

1 **Tissue nonspecific alkaline phosphatase improves bone**
2 **quality but does not alleviate craniosynostosis in the**
3 **FGFR2^{C342Y/+} mouse model of Crouzon syndrome**

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20 **Abstract**

21 Crouzon syndrome is a congenital disorder characterized by craniosynostosis, the premature
22 fusion of cranial bones. Craniosynostosis leads to high intracranial pressure and abnormal skull
23 and facial shapes that are relieved by surgery. Crouzon syndrome is caused by activating
24 mutations in fibroblast growth factor receptor 2 (FGFR2). The goal of this study was to
25 determine if delivery of recombinant tissue nonspecific alkaline phosphatase (TNAP) could
26 prevent or diminish the severity of craniosynostosis in post-natal craniosynostosis onset BALB/c
27 and/or peri-natal craniosynostosis onset C57BL/6 FGFR2^{C342Y/+} mouse models of Crouzon
28 syndrome. Mice were injected with a lentivirus encoding a mineral targeted form of TNAP
29 immediately after birth. Cranial bone fusion as well as cranial bone volume, mineral content
30 and density were assessed by micro computed tomography. Craniofacial shape was measured
31 with calipers using previously established landmarks and measurements. Alkaline phosphatase
32 activity levels were measured in serum. Results show that postnatal delivery of TNAP increases
33 serum levels of alkaline phosphatase activity and improves bone volume, density and mineral
34 content, but does not alleviate craniosynostosis, craniofacial shape or cranial base
35 abnormalities in FGFR2^{C342Y/+} Crouzon mice. These results indicate that post-natal recombinant
36 TNAP enzyme therapy is therapeutic for bone mineralization but not efficacious for relief of
37 FGFR-associated craniosynostosis and associated craniofacial shape defects.

38

39 **Introduction**

40 Craniosynostosis is the pediatric condition of premature cranial bone fusion. This condition
41 can lead to high intracranial pressure, abnormal skull and facial shapes, blindness, seizures and
42 brain abnormalities [1-6]. Because the sole treatment is surgery, even with appropriately early
43 diagnosis patients can suffer high morbidity [7-9]. Surgical approaches also do not fully correct
44 abnormal skull and facial shapes, which contribute to social challenges. Previous studies
45 showed that the pathogenesis of craniosynostosis can include abnormal boundary
46 formation/maintenance, lineage commitment, proliferation and/or apoptosis of cranial
47 progenitor cells [10-23]. Despite these important advancements, a pharmaceutical treatment
48 for craniosynostosis is not yet realized.

49 Craniosynostosis occurs in association with activating mutations in *Fgfr2* [10, 12, 24, 25].
50 Craniosynostosis also occurs at high incidence in infants with hypophosphatasia, a metabolic
51 disorder that occurs due to inactivating mutations in *Alpl*, the gene for tissue nonspecific
52 alkaline phosphatase (TNAP) [26-29]. We previously demonstrated that FGF signaling decreases
53 TNAP expression [14, 30, 31]. TNAP expression is also reduced in primary cells isolated from
54 FGFR2^{C342Y/+} mice that have been induced to differentiate into osteoblasts when cultured *in*
55 *vitro* or in a 3D collagenous matrix *in vivo* [14, 15]. These results indicate that one of the
56 mechanisms by which FGF signaling influences craniofacial skeletal development may involve
57 reduced TNAP. Notably, postnatal delivery of a recombinant mineral-targeted form of TNAP did
58 prevent craniosynostosis in the TNAP^{-/-} mouse model of hypophosphatasia [32]. The objective
59 of this study was to determine if postnatal delivery of recombinant TNAP could prevent or
60 diminish the severity of craniosynostosis and associated craniofacial shape defects in the
61 FGFR2^{C342Y/+} mouse model of Crouzon syndrome.

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63 **Materials and methods**

64 **TNAP Lentivirus**

65 Recombinant mineral-targeted TNAP lentivirus was generously provided by Dr. Jose Luis Millán
66 (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA). This virus expresses a
67 mineral-targeted protein that is composed of soluble human TNAP enzyme fused to the
68 constant region of human IgG1 and a C-terminal deca-aspartate motif to confer targeting to
69 hydroxyapatite. The aspartate tag confers 30x higher affinity for hydroxyapatite than untagged
70 enzyme [33]. Production and titer of the lentivirus was performed by the University of Michigan
71 Vector Core. Treatment with this recombinant form of TNAP was previously shown to increase
72 serum alkaline phosphatase levels and rescue long bone and craniofacial defects seen in
73 hypophosphatasia [26, 32, 34, 35].

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75 **Animal Procedures**

76 Because severity of craniosynostosis and associated craniofacial shape defects are variable on
77 the mixed genetic background, $FGFR2^{C342Y/+}$ mice were backcrossed with BALB/c and C57BL/6
78 mice (obtained from Charles River Laboratories) for at least fifteen generations prior to
79 experiments. BALB/c $FGFR2^{C342Y/+}$ mice have a moderate form of Crouzon syndrome with
80 craniosynostosis apparent between three and four weeks after birth [15]. C57BL/6 mice have a
81 severe form of Crouzon syndrome with craniosynostosis first apparent in neonatal mice (data not
82 shown). Genotyping was performed as previously described [12, 15]. Briefly, DNA from tail

