De novo mutations in inhibitors of Wnt, BMP, and Ras/ERK signaling pathways in non-syndromic midline craniosynostosis

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Non-syndromic craniosynostosis (NSC) is a frequent congenital malformation in which one or more cranial sutures fuse prematurely. Mutations causing rare syndromic craniosynostoses in humans and engineered mouse models commonly increase signaling of the Wnt, bone morphogenetic protein (BMP), or Ras/ERK pathways, converging on shared nuclear targets that promote bone formation. In contrast, the genetics of NSC is largely unexplored. More than 95% of NSC is sporadic, suggesting a role for de novo mutations. Exome sequencing of 291 parent–offspring trios with midline NSC revealed 15 probands with heterozygous damaging de novo mutations in 12 negative regulators of Wnt, BMP, and Ras/ERK signaling (10.9-fold enrichment, $P = 2.4 \times 10^{-11}$). *SMAD6* had 4 de novo and 14 transmitted mutations; no other gene had more than 1. Four familial NSC kindreds had mutations in genes previously implicated in syndromic disease. Collectively, these mutations contribute to 10% of probands. Mutations are predominantly loss-of-function, implicating haploinsufficiency as a frequent mechanism. A common risk variant near *BMP2* increased the penetrance of *SMAD6* mutations and was overtransmitted to patients with de novo mutations in other genes in these pathways, supporting a frequent two-locus pathogenesis. These findings implicate new genes in NSC and demonstrate related pathophysiology of common non-syndromic and rare syndromic craniosynostoses. These findings have implications for diagnosis, risk of recurrence, and risk of adverse neurodevelopmental outcomes. Finally, the use of pathways identified in rare syndromic disease to find genes accounting for non-syndromic cases may prove broadly relevant to understanding other congenital disorders featuring high locus heterogeneity.

de novo mutation | craniosynostosis | BMP signaling | Wnt signaling | Ras/ERK signaling

Craniosynostosis affects 1 in 2,000 live births, constituting the second most-common craniofacial birth defect following orofacial clefts (1). Premature fusion of any cranial suture is treated surgically in infancy to prevent neurological impairment, with cases affecting the midline (sagittal and metopic) sutures accounting for more than half of all disease (2, 3). Fifteen percent of cases are syndromic, with extracranial malformations, while the remaining 85% are non-syndromic with isolated craniosynostosis (4).

Many genes have been implicated in human syndromic craniosynostoses, with at least 40 showing significant statistical support or having been identified in more than one study (5). Many genes have also been implicated by studies of mouse genetics (4, 6–9). These genes are involved in signaling pathways that converge on promotion of osteoblast differentiation and bone formation. The most prominent of these are the Ras/ERK, bone morphogenetic protein (BMP), and Wnt pathways, with ephrin, smoothened/hedgehog, STAT, and retinoic acid signal-

ing pathways being implicated at lower frequency (5). Examples include gain-of-function (GOF) mutations in FGF receptors 1–3, which present with craniosynostosis of any or all sutures with variable hypertelorism, proptosis, midface abnormalities, and syndactyly, and loss-of-function (LOF) mutations in *TGFBR1/2* that present with craniosynostosis in conjunction with severe cardiac/vascular abnormalities and other extracranial defects.

Whereas most syndromic craniosynostoses demonstrate patent Mendelian inheritance (5), the vast majority of non-syndromic cases are sporadic, suggesting a possible role for de novo mutation. To test this possibility, in a prior study of whole-exome sequencing of 191 probands with midline non-syndromic craniosynostosis (NSC), including 132 case-parent trios, we found a significant excess of damaging protein-altering de novo mutations, likely accounting for ~11% of cases. One gene, *SMAD6*,

Significance

Craniosynostosis is a common congenital malformation resulting from premature fusion of the bones that comprise the cranial vault, requiring surgery in infancy to prevent adverse neurologic outcomes. Eighty-five percent of cases are non-syndromic and of unknown cause. By exome sequencing of families with non-syndromic midline craniosynostosis, we show that 5% of cases have de novo damaging mutations in negative regulators of the Wnt, bone morphogenetic protein (BMP), and Ras/ERK signaling pathways, developmental cascades that converge on common nuclear targets to promote bone formation. Another 5% have transmitted mutations in these pathways. Common variants near *BMP2* show genetic interaction with these rare mutations. The results provide insight into pathophysiology and have immediate implications for the diagnosis and genetic counseling of families with craniosynostosis.

surpassed genome-wide thresholds for significance, with three damaging de novo mutations and 10 rare independent transmitted LOF or damaging missense (D-mis) mutations (10). These *SMAD6* mutations showed striking incomplete penetrance, which was explained by epistatic interaction with a previously identified (11) common risk allele near the BMP signaling ligand, *BMP2* (10). No other gene had more than one damaging de novo mutation. In this prior study, the power to determine which other de novo mutations were pathogenic was limited by the study size and the apparent high degree of locus heterogeneity. We now report results of whole-exome sequencing of a much larger cohort of NSC families.

Results

Exome Sequencing of Midline NSC. We studied a cohort of 384 probands with NSC affecting the sagittal ($n = 237$), metopic ($n = 136$), combined sagittal and metopic ($n = 10$), or combined sagittal and coronal ($n = 1$) sutures. These included 291 parent–offspring trios, 132 of which were previously reported (10). Whole-exome sequencing was performed as described in *Methods*. Of the targeted bases, 96.6% had 8 or more independent reads, and 88.6% had 20 or more (Table S1). In parallel, we analyzed de novo variants in parents and healthy siblings of 1,789 autism probands from the Simons simplex collection sequenced on the same platform. Variants in both cohorts were called using the same GATK pipeline, and de novo mutations were identified using TrioDeNovo (12); the impact of missense mutations was inferred using MetaSVM (*Methods*).

