# Characterization of craniofacial defects in a novel mouse model for Alagille syndrome

*2020 Grants*

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# FollowUp Form

## *Award Information*

*In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:*

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
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### Title of Project*

Characterization of craniofacial defects in a novel mouse model for Alagille syndrome

### Award Type

Biomedical Research Award (BRA)

### Period of AAOF Support

July 1, 2020 through June 30, 2023

### Institution

University of California, San Francisco

### Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)

NA

### Amount of Funding

$30,000.00

### Abstract

(add specific directions for each type here)

# *Respond to the following questions:*

## Detailed results and inferences:*

If the work has been published, please attach a pdf of manuscript below by clicking "Upload a file".
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Use the text box below to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should also be attached by clicking "Upload a file".

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Attached is a draft of our manuscript for submission. Unfortunately, the 2MB limit is too low to upload figures. Can you provide an email address for me to send in the complete manuscript?

## Were the original, specific aims of the proposal realized?*

Yes, the 2 subaims of the original proposal was completed.
The 3rd subaim to generate an Ndr mouse was not completed due to a lack of funding. However, my collaborator in Sweden (Emma Andersson) is happy to continue to provide mice for our studies.

## Were the results published?*

No

## Have the results of this proposal been presented?*

No

## To what extent have you used, or how do you intend to use, AAOF funding to further your career?*

We will submit our Ndr manuscript soon for publication. I have used AAOF funding to generate preliminary data to submit NIH grants. The funding provided by the AAOF is very helpful and an incredible resource for orthodontic research. Thank you!

## Accounting: Were there any leftover funds?

$0.00

# *Not Published*

## Are there plans to publish? If not, why not?*

Yes, we are close to submitting our manuscript. We will do so once we complete one final study (measurement of snout lengths in P30 mice is almost completed).

# *Not Presented*

## Are there plans to present? If not, why not?*

Yes, this work will be presented at the AAO or IADR conference, depending on which I will attend in the near future.

# *Internal Review*

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## *Applicant File Uploads*

- NDR manuscript final.pdf

**Craniofacial and dental anomalies in a mouse model for Alagille Syndrome**

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Abstract**

Alagille syndrome (ALGS1; Online Mendelian Inheritance in Man/OMIM no. 118450, and ALGS2, OMIM no. 610205) is a multisystem, genetic disorder characterized by chronic cholestasis, cardiovascular anomalies, ocular abnormalities, vertebral defects, and characteristic facial features. Despite the associated facies with Alagille syndrome, little is known regarding any changes to skull morphology or to teeth. Notch signaling is a highly conserved and cell-contact-dependent pathway that is important in development and homeostasis. Mutations within the Notch ligand JAGGED1 (*JAG1*) may lead to ALGS1. The *Jag1*$^{Ndr/Ndr}$ missense (H268Q) mouse model was generated that closely phenocopied ALGS1 (1). To better understand the craniofacial and dental manifestations of ALGS1, we analyzed 8-month-old *Jag1*$^{Ndr/Ndr}$ (also known as Ndr) mice using micro-computed tomography (microCT) and geometric morphometrics (GMA) to determine changes to skull morphology. We used histology and scanning electron microscopy (SEM) to analyze the dentition and their development. Ndr mice showed less convexity and vertically shorter crania, decreased transverse widths, and the intersection between parietal, occipital, and squamosal bones, as well as the joint of the squamosal body to the zygomatic bone, were both located more posteriorly. The dentition in Ndr mice showed numerous defects, such as changes in molar morphology and partial detachment of the ameloblast-enamel matrix and ameloblast-stratum intermedium (SI) interactions in incisors and molars. Interestingly, we did not observe any obvious enamel rod mineralization defects using SEM, although subtle differences were noted near the dentin-enamel junction (DEJ). Our study demonstrates the roles of the Notch signaling pathway on craniofacial and dental development.

## Introduction

Alagille Syndrome (ALGS) is an incurable, multisystem, autosomal-dominant genetic disorder (2) with an incidence of ~1 in 70,000 (3). ALGS1 (OMIM no. 118450) occurs in ~90% of patients due to mutations in *JAGGED1* (*JAG1*) (4,5) and ALGS2 (OMIM no. 610205) occurs in ~1% due to *NOTCH2* mutations (6). Diagnosis of ALGS is based on histological findings of bile duct paucity, as well as three or more of the following 5 major features - chronic cholestasis, cardiovascular anomalies, ocular abnormalities, skeletal defects, and characteristic facial features (2,7). ALGS is distinguished from other liver disorders because of abnormalities observed in multiple systems (8). ALGS with severe liver or cardiac involvement is most often diagnosed in infancy with a 25% mortality rate before the age of five. However, the severity and clinical significance of ALGS phenotype are highly variable (7). Despite the potential use of characteristic skeletal defects and facies as clinical presentations