Co-occurrence of frameshift mutations in SMAD6 and TCF12 in a child with complex craniosynostosis

Andrew T. Timberlake1,2, Robin Wu2, Carol Nelson-Williams1, Charuta G. Furey1, Kristi I. Hildebrand3, Scott W. Elton3, Jeyhan S. Wood4, John A. Persing2 and Richard P. Lifton1,5

Abstract
Non-syndromic craniosynostosis (CS) affects 1 in 2350 live births. Recent studies have shown that a significant fraction of cases are caused by de novo or rare transmitted mutations that promote premature osteoblast differentiation in cranial sutures. Rare heterozygous loss-of-function (LOF) mutations in SMAD6 and TCF12 are highly enriched in patients with non-syndromic sagittal and coronal CS, respectively. Interestingly, both mutations show striking incomplete penetrance, suggesting a role for modifying alleles; in the case of SMAD6, a common variant near BMP2 drastically increases penetrance of sagittal CS. Here, we report a proband presenting with both sagittal and coronal craniosynostosis with the highly unusual recurrence of CS within two months of initial surgery, requiring a second operation to re-establish suture patency at six months of age. Exome sequencing revealed a rare transmitted frameshift mutation in SMAD6 (p. 152 fs*27) inherited from an unaffected parent, absence of the common BMP2 risk variant, and a de novo frameshift mutation in TCF12 (p.E548fs*14). SMAD6 and TCF12 independently inhibit transcriptional targets of BMP signaling. The findings are consistent with epistasis of these mutations, increasing penetrance and severity of CS in this proband. They also add to the list of composite phenotypes resulting from two Mendelian mutations, and support the utility of exome sequencing in atypical CS cases.

The female proband was delivered at term after an uncomplicated pregnancy, and was referred to the pediatric neurosurgical service at birth due to her abnormal head shape. On physical exam, the patient had flattening of the left frontal bone with contralateral frontal bossing and associated harlequin deformity of the left orbit, consistent with left coronal synostosis. In addition, the calvarium posterior to the coronal sutures was elongated and narrow (scaphocephalic), consistent with sagittal synostosis (Fig. 1a, b). A CT scan confirmed sagittal and left coronal synostosis, and the child underwent endoscopic strip craniectomy at nine weeks of age.

The child was subsequently followed biweekly in neurosurgery clinic and by an orthotist for helmet adjustments to shape skull growth. Two months after surgery, the orthotist noted that the child’s left forehead remained flattened and was not rounding as expected. At the child’s subsequent neurosurgical clinic visit, the parietal and occipital regions had rounded and expanded as expected; however, the left frontal region had stopped rounding. A repeat head CT was performed, which demonstrated rapid healing and patency of the sagittal suture and re-fusion of the left coronal suture along with complete fusion of the right coronal suture (Fig. 1c–e). Such rapid recurrence of craniosynostosis is extremely unusual. To correct the anterior deformity, the child underwent a cranial vault reconstruction with fronto-orbital...
advancement at six months of age. The child is developing normally to date, and it is unknown at present if she will need further surgical cranial reconstruction.

To explore potential genetic contributions to her condition, we performed whole exome sequencing of the case-parent trio using DNA prepared from buccal swab samples according to standard protocols. Exome capture was performed using the IDT xGen capture reagent, which was followed by 99 base paired-end sequencing on the Illumina HiSeq 2000 instrument. Sequence reads were aligned to the GRCh37/hg19 human reference genome using BWA-Mem. Local realignment and quality score recalibration were performed using the GATK pipeline, after which variants were called using the GATK Haplotype Caller. A Bayesian algorithm, TrioDeNovo, was used to call de novo mutations. VQSR ‘PASS’ variants with an ExAC allele frequency \( \leq 10^{-3} \) sequenced to a depth of eight or greater in the proband and 10 or greater in each parent with Phred-scaled genotype likelihood scores >30 and de novo quality scores (\( \log_{10}(\text{Bayes factor}) \)) >6 were considered. Independent aligned reads at variant positions were visualized in silico to remove false calls. All retained calls had de novo genotype quality scores of 100. Transmitted variants were called as per above, and all variants were annotated using ANNOVAR with allele frequencies assigned to each variant from the ExAC database.