The results identified an average of 1.12 de novo mutations per proband, and closely approximated the expected Poisson distribution (Fig. S1). The observed mutation rate (1.69×10^{-8} per base pair) closely matched both expectation and prior experimental results (Table 1). The total burden of de novo mutations in controls was similar (Table 1).

Significant Burden of Damaging de Novo Mutations. The distribution of types of de novo coding mutations observed in probands was compared with that expected from the probability of mutation of each base in the coding region and flanking splice sites (13). Probands showed no significant enrichment of synonymous or missense mutations predicted to be tolerated; however, there was significant enrichment of inferred damaging missense (D-mis) and LOF mutations (Table 1). In contrast, no enrichment was observed in any class of coding region mutation in control subjects. From the observed excess of damaging de novo mutations in probands compared with expectation in this set, we infer that damaging protein-altering de novo mutations contribute to ∼8% of cases. *SMAD6*, with three different LOF and one D-mis de novo mutation, was the only gene with more than one damaging de novo mutation (probability of three or more de novo LOF mutations in *SMAD6* by chance = 4.0×10^{-10}) (Dataset S1).

Enrichment of Mutations in Negative Regulators of Wnt, BMP, and Ras/ERK Signaling. As discussed above, mutations that cause rare syndromic forms of craniosynostosis in humans and in mouse cluster in developmental signaling pathways that converge on promotion of osteoblast differentiation and bone formation (5). These are encompassed by the Ras/ERK, Wnt, BMP, hedgehog, ephrin, STAT, and retinoic acid signaling pathways (5). We considered whether de novo mutations in these pathways are enriched in NSC. We anticipated that mutations would most frequently be LOF mutations in negative regulators of signaling rather than GOF in promoters of signaling, because of the generally much larger target size for mutations producing LOF rather than GOF. We first compared the observed and expected burden of damaging de novo mutations in all genes in these pathways ($n = 963$) as defined by Gene Ontology (GO) (Table S2). There were 19 damaging de novo mutations in cases compared with 4.7 expected from the Poisson distribution, a fourfold enrichment and highly statistically significant ($P = 6.7 \times 10^{-7}$) (Table S3). All 19 of these were confirmed as de novo mutations by Sanger sequencing. There was no enrichment of mutations in this gene set in controls, with 27 damaging mutations observed compared with 29.2 expected (enrichment = 0.92; $P = 0.68$).

Notably, all 19 of these mutations occurred in either the Wnt, BMP, or Ras/ERK pathway (4.8-fold enrichment, $P = 4.8 \times 10^{-8}$),

Table 1. Burden of de novo mutations in 291 probands with midline craniosynostosis and 1,789 controls

| Mutation class | Cases, $n = 291$ | | | | | | Controls, $n = 1,789$ | | | | | |
| | Observed | | Expected | | | | Observed | | Expected | | | |
	n	Rate	n	Rate	Enrichment	P	n	Rate	n	Rate	Enrichment	P
All genes												
Total	325	1.12	325.6	1.12	1.00	0.51	1,830	1.02	1,999.5	1.12	0.92	1.00
Synonymous	61	0.21	92.3	0.32	0.66	1.00	484	0.27	567.6	0.32	0.85	1.00
Protein altering	264	0.91	232.9	0.80	1.13	**0.024**	1,346	0.79	1,431.9	0.80	0.94	0.99
Total missense	225	0.77	204.3	0.70	1.10	0.08	1,196	0.67	1,255.9	0.70	0.95	0.96
T-mis	175	0.60	166.0	0.57	1.05	0.28	974	0.54	1020.4	0.57	0.96	0.80
D-mis	50	0.17	38.3	0.13	1.31	**0.040**	222	0.12	235.5	0.13	0.94	0.82
LOF	39	0.13	28.6	0.10	1.36	**0.037**	150	0.08	175.9	0.10	0.85	0.98
Damaging	89	0.31	66.9	0.23	1.33	**0.006**	372	0.21	411.5	0.23	0.90	0.98
Negative regulators of Ras/ERK, BMP, and Wnt signal transduction												
Total	19	0.07	5.46	0.02	3.48	**4.90×10^{-6}**	31	0.02	33.6	0.02	0.92	0.69
Synonymous	2	0.007	1.60	0.005	1.26	0.47	12	0.007	9.8	0.005	1.22	0.28
Protein altering	17	0.058	3.87	0.013	4.39	**7.35×10^{-7}**	19	0.011	23.8	0.013	0.80	0.86
Total missense	9	0.031	3.42	0.012	2.63	**8.56×10^{-3}**	17	0.010	21.0	0.012	0.81	0.84
T-mis	2	0.007	2.50	0.009	0.80	0.54	14	0.008	15.4	0.009	0.97	0.55
D-mis	7	0.024	0.92	0.003	7.65	**4.82×10^{-5}**	3	0.002	5.6	0.003	0.53	0.92
LOF	8	0.027	0.45	0.002	17.6	**3.04×10^{-8}**	2	0.001	2.8	0.002	0.72	0.77
Damaging	15	0.052	1.37	0.005	10.9	**2.40×10^{-11}**	5	0.003	8.42	0.005	0.59	0.92

