s parent or guardian. Venous blood was used for DNA extraction and fibroblast cultures were established from skin biopsies taken from scalp incisions during surgical intervention. Intracranial pressures in subject 1 were documented by 24–48 hr direct recording with an intraparenchymal Codman Microsensor.\textsuperscript{21} The screening panel comprised samples from 307 individuals with syndromic or non-syndromic craniosynostosis. All DNA samples were previously tested for mutation hotspots in \textit{FGFR2}, \textit{FGFR3}, \textit{TWIST1}, and \textit{TCF12}.\textsuperscript{3,4} Significant chromosome aneuploidy in individuals with \textit{ZIC1} mutations was excluded by karyotyping and/or array comparative genomic hybridization. Where necessary, correct biological relationships were confirmed by segregation analysis of a panel of 13 microsatellites (D1S2868, D3S1311, D4S403, D5S2027, D6S1610, D7S19, D9S158, D10S548, D11S898, D13S1265, D14S280, D16S5415, and D18S474).

Whole Genome/Exome Sequencing and Mutation Screening of \textit{ZIC1}

Whole genome sequencing (WGS) of the male proband subject 1 and his parents was performed as part of the WGS500 clinical genome sequencing initiative.\textsuperscript{22} In brief, 3–5 μg DNA was used to prepare libraries for 100 bp paired-end sequencing to generate a mean coverage of 30× using the Illumina HiSeq2000 platform. Sequence reads were mapped to the human reference GRCh37d5 using Stampy (v1.0.12–1.0.22) and variants called with Platypus (v0.2.4).\textsuperscript{23,24} To identify de novo mutations, we prioritized variants within coding regions that were called as absent in both parents and in dbSNP135, generating a list of 203 variants in 177 genes, of which 39 were classified as protein altering. Visualization of the trio read alignments revealed a single bona fide change in \textit{ZIC1} (12 of 27 reads), which was absent in both the paternal (19 reads) and maternal (31 reads) samples; the other 38 variants were either in fact present in one of the parents or were artificial (Table S1). Possible recessive inheritance was analyzed with an in-house perl script to list homozygous, compound heterozygous, and hemizygous X chromosomes in subject 1, with a frequency cut-off of 0.003 in either 1000 Genomes or Exome Variant Server; variants in two genes fitted the criteria (Table S1). Whole genome sequencing of genomic DNA from four subjects in family 5 (affected: 5:II.2, 5:III.3, 5:III.6; unaffected: 5:III.5) was performed by BGI Complete Genomics.\textsuperscript{25,26} Filtering based on a list of genes mutated in craniosynostosis identified a predicted missense substitution encoded by \textit{ZIC1}, present only in the three affected individuals. Exome sequencing of subject 3 was performed on genomic DNA (extracted from whole blood) using an Agilent SureSelect Human All Exon Kit (v5; 50 Mb) on the Illumina HiSeq2000 platform. Reads were mapped to hg19 with Novelalign (Novocraft Technologies) and variants called with SAMtools and annotated by ANNOVAR.

To investigate further the significance of the \textit{ZIC1} mutations, primers were designed for amplification of genomic DNA (GenBank: NT_005612.17) and cDNA (GenBank: NM_003412.3), for multiplex ligation-dependent probe amplification (MLPA) analysis, and for deep sequencing (Table S2, which provides details of experimental conditions). Variant screening of all three exons of \textit{ZIC1} was performed by dideoxy sequencing on PCR amplification products from genomic DNA by BigDye Terminator v3.1 (Applied Biosystems). Copy-number variation was analyzed by MLPA using probes to each exon, according to the manufacturer’s instructions (MRC Holland). RNA was extracted from fibroblasts (Trizol, Invitrogen), cDNA synthesized with RevertAid first strand cDNA kit (Thermo Scientific), and the samples analyzed by agarose gel electrophoresis after digestion with BfaI. To quantify the proportions of wild-type to mutant allele in cDNA, an amplification product spanning exons 2–3 was used as a template for PCR to add Ion Torrent P1 and A adapters, and the resulting product
was purified with AMPure beads (Beckman Coulter). Emulsion PCR and enrichment were performed with the Ion PGM Template OT2 200 Kit (Life Technologies) according to the manufacturer’s instructions and sequencing of enriched templates performed on the Ion Torrent PGM (Life Technologies) for 125 cycles with the Ion PGM Sequencing 200 kit v2. Data were processed with Ion Torrent platform-specific pipeline software v.4.2.1.

