

A. Specific Aims:

The presentation of craniosynostosis, the premature fusion of cranial sutures, can involve a single suture or multiple sutures and can be associated with other malformations. Midface hypoplasia (underdevelopment of the midface) is a significant co-morbidity of craniosynostosis. A recent review of over 769 craniosynostosis cases seen at the Seattle Craniofacial Center has found that ~40% of patients with complex craniosynostosis (excluding the known syndromic craniosynostoses) and ~12% of cases with isolated single suture craniosynostosis have midfacial involvement. These data suggest a common genetic basis yet little is known about the genes involved.

Specific Aim 1: To quantitatively characterize the appearance and progression of midface hypoplasia and other cranial deformations in seven new mutant mouse lines as potential new models of craniosynostosis-associated midface hypoplasia.

Specific Aim 2: To determine the underlying genetic mutation responsible for each new mutant using exome sequencing technology.

B. Studies and Results:

Specific Aim 1 – Since the initial progress report, three of the remaining four lines of mutant mice have been recovered from cryopreservation at the Jackson Laboratories and are now successfully maintained in the vivarium at the Seattle Children’s Research Institute. The final line to be obtained from the Jackson died out prior to shipment to SCRI due to breeding difficulties resulting from a fertility issue. As a substitute, we therefore characterized the craniofacial phenotype in the *bat* mutant line. The *bat* line carries a splice site mutation in *Frem1*, a gene that we identified as the likely cause of a form of metopic craniosynostosis in humans that is also associated with midface hypoplasia (Vissers et al, 2011).

High resolution microCT phenotyping has been conducted on ~180 new mutant (*sbse*, *snol*, *shsn*, *frg*, *Sofa*, and *stn*) and genetic background controls (C57Bl/6J, A/J, and C3H/HeJ). In addition, nearly 50 homozygous *bat*, heterozygous *bat*, and their controls were also scanned. To facilitate simple detection of pathogenic premature fusions, the majority of the scans have been conducted between at postnatal day 23 and day 28, as all cranial and facial sutures are normally patent at these ages. The craniofacial suture fusions and other craniofacial findings recorded to date in each of the six mutant strains are summarized in Table 1.

Ongoing imaging and phenotyping is being performed for some mutants to ensure the spectrum of variable features is

Table 1: Craniofacial suture fusions in the six mouse mutants.

	Sofa	stn	shsn	sbse	frg	snol
suture fusion						
<i>premax-frontal</i>	?		+	+	+	+
<i>premax-max</i>	+		+	+		
<i>max-frontal</i>		+	+	+		
<i>nasal-frontal</i>	+	+				
<i>metopic</i>				+	+	
<i>interfrontal</i>	+*	+*	+**	+	+*	
<i>coronal</i>	?	+	+	+		
<i>sagittal</i>	?					
<i>lambdoid</i>				+		
<i>cranial base</i>			+		+	
<i>zygomatic</i>				+		
midface hypoplasia/asymmetry	+	+	+	+	+	+***
bulbous skull				+		+
nasal bone hypoplasia	+	+	+	+	+	
nasal bone anomaly					+	
other metopic anomaly		+	+			
malocclusion			+			+
mandible hypoplasia/asymmetry				+	+	
hydrocephaly			+			
tooth root exposed			+	+		
cervical anomalies				+		
abnormally positioned ears				+	+	
gene	<i>Pfas</i>	ND			ND	

*wide, diamond shaped interfrontal; ** wide triangular shaped interfrontal

fully appreciated. In addition, mutants of other ages, specifically neonates and late stage embryos, will be assessed for certain mutants to determine the timing of appearance of the various anomalies and to provide insight into the nature of the pathology.