83 digests was amplified by polymerase chain reaction using 5'-gagtaccatgctgactgcatgc-3' and 5'-
84 ggagaggcatctctgtttcaagacc-3' primers to yield a 200 base pair band for wild type FGFR2 and a 300
85 base pair band for mutant FGFR2^{C342Y}. Mice were fed ad libitum and housed under standard 12
86 hour dark/light cycles. Litters were randomly assigned to treatment/no treatment groups.
87 Treated mice were injected with 1.0×10^7 transforming units lentivirus or an equivalent volume
88 of phosphate buffered saline via the jugular vein two days after birth. BALB/c mice (n=12 FGFR2^{+/+}
89 control mice, n=14 FGFR2^{C342Y/+} control mice, n=16 FGFR2^{C342Y/+} TNAP lentivirus treated mice)
90 were euthanized by CO₂ overdose at four weeks post-natal and C57BL/6 mice (n=7 FGFR2^{+/+}
91 control mice, n=7 FGFR2^{C342Y/+} control mice, n=14 FGFR2^{C342Y/+} TNAP lentivirus treated mice) were
92 euthanized by CO₂ overdose at three weeks post-natal for analyses. BALB/c mice were sacrificed
93 at a later age than C57BL/6 mice because craniosynostosis onset occurs later in BALB/C than in
94 C57BL/6 FGFR2^{C342Y/+} mice. Blood was collected by aortic puncture under surgical anesthesia.
95 Mice were weighed, and body length was measured for each animal. All animal procedures were
96 prospectively approved of by the University of Michigan's University Committee on Use and Care
97 of Animals (UCUCA, protocol PRO00006815). All samples were de-identified as to genotype and
98 treatment group, and each analysis was performed on all BALB/c mice or on all C67BL/6 mice at
99 one time. The primary outcome assessment was craniosynostosis incidence. Secondary outcome
100 assessments included cranial bone density measurements, craniofacial shape measurements,
101 cranial base synchondrosis fusions and cranial base bone lengths.

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103 **Serum Analyses**

104 Mice were fasted for six hours prior to blood collection. Alkaline phosphatase activity (AP) in
105 serum was quantified using the colorimetric reagent 4-nitrophenyl-phosphate disodium
106 hexahydrate (Sigma), as compared to a standard curve using commercially available alkaline
107 phosphatase enzyme (Sigma). Inorganic phosphate quantifications were performed using
108 commercially available kits (Pointe Scientific), also as compared to standard curves.

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110 **Micro Computed Tomography**

111 Whole skulls were scanned at an 18 μm isotropic voxel resolution using the eXplore Locus SP
112 micro-computed tomography imaging system (GE Healthcare Pre-Clinical Imaging, London, ON,
113 Canada). Regions of interest (ROI's) for parietal and frontal bones were established as 1 mm in
114 length, 1 mm in width and depth equivalent to thickness of bone, as previously described [15,
115 29]. Density, volume and mineral content of cranial bones from mice were measured using
116 previously established methods using Microview version 2.2 software (GE Healthcare Pre-Clinical
117 Imaging, London, ON) and established algorithms [36, 37].

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119 **Cranial Suture Assessment**

120 Fusion between cranial bones (fusion of coronal suture, lambdoid suture and sagittal suture) plus
121 fusion of the inter-sphenoidal (ISS) and spheno-occipital (SOS) synchondroses were identified on
122 micro CT scans of skulls dissected mice. Cranial sutures were viewed using the two-dimensional
123 micro CT slices in an orthogonal view across the entire length of the suture or synchondrosis, as
124 previously described [15, 29].

125 Reliability of suture fusion assessment was verified by both intra-operator and inter-
126 operator reliability statistics by calculating intraclass correlation coefficients (ICC). Intra-
127 operator reliability statistics was carried out by assessing suture fusion status of the coronal,
128 sagittal and lambdoid sutures as well as the inter-sphenoidal (ISS) and spheno-occipital (SOS)
129 synchondroses on fifteen micro CT scans by one investigator two times separated by a two-
130 month period. Inter-operator reliability was carried out by analyzing fifteen micro CT scans by a
131 second investigator. The ICC for intraoperator reliability for suture fusion assessment is .970
132 ($p \leq .0001$) and the ICC for interoperator reliability is .972 ($p \leq .0001$). Thus, there is high
133 intraoperator and interoperator reliability for suture fusion assessment.

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135 **Linear Measurements**

136 Craniofacial linear skeletal measurements were taken using digital calipers on dissected skulls.
137 Linear measurements were calculated using previously reported craniofacial skeletal landmarks
138 [15, 38, 39], including standard measurements currently in use by the Craniofacial Mutant Mouse
139 Resource of Jackson Laboratory (Bar Harbor, ME). Linear measurements were normalized to total
140 skull length (measured from nasale to opisthion) to account for size differences between
141 $FGFR2^{+/+}$ and $FGFR2^{C342Y/+}$ mice. Measurements were performed twice and an average of the two
142 measurements was utilized for statistical comparison by genotype and treatment. Cranial base
143 anterior-posterior bone lengths were measured on micro CT scans using *Dolphin Imaging 11.0*
144 software (Dolphin Imaging and Management Solutions, Chatsworth, CA), as previously described
145 [40].

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147 **Statistics**

148 An Analysis of Variance (ANOVA) was performed to compare groups by gender, genotype and
149 treatment group. Because serum AP levels varied in mice injected with the lentivirus, linear
150 regressions were also performed to determine if, and to what extent serum AP levels
151 associated with changes in measured phenotypes. The incidence of cranial suture fusion, and
152 cranial base synchondrosis fusion was analyzed by the Fishers exact test.

153

154 **Results**

155 Injection with the TNAP expression lentivirus significantly increased serum alkaline phosphatase
156 (AP) levels in all of the treated mice (Table 1). BALB/c Crouzon mice injected with the lentivirus
157 increased serum AP levels by 1.2 U/mL when compared to control Crouzon mice ($p < .0001$)
158 when measured at four weeks old. C57BL/6 Crouzon mice injected with the TNAP expression
159 lentivirus increased serum AP levels by 1.8 U/mL when compared to control Crouzon mice
160 ($p < .0001$) when measured at three weeks old. No significant difference in serum AP levels were
161 seen between untreated Crouzon and wild type mice on the BALB/c or C57BL/6 backgrounds.
162 As expected, injection with the lentivirus did not alter serum inorganic phosphate (P_i) levels.