Xenopus Assays

All experiments using Xenopus were approved by the Institutional Animal Care and Use Committee of Montana State University. Xenopus full-length zic1 and zic1ΔC constructs were described previously (zic1ΔC was originally termed oplΔC). The zic1ΔC construct was made by PCR amplification of the portion of zic1 cDNA encoding the N terminus and zinc finger domains, including four amino acids of the C-terminal region, followed by cloning into the EcoRI and XbaI sites of pCS2+ATG.27 The human ZIC1 cDNA pCR4-Topo-ZIC1 (ThermoFisher) was subcloned into pcDNA3 and six different nucleotide substitutions—c.895G>T (p.Glu299*), c.1163C>A (p.Ser388*), c.1198G>C (p.Gly402*), c.1204G>T (p.Glu400Arg), c.1240A>G (p.Glu402*), and c.1309_1310GC>TA (p.Ser388*)—were introduced by PCR mutagenesis using the primer sequences and experimental conditions provided in Table S2. The human ZIC1 constructs were subsequently digested with EcoRI and XbaI and ligated into the pCS2+ plasmid. Capped sense RNAs for microinjection were synthesized from the Xenopus and human pCS2+ constructs by SP6 transcription of NarI linearized plasmids. Xenopus laevis eggs were collected and fertilized as previously described28 and embryos were staged according to Nieuwkoop and Faber.29 Embryos at the two-cell stage were injected into a single cell with 200 pg sense RNA synthesized from cDNA constructs, together with 25 pg lacZ RNA as tracer. After β-galactosidase staining,30 wild-type embryos were bleached by exposing the embryos to fluorescent light in hybridization buffer containing 1% H2O2. Expression of en-2 was determined in neurula stage 15–17 albino and wild-type embryos by in situ hybridization with digoxigenin-labeled antisense RNA en-2 probe as described.32 An anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche) and the alkaline phosphatase substrate NBT/BCIP (Fisher Scientific) were used for color detection. Embryos were scored double-blind to determine changes in en-2 expression in comparison to the uninjected side. Results using wild-type and mutant constructs were compared by Fisher’s exact test with Bonferroni correction for multiple comparisons (n = 9).

RNA In Situ Hybridization of Mouse Embryos

Experimental procedures were performed in accordance with UK Animals (Scientific Procedures) Act, 1986 (PPL 70/7194). For whole-mount embryo in situ hybridization, embryos were dissected, fixed overnight in 4% paraformaldehyde in phosphate-buffered saline, and dehydrated through graded methanol solutions. Non-radioactive RNA in situ hybridization was performed as described31 before vibratome sectioning. RNA probes for Zic133 and En134 were digoxigenin labeled with the In Vitro Transcription kit (Roche Applied Science) followed by anti-digoxigenin-AP antibody (1:1,000) (Roche Applied Science) and NBT/BCIP (Sigma) staining to detect the hybridization signals.

Results

Identification of ZIC1 Mutations

The proband (subject 1) presented at birth with severe brachycephaly (figure 1A), which was shown by three-
dimensional computed tomographic reconstruction (3D-CT) to be caused by bicoronal synostosis. He required three major craniofacial surgical procedures (at the ages of 7 months, 2.4 years, and 4.8 years), the latter two because of raised intracranial pressure. In addition he had autistic traits and moderate-severe learning disability, features that are rarely associated with coronal synostosis. Genetic testing for mutations known to be associated with coronal synostosis was negative; his phenotype is summarized in Table 1 and more detailed descriptions of all subjects are provided in the Supplemental Data (Case Reports).

We undertook WGS of the parent-child trio and analyzed the data for variants consistent with either (1) autosomal or X-linked recessive inheritance or (2) a new dominant mutation (Table S1). After filtering, variants in two genes were consistent with the recessive disease model, but these genes, CNGA3 (MIM: 600053) and NEB (MIM: 161650), are associated with achromatopsia 2 (MIM: 216900) and nemaline myopathy 2 (MIM: 256030), respectively, disorders without a craniofacial phenotype, and thus were not considered further. The single dominant candidate was a heterozygous c.1163C>A mutation in ZIC1 (GenBank: NM_003412.3), predicting the nonsense change p.Ser388*. This variant was absent in the parental samples and was confirmed by dideoxy sequencing (Figure 2A).

The significance of this de novo ZIC1 mutation was initially uncertain. Contiguous heterozygous deletions of ZIC1 and its adjacent paralog ZIC4 were previously described in DWM,18 although a few deletion cases have lacked this characteristic phenotype.19,20 Review of the CT scan in subject 1 showed no brain malformation, and on a later magnetic resonance imaging (MRI) scan, only minor abnormalities of configuration of the ventricles and corpus callosum were evident (not shown).