Specific Aim 2 – During the course of this reporting period, genomic DNAs from three of the mouse mutants (*Sofa*, *stn* and *frg*) were utilized for the development of a sequencing platform suitable for assessing the entire mouse exome (the exome representing the coding portions of all genes within the genome). In the last few years whole exome sequencing of human genomes has proven a fruitful approach to identifying disease-causing genes, as aptly demonstrated by our collaborators in the Department of Genome Sciences, University of Washington. Given the high conservation of most genes between mouse and humans, we initially assessed the suitability of using human exome arrays to capture corresponding murine sequences. This approach proved insufficient for complete capture and sequencing of the mouse DNA. Consequently, we worked with Roche-NimbleGen and the Jackson Laboratories to develop specific murine exome capture arrays. This new approach was very successful as demonstrated by the high level coverage of sequences across all mouse genes. Using this approach, we identified a 15 bp deletion in the *Pfas* gene (Figure 1A; Fairfield et al, 2011). *Pfas* encodes the multi-domain enzyme, phosphor-ribosyl-formyl-glycinamide synthase, which catalyzes the fourth step in *de novo* purine biosynthesis. Purines play a number of essential cellular roles aside from providing the precursors for RNA and DNA synthesis, including functioning in energy transfer, and as coenzymes and regulatory molecules (Alexiou & Leese, 1992; Brodsky et al, 1997).

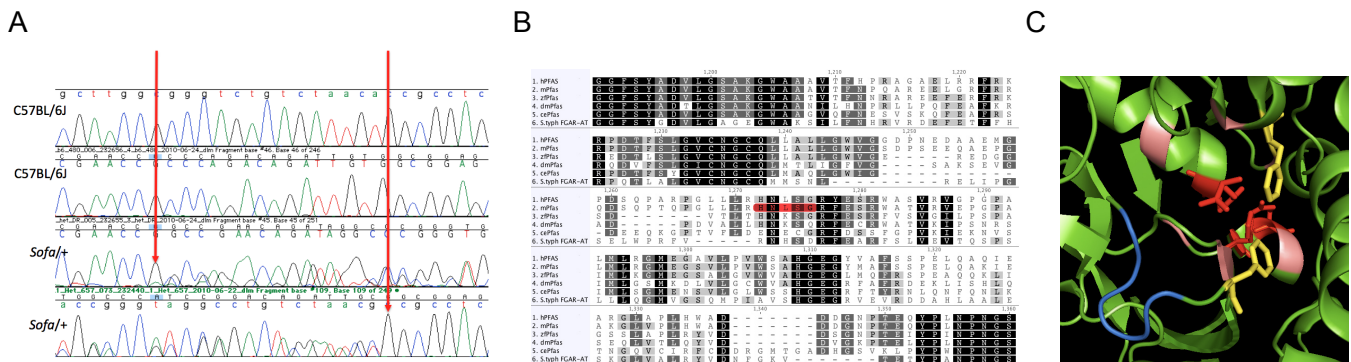


Figure 1: Sofa is caused by an in frame deletion in *Pfas*. (A) Alignment of C57Bl/6J control and *Sofa* heterozygote DNA sequence over part of the *Pfas* gene reveals a 15bp deletion, defined by red arrows. (B) The mouse *Pfas* protein sequence was aligned with the *Pfas* sequences from humans, zebrafish, *D.melanogaster*, and *C.elegans*, as well as with the FGAR-AT enzyme from *S.typhimurium*. The mutation in the *Sofa* mutant is an in frame deletion of 5 amino acids (highlighted in red) in a region of only moderate evolutionary conservation. Residues highlighted in black are completely conserved, those in dark grey are conserved in 5 of the 6 species, while those in light grey are conserved in 4 of the 6 species. (C) The 5 amino acids homologous to those deleted in the *Sofa* mutant were located on the modeled 3D crystal structure of the *S.typhimurium* PurL enzyme. The central glutaminase catalytic site is shown. The defining catalytic triad of residues (Cys-His-Glu) are highlighted in red. Other residues of the catalytic site are in pink and yellow. The glutaminase active site converts L-glutamine to L-glutamate, releasing ammonium, which is 'channeled' within the enzyme to the adjacent FGAR synthetase catalytic site (not shown). The two phenylalanine residues that form the 'gate' to the proposed ammonium channel are indicated in yellow. The homologous residues to those deleted in the *Sofa* mutant are indicated in blue. This five amino acid deletion is expected to impact the positioning of the adjacent phenylalanine and hence ammonium channeling and overall enzyme activity.

