A New Point Mutation in *Col2a1* Causes a Shortened Nose and Cleft Palate

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Mutation (allele) symbol: *Col2a1*^{M2J}

Mutation (allele) name: mutation 2 Jackson

Strain of origin: (C3HeB/FeJ x C57BL/6J)F1

Current strain name: B6;C3Fe-*Col2a1*^{M2J}/GrsrJ

Stock #012724 (jaxmice.jax.org)

Phenotype categories: craniofacial, cleft palate

Origin and Description

A mutant female with a shortened face was identified in the F1 offspring of a C3HeB/FeJ female bred to an N-ethyl-N-nitrosourea treated C57BL/6J male. This mutant female was bred to a C57BL/6J male and the mutant offspring proved this phenotype dominant. The mutant line was then maintained by sibling intercrossing for 4 generations, and then backcrossed once to C57BL/6J due to a breeding slump. The phenotype followed for breeding was a dominant short nose, but embryonic cleft palate was also observed in some mutants. Scoring 122 offspring from wildtype x heterozygous matings, 69 of the offspring (57%) had the short nose. None of 42 embryos at E16.5 from wildtype x heterozgyous matings displayed cleft palate, but 14 of 67 embryos at E16.5 or E17.5 from heterozygous intercrosses did display cleft palate. Both female and male heterozygotes are fertile and no evidence of incomplete penetrance was found.

Genetic analysis

To determine the genetic map position, a heterozygote was outcrossed to BALB/cByJ and affected F1 offspring were mated to unrelated C57BL/6J. DNA from sixteen affected and nine unaffected N2 offspring was sent to the Fine Mapping Laboratory at The Jackson Laboratory. This mutation was found to map to Chromosome 15 between *D15Mit159* at 87 Mb and *D15jmp37* at 99 Mb. The *Col2a1* gene is at 97.8 Mb and whole exome sequencing produced two SNPs in this gene that were candidates for validation. The most likely causative lesion is a nonsense mutation in exon 33 that introduces a premature stop codon. The second closely linked SNP is an A to T transversion in intron 12 that could potentially act as a cryptic splice site[1].

Homozygous and heterozygous embryos from the F2 mapping cross were used for RT-PCR to see if the missense mutation in intron 12 was a cryptic splice site. No differences were found that would rule out the potential splice site as causative. Using the Primer3 Design program, primers were designed around the nonsense mutation in exon 33. When sequenced, seven phenotypic heterozygotes typed positive for both A and G at that locus,

and eight phenotypic wild-type mice typed only G (matching the published Ensembl sequence), confirming exon 33 as the location of the *M2J* mutation.

To further examine potential embryonic lethal phenotypes, a heterozygote was mated with a BALB/cByJ and affected F1s were subsequently mated in sibling pairs. Some E.16.5 F2 embryos had cleft palate. However of seven affected F2 embryos genotyped for *D15Mit242* at 90 Mb in the non-recombinant region, 5 cleft palate mice genotyped as heterozygous and 2 as homozygous. After the mutation was identified and confirmed to be in *Col2a1* we sequenced seven more cleft palate embryos for the exon 33 point mutation and three typed as heterozygotes and four as homozygotes.

Biological characterization

Craniofacial morphology of twelve-week-old mice

Skulls were prepared by incomplete maceration in potassium hydroxide, stained with alizarin red, and stored in undiluted glycerin (Green, 1952). Morphological measurements of the skull (see protocols below) were made using digital calipers (Stoelting, Wood Dale, Ill) with previously established landmarks (Richtsmeier, 2000).