Damaging and tolerated missense called by MetaSVM (D-mis and T-mis, respectively). LOF denotes premature termination, frameshift, or splice-site mutation; damaging denotes LOF and D-mis mutations; n, number of de novo mutations; rate, number of de novo mutations per subject. P values represent the upper tail of the Poisson probability density function. Bold text indicates significant P values.
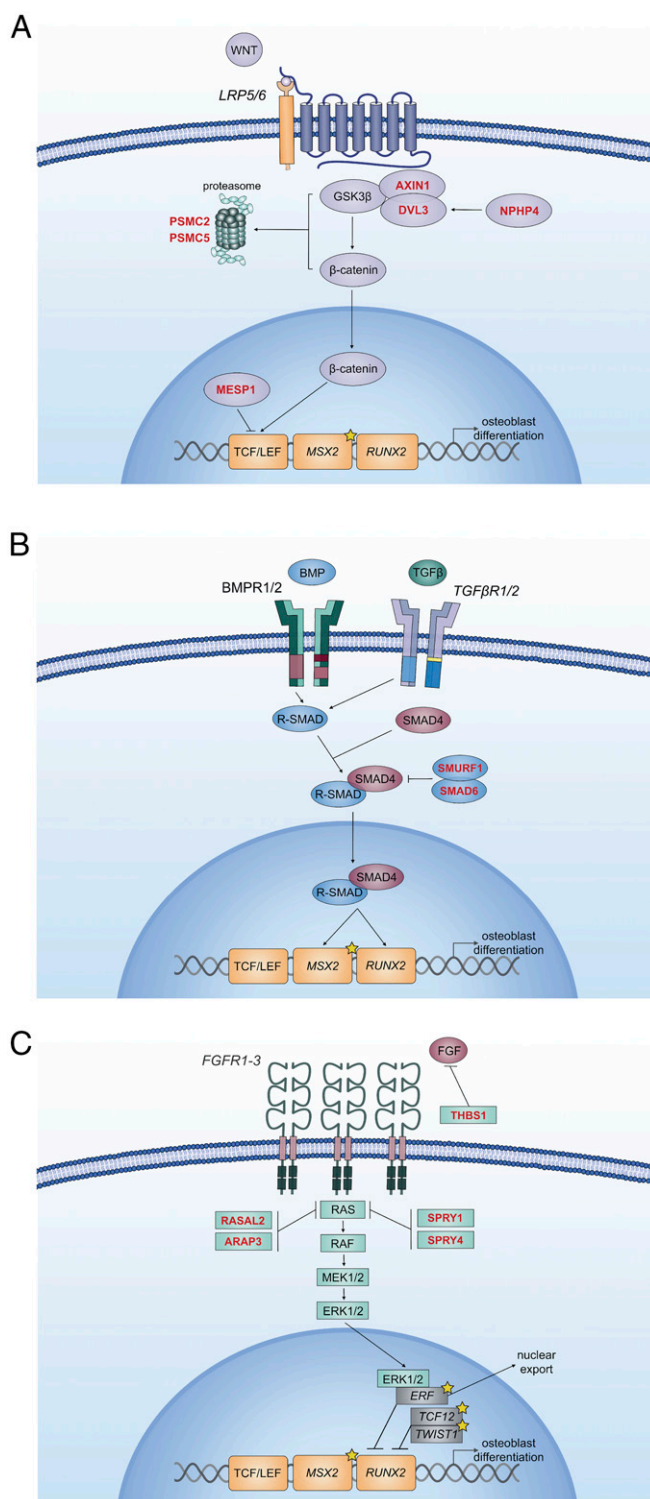
Table 2. Damaging de novo mutations in negative regulators of Wnt, BMP, and Ras/ERK signal transduction in 291 midline NSC probands

Craniosynostosis type	Gene	Mutation	ExAC frequency	pLI
Metopic	*ARAP3*	IVS6+1delGT	Novel	0.97
Sagittal	*AXIN1*	E322G	Novel	0.25
Sagittal	*DVL3*	G327fs	Novel	1.00
Sagittal	*MESP1*	E104*	8.92×10^{-5}	0
Sagittal	*NPHP4*	E453K	0.0002	0
Metopic	*PSMC2*	R297G	Novel	1.00
Metopic	*PSMC5*	R317W	Novel	0.97
Sagittal	*RASAL2*	R571P	Novel	0.23
Sagittal+Metopic	*SMAD6*	Q78fs	Novel	0
Metopic	*SMAD6*	G88fs	Novel	0
Sagittal	*SMAD6*	E374*	Novel	0
Metopic	*SMAD6*	G390C	Novel	0
Metopic	*SMURF1*	R468W	Novel	1.00
Sagittal	*SPRY1*	Q6fs	Novel	0
Sagittal	*SPRY4*	E160*	Novel	0.11