Nonsense mutations of ZIC1 have not previously been reported, but the location of the nucleotide substitution in the terminal (third) exon (Figure 2A) predicted that it would escape nonsense-mediated decay.21 We digested ZIC1 cDNA generated from scalp fibroblasts with the restriction enzyme BfaI, which cuts the mutant allele, and on a later magnetic resonance imaging (MRI) scan, only minor abnormalities of configuration of the ventricles and corpus callosum were evident (not shown).

Nonsense mutations of ZIC1 have not previously been reported, but the location of the nucleotide substitution in the terminal (third) exon (Figure 2A) predicted that it would escape nonsense-mediated decay.21 We digested ZIC1 cDNA generated from scalp fibroblasts with the restriction enzyme BfaI, which cuts the mutant allele, and on a later magnetic resonance imaging (MRI) scan, only minor abnormalities of configuration of the ventricles and corpus callosum were evident (not shown).

To search for further evidence that ZIC1 mutations cause craniosynostosis, we screened a panel comprising 307 unrelated subjects with synostosis affecting any combination of sutures (including 45 and 112 with exclusively bilateral and unilateral coronal synostosis, respectively) and for whom no genetic diagnosis had been made. Initially we identified a single heterozygous nonsense mutation in this panel (subject 2: c.1204G>T encoding p.Glu402*); neither parent had the mutation, indicating that it had arisen de novo (sample relationships were confirmed by microsatellite analysis). We did not identify any ZIC1 copy-number changes in this craniosynostosis panel by MLPA (data not shown). Later, we discovered by exome sequencing that a second individual included on the panel (subject 3) had the identical mutation, but present in mosaic state (see legend to Figure 2A for details). Strikingly, review of the phenotypes of subjects 2 and 3 revealed that both had bicoronal synostosis with severe brachycephaly (Figures 1B–1D); in addition, both had learning disability, which was milder in subject 3 who had the mosaic mutation. Review of CT brain scans showed that subject 2 had agenesis of corpus callosum and dilated lateral ventricles, but neither subject 2 or 3 had DWM (not shown).

In an attempt to further replicate these findings, we examined DNA samples collected at a second craniofacial unit (Rotterdam), specifically where the combination of both coronal synostosis and significant learning disability was present. Only three samples were available for analysis, which reflects the rarity of this combination of phenotypes; remarkably, however, dideoxy sequencing showed that one of these samples harbored a heterozygous nonsense mutation in ZIC1 (c.1165C>T encoding p.Gln389*) at the codon adjacent to that affected in subject 1 (Figure 2A). This child had presented with bicoronal and unilateral lambdoid synostosis (Figures 1E and 1F) and had significant learning problems (Table 1). An MRI scan identified several cerebral anomalies including a short corpus callosum, mildly enlarged lateral ventricles, peaked tentorium, hypoplastic pons, and cerebellum with prominent cerebellar folia and enlarged foramen magnum with signal void near the cervical cord (Figure 1G).

The final family (family 5) consisted of six affected individuals in three generations (Figure 1H). Two cousins (subjects 5:III.3 and 5:III.6) had bicoronal synostosis (Figures 1I and 1J) and a further individual (subject 5:III.1, a half-brother of 5:III.3) had a DWM but no craniosynostosis (not shown). All three, and their respective mothers (5:II.2 and 5:II.4), had mild learning disability. Whole genome sequencing of four individuals (subjects 5:II.2, 5:II.3, 5:III.3, 5:III.6) identified a heterozygous variant in ZIC1, c.1198G>C encoding p.Gly400Arg, present in the three affected individuals (5:II.2, 5:III.3, 5:III.6) but not in the unaffected spouse (5:II.3). These results were confirmed by dideoxy sequencing, which showed that the variant was also present in 5:III.1 (the individual with DWM) and in 5:II.4 (the obligate transmitting mother) but not in her two unaffected children (5:III.4).
| Subject ID | Reference ID | Gender | Mutation (cDNA) and Alteration (Protein) | Cranial Sutures | Number of Major Craniofacial Procedures | Other Brain Abnormalities on CT/MRI Scanning | Strabismus/Ptosis | Learning Disability | Other Major Clinical Features |
|------------|--------------|--------|-----------------------------------------|----------------|---------------------------------------|---------------------------------------------|------------------|------------------|-----------------------------|
| 1          | 4447         | M      | c.1163C>A (p.Ser388*)                   | bicoronal synostosis | 3                                     | abnormal configuration of ventricles and corpus callosum | –                | moderate-severe | scoliosis, foreskin stricture |
| 2          | 4098         | F      | c.1204G>T (p.Glu402*)                  | bicoronal synostosis | 0                                     | agenesis of corpus callosum, dilated lateral ventricles | divergent strabismus | moderate         | scoliosis |
| 3          | 4133/5847    | M      | c.1204G>T (p.Glu402*) $^a$             | bicoronal synostosis, bony defect of sagittal suture | 2                                     | normal on CT scan | –                | mild             | – |
| 4          | 12D11570     | M      | c.1165C>T (p.Gln389*)                 | bicoronal synostosis, partial R lambdoid synostosis, bony defect of metopic and sagittal sutures | 1                                     | mildly enlarged lateral ventricles, shortened corpus callosum, hypoplastic pons, enlarged foramen magnum | strabismus sursoadductorius | moderate-severe | – |
| S:II.2     | 12D15615     | F      | c.1198G>C (p.Gly400Arg)               | brachycephaly, delayed closure anterior fontanelle | 0                                     | atrophy of the rostral part of the cerebellum and pons | –                | mild             | – |
| S:II.4     | 08D1850      | F      | c.1198G>C (p.Gly400Arg)               | plagiocephaly | 0                                     | reduced dorsum of pons, minor posterior fossa abnormalities | strabismus correction, ptosis L eye | below average | – |
| S:III.1    | 14D6457      | M      | c.1198G>C (p.Gly400Arg)               | delayed closure anterior fontanelle (4 years) | 0                                     | DWM                          | –                | mild             | 50 dB sensorineural hearing loss R; spina bifida occulta; LHRH deficiency |
| S:III.3    | 12D15613     | M      | c.1198G>C (p.Gly400Arg)               | bicoronal synostosis, patent metopic, bilateral bony defect of lambdoid sutures | 0                                     | normal on CT scan | bilateral convergent strabismus + ptosis | mild | – |
| S:III.6    | 10D5797      | M      | c.1198G>C (p.Gly400Arg)               | bicoronal synostosis, bilateral parietal foramina | 1                                     | reduced dorsum of pons, minor posterior fossa abnormalities | bilateral divergent strabismus + ptosis R eye | below average | – |