Table 1: Measurements and Calculated Ratios of Twelve-week-old B6;C3Fe-*Col2a1* ^{M2J}/GrsrJ skulls stained with Alizarin Red
(n=6; mean± SEM; $^{ab}p \leq 0.05$)

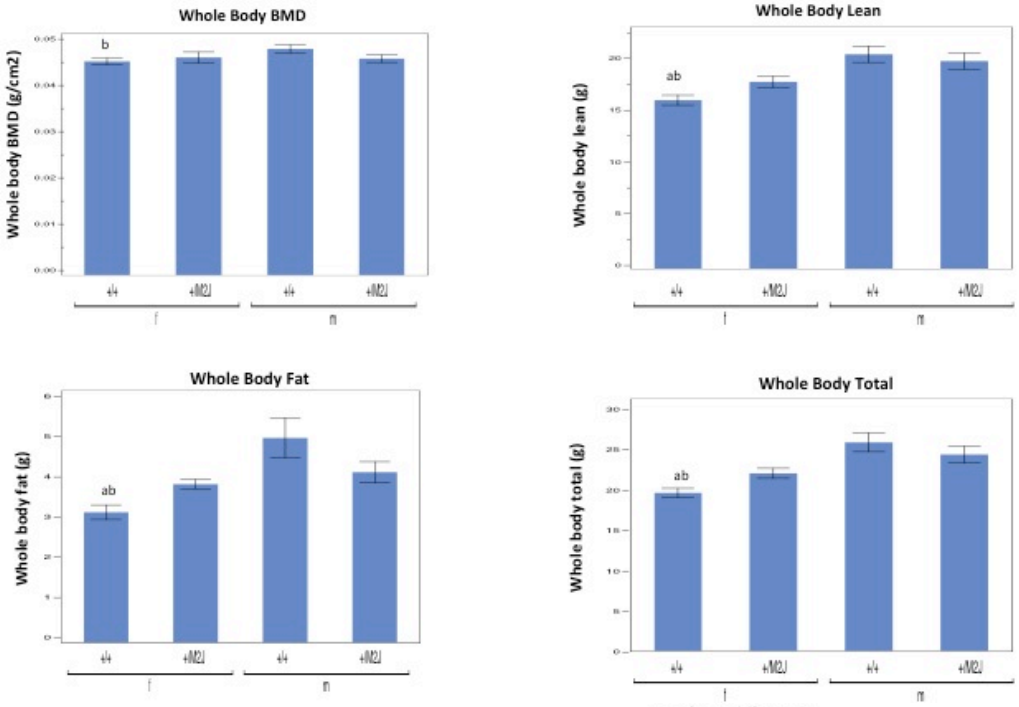
Measurements	Male +/+	Male *+/M2J*	Female +/+	Female *+/M2J*
Skull Length	22.48± 0.264[a]	20.58± 0.193	22.38± 0.244[a]	20.91± 0.320
Nose Length	15.72± 0.133[a]	13.99± 0.249	15.53± 0.077[a]	14.55± 0.336
Skull Height	10.66± 0.119	10.35± 0.324	10.45± 0.133	10.17± 0.232
Skull Width	10.67± 0.171	10.86± 0.163	10.73± 0.081	10.54± 0.174
Inner Canthal Distance	6.62± 0.106	6.97± 0.269	6.15± 0.190	6.31± 0.430
Lower Jaw Length	11.09± 0.168	10.66± 0.137	11.06± 0.092	10.89± 0.139
Upper Jaw Length	16.54± 0.147[a]	15.07± 0.184	16.42± 0.162[a]	15.33± 0.415
Jaw Length Ratio	1.49± 0.023[a]	1.42± 0.023	1.49± 0.019	1.41± 0.037
Skull/Nose Length Ratio	1.43± 0.024	1.47± 0.018	1.44± 0.017	1.44± 0.045
Skull Height/ Length Ratio	0.48± 0.007[a]	0.51± 0.007	0.47± 0.010	0.49± 0.011
Skull Length/ Width Ratio	2.11± 0.035[a]	1.90± 0.028	2.09± 0.023	1.99± 0.049
Skull Height/Width Ratio	1.00± 0.015[a]	0.96± 0.010	0.97± 0.012	0.97± 0.243
Right Ear Pinna Length	13.92± 0.433	13.71± 0.109	14.01± 0.175	13.91± 0.158

The differences in skull, nose and upper jaw lengths in male and female controls versus mutants were all statistically significant. The ratios of skull height to length, length to width and height to width were statistically significant in only the males (graphs 2, 3, 4).