pLI, measure of intolerance to LOF mutation. Most intolerant score is 1.0

again with no enrichment in controls (enrichment = 0.90; $P = 0.72$) (Table S4). We used GO annotation to partition these genes into positive regulators ($n = 491$) and negative regulators ($n = 281$) of Wnt, BMP, and Ras/ERK signal transduction, and assessed the burden of de novo mutation in each (Table S4). Fifteen of the damaging de novo mutations were in negative regulators of this pathway, compared with the 1.4 expected (Table 1), a highly significant result ($P = 2.40 \times 10^{-11}$) with extremely strong enrichment (10.9-fold). Among these 15 damaging de novo mutations, there were 8 LOF (17.6-fold enrichment), and 7 D-mis (7.7-fold enrichment); in contrast there were only 2 synonymous and 2 tolerated missense (T-mis) mutations (enrichment 1.0), demonstrating selective enrichment for damaging mutations (Tables 1 and 2). There was no significant enrichment of damaging de novo mutations in this gene set in controls (enrichment 0.6) (Table 1) or in positive regulators of these pathways (Table S4). Enrichment of damaging de novo mutations in negative regulators of these pathways remained highly significant after exclusion of mutations in the previously known gene *SMAD6* (8.4-fold enrichment, $P = 1.4 \times 10^{-7}$) (Table S5). Moreover, negative regulators of each of the Wnt, BMP, and Ras/ERK pathways were individually enriched (Wnt enrichment 7.5-fold, $P = 1.8 \times 10^{-4}$; BMP enrichment 19-fold, $P = 8.4 \times 10^{-6}$; Ras/ERK enrichment 10.1-fold, $P = 7.4 \times 10^{-4}$) (Table S6). From the overall enrichment of these mutations, we infer that >90% contribute to NSC risk. We also infer that damaging de novo mutations in negative regulators of Wnt, BMP, or Ras/ERK signaling account for 4.7% of all midline NSC cases studied, and account for 62% of the total excess burden of damaging de novo mutations contributing to NSC in this cohort.

The finding that mutations in these pathways are highly enriched among negative regulators of Wnt, BMP, or Ras/ERK signaling is consistent with the known role of these signaling pathways in promoting osteoblast differentiation and premature bone formation (Fig. 1 and Table 2). In this cohort, damaging de novo mutations in negative regulators of BMP signaling included those in *SMAD6* and its binding partner, *SMURF1*. *SMAD6* is an inhibitory SMAD that inhibits nuclear translocation of phosphorylated receptor-SMADs, and *SMURF1* is a ubiquitin ligase that ubiquitylates receptor-SMADs and activated BMP receptors, targeting them for proteasomal degradation. Mice with genetic deficiency for *SMAD6* or *SMURF1*, or activation of upstream BMP-receptors each exhibit midline craniosynostosis phenotypes, supporting the human genetic evidence for these two genes (6, 14, 15).

Fig. 1. De novo mutations in craniosynostosis probands identified in negative regulators of Wnt, BMP, and Ras-ERK signal transduction. Schematic of signaling in Wnt (*A*), BMP (*B*), and RAS/ERK (*C*) pathways are shown. All contribute to osteoblast differentiation and bone formation via common transcriptional targets. Genes in italics are mutated in syndromic craniosynostosis. Genes in red font are negative regulators of signaling that have damaging de novo mutations in probands. Genes noted with yellow stars are known syndromic genes found mutated in kindreds with midline NSC.

Damaging de novo mutations in negative regulators of Wnt signaling included those in *AXIN1, MESP1, NPHP4, PSMC2, PSMC5,* and *DVL3.* AXIN1 is part of the complex that constitutively degrades β-catenin, inhibiting Wnt signaling. *MESP1* encodes a transcription factor that induces expression of DKKs, inhibitors of canonical Wnt signaling (16). Knockdown of NPHP4 results in accumulation of β-catenin, an effector of canonical Wnt signaling (17). DVL3 is part of a complex that recruits AXIN1 to the nuclear membrane upon Wnt activation of LRP5/6 signaling; however, DVL3 is also required for Wnt signal propagation (18, 19). PSMC2 and PSMC5 are components of the regulatory subunit of the 26S proteasome, and are known to bind ubiquitylated Wnt signaling substrates, promoting their degradation (20, 21). Thus, damaging mutations in these genes can plausibly increase signaling, contributing to NSC.
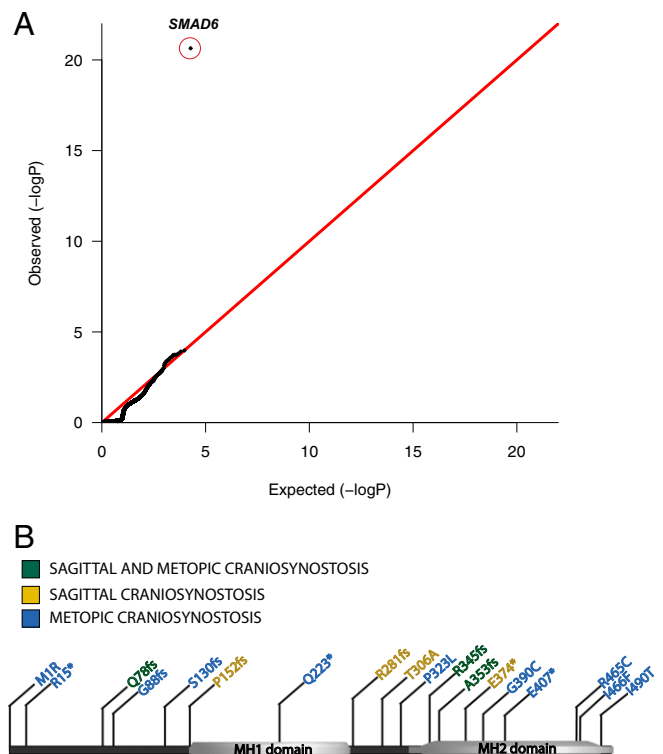
Damaging mutations in negative regulators of Ras/ERK signaling include those in *SPRY1, SPRY4, RASAL2,* and *ARAP3.* *SPRY1* and *SPRY4* are developmental regulators of EGF and FGF signaling that inhibit Ras activation. *RASAL2* is also an inhibitor of the Ras-cAMP pathway. *ARAP3* is an Arf and Rho GAP with a Ras-associating domain; however, its biological function is poorly characterized.