$^a$Mutation present in mosaic state.
and 5:III.5). This variant is absent from more than 120,000 alleles in the Exome Aggregation Consortium (ExAC). The combination of phenotypic features, the segregation of the variant, its location in the region where the previously identified nonsense mutations all clustered (Figure 2A), and absence in large databases of variation suggest that this variant is causative of the phenotype.

**Functional Consequence of ZIC1 Mutations in Xenopus Embryo Assay**

The function of the Xenopus zic1 ortholog has been studied in detail, where it was shown to act together with Pax3 as a key transcription factor required for the initiation of neural crest formation. In Xenopus embryos, a signaling cascade has been proposed in which inhibition of bone

---

**Figure 2. Molecular Genetic Analysis of Individuals with ZIC1 Mutations**

(A) Cartoon showing exon organization (white boxes denote non-coding regions) and previously identified conserved domains (Zic opa conserved motif [ZOC], zinc finger N-flanking conserved region [ZF-NC], and five zinc fingers [1–5, blue boxes]) of human ZIC1. Also indicated is the C-terminal NEWYV motif conserved in all family members except ZIC4. Above the cartoon are the positions of the five independent ZIC1 mutations described in this report, and dideoxy-sequence traces showing comparison of normal sequence (above) and mutant sequence (below). Note, in the case of subject 3, the mutation was not evident in the DNA sample (sourced from scalp fibroblasts; not shown) originally analyzed; however, in the exome sequence of DNA sourced from blood of the same individual, 63 of 183 (34%) reads showed the c.1204G>T mutation, which is also readily apparent on the dideoxy sequence. The relative heights of mutant and wild-type peaks differ between samples from subject 3 and subject 2, who is constitutionally heterozygous for the identical mutation, corroborating that in subject 3 the mutation is present in high-level mosaic state.

(B) Agarose gel analysis of ZIC1 cDNA obtained from RNA extracted from scalp fibroblasts of subject 1 and digested with BfaI. The fragments yielded by digestion of the mutant allele are indicated with asterisks.

(C) Amino acid sequence encoded by 30-terminal exon of ZIC1 and comparison with the paralogous human proteins ZIC2-ZIC5, showing conservation including the NEWYV motif (bracket). The end of the fifth zinc finger (Zn5) is shown above the sequence, as are the positions of the four different pathogenic variants (red symbols) (triangle, nonsense; circle, missense) identified in this study. The positions at which the Xenopus constructs zic1DC2 and zic1DC are truncated, relative to the human sequence, are indicated by blue arrows (note zic1DC2 is equivalent to p.Gln389*). Additional human constructs tested in the Xenopus assay are indicated by green symbols.
almost the entire region C-terminal to the zinc fingers. We next examined the activity of human ZIC1, whereas injection of wild-type ZIC1 morphogenetic proteins (Bmps) activates zic1, which in turn activates members of the Wnt family including wnt1, which in turn activates the transcription factor engrailed-2 (en-2). A previously designed assay had shown that injection of zic1ΔC RNA encoding a truncated ZIC1 into a single cell of 2-cell stage embryos led to increased en-2 expression in stage 15–17 Xenopus embryos, whereas injection of wild-type zic1 construct had no effect. We next examined the activity of human ZIC1 and various mutant constructs with this assay.