Graph 1

12 Week old B6;C3Fe-*Col2a1* ^{M2J}/GrsrJ
split by sex and genotype (n=6; mean± SEM p≤ 0.05)

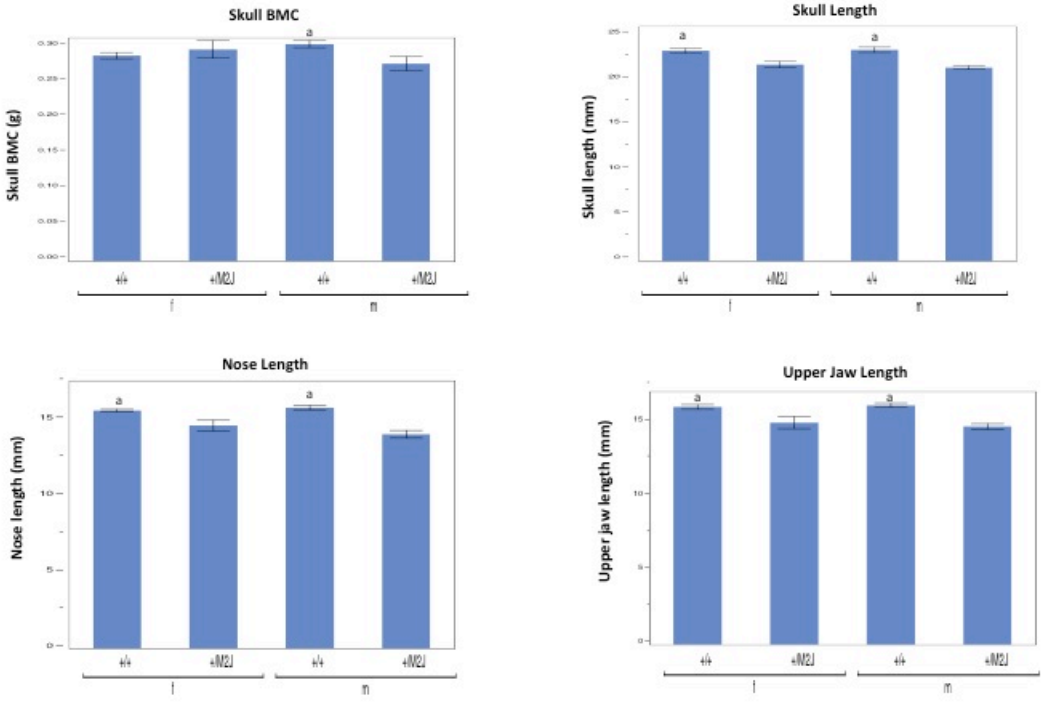
Significant differences
a *+/M2J* vs. *+/+* within sex
b male vs. female within genotype