In addition to the above mutations there are several others of interest. Two probands with metopic NSC had de novo LOFs in chromatin modifiers (*SUV420H1* and *SMARCD2*); de novo LOFs in these two genes, as well as other chromatin modifiers, have previously been implicated in the pathogenesis of other congenital disorders, including autism and congenital heart disease (22–24), and de novo LOFs in other chromatin modifiers, including *ASXL1, KAT6A,* and *KMT2D* have been reported in rare cases of syndromic craniosynostosis (5), suggesting that this gene set plays a role in a smaller fraction of NSC cases. Additional interesting mutations include a de novo D-mis variant in *THBS1,* a gene with very high expression in midline sutures that regulates the bioavailability of FGF signaling substrates at the plasma membrane (25), and a de novo D-mis variant in *MAPK7,* a nuclear effector of several RTK signaling pathways (Table 3).

Transmitted Mutations in *SMAD6* and Epistasis with a Common Risk Allele. We previously reported a highly significant burden of de novo and rare [Exome Aggregation Consortium (ExAC) minor allele frequency (MAF) $< 2 \times 10^{-5}$] transmitted damaging alleles in *SMAD6.* Among 193 probands not previously reported, there were 5 novel *SMAD6* damaging alleles, including 4 LOF and 1 D-mis; 4 of these were transmitted and 1 was de novo. This frequency of rare transmitted and de novo mutations was not expected by chance ($P = 2.7 \times 10^{-7}$ by binomial test). As before, all four transmitting parents had no sign of craniosynostosis or other congenital anomalies. These results strongly replicate our prior finding. In the combined set of 384 probands there were 18 independent damaging *SMAD6* mutations (12 LOFs; LOF $P = 2.3 \times 10^{-21}$ by binomial test) (Fig. 2). In contrast, there were 0 LOFs among 3,337 parental controls and only 5 LOFs among ~59,000 non-Finnish European

Fig. 2. Quantile-quantile plot of *P* values for LOF alleles in all protein-coding genes in 384 craniosynostosis probands. Rare (ExAC frequency $< 2 \times 10^{-5}$) LOF alleles were identified in probands. The probability of the observed number of variants in each gene occurring by chance was calculated from the total number of observed variants and the proportion of the coding length of the exome comprising each gene using the binomial test. (*A*) The observed distribution of *P* values matches the expected binomial distribution with the exception of *SMAD6,* in which 12 LOF alleles are observed compared with the expected 0.10. (120-fold enrichment; $P = 2.28 \times 10^{-21}$). (*B*) Distribution of all damaging mutations in *SMAD6.*

alleles in ExAC. These *SMAD6* LOFs are thus enriched more than 100-fold versus controls, and damaging *SMAD6* alleles comprise 5% of all midline NSC: 8% of all metopic, 2% of all sagittal, and 30% of cases with both sagittal and metopic NSC.

We previously demonstrated incomplete penetrance of *SMAD6* mutations, and showed epistasis between these rare mutations and common risk alleles near *BMP2* that were the strongest signal in a prior genome-wide association study (GWAS) of sagittal NSC (10, 11). Following addition herein of five new kindreds with a de novo or rare transmitted *SMAD6* mutation, we reevaluated penetrance and interaction of *SMAD6* and *BMP2* alleles by association and parametric analysis of linkage under a two-locus model considering rare damaging alleles of *SMAD6* and the common risk allele of SNP rs1884302. The overall estimate of penetrance of rare damaging SMAD6 alleles, excluding probands, is 17%. Among all *SMAD6* mutation carriers, 24% of those without the common rs1884302 *BMP2* risk allele had NSC, whereas 94% with the *BMP2* risk allele had NSC ($P = 1.4 \times 10^{-5}$) (Table S7). There was also highly significant linkage of rare *SMAD6* mutations and common *BMP2* risk alleles under a parametric two-locus model [logarithm of the odds (LOD) score 8.2, odds >100 million:1 in favor of linkage], which is >1,400× more likely than the best single-locus model (Fig. S2 and Tables S8 and S9) (10).

No other individual gene approached genome-wide significance in analysis of LOF dominant alleles, and no gene had more than one rare recessive genotype. Among genes in these pathways with a damaging de novo mutation, *AXIN1* had two rare (MAF $< 2 \times 10^{-5}$) heterozygous transmitted damaging mutations

Table 3. Additional damaging de novo mutations of interest

Craniosynostosis type	Gene	Mutation	ExAC frequency	pLI
Sagittal	*ARHGEF18*	F1135fs	8.70×10^{-6}	0.91
Sagittal	*MACF1*	IVS89+1G > A	Novel	1.00
Sagittal	*MAPK7*	R235W	8.26×10^{-6}	0.23
Sagittal	*NAA25*	F359fs	Novel	1.00
Metopic	*SMARCD2*	R73*	Novel	0.98
Metopic	*SUV420H1*	T97fs	Novel	1.00
Sagittal + Left Coronal	*TCF12*	K287fs	Novel	0.97
Sagittal	*THBS1*	R980C	8.47×10^{-6}	1.00
Metopic	*ZCCHC11*	E1275fs	Novel	1.00

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(one LOF, one D-mis) and *RASAL2* had one. Interestingly, mice with genetic deficiency for the homolog *AXIN2* have midline craniosynostosis (26).