163 Initial statistical comparison of groups by ANOVA showed that Crouzon mice weigh less and
164 are shorter in body length than their wild type littermates, regardless of genetic background
165 (Table 1). Linear regression performed to account for serum AP level variability in the lentivirus
166 injected mice showed that, on the BALB/c background, serum AP levels did not alter weight in

167 wild type mice but did decrease weight in Crouzon mice by 0.9 g per U/ml which accounted for
168 37% of the weight variability in in these mice ($p < .03$). On the C57BL/6 background, linear
169 regression showed no impact of serum AP level on weight, regardless of genotype. Serum AP
170 levels did not alter body length, regardless of genetic background or genotype.

171 **Table 1.** Serum and Body Measurements in TNAP vs. untreated Balb/C and C57Bl/6mice.

Strain	Genotype	Treatment	Body Weight (g)	Body Length (mm)	Serum AP Level (units/ml)	Serum Pi Level (mg/dl)
Balb/C	FGFR2 ^{+/+}	no	13.5 +/- 2.2*	7.4 +/- 0.3*	0.03 +/- 0.01	10.9 +/- 0.8
Balb/C	FGFR2 ^{C342Y/+}	no	9.2 +/- 1.7	6.5 +/- 0.3	0.03 +/- 0.78	9.9 +/- 1.4
Balb/C	FGFR2 ^{C342Y/+}	yes	8.9 +/- 2.6	6.5 +/- 0.7	1.30 +/- 0.54 [#]	9.8 +/- 1.1
C57Bl/6	FGFR2 ^{+/+}	no	8.7 +/- 0.1*	6.9 +/- 0.3*	0.01 +/- 0.01	9.5 +/- 1.0
C57Bl/6	FGFR2 ^{C342Y/+}	no	5.8 +/- 1.1	5.8 +/- 0.6	0.02 +/- 0.01	8.7 +/- 1.1
C57Bl/6	FGFR2 ^{C342Y/+}	yes	6.6 +/- 1.0	5.9 +/- 0.4	1.93 +/- 0.77 [#]	9.0 +/- 0.6

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* p value < 0.01 between genotypes

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[#] p value < 0.01 between treatment groups

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Qualitative analysis of craniofacial skeletal shape suggested that Crouzon mice differ in

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morphology from their wild type counterparts, and that post-natal delivery of mineral-targeted

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TNAP via lentivirus did not impact morphology (Figs 1,2). Craniofacial skeletal linear

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measurements normalized to total skull length revealed many differences between FGFR2^{342Y/+}

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Crouzon and FGFR2^{+/+} wild type mice on both congenic backgrounds (Table 2). BALB/c Crouzon

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mice had increased cranial height, cranial width, inner canthal distance, parietal bone length

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and cranial height to width ratios, with decreased nasal bone length. C57BL/6 Crouzon mice

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had increased cranial height, cranial width, inner canthal distance, frontal bone length, parietal

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bone length and cranial height to width ratios, with decreased nose and nasal bone lengths.

185 Treatment with the TNAP lentivirus did not alter craniofacial skeletal measurements in Crouzon
 186 mice on either genetic background.

187 **Table 2.** Linear Craniofacial Skeletal Measurements in
 188 TNAP vs. untreated BALB/c and C57Bl/6 mice.

Strain	Measurement	FGFR2 ^{+/+} vehicle	FGFR2 ^{C342Y/+} vehicle	FGFR2 ^{C342Y/+} TNAP
BALB/c	Cranial Height	.36 +/- .01*	.45 +/- .01	.45 +/- .01
BALB/c	Cranial Width	.55 +/- .01*	.63 +/- .01	.62 +/- .01
BALB/c	Inner Canthal Distance	.20 +/- .01*	.25 +/- .01	.26 +/- .01
BALB/c	Nose Length	.65 +/- .01	.65 +/- .01	.65 +/- .01
BALB/c	Nasal Bone Length	.33 +/- .02*	.32 +/- .01	.32 +/- .03
BALB/c	Frontal Bone length	.33 +/- .02	.33 +/- .01	.34 +/- .03
BALB/c	Parietal Bone Length	.20 +/- .01*	.25 +/- .02	.26 +/- .01
BALB/c	Ratio Height to Width	.66 +/- .02*	.72 +/- .02	.72 +/- .02
C57Bl/6	Cranial Height	.38 +/- .01*	.52 +/- .01	.52 +/- .03
C57Bl/6	Cranial Width	.55 +/- .01*	.64 +/- .01	.64 +/- .02
C57Bl/6	Inner Canthal Distance	.23 +/- .01*	.29 +/- .01	.29 +/- .01
C57Bl/6	Nose Length	.65 +/- .01*	.62 +/- .01	.63 +/- .03
C57Bl/6	Nasal Bone Length	.32 +/- .01*	.23 +/- .02	.23 +/- .01
C57Bl/6	Frontal Bone length	.35 +/- .01*	.40 +/- .02	.41 +/- .03
C57Bl/6	Parietal Bone Length	.22 +/- .01*	.26 +/- .02	.27 +/- .02
C57Bl/6	Ratio Height to Width	.38 +/- .01*	.52 +/- .01	.52 +/- .03