Graph 2

12 Week old B6;C3Fe-*Col2a1* ^{M2J}/GrsrJ
split by sex and genotype (n=6; mean± SEM p≤ 0.05)

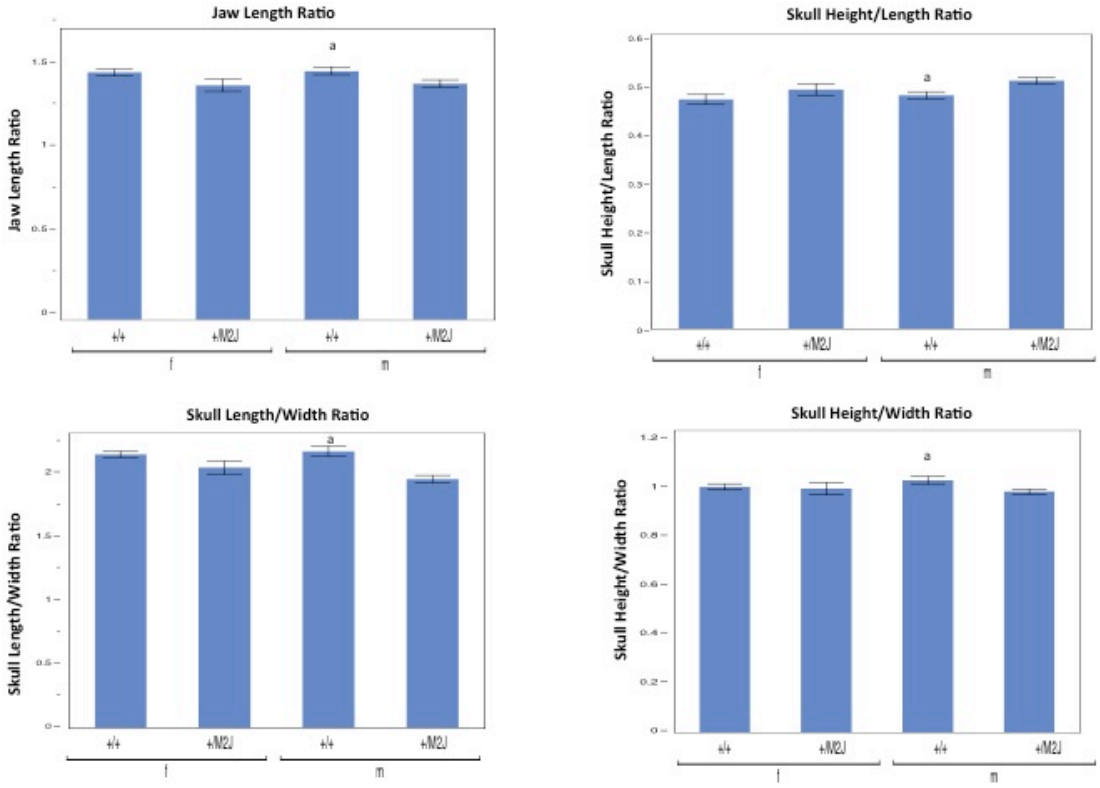
Significant differences
a *+/M2J* vs. *+/+* within sex
b male vs. female within genotype



Graph 3

12 Week old B6;C3Fe-*Col2a1* ^{M2J}/GrsrJ
split by sex and genotype (n=6; mean± SEM p≤ 0.05)

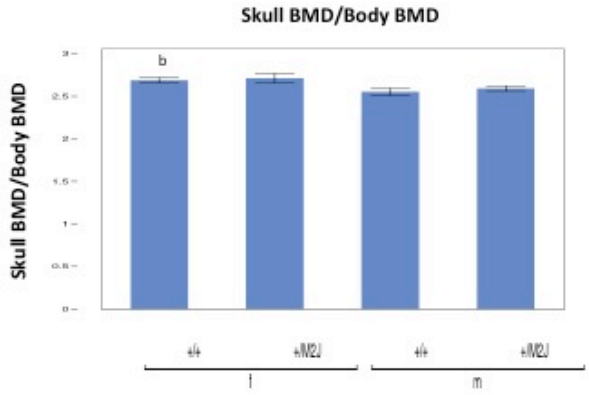
Significant differences
a *+/M2J* vs. *+/+* within sex
b male vs. female within genotype



Graph 4

12 Week old B6;C3Fe-*Col2a1* ^{M2J}/GrsrJ
split by sex and genotype (n=6; mean± SEM p≤ 0.05)

Significant differences
a *+/M2J* vs. *+/+* within sex
b male vs. female within genotype



DEXA analysis of whole body aBMD and body composition of twelve-week-old mice
Whole body, areal bone mineral density (aBMD), bone mineral content (BMC) and body composition (lean, fat and % fat mass) were assessed by PIXImus densitometry (GE LUNAR, Madison, WI)

Table 2: PIXImus Densitometric Measurements of
Twelve-week-old B6;C3Fe-*Col2a1* ^{M2J}/GrsrJ
(n=6; mean± SEM; $^{ab}p \leq 0.05$)

Measurements	Male +/+	Male +/*M2J*	Female +/+	Female +/*M2J*
Whole Body BMD (g/cm2)	0.0467± 0.00082[b]	00447± 0.00076	0.04413± 0.00060	0.0450± 0.00110
Whole Body BMC (g)	0.454± 0.0126	0.454± 0.0308	0.422± 0.0137	0.429± 0.0127
Whole Body Lean (g)	19.93± 0.754[b]	19.30± 0.742	15.67± 0.463[a]	17.37± 0.506
Whole Body Fat (g)	4.9± 0.48[b]	4.1± 0.25	3.1± 0.17[a]	3.8± 0.11
Total Mass (g)	24.78± 1.113[b]	23.35± 0.975	18.80± 0.541[a]	21.12± 0.589
% Fat	20	18	17	18
Skull BMD (g/cm2)	0.1165± 0.00118	0.1134± 0.00176	0.1160± 0.00132	0.1191± 0.00325
Skull BMC (g)	0.291± 0.0047[a]	0.265± 0.0093	0.276± 0.0042	0.284± 0.01178
Skull BMD/Body BMD	2.4983± 0.03851[b]	2.5367± 0.02906	2.6300± 0.02840	2.6533± 0.05251