Because of the role of the *BMP2* risk allele at rs1884302 in modifying the phenotype of rare *SMAD6* mutations, we considered whether it might play a similar role with rare damaging alleles in other genes in these pathways. We used the transmission disequilibrium test to determine whether the *BMP2* risk allele was transmitted from heterozygous parents to affected offspring significantly more frequently than expected by chance in these 15 kindreds. Among 17 possible transmissions from heterozygous parents, the risk SNP was transmitted in 13 ($P = 0.03$). Considering only those kindreds with damaging de novo mutations in genes proposed to be more tolerant to heterozygous LOF mutation [measure of intolerance to LOF mutation (pLI) < 0.9; $n = 10$]—and perhaps in greater need of contribution from additional genetic or environmental factors to produce craniosynostosis—the risk SNP was transmitted in 10 of 11 possible transmissions (transmission disequilibrium test $P = 0.007$), including all 7 informative transmissions after exclusion of kindreds with de novo *SMAD6* mutations ($P = 0.008$). Although the numbers of events are small, this result suggests that the *BMP2* risk SNP might commonly interact with rare alleles in multiple pathways to produce midline craniosynostosis.

Variable Expressivity of Mutations in Syndromic Craniosynostosis Genes. There were 49 probands with 1 or more additional relatives with craniosynostosis in our cohort, including 45 probands without *SMAD6* mutations who had 49 affected first-degree relatives and 12 more distant relatives. Among these families, four had previously undescribed transmitted variants (absent in ExAC and other public databases) among 57 genes previously implicated in syndromic craniosynostoses (*TWIST1*, *TCF12*, *ERF*, and *MSX2*) (Table 4). By comparison, among 3,337 parental controls, there were only 9 damaging variants in 57 genes proposed to contribute to syndromic craniosynostosis (5) ($P = 1.9 \times 10^{-5}$ Fisher's exact test). All four mutations found in probands were shared by all affected members of these kindreds, an event not likely to occur by chance (probability of cosegregation by chance 1 in 64). These findings make it highly likely that these novel variants are causally related to disease in these families.

The novel damaging variant in *TWIST1* (p.Y160C) was identified in a parent and child who both had isolated sagittal craniosynostosis with no syndromic features. LOF mutations or missense mutations in the helix–loop–helix domain of *TWIST1* cause Saethre-Chotzen syndrome, which almost always involves either uni- or bicoronal craniosynostosis in conjunction with any combination of ptosis, hypertelorism, strabismus, epicanthal folds, syndactyly, dental malocclusion, and mild-to-moderate learning disability (27). P.Y160 lies at the border of the helix–loop–helix domain (amino acids 108–159).

The *TCF12* mutation (IVS12+2 > insT) was a "T" insertion that altered the splice donor site of intron 12 from GTGAG to GTTGAG. Multiple in silico splice predictors suggest that this mutant slice donor is less favorable than an alternative GTGAT sequence 29 bases upstream in exon 12 (*Methods*). This mutation was shared by a parent–offspring pair with isolated sagittal craniosynostosis. LOF mutations in *TCF12*, a transcription factor that

heterodimerizes with *TWIST1*, cause a syndrome with considerable phenotypic overlap with Saethre-Chotzen syndrome, almost always involving the coronal sutures (28). To our knowledge, there are no previously described cases of isolated sagittal NSC in conjunction with *TCF12* mutation.

The mutation in the transcription factor ERF (p.S532fs*3) was a novel frameshift and was shared among a proband, a sibling and their father who all had isolated metopic NSC with no syndromic features. LOF variants in *ERF* cause multisuture craniosynostosis with variable midface hypoplasia (29). To our knowledge, no cases of isolated metopic NSC have previously been described in conjunction with LOF mutation in *ERF*.

Finally, the mutation in the homeobox transcription factor *MSX2* (p.P148S) was a novel missense variant found in a proband with sagittal NSC and an aunt with bicoronal NSC. Remarkably, this mutation introduces a different substitution at the same codon in the homeodomain as two different mutations previously reported (p.P148L and p.P148H) in two large kindreds with autosomal dominant multisuture craniosynostosis, brachydactyly, and neurologic features, including headache and seizure (Boston-type craniosynostosis) (30, 31). Functional studies of the previously identified missense variants demonstrated that they induced a GOF effect via increased affinity of *MSX2* for its target DNA sequence (30, 32). Affected members of our kindred did not have brachydactyly or other syndromic features, and penetrance was incomplete, with an unaffected mother.

Given the atypical sutures involved and completely non-syndromic presentations in these four kindreds, these cases support variable expressivity of known disease loci (Fig. S3). These four genes are all nuclear effectors that modulate signaling downstream of Wnt, BMP, and Ras/ERK signaling, and strengthen the genetic evidence linking syndromic and NSC (Fig. 1).

Discussion

These results demonstrate that de novo damaging mutations with large effect in negative regulators of Wnt, BMP, and Ras/ERK pathways play a frequent role in midline NSC. The evidence supporting this conclusion is extremely strong with highly significant P values along with very strong prior biological support of the role of these pathways in craniosynostosis (1, 5). These findings provide strong genetic evidence linking the biology underlying non-syndromic and syndromic forms of craniosynostosis. Among the 12 genes in these pathways with de novo damaging mutations, >90% are expected to be disease-related. The large fraction of mutations that are LOF implies haploinsufficiency as the mechanism of genetic effect, with half the normal gene dose insufficient to provide normal suppression of signaling in development. This suggests these mutations increase signaling, a known mechanism for promoting bone formation and premature suture closure. We presume that syndromic genes act in multiple developmental processes and tissues, whereas non-syndromic genes are either sufficiently expressed in other tissues to not be dose-limited or can be compensated.