189 Measures are reported as normalized to total skull length.

190 * p value < 0.01 between genotypes

191 # p value < 0.01 between treatment groups

192 Analysis of cranial bone fusions revealed high incidences of premature fusion of the coronal
 193 and lambdoid sutures in both BALB/c and C57Bl/6 Crouzon mice, with no fusions evident in
 194 wild type mice (Fig. 3). Although 100% of BALB/c and C57Bl/6 Crouzon mice exhibited coronal
 195 suture fusion, fusions in BALB/c mice tended to be point fusions as opposed to fusion of
 196 approximately 1/3 of the coronal suture in the C57Bl/6 C mice. Analysis of cranial base
 197 synchondrosis fusions also revealed high incidences of fusion of the inter-sphenoidal

198 synchrondrosis (ISS) in both strains of Crouzon mice, with no fusions evident in wild type mice.
199 The incidence of fusion of the spheno-occipital synchrondrosis (SOS) was higher in C57BL/6
200 Crouzon mice than BALB/c Crouzon mice, despite the younger age of the C57BL/6 mice. No
201 cranial base fusions were evident in the wild type littermate mice. While some trends, including
202 diminished incidence of lambdoid suture fusion and increased incidence of spheno-occipital
203 synchrondrosis (SOS) fusion are seen upon treatment, these differences were not statistically
204 significant. Measurement of cranial base bones demonstrated decreased length of the basis-
205 sphenoid and pre-sphenoid bones in BALB/c and C57BL/6 Crouzon as compared to wild type
206 mice (Table 3). Treatment with TNAP did not increase length of the basis-sphenoid bone in
207 Crouzon mice. Treatment with TNAP did increase length of the pre-sphenoid bone in BALB/c
208 Crouzon mice, but not to the equivalent of sphenoid bone length seen in wild type mice.
209 Together, the data indicates that delivery of mineral-targeted TNAP via lentivirus shortly after
210 birth does not impact cranial bone or cranial base bone fusions in these mice. Treatment with
211 the mineral-targeted TNAP may enhance growth of anterior cranial base bones in the model of
212 more moderate Crouzon syndrome (BALB/c strain), but not to the extent seen in control wild
213 type mice.

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220 **Table 3.** Cranial base measurements in TNAP vs. untreated Balb/C and C57Bl/6 mice.

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Strain	Genotype	Treatment	Basis Occipitus (mm)	Basis Sphenoid (mm)	Pre-Sphenoid (mm)
Balb/C	FGFR2 ^{+/+}	vehicle	3.1 +/- 0.1	2.9 +/- 0.1*	2.5 +/- 0.1*
Balb/C	FGFR2 ^{C342Y/+}	vehicle	2.9 +/- 0.3	2.4 +/- 0.3	1.8 +/- 0.1
Balb/C	FGFR2 ^{C342Y/+}	TNAP	2.9 +/- 0.2	2.5 +/- 0.3	1.9 +/- 0.1#
C57Bl/6	FGFR2 ^{+/+}	vehicle	2.8 +/- 0.2	2.9 +/- 0.2*	2.2 +/- 0.1*
C57Bl/6	FGFR2 ^{C342Y/+}	vehicle	2.8 +/- 0.1	2.7 +/- 0.1	1.7 +/- 0.1
C57Bl/6	FGFR2 ^{C342Y/+}	TNAP	3.0 +/- 0.2	2.6 +/- 0.1	1.7 +/- 0.1

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235 * p value < 0.01 between genotypes

236 # p value < 0.01 between treatment groups

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238 Micro CT based analyses of cranial bones demonstrated significantly diminished bone

239 mineral density, tissue mineral content, tissue mineral density and bone volume fraction in

240 frontal bones, plus significantly diminished tissue mineral density and bone volume fraction in

241 parietal bones of Crouzon mice when compared to wild type littermates on both BALB/c and

242 C57BL/6 backgrounds (Table 4). Injection with the TNAP lentivirus significantly increased

243 frontal bone mineral density, tissue mineral content and bone volume fraction, plus parietal

244 bone volume fraction in Crouzon mice on the BALB/c background. Injection with the TNAP

245 lentivirus did not significantly impact any of the cranial bone parameters in Crouzon mice on

246 the C57BL/6 background.

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251 **Table 4.** Cranial bone volume, density and mineral content
 252 in TNAP vs. untreated congenic BALB/c and C57BL/6 mice.

Strain	Genotype	Treatment	Cranial Bone	Bone Mineral Content (mg)	Bone Mineral Density (mg/cc)	Tissue Mineral Content (mg)	Tissue Mineral Density (mg/cc)	Bone Volume Fraction
BALB/c	FGFR2 ^{+/+}	PBS	Frontal	.035 +/- .004	405 +/- 14*	.028 +/- .007*	692 +/- 14*	0.41 +/- .03*
BALB/c	FGFR2 ^{C342Y/+}	PBS	Frontal	.031 +/- .008	361 +/- 63	.020 +/- .006#	671 +/- 18	0.36 +/- .0
BALB/c	FGFR2 ^{C342Y/+}	TNAP	Frontal	.035 +/- .005	401 +/- 26#	.026 +/- .015#	683 +/- 29	0.42 +/- .06#
BALB/c	FGFR2 ^{+/+}	PBS	Parietal	.034 +/- .004	405 +/- 12	.023 +/- .005	693 +/- 15*	0.43 +/- .03*
BALB/c	FGFR2 ^{C342Y/+}	PBS	Parietal	.031 +/- .007	396 +/- 39	.020 +/- .005	669 +/- 24	0.36 +/- .06
BALB/c	FGFR2 ^{C342Y/+}	TNAP	Parietal	.034 +/- .006	403 +/- 27	.025 +/- .012	691 +/- 36	0.42 +/- .07#
C57BL/6	FGFR2 ^{+/+}	PBS	Frontal	.017 +/- .003	245 +/- 25*	.006 +/- .001*	570 +/- 18*	0.12 +/- .01*
C57BL/6	FGFR2 ^{C342Y/+}	PBS	Frontal	.013 +/- .002	209 +/- 25	.004 +/- .001	519 +/- 26	0.10 +/- .01
C57BL/6	FGFR2 ^{C342Y/+}	TNAP	Frontal	.016 +/- .004	225 +/- 30	.004 +/- .001	553 +/- 40	0.11 +/- .01
C57BL/6	FGFR2 ^{+/+}	PBS	Parietal	.015 +/- .001	237 +/- 19	.005 +/- .001	590 +/- 13*	.121 +/- .023*
C57BL/6	FGFR2 ^{C342Y/+}	PBS	Parietal	.012 +/- .003	217 +/- 18	.004 +/- .001	558 +/- 36	.098 +/- .006
C57BL/6	FGFR2 ^{C342Y/+}	TNAP	Parietal	.013 +/- .004	232 +/- 42	.004 +/- .001	575 +/- 32	.105 +/- .009