Whole body lean, mass and fat differences were all statistically significant between female mutant and controls and skull BMC differed significantly between male mutants versus controls (graphs 1 and 2).

Pathology

A standard pathology screen of a 7-week-old female mutant and heterozygote littermate revealed no lesions. Hearing was assessed by auditory brainstem response testing. A one-month-old mutant and two wild-type mice all had normal hearing. In three-month-old mice, one mutant and one control had some hearing loss while another mutant had normal hearing. There appear to be no hearing problems associated with the *Col2a1*^{M2J} allele. A clinical eye exam on female and male mutants and controls at 7 weeks of age concluded that all had normal eyes.

Discussion

This ENU induced mutation has a phenotype consistent with that of other Col2a1 mutants, which often have complete perinatal lethality and some cleft palate occurring in homozygotes and craniofacial defects in heterozygotes. Cleft palate has been reported in *Col2a1*^{tm1Prc} heterozygotes with incomplete penetrance, consistent with our findings for *Col2a1*^{M2J}.

Acknowledgements
For their expertise and assistance, we wish to acknowledge: Coleen Kane for preparation of tissues for histological assessment; Roderick T. Bronson, Ph.D. for pathological evaluation; Chantal Longo-Guess for ABR analysis; Bo Chang, M.D. for eye examination; Griff Gilbert for RT-PCR; and Heather Fairfield for sequencing and analysis.

References
1) Fairfield H et al. 2011. Mutation discovery in mice by whole exome sequencing. Genome Biol. 12(9):R86. PMCID: PMC3308049.

Protocols
Standard Protocols and Procedures of the JAX® Craniofacial Resource
Mouse Colony Maintenance
Craniofacial Resource mice are housed in 51 square inch polycarbonate boxes, on bedding composed of sterilized shavings of Northern White Pine, under 14:10 hour light:dark cycles. A diet of autoclaved NIH 31 (6% fat diet, Ca:P of 1.15:0.85, 19% protein, vitamin and mineral fortified; Purina Mills International, Richmond, IN) and water acidified with HCl to achieve a pH of 2.8-3.2 (which prevents bacterial growth) are freely available. Mouse colony maintenance and use is reviewed and approved by The Jackson Laboratory Institutional Animal Care and Use Committee and is in accordance with The National Institutes of Health guidelines for the care and use of animals in research.

PIXImus Densitometry
PIXImus scans (PIXImus, LUNAR, Madison, WI) which provide skeletal and body composition data such as Bone Mineral Density (BMD, g/cm2), Bone Mineral Content (BMC, g), body mass (g), lean mass (g), fat mass (g), and % fat mass, are completed on groups of 6 male and 6 female 12 week old mutant and control mice. The skulls and bodies are scanned separately to provide independent data on skull BMD and BMC and body BMD and BMC. The PIXImus small animal densitometer (DEXA) has a resolution of 0.18 x 0.18 mm pixels and is equipped with software version 1.46 . The PIXImus is calibrated routinely with a phantom utilizing known values, and a quality assurance test is performed daily. The variability in precision for measuring total body BMD is, less than 1%, and approximately 1.5% for specialized regions such as the skull. The correlation between PIXImus BMD measurements of 614 lumbar vertebrae compared to peripheral quantitative computerized tomography (pQCT) measurements was found to be significant ($p<0.001$; $r=.704$) (Donahue, 1999) .

Faxitron X-rays
X-rays at 5X magnification of the skull and at 3X magnification of the body of a male and female mutant and control at 12 weeks of age are obtained using a Faxitron MX20 cabinet X-ray (Faxitron X-Ray Corp., Wheeling, IL. USA) and Kodak Min-R 2000 mammography film (Eastman Kodak Co., Windsor, CO, USA). X-rays are then analyzed to determine the specificity of the skeletal phenotype.