Analysis showed that the proband had rare heterozygous LOF mutations in both of the two predominant non-syndromic CS genes, SMAD6 and TCF12. The mutation in SMAD6 was an early frameshift mutation (p. L152fs*27), which was transmitted from an unaffected parent (Fig. 2, Table 1). The mutation in TCF12 was also a frameshift mutation (p.K548fs*14), which was de novo. Both mutations were absent from the ExAC and GnomAD databases, which contain >240,000 alleles, and both mutations were confirmed by Sanger sequencing (Fig. 2). No other compelling heterozygous rare LOF or damaging missense variants were identified, and no rare recessive genotypes were identified (Table 1, Supplementary Table 1).
Fig. 2 Heterozygous LOF mutations in SMAD6 and TCF12 in a proband with complex craniosynostosis. a Pedigree and genotypes. Genotypes of each subject are shown: SMAD6 genotypes are in blue, and TCF12 genotypes are in red. “+” and “D” denote the wild-type and indicated frameshift alleles, respectively. No member of the trio harbored the BMP2 risk SNP ‘C’ at rs1884302. The SMAD6 p.152fs*27 mutation was transmitted from an unaffected parent, and the TCF12 p.E548fs*14 mutation arose de novo in the proband. b Confirmation of the de novo TCF12 mutation. Sanger sequencing traces of PCR amplicons containing the TCF12 mutation identified by exome sequencing are shown. The mutation identified in the DNA sequence and its impact on TCF12 protein (in single letter code) are indicated above the trace. The deleted bases are denoted, and they result in an overlap of wild-type and mutant sequences. Both the mother and father’s traces demonstrate the wild-type TCF12 sequence, whereas the proband has a de novo 4-bp deletion that results in a frameshift. c In silico visualization of the SMAD6 frameshift deletion in the proband. Sequence reads derived from single molecules on the Illumina platform are shown. The reference sequence of a segment of SMAD6 that includes base 15:66996051 (denoted by arrow) is shown in the top row, and red, blue, green and yellow squares represent the bases A, C, G, and T, respectively. Below, all independent reads that map to this interval are shown. The results show that the proband and father both have a 7-bp deletion that causes a frameshift in the SMAD6 coding sequence.

| Gene name | Chrom | Position | Ref | Alt | Mutation class | Impact | ExAC frequency | pLI |
|-----------|-------|----------|-----|-----|----------------|--------|----------------|-----|
| RIIAD1    | 1     | 151694016| G   | T   | stopgain       | p. E2X | Novel          | NA  |
| ENPP6     | 4     | 185033931| AT  | —   | frameshift deletion | p. M296fs | Novel     | 0   |
| SMO2C1    | 14    | 70418934 | GCAGGTCTCTAC | — | frameshift deletion | p. G60fs | Novel | 0.01 |
| TCF12     | 15    | 57555366 | AAAG | — | frameshift deletion | p. E548fs | Novel | 0.97 |
| SMAD6     | 15    | 66996051 | CGGCGGG | — | frameshift deletion | p. P152fs | Novel | 0   |
| ZNF551    | 19    | 58199463 | G   | —   | frameshift deletion | p. R579fs | Novel | 0   |

Table containing all rare (ExAC frequency < 2 × 10⁻⁵) LOF variants identified in a child with complex craniosynostosis. Two novel LOF variants in previously identified craniosynostosis genes (SMAD6 and TCF12) were identified. The novel TCF12 frameshift mutation was discovered to have arisen de novo in the proband (Fig. 2).
Heterozygous TCF12 mutations have been previously shown to cause coronal CS with considerable phenotypic overlap with Saethre–Chotzen syndrome, which is caused by LOF mutation in TWIST1, which heterodimerizes and inhibit transcription downstream of BMP signaling. The co-occurrence of loss-of-function mutations in two master regulators of this signaling pathway that can cause craniosynostosis independently was predicted to result in severely impaired inhibition of BMP/SMAD signaling and excessive osteogenic drive.

Fig. 3 The BMP signaling cascade in osteoblast differentiation. BMP receptors phosphorylate receptor-SMADs upon ligand binding. SMAD6 is a member of the inhibitory-SMAD family that prevents nuclear translocation of activated SMAD4/receptor-SMAD complexes. TCF12 and TWIST1 are basic helix-loop-helix transcription factors that heterodimerize and inhibit transcription downstream of BMP signaling. The co-occurrence of loss-of-function mutations in two master regulators of this signaling pathway that can cause craniosynostosis independently was predicted to result in severely impaired inhibition of BMP/SMAD signaling and excessive osteogenic drive.