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 254 * p value < 0.01 between genotypes
 255 # p value < 0.01 between treatment groups

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257 Discussion

258 In this study we sought to determine if treatment with recombinant mineral targeted TNAP
 259 could rescue the craniofacial skeletal phenotype of FGFR2^{C342Y/+} Crouzon mice when delivered
 260 post-natal with lentivirus. The FGFR2^{C342Y/+} mutation was previously demonstrated to cause
 261 ligand independent signaling and is therefore widely considered to be an activating mutation
 262 leading to increased FGF signaling [41-44]. We pursued this investigation because we previously
 263 demonstrated that FGF signaling diminishes TNAP expression [14, 30, 31], and showed that

264 TNAP deficiency in mice leads to a similar craniofacial phenotype to that seen in $FGFR2^{C342Y/+}$
265 Crouzon mice including coronal and lambdoid but not sagittal craniosynostosis, fusion of cranial
266 base synchondroses and abnormal brachycephalic/acrocephalic head shapes [12, 15, 29, 40].
267 Additionally, in a previous study using archival aliquots of lentivirus expressing the mineral
268 targeted recombinant form of TNAP that resulted in increases in serum AP activity in only a
269 small number of the treated mice, we found statistical differences in the morphology of the
270 inferior skull surface and skull height in treated vs. untreated $FGFR2^{C342Y/+}$ mice [45].

271 Here we found that post-natal lentiviral delivery of recombinant TNAP rescued cranial bone
272 density, mineral content and volume fraction but not craniosynostosis or craniofacial shape in
273 $FGFR2^{C342Y/+}$ Crouzon mice. Improvement of cranial bone density, mineral content and volume
274 fraction by TNAP treatment in the Crouzon mice is consistent with results showing that
275 recombinant mineral targeted TNAP treatment rescues mineralization of craniofacial and long
276 bones in in the $Alpl^{-/-}$ mouse model of infantile HPP and humans with infantile and childhood
277 HPP [32, 34, 46, 47]. Treatment with mineral targeted TNAP via lentivirus did not rescue
278 craniosynostosis or craniofacial shape defects in the Crouzon mice. This result is inconsistent
279 with the rescue of craniosynostosis seen in $Alpl^{-/-}$ mice treated with mineral targeted
280 recombinant TNAP protein [32] but is consistent with results in human studies which indicate
281 that post-natal treatment with recombinant TNAP protein does not rescue craniosynostosis [26,
282 47]. While lentiviral TNAP treatment did not rescue cranial base synchondrosis fusion in the
283 Crouzon mice, length of the pre-sphenoid bone was increased in treated mice on the BALB/c
284 genetic background. This result is consistent with our previous study using archival lots of the
285 lentivirus [45] which suggested changes in inferior skull morphology and may indicate that

286 TNAP can promote cranial base growth in more moderate presentations of Crouzon syndrome
287 to some extent.

288 Lack of rescue of craniosynostosis, cranial base synchondrosis fusions and craniofacial shape
289 abnormalities by lentiviral delivery of mineral targeted TNAP indicates that TNAP is not
290 essential for these characteristics of Crouzon syndrome. This could be due to the fact that TNAP
291 levels are not decreased at all stages in $FGFR2^{C342Y/+}$ mice [44] and is consistent with our finding
292 in this study that serum AP levels are similar in untreated Crouzon and wild type mice. It is also
293 possible that TNAP is simply not efficacious for preventing FGFR-associated cranial bone and
294 cranial base bone fusions, despite being decreased in cranial bone progenitor cells [14, 15].
295 More recently we showed that TNAP regulates expression of FGFR2 and Erk1,2 activity [48].
296 This latter data suggests the alternative hypothesis that FGF and Erk1,2 signaling changes cause
297 craniosynostosis in TNAP deficiency as opposed to TNAP deficiency causing craniosynostosis in
298 Crouzon syndrome.

299

300 **Acknowledgments**

301 Lentiviral vector encoding the mineral targeted form of TNAP was generously provided by Dr.
302 José Luis Millán (Sanford Burnham Prebys Medical Discovery Institute, Human Genetics
303 Program, San Jose, CA).

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Figure Legends:

489

490 **Figure 1. Micro CT images of BALB/c congenic untreated and treated *FGFR2*^{C342Y/+} mice.** Micro
491 CT isosurface images of P28 *FGFR2*^{C342Y/+} Crouzon (CZ) and wild type (WT) mice on the BALB/c
492 congenic background are shown in axial view from above (A,B,C) and lateral view (B,D,F).
493 Darker bone is bone of diminished density.