Skull Preparation
Skulls of 6 male and 6 female mutants and controls are collected at 12 weeks of age, prepared by incomplete maceration in potassium hydroxide, stained with alizarin red, and stored in undiluted glycerin (Green, 1952). During the collection process, right ear pinnae are measured with digital hand calipers (Stoelting, Wood Dale, IL, USA).

Hand Caliper Skull Measurements
Seven measurements taken with hand held digital calipers are used routinely to define skull morphology at the Lab's craniofacial resource. These measures have a high degree of accuracy and precision in our hands and are able to discriminate differences between mutant and control skull characteristics. Our linear measures have been added to those illustrated by Dr. Joan Richtsmeier in

her paper characterizing craniofacial differences in mouse models of Down Syndrome using three dimensional anatomical landmarks (Richtsmeier, 2000. Dev. Dyn. Feb; 217(2):137-45). Skulls are cleared with potassium hydroxide and stained with alizarin red dye in preparation for caliper measurements to be taken.

Skeletal Preps

In many cases whole skeletons of mutant and control mice are cleared in 1% KOH, stained with alizarin, stored in glycerin (Green, 1952) and then evaluated for skeletal malformations. Malformations found can indicate that the craniofacial phenotype is part of a greater syndrome.

Data Analysis

Hand caliper skull measurements and PIXImus skeletal and body composition data are evaluated using StatView 4.5 software (Abaccus Cary, NC) for Macintosh computers. Differences are considered significant when $p < 0.05$.

Molecular Mapping

Mutations resulting in unique phenotypes are genetically mapped to establish the chromosomal location of the causative gene. In the past, the Craniofacial Mutant Resource relied on high-resolution mapping to narrow the genetic interval to a manageable size for candidate gene analysis. With the advent of sequence capture and high throughput sequencing (HTPS) techniques, this level of resolution is no longer required. Genetic mapping to rough chromosomal position still provides a number of useful advantages however, such as a reduced computational burden, fewer variants to validate, and greater confidence in variant causality. To identify causative mutations we use a combination of genetic mapping and HTPS. We have found that mapping a gene at least to a chromosome greatly facilitates the analyses of HTPS. Our studies use a combination of 1-10 Mb interval-specific, gene-specific, and whole exome approaches to identify a wide spectrum of mutation types across diverse genetic backgrounds.

Sequencing

Exome capture and sequencing

Once linkage is established and a broad chromosomal location identified, we employ whole exome capture and HTPS to identify potential causative variants. Whole DNA exomes from mutant samples are captured using an in-solution, hybridization-based probe pool developed in our group in collaboration with Roche-Nimblegen. The content of the probe pool is defined by the unified mouse gene catalog which, excluding UTR sequences, olfactory receptors and pseudogenes, encompasses approximately 50 Mb of genomic sequence. Our preliminary exome data indicate high capture sensitivity and specificity, >96.7% of the targeted bases covered with just one lane of 75 bp paired-end on the Illumina GAIIx. Our primary sequencing approach is to sequence whole exomes from enriched mutant DNA samples and to multiplex where possible (Fairfield, et al., 2011). An additional advantage of the paired end sequencing approach is that it provides positional information that is critical for the identification of spontaneous mutations that are due to genome rearrangements (larger insertions or deletions).

Analysis and validation

All raw sequence data analysis, including read mapping and SNP/mutation calling are performed by the Computational Science service at JAX, using Galaxy sequence analysis tools. Multiple candidate variations are detected in each strain, but most are eliminated upon validation. For validation, each candidate mutation is PCR amplified from up to 10 other individuals within the same mutant pedigree. Each PCR amplimer is subjected to Sanger sequencing. In the majority of the cases, non-mutagenic variants will not segregate with the phenotype, but bona fide mutations will. Validation is not attempted until sufficient sequencing coverage has been obtained, as indicated by comparison of computational analysis of parameters like '% target bases covered' and by comparison of the variant profiles obtained to the Sanger whole genome sequencing data.

Protocol References

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