Rather than causing a frameshift, this mutation in *Pfas* causes a 5 amino acid in frame deletion in the glutaminase domain at the C-terminus of the enzyme (Figure 1B). To determine whether this in frame deletion is likely causative, we aligned the amino acid sequence with that of the orthologous *Salmonella typhimurium* PurL enzyme, a dual catalytic enzyme for which the 3D crystal structure has been determined. This analysis revealed that the homologous mPfas residues line the proposed central channel of PurL/Pfas through which ammonia, the product of the glutaminase reaction, is transferred to the FGAM synthetase catalytic site (Figure 1C). It is therefore likely that loss of these five residues would impact the channeling of ammonia between the two active sites of the enzyme and thus affect enzyme function. In this regard, homozygotes for the *Sofa* mutation are embryonic lethal at an as yet undefined developmental stage, consistent with the importance of the *de novo* pathway for purine biosynthesis in

early mammalian development (Alexiou & Leese, 1992). The dominant nature of the *Sofa* mutation likely reflects the many important roles of purines in these early proliferating tissues. *Sofa* is the first mouse phenotype to be associated with perturbed purine biosynthesis.

In contrast to the dominant *Sofa* mutant, we surprisingly did not identify any causative changes in the exomes of either of the two recessive mutations, *stn* and *frg*. This may indicate that such recessive mutations are caused by regulatory changes that lie outside the exomic regions captured and analyzed in these initial studies. Nevertheless, we have demonstrated for the first time that whole exome sequencing can be successfully applied to identify the cause of specific mouse mutants.

As the remaining mutants, including *stn* and *frg*, are all recessive, we have chosen not to pursue these with further exome-based sequencing. Rather, we will perform either mapping interval-specific or whole genome sequencing to cover the likelihood of non-coding changes being responsible for the phenotypes in these craniofacial mutants. The cost of this option has reduced considerably and our Genome Sciences colleagues are keen to help develop this for the mouse.

C. Significance

Our unique capabilities to quantitatively image the developing skull in mice, combined with advances in high-throughput genetic analysis, has enabled the identification of new models of craniosynostosis-associated midface hypoplasia that will provide valuable tools by which to understand both the genetic and ultimately the 'environmental' factors contributing to the susceptibility and severity in presentation of various types of craniosynostosis. Our investigations will continue to complement the ongoing genetic studies being conducted with the participation of a large number of patients with craniosynostosis being seen at Seattle Children's Craniofacial Center and promise to identify new causes of human craniosynostosis, and therefore more accurate diagnosis and better prognostic information for families. Our results to date provide one new candidate gene and implicate a previously unsuspected pathway in craniosynostosis-associated midface hypoplasia.

D. Plans

There are no major modifications to our original plans.

E. Publications

1. *Vissers, L.E.L.M., ***COX, T.C.**, *Maga, A.M., Short, K.M., Wirdajaja, F., Janssen, I.M., Jehee, F., Bertola, D., Liu, J., Yagnik, G., Sekiguchi, K., Kiyozumi, D., van Bokhoven, H., Marcelis, C., Cunningham, M.L., Anderson, P.J., Boyadjiev, S., Passos-Buenos, M-R., Veltman, J.A., Smyth, I., Buckley, M.F., & Roscioli, T. (2011) Heterozygous mutations of *FREM1* are associated with an increased risk of isolated metopic craniosynostosis in humans and mice. ***PLoS Genetics*** 7(9): e1002278. doi:10.1371/journal.pgen.1002278.
**authors contributed equally to this work.*
2. Fairfield, H., Gilbert, G., Barter, M., Corrigan, R., Curtain, M., Ding, Y., D'Ascenzo, M., Gerhardt, D., He, C., Huang, W., Richmond, T., Rowe, L., Probst, F.J., Bergstrom, D., Murray, S., Bult, C., Richardson, J., Cunningham, M.L., **COX, T.C.**, Justice, M., Spector, M.S., Lowe, S.W., Albert, T., Kile, B., Gut, I., Hager, J., Donahue, L-R., Jeddloh, J., Shendure, J., Reinholdt, L.G. (2011) Mutation discovery in mice by whole exome sequencing. ***Genome Biology*** 12:R86 doi:10.1186/gb-2011-12-9-r86.