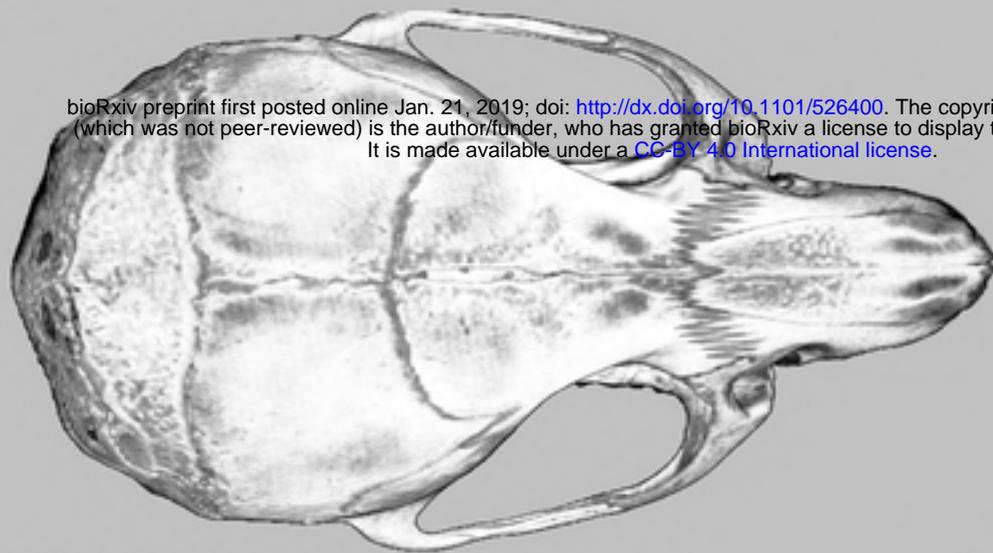
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495 **Figure 2. Micro CT images of C57BL/6 congenic untreated and treated *FGFR2*^{C342Y/+} mice.** Micro
496 CT isosurface images of P21 *FGFR2*^{C342Y/+} Crouzon (CZ) and wild type (WT) mice on the C57BL/6
497 congenic background are shown in axial view from above (A,B,C) and lateral view (B,D,F).
498 Darker bone is bone of diminished density. Cranial bone density is diminished in C57BL/6
499 *FGFR2*^{C342Y/+} mice to the extent that cranial base bones show through the translucent cranial
500 bones.

501

502 **Figure 3. Incidence of craniosynostosis and cranial base synchondrosis fusions in untreated**
503 **and treated $FGFR2^{C342Y/+}$ mice.** No fusions are evident in wild type mice on either the BALB/c or
504 C57BL/6 backgrounds. A high incidence of coronal and lamboid suture fusion but no fusion of
505 the sagittal suture is evident in Crouzon mice on both the BALB/c and C57BL/6 backgrounds. A
506 high incidence of intersphenoidal synchondrosis (ISS) is seen in Crouzon mice on both
507 backgrounds. Spheno-occipital synchondrosis (SOS) is seen approximately half of C57BL/6
508 Crouzon mice but rarely in BALB/c Crouzon mice. Treatment with TNAP does not significantly
509 influence fusion of any cranial suture or cranial base synchondroses on either genetic
510 background.