Heterozygous TCF12 mutations have been previously shown to cause coronal CS with considerable phenotypic overlap with Saethre–Chotzen syndrome, which is caused by LOF mutation in TWIST1, which heterodimerizes and inhibit transcription downstream of BMP signaling. The co-occurrence of loss-of-function mutations in two master regulators of this signaling pathway that can cause craniosynostosis independently was predicted to result in severely impaired inhibition of BMP/SMAD signaling and excessive osteogenic drive.

While several factors, such as patient age at presentation, suture fusion pattern, and patient co-morbidities, play a role in which type of surgery (endoscopic versus open) is offered to craniosynostosis patients, knowing the genetic results for specific patients could prove useful in guiding operative planning for this complex patient population. The goals of cranial vault reconstruction are to obtain an aesthetically pleasing shape of the skull that will allow adequate growth of the brain in ideally one operation. Identification of high risk genotypes prior to surgery—particularly in cases with unusual clinical features—may prove useful in guiding surgical management in the future and may enable more informed discussions with patients' families.

HGV Database
The relevant data from this Data Report are hosted at the Human Genome Variation Database at https://doi.org/10.6084/m9.figshare.hgv.2330 https://doi.org/10.6084/m9.figshare.hgv.2333.
Acknowledgements
The study protocol was approved by the Yale Human Investigation Committee Institutional Review Board, and consent for use of patient photographs was obtained by the treating physicians. This project was supported by the Yale Center for Mendelian Genomics (NIH Grant M#UM1HG006504-05), the NIH Medical Scientist Training Program (NIH/National Institute of General Medical Sciences Grant T32GM007205), and the Howard Hughes Medical Institute.

Author details
1Department of Genetics, Yale University School of Medicine, New Haven, CT, USA. 2Section of Plastic and Reconstructive Surgery, Yale University School of Medicine, New Haven, CT, USA. 3Division of Pediatric Neurosurgery, University of North Carolina School of Medicine, Chapel Hill, NC, USA. 4Division of Plastic and Reconstructive Surgery, University of North Carolina School of Medicine, Chapel Hill, NC, USA. 5Laboratory of Human Genetics and Genomics, The Rockefeller University, New York, NY, USA.

Conflict of interest
The authors declare that they have no conflict of interest.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information is available for this paper at https://doi.org/10.1038/s41439-018-0014-x.

References
1. Wei, Q. et al. A Bayesian framework for de novo mutation calling in parent-offspring trios. Bioinformatics 31, 1375–1381 (2015).
2. Wang, K., Li, M. & Hakonarson, H. ANNOVAR functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 38, e164 (2010).
3. Lek, M. et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature 536, 285–291 (2016).
4. Sharma, V. P. et al. Mutations in TCF12, encoding a basic helix-loop-helix partner of TWIST1, are a frequent cause of coronal craniosynostosis. Nat. Genet. 45, 304–307 (2013).
5. Timberlake, A. T. et al. Two locus inheritance of non-syndromic midline craniosynostosis via rare SMAD6 and common BMP2 alleles. Elife 5, e20125 (2016).
6. Wilkie, A. O. M., Johnson, D. & Wall, S. A. Clinical genetics of craniosynostosis. Curr. Opin. Pediatr. 29, 622–628 (2017).
7. Timberlake, A. T. et al. De novo mutations in inhibitors of Wnt, BMP, and Ras/ERK signaling pathways in non-syndromic midline craniosynostosis. Proc. Natl Acad. Sci. USA 114, E7341–E7347 (2017).
8. Posey, J. E. et al. Resolution of disease phenotypes resulting from multilocus genomic variation. N. Engl. J. Med. 376, 21–31 (2017).
9. Yi, S., Yu, M., Yang, S., Miron, R. J. & Zhang, Y. Tcf12, a member of basic helix-loop-helix transcription factors, mediates bone marrow mesenchymal stem cell osteogenic differentiation in vitro and in vivo. Stem Cells 35, 386–397 (2017).