Mutations in these three pathways account for 10% of probands in this cohort, with rare damaging de novo or transmitted mutations in *SMAD6* found in 5%, damaging de novo mutations in 11 other negative regulators of these pathways in another 4%, and transmitted mutations in 4 genes previously implicated in syndromic craniosynostosis in 1%. De novo mutations in these pathways account for more than 60% of the excess of damaging de novo mutations seen in this cohort. GO analysis of pathway enrichment following removal of genes in these pathways identifies no additional pathways approaching significant enrichment.

These findings have implications for establishing genetic diagnosis and assessing risk of recurrent disease among NSC families. Among the 49 kindreds with familial disease, a likely genetic cause was identified in 16%, with 8% explained by rare

Table 4. Mutations in known syndromic genes in multiplex NSC kindreds

Craniosynostosis type	Gene	Mutation	ExAC frequency	pLI
Metopic	*ERF*	S532fs	Novel	0.81
Sagittal, Coronal	*MSX2*	P148S	Novel	0.42
Sagittal	*TCF12*	IVS12+2 > insT	Novel	0.97
Sagittal	*TWIST1*	Y160C	Novel	0.18

damaging transmitted variants in *SMAD6*, and another 8% with transmitted mutations in genes previously implicated in syndromic disease. As more subjects are sequenced and individual genes are more firmly implicated, the finding of a likely causal de novo mutation in an affected child will provide reassurance to parents that recurrence is unlikely. Similarly, the finding of causal transmitted genotypes can be used for prospective counseling.

Our finding of mutations in genes that characteristically cause syndromic disease in probands with NSC demonstrates variable phenotypic expression of mutations. In the case of *MSX2*, the finding of a distinct phenotype from a different substitution at P148 in *MSX2* strongly suggests an allelic effect; whether others are the result of stochastic factors, allele-specific effects, or genetic modifiers remains to be determined.

Like autism (24, 33–35) and congenital heart disease (22, 23), damaging mutations in many different genes contribute to NSC. From the observed excess of damaging de novo mutations and the number of genes mutated more than once, we estimate that the number of different genes that contribute to NSC is ~190 (Fig. S4). Because only *SMAD6* has more than one damaging de novo mutation, the confidence intervals of this estimate remain very large. This observation indicates that a large number of cases will need to be sequenced to approach acquisition of a complete set of midline craniosynostosis genes.

Although de novo mutations in these pathways impart very high relative risks of disease, the data provide significant evidence that the common risk allele near *BMP2* modifies disease risk for many of these genes, as it does for *SMAD6*. While the results for these other genes are statistically significant, they are based on a small number of observations. Genes with high pLI (e.g., >0.95) are highly predictive of those in which heterozygous LOF mutations impair reproductive fitness and impart disease phenotypes. This is not generally expected for LOF mutations in genes with low pLI. Interestingly, six of the genes with heterozygous de novo LOF mutations among negative regulators of the Wnt, BMP, and Ras/ERK pathways have low pLI (all <0.25); probands with mutations in these genes are particularly enriched for the common *BMP2* risk allele, consistent with these rare mutations being insufficient to consistently produce a disease phenotype without additional genetic contributions. This observation raises the broader question of whether other fitness-impairing traits associated with haploinsufficient mutations in genes with low pLI may also frequently require genetic or environmental modifiers to produce disease.

Whereas fusion of a cranial suture is the only physical manifestation of disease in NSC, more than one-third of all cases have subtle yet clinically recognizable neurocognitive deficits (36–38). The etiology of these deficits remains enigmatic to date. Precise regulation of BMP, Wnt, and Ras/ERK signaling is crucial to normal brain development (39–42), raising the possibility that genotype might influence neurocognitive outcome, as appears to be the case for congenital heart disease (22, 23). The results provide a means of stratifying non-syndromic patients to assess the extent to which specific genotypes contribute to neurocognitive outcome.

Finally, the genetic similarities and differences between syndromic and NSC are striking. Probands with craniosynostosis and distinctive extracranial phenotypes predictably have mutation in specific genes. In contrast, the genetic causes in probands with NSC are thus far explained in a small fraction of cases that are highly genetically heterogeneous, making their identification more challenging. Nevertheless, shared biological pathways provide genetic contributions to both rare syndromic and more common non-syndromic probands. Without the prior study of syndromic craniosynostosis, the pathway analysis that led to the current results would not have been as statistically powerful. This observation underscores the importance of solving all rare

syndromic cases, as they commonly prove relevant to identification of pathways relevant to non-syndromic disease. These considerations also support the general use of rigorous pathway analysis in the study of non-syndromic diseases that are likely highly genetically heterogeneous.

Methods

Subjects and Samples. Participants for this study were ascertained from the Yale Pediatric Craniofacial Clinic, the Pediatric Neurosurgery Clinic at the Medical University of Silesia, Poland, or by responding to an invitation posted on the Cranio Kids–Craniosynostosis Support and Craniosynostosis–Positional Plagiocephaly Support Facebook pages. All participating individuals or their parents provided written informed consent to participate in a study of the genetic cause of craniosynostosis in their family. Inclusion criteria included a diagnosis of sagittal or metopic craniosynostosis in the absence of known syndromic forms of disease by a craniofacial plastic surgeon or pediatric neurosurgeon. All probands had undergone reconstructive surgery. Participating family members provided buccal swab samples (Isohelix SK-2S buccal swabs) or saliva samples (DNA Genotek OGR-575), craniofacial phenotype data, medical records, operative reports, and imaging studies. The study protocol was approved by the Yale Human Investigation Committee Institutional Review Board.