In initial experiments, we replicated the previously reported results of the Xenopus assay. Although very few (3%) embryos were disrupted by injection of the wild-type zic1 construct, this increased to 31% using the previously published zic1ΔC. Moreover, a construct deleting almost the entire region C-terminal to the zinc fingers (zic1ΔC2: Figure 2C) showed an even higher proportion (61%) of disrupted embryos (Figure 3). Using constructs encoding human ZIC1, injection of full-length ZIC1 RNA yielded only 8% disrupted embryos, but this was increased to 79% and 68% with constructs corresponding to two of the observed truncations, p.Glu402* (subject 1) and p.Glu402* (subjects 2 and 3), respectively. Strikingly, a similar magnitude of effect (66%) was found with the most C-terminal truncation construct (p.Ala437*, which does not correspond to an observed mutation), highlighting the importance of the terminal 11 amino acids, which includes a 5-amino-acid motif (NEWYV) of unknown function that is conserved in human ZIC2, ZIC3 (isoform A), and ZIC5 (Figure 2C) but is not present in any other human protein. Two constructs encoding missense substitutions were studied with the same assay: p.Gly400Arg present in family 5 and p.Thr414Ala, corresponding to a rare SNP (dbSNP rs143292136), present in 104/121,380 alleles in ExAC, which we had identified in a child with bicornal synostosis and her unaffected father (data not shown); 60% of embryos were disrupted in both cases. Although producing a slightly milder effect than the nonsense mutations, these results suggest that residues in the C-terminal domain in addition to the NEWYV motif contribute to function. Importantly, using a truncated control construct (p.Glu299*) missing the last three of the five zinc fingers, a much lower proportion of embryos (20%) was disrupted, which did not differ significantly from the full-length ZIC1 RNA (Figure 3C). This indicates that intact zinc fingers are required to induce consistently abnormal en-2 expression in this assay.

**Zic1 Expression in Mouse Embryos**

Of note in the above experiments, the effect of the ZIC1 C-terminal mutants was to increase the expression of the Xenopus target gene en-2 (Figures 3B and 3C). Given previous evidence that the paralogous gene En1 is critical for early biogenesis of the murine coronal suture,11 we asked whether Zic1 might also be expressed in relevant cells. Although the neural pattern of Zic1 expression20,41 and loss-of-function phenotypes19,20 were previously described in the mouse, no evidence has linked Zic1 expression to coronal suture development. Therefore, we analyzed expression patterns of Zic1 in embryonic mouse heads between E11.5 and E17.5. Between E11.5 and E12.5, a distinct domain of Zic1 expression was observed in the supraorbital region and cephalic mesoderm, which appeared to precede and partly overlap En1 expression (Figure 4). By contrast, no Zic1 expression was observed in the calvaria at E14.5 and E17.5 (not shown).

**Discussion**

Given the homology to one of the classical early patterning genes of the Drosophila embryo, the function of the vertebrate Zic family has been the focus of sustained...
interest. Highlighting their importance for development, mutations in the related genes ZIC2 (MIM: 603073) and ZIC3 (MIM: 300265) were previously described in holo-prosencephaly (MIM: 609637) and X-linked visceral heterotaxy (MIM: 306955), respectively. In the case of ZIC1, the observation that heterozygous deletions in tandem with ZIC4 cause DWM has supported work in Xenopus and mouse, indicating key roles for the Zic1 ortholog in neurogenesis (in the mouse, deficiency of Zic1 and Zic4 contribute additively to cerebellar hypoplasia). Our data now highlight a previously unsuspected subsidiary role for ZIC1 in early patterning events in the coronal suture.

The nonsense mutations of ZIC1 present in subjects 1 to 4 all arose de novo (post-zygotically in the case of subject 3, who has an attenuated phenotype) and are associated with consistent features comprising bicoronal synostosis, moderate to severe learning disability, and subtle abnormalities in brain anatomy including variable deficiency of the corpus callosum and abnormal conformation of the ventricles and posterior fossa. The progressive scoliosis in two of these individuals is likely to be causally related to the mutation, because vertebral and thoracic defects were also observed in mouse mutants homozygous for the null mutation in Zic1. In family 5, a heterozygous missense variant p.Gly400Arg, present in the same C-terminal region of the ZIC1 protein, segregated through three generations in six affected individuals. The phenotype in this family was variable, with two individuals having documented bicoronal synostosis and another a DWM. Associated learning disabilities were less severe than in subjects with truncations. Interestingly, MRI scans showed that several individuals from this family not formally diagnosed with DWM nevertheless had more subtle abnormalities in the conformation of the posterior fossa (Figure 1J). Hence the cerebral features were reminiscent of, but milder than, the malformations described in the previous cases with contiguous ZIC1-ZIC4 deletions. In contrast to many craniosynostosis syndromes, no diagnostic limb anomalies were apparent in any of the affected individuals.