A



WT BALB/c 4wk no tx

B

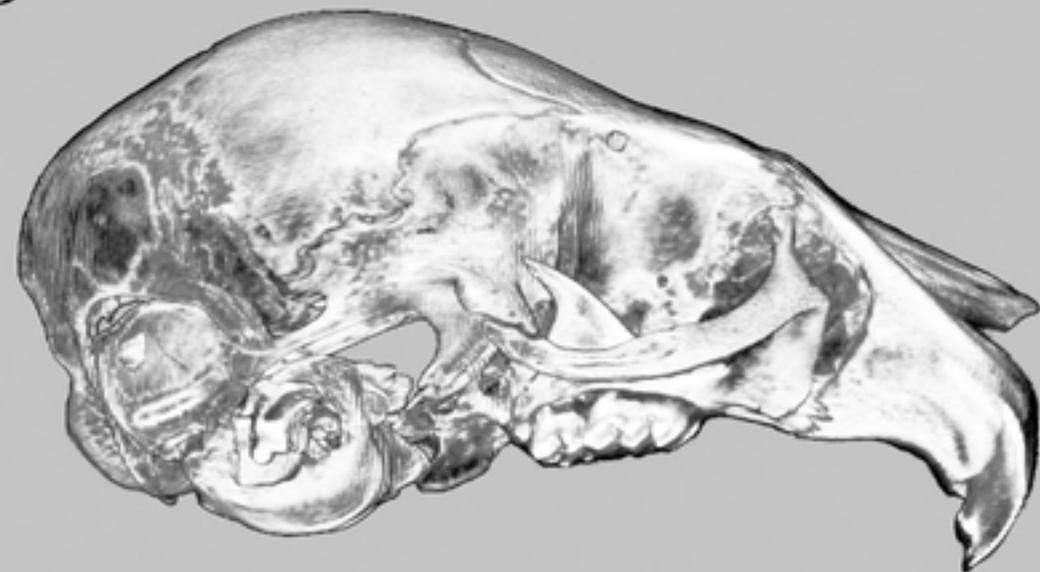


C



Cz BALB/c 4wk no tx

D



E



Cz BALB/c 4wk tx

F

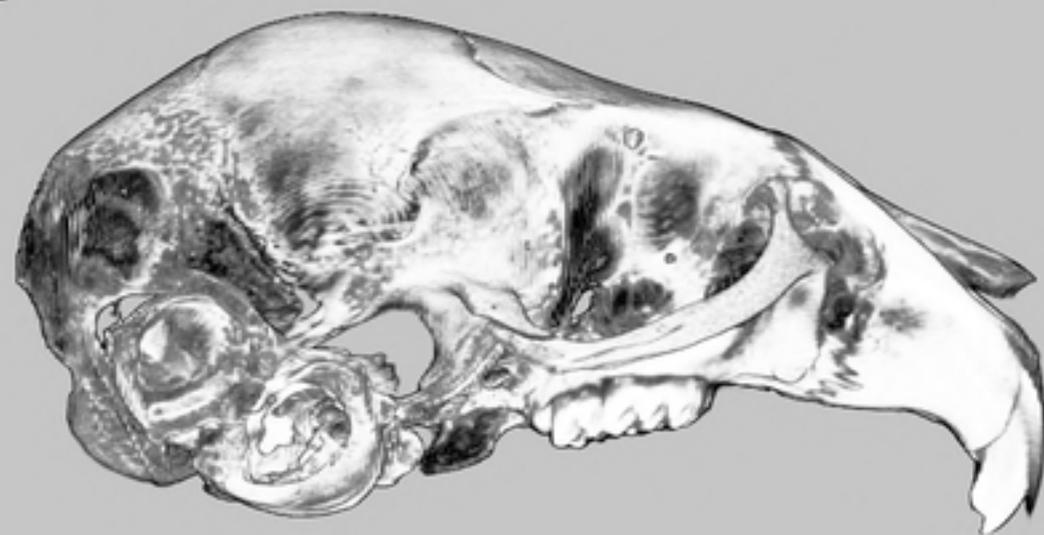
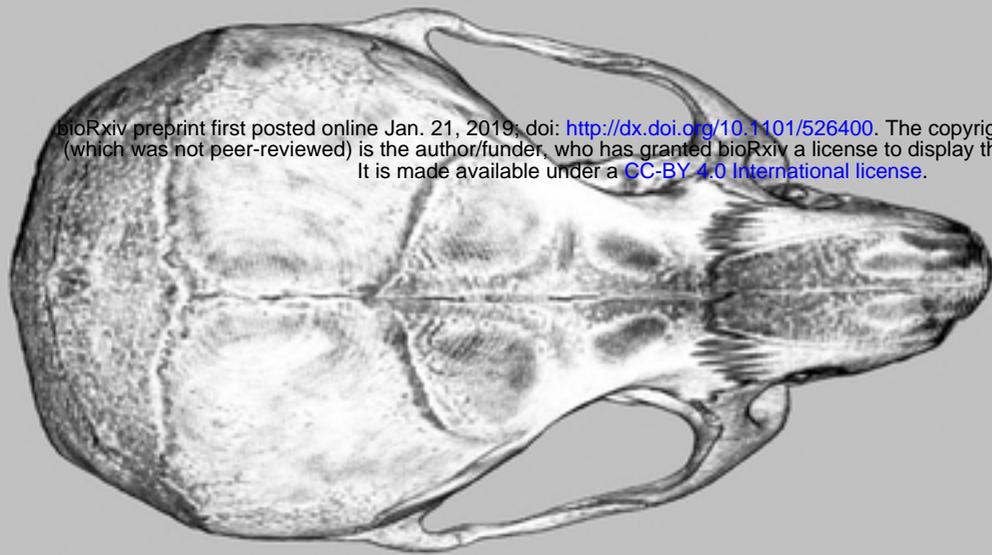