Control trios were those sequenced from the Simons Foundation Autism Research Initiative Simplex Collection (24, 35, 43). Simplex families composed of two unaffected parents, one child with autism, and one unaffected sibling, underwent whole-exome sequencing, with 1,789 trios of unaffected family members serving as controls for this study.

Exome Sequencing and Analysis. DNA was prepared from buccal swab or saliva samples according to the manufacturer's protocol. Exome sequencing was performed by exon capture using the Roche MedExome, Roche V2, or IDT xGen capture reagent, followed by either 74 or 99 base paired-end sequencing on the Illumina HiSEq. 2000.

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 (12). VQSR "PASS" variants with ExAC allele frequency $\leq 10^{-3}$ sequenced to a depth of 8 or greater in the proband and 10 or greater in each parent with Phred-scaled genotype likelihood scores >30 and de novo quality scores [$\log_{10}$(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. Fifty de novo mutations were randomly selected for validation by bidirectional Sanger sequencing of the proband and both parents; 100% of these tests confirmed de novo mutation in the proband. All de novo variants contributing to significant results, specifically each of the damaging variants identified in the gene set for negative regulation of Wnt, BMP, and Ras/ERK signaling, were confirmed by Sanger sequencing. Transmitted variants were called as per above. All de novo and transmitted variants were annotated using ANNOVAR (44). Allele frequencies of identified variants were taken from the ExAC database. The impact of nonsynonymous variants was predicted using the MetaSVM rank score, with scores greater than 0.83357 serving as a threshold for predicting that the mutation was deleterious (MetaSVM "D", D-mis) (45). Transmitted variants identified in known craniosynostosis genes (Table 4) were confirmed by Sanger sequencing.

Burden of de Novo Mutations. Statistical analysis of the burden of de novo mutations in craniosynostosis cases and autism controls were performed in R using the denovolyzeR package, as previously described (10, 46). The expected number of de novo mutations in case and control cohorts across variant classes was calculated, and this value was compared with the observed number in each cohort using Poisson statistics (13). For gene-set enrichment analyses, only mutations observed or expected in genes within the specified gene set were included in each statistical test.

Contribution of de Novo Mutation to Craniosynostosis. The number of cases with protein-altering de novo mutations genes was calculated. This number ($n = 89$), minus the number expected by chance ($n = 66.9$), is the number of mutations expected to contribute to craniosynostosis risk ($n = 22.1$). Dividing this number by the total number of trios ($n = 291$) provides the percentage of cases in which damaging de novo mutations genes are expected to cause craniosynostosis (7.6%).

Binomial Test. The observed distribution of rare (ExAC frequency $< 2 \times 10^{-5}$) LOF alleles was compared with the expected distribution using the binomial test. The total number of LOF alleles in 384 probands was tabulated ($n = 2,260$), and the expected number for each gene was calculated from the proportion of the exome comprising the coding region of each gene, multiplied by the total number of LOF alleles identified in cases. Enrichment was calculated as the number of observed mutations divided by the expected number.

In Silico Splice-Site Prediction. Splice-site predictors Human Splicing Finder (47) and Genie (48) were used to assess the impact of a single nucleotide insertion at the splice donor site of intron 12 of *TCF12* that changed the donor site sequence from GTGAG to GTTGAG. Both programs predicted that the insertion resulted in a more favorable splice site 29 bases upstream in exon 12. Genie assigned a score of 0.55 to the wild-type canonical splice donor site; however, after mutation of the wild-type sequence to GTTGAG, the alternate splice donor 29 bases upstream was assigned a score of 0.52, while the GTTGAG sequence had a score <0.1. Using Human Splicing Finder, the wild-type donor-site sequence was deemed the most likely donor site with a MaxENT score of 7.0; however, after mutation of this sequence to GTTGAG, the alternative sequence 29 bases upstream became the most likely donor site with a MaxENT score of 5.99.

Estimating the Number of Risk Genes in Midline Craniosynostosis. We followed a previously described method to estimate the number of genes in which damaging de novo mutations contribute risk to craniosynostosis (23). In summary, the number of observed damaging mutations in 291 craniosynostosis cases (K), the number of those genes mutated twice ($R1$), and number of those genes mutated at least three times ($R2$) was tabulated. We next estimated the fraction of damaging alleles in risk genes based on observed enrichment in cases compared with expectation [$E = (M1 - M2)/M1$, where $M1$ and $M2$ are the numbers of damaging mutations in cases and expectation, respectively]. The likelihood of risk gene number $L(G)$ was simulated, with the number of damaging mutations (K) fixed at the observed number in cases. G risk genes were selected from all genes with equal probability, and the number of contributing damaging mutations in risk genes ($C1$) was established by sampling from a binomial distribution Binom(K,E). The number of noncontributing damaging mutations was set as $C2 = K - C1$. We then simulated $C1$ contributing damaging mutations by sampling with replacement from G genes and $C2$ noncontributing mutations from all genes using their background mutation rate as probability weights. In this process, 20,000 simulations were performed for every G from 1 to 1,000, and $L(G)$ was set to be the proportion of simulations in which the number of genes with two damaging mutations was exactly $R1$ and the number of genes with more than two damaging mutations was exactly $R2$.

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