In assessing the association of ZIC1 mutations with craniosynostosis, two features are particularly striking: the highly localized distribution of the mutations and the severity of the phenotype. All five mutations predict protein alterations within a 15-residue stretch encoded by the final exon. The occurrence of distinct phenotypes and/or patterns of inheritance associated with truncations localized to the terminal exon is a well-recognized indication that escape from nonsense-mediated decay might be responsible. Indeed, we were able to demonstrate that the transcript carrying the nonsense mutation was stable in a fibroblast cell line from the individual (subject 1) with the most N-terminal truncation (Figure 2B). The qualitative difference in phenotype associated with

![Figure 4. Expression of Zic1 in E11.5–E12.5 Mouse Embryos Analyzed by RNA In Situ Hybridization and Comparison with En1](image-url)
heterozygous truncating mutations, compared to previously reported heterozygous deletions, supports a gain-of-function mechanism (see also below). In the four case subjects with truncating mutations, the coronal synostosis was always bilateral and associated with marked brachycephaly (Figures 1A–1G), consistent with a severe, early effect on cranial suture formation; localized sutural ossification defects also occurred frequently. Craniosynostosis accompanied by DWM is rare, although an association with sagittal synostosis was reported previously.

To investigate the mechanism by which these mutations could disturb coronal suture biogenesis, we first explored their functional effect in a previously established Xenopus assay. Injection of several constructs truncated at different positions in the C-terminal region led to disrupted en-2 expression in a majority of treated embryos, provided that the zinc finger domain remained intact (Figure 3). Mostly the disrupted expression pattern involved combinations of upregulation, shifting, and expansion of en-2 expression (for examples see Figure 3B), although with the larger truncations retaining all Zn fingers, some embryos showed reduced en-2 expression. The zic1ΔC construct has been used previously in Xenopus to obtain enhanced biological responses to zic1 in several assays in which sensitization of the ectoderm to Bmp inhibition led to activation of genes expressed in the neural crest and neural tube; this was interpreted as showing that the C-terminal region has negative regulatory activity. The biological basis of this activity remains unknown; based on the enhanced en-2 expression associated with both the very C-terminal truncation p.Ala437* and the two missense substitutions, this might involve protein interaction over several parts of the ZIC1 C-terminal region, which shows several patches that are conserved between multiple ZIC paralogs (Figure 2C). Although the Xenopus assay supports the general evidence that the c.1198G>C (p.Gly400Arg) substitution is causative of the phenotype in family 5, a cautionary note is provided by the finding that p.Thr414Ala also disrupted en-2 expression, because this variant occurs too frequently (at 1 in 1,167 alleles) to be penetrant for craniosynostosis in more than a small proportion of individuals who carry this variant.

In a second approach to understand the pathogenic mechanisms, we asked whether the pattern of Zic1 expression was consistent with a specific role in coronal suture biogenesis. We found a previously undescribed, transient zone of Zic1 expression in the supraorbital regulatory center at E11.5 (Figure 4) that is spatially and temporally overlapping with that of En1 (Figure 4) and is consistent with an early instructive role for Zic1 in the supraorbital regulatory center; combining these observations with the finding of increased en-2 expression driven by mutant ZIC1 constructs in the Xenopus experiments, we propose that the coronal synostosis phenotype associated with the human mutations might be attributable to alteration of EN1 expression in the supraorbital regulatory center, thus disrupting the patterning of the coronal suture at a very early stage in its development. The induction of en-2

expression by ZIC1 orthologs is well characterized in both Drosophila and Xenopus and is thought to act through the Wnt signaling pathway. A further component of this signaling network is likely to be Lmx1b, which encodes a LIM-homeodomain protein and is upregulated in early neural crest of Xenopus. In the mouse, Lmx1b is prominently expressed in the supraorbital region at E11.5, and homozygous mutants have severely abnormal cranial sutures. In humans, heterozygous mutations in LMX1B usually cause nail-patella syndrome (MIM: 161200), but a specific missense mutation in the N-terminal arm of the homeodomain has been associated with craniosynostosis.