Figure 1. Micro CT images of BALB/c congenic untreated and treated

A



WT C57BL6 3 wk no tx

B



C



Cz C57BL6 3 wk no tx

D



E



Cz C57BL6 3 wk tx

F



Figure 2. Micro CT images of C57BL/6 congenic untreated and treated

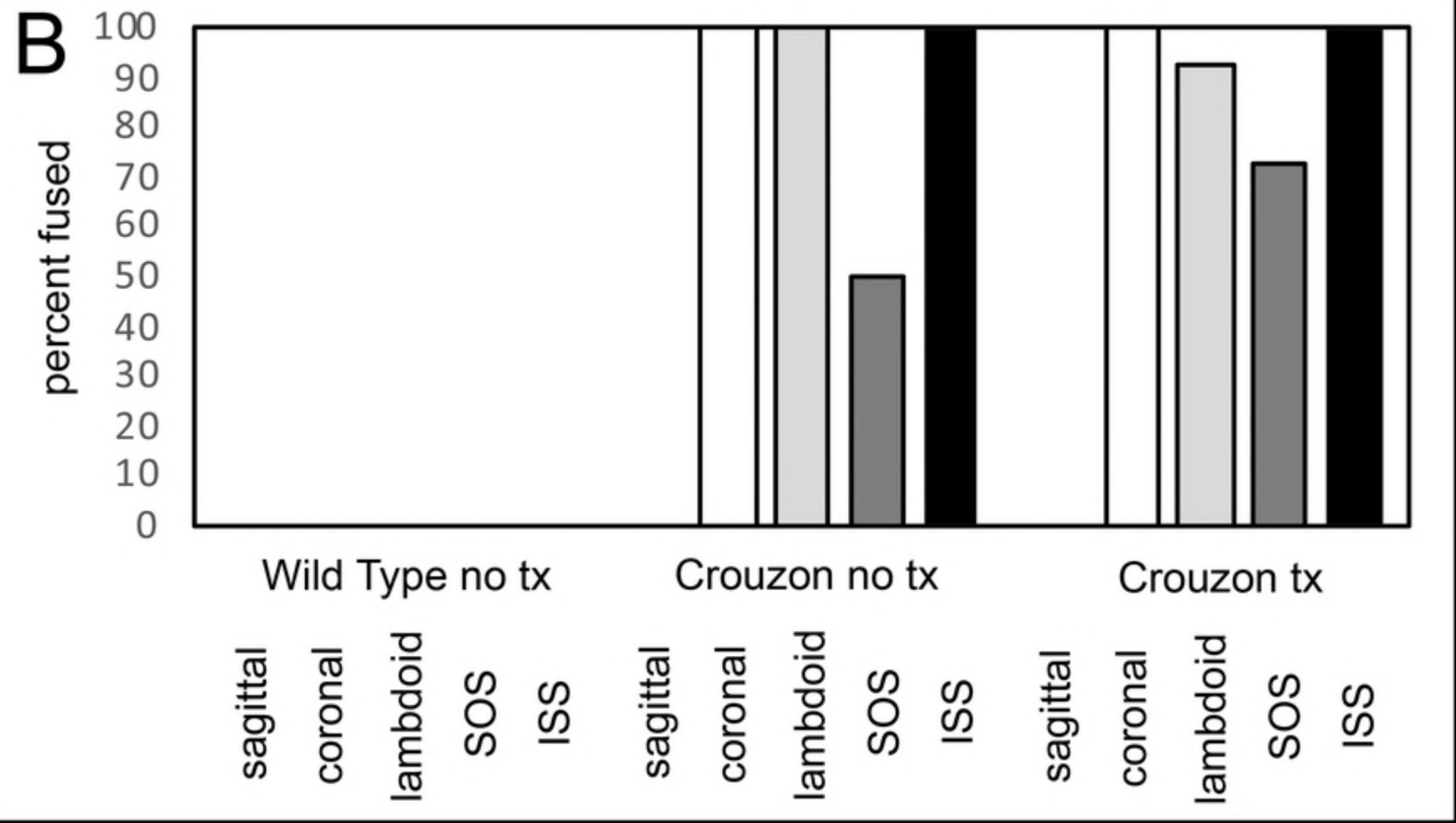
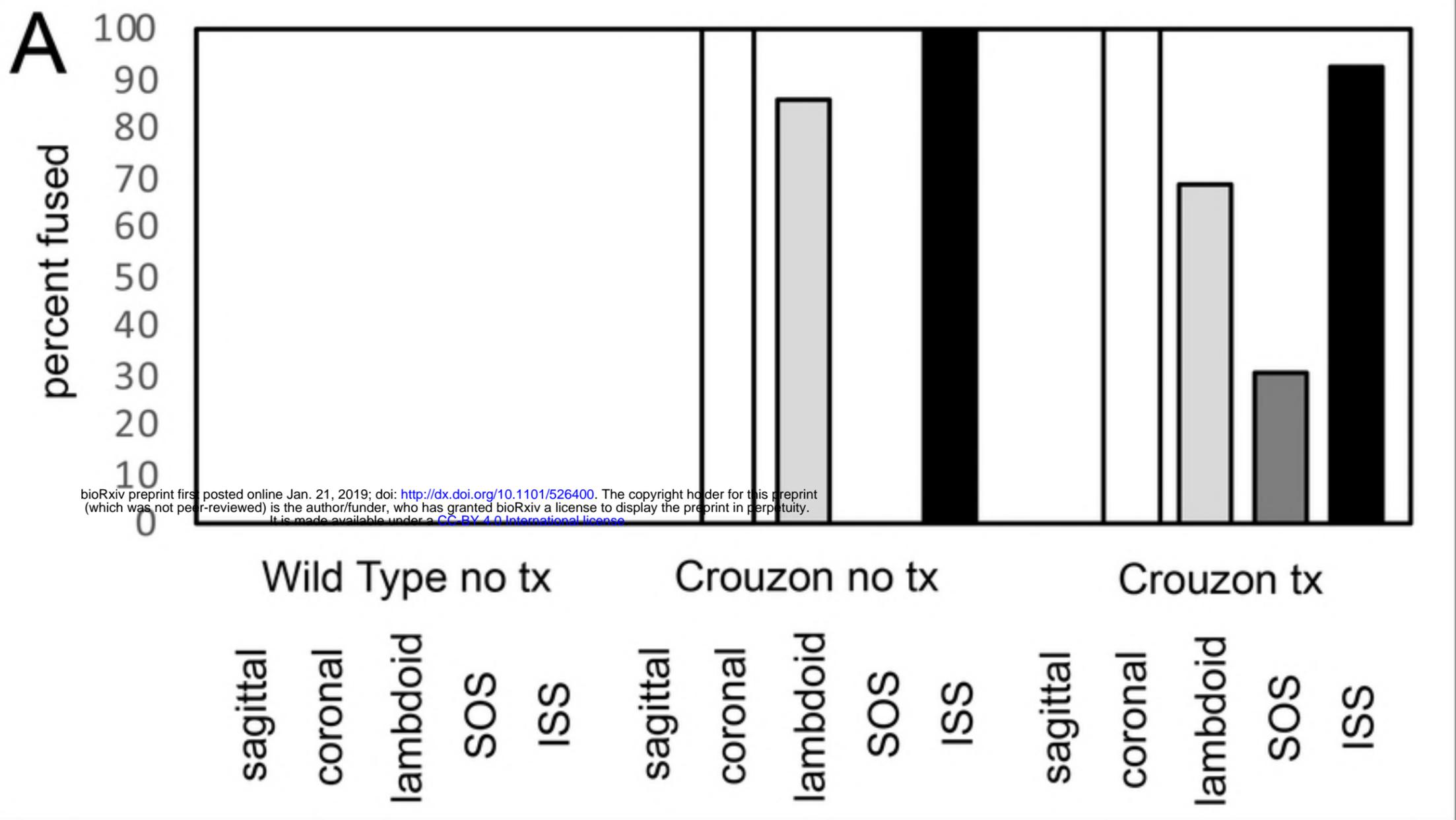


Figure 3. Incidence of craniosynostosis and cranial base synchondrosis