Putting the evidence together, we propose that the ZIC1 mutations we have described serendipitously uncover an important role for this transcription factor in early lineage commitment at the supraorbital regulatory center and that En1 is likely to represent a key target gene. This will stimulate further work to define more precisely the role of Zic1 in the coronal suture and to delineate the function of the conserved C-terminal domain. In ZIC2, constructs with holoprosencephaly-associated mutations C-terminal to the zinc fingers showed variable loss of transactivation activity, whereas in ZIC3, the single missense mutation beyond the zinc fingers reported to date (in a simplex case subject with congenital heart disease) was uniquely associated with increased transactivation activity. Collectively these data highlight the distinct properties of the C-terminal domain of ZIC proteins. The sequence conservation identified in this region (Figure 2C) suggests that a shared but currently unexplored mechanism exists for their regulatory function. Finally, although ZIC1 mutations are rare because the genetic target is localized, the complications for intellectual and skeletal development are more serious than is usually the case with coronal synostosis; therefore, genetic testing is recommended when the more common diagnostic possibilities (including mutations in TWIST1, FGFR3, FGFR2, and TCF12) have been excluded.

Supplemental Data

Supplemental Data include two tables, supplemental case reports, and WGSS500 member list and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2015.07.007.

Acknowledgments

We thank Michael Parker and Julie Phipps for assistance with subject recruitment, Sue Butler for cell culture work, and Geoff Maher and Yan Zhou for preparing and running the PGM libraries. This work was funded by the National Science Foundation (DBI-1309250 to J.F. and IOS-0846168 to C.S.M.), the Wellcome Trust (Project Grant 093329 to A.O.M.W. and S.R.F.T., Senior Investigator award 102731 to A.O.M.W.), and the Oxford NIHR Biomedical Research Centre. Members of the 500 Whole-Genome Sequences (WGSS500) Consortium are listed in the Supplemental Data.
Web Resources
The URLs for data presented herein are as follows:
1000 Genomes, http://browser.1000genomes.org
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
ExAC Browser, http://exac.broadinstitute.org/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server,
http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

References
1. Wilkie, A.O.M., Byren, J.C., Hurst, J.A., Jayamohan, J., John-
son, D., Knight, S.J.L., Lester, T., Richards, P.G., Twigg, S.R.F.,
and Wall, S.A. (2010). Prevalence and complications of
single-gene and chromosomal disorders in craniosynostosis.
Pediatrics 126, e391–e400.
2. Lajenie, E., Le Merer, M., Bonaiti-Pellie, C., Marchac, D.,
and Renier, D. (1995). Genetic study of nonsyndromic coronal
craniosynostosis. Am. J. Med. Genet. 55, 500–504.
3. Johnson, D., and Wilkie, A.O.M. (2011). Craniosynostosis.
Eur. J. Hum. Genet. 19, 369–376.
4. Sharma, V.P., Fenwick, A.L., Brockop, M.S., McGowan, S.J.,
Goos, J.A., Hoogeboom, A.J., Brady, A.E., Jeelani, N.O., Lynch,
S.A., Mulliken, J.B., et al.; 500 Whole-Genome Sequences
(WGS500) Consortium (2013). Mutations in TCF12, encoding
a basic helix-loop-helix partner of TWIST1, are a frequent
cause of coronal craniosynostosis. Nat. Genet. 45, 304–307.
5. Jiang, X., Iseki, S., Maxson, R.E., Sucov, H.M., and Morrius-Kay,
G.M. (2002). Tissue origins and interactions in the mamma-
lian skull vault. Dev. Biol. 241, 106–116.
6. Yoshida, T., Vivatbutsiri, P., Morrius-Kay, G., Saga, Y., and Iseki,
S. (2008). Cell lineage in mammalian craniofacial mesen-
chyme. Mech. Dev. 125, 797–808.
7. Twigg, S.R.F, and Wilkie, A.O.M. (2015). A genetic-pathophys-
iological framework for craniosynostosis. Am. J. Hum. Genet.
97, this issue, 359–377.
8. Merrill, A.E., Bochukova, E.G., Brugger, S.M., Ishii, M., Pilz,
D.T., Wall, S.A., Lyons, K.M., Wilkie, A.O.M., and Maxson,
R.E., Jr. (2006). Cell mixing at a neural crest-mesoderm bound-
ary and deficient ephrin-Eph signaling in the pathogenesis of
craniosynostosis. Hum. Mol. Genet. 15, 1319–1328.
9. Hajihosseini, M.K., Duarte, R., Pegrum, J., Donjacour, A.,
Lana-Elola, E., Rice, D.P., Sharpe, J., and Dickson, C. (2009). Evidence that Fgf10 contributes to the skeletal and visceral
defects of an Apert syndrome mouse model. Dev. Dyn. 238,
376–385.
10. Holmes, G., Rothschild, G., Roy, U.B., Deng, C.X., Mansuk-
hani, A., and Basilico, C. (2009). Early onset of craniosyn-
ostosis in an Apert mouse model reveals critical features of this
pathology. Dev. Biol. 328, 273–284.
11. Deckelbaum, R.A., Holmes, G., Zhao, Z., Tong, C., Basilico, C.,
and Loomis, C.A. (2012). Regulation of cranial morphogenesis
and cell fate at the neural crest-mesodern boundary by
engrailed 1. Development 139, 1346–1358.
12. Ting, M.C., Wu, N.L., Roybal, P.G., Sun, J., Liu, L., Yen, Y., and
Maxson, R.E., Jr. (2009). EphA4 as an effector of Twist1 in
the guidance of osteogenic precursor cells during calvarial
bone growth and in craniosynostosis. Development 136,
855–864.
13. Deckelbaum, R.A., Majithia, A., Booker, T., Henderson, J.E.,
and Loomis, C.A. (2006). The homeoprotein engrailed 1 has
pleiotropic functions in calvarial intramembranous bone for-
mation and remodeling. Development 133, 63–74.
14. Ali, R.G., Bellchambers, H.M., and Arkell, R.M. (2012). Zinc
fingers of the cerebellum (Zic): transcription factors and co-
factors. Int. J. Biochem. Cell Biol. 44, 2065–2068.
15. Benedyk, M.J., Mullen, J.R., and DiNardo, S. (1994). odd-paired:
a zinc finger pair-rule protein required for the timely activa-
tion of engrailed and wingless in Drosophila embryos. Genes
Dev. 8, 105–117.
16. Aruga, J., Kamiya, A., Takahashi, H., Fujimi, T., Shimizu, Y.,
Ohkawa, K., Yazawa, S., Umesono, Y., Noguchi, H., Shimizu,
T., et al. (2006). A wide-range phylogenetic analysis of Zic
proteins: implications for correlations between protein struc-
ture conservation and body plan complexity. Genomics 87,
783–792.
17. Merzdorf, C.S. (2007). Emerging roles for zic genes in early
development. Dev. Dyn. 236, 922–940.
18. Grinberg, I., Northrup, H., Arding, H., Prasad, C., Dobyns,
W.B., and Millen, K.J. (2004). Heterozygous deletion of the
linked genes ZIC1 and ZIC4 is involved in Dandy-Walker
malformation. Nat. Genet. 36, 1053–1055.
19. Aruga, J., Minowa, O., Yaginuma, H., Kuno, J., Nagai, T., Noda,
T., and Mikoshiba, K. (1998). Mouse Zic1 is involved in cere-
bellar development. J. Neurosci. 18, 284–293.
20. Blank, M.C., Grinberg, I., Aryee, E., Laliberte, C., Chizhikov,
V.V., Henkelman, R.M., and Millen, K.J. (2011). Multiple
developmental programs are altered by loss of Zic1 and Zic4
to cause Dandy-Walker malformation cerebellar pathogenesis.
Development 138, 1207–1216.
21. Eley, K.A., Johnsson, D., Wilkie, A.O.M., Jayamohan, J.,
Richards, P., and Wall, S.A. (2012). Raised intracranial pressure
is frequent in untreated nonsyndromic unicoronal synostosis
and does not correlate with severity of phenotypic features.
Plast. Reconstr. Surg. 130, 690e–697e.
22. Taylor, J.C., Martin, H.C., Lise, S., Broxholme, J., Cazier, J.-B.,
Rimmer, A., Kanapin, A., Lunter, G., Fiddy, S., Allan, C., et al.
(2015). Factors influencing success of clinical genome
sequencing across a broad spectrum of disorders. Nat. Genet.
47, 717–726.
23. Rimmer, A., Phan, H., Mathissons, I., Iqbal, Z., Twigg, S.R.F,
Wilkie, A.O.M., McVean, G., and Lunter, G.; WGS500
Consortium (2014). Integrating mapping-, assembly- and
haplotype-based approaches for calling variants in clinical
sequencing applications. Nat. Genet. 46, 912–918.
24. Lunter, G., and Goodson, M. (2011). Stampy: a statistical algo-
rithm for sensitive and fast mapping of Illumina sequence
reads. Genome Res. 21, 936–939.
25. Drmanac, R., Sparks, A.B., Callow, M.J., Halpern, A.L., Burns,
N.L., Kermani, B.G., Pant, K.P., Ebert, J.C., Brownley, A., Morenzoni, M., Kar-
pinchy, V., et al. (2012). Computational techniques for

The American Journal of Human Genetics 97, 378–388, September 3, 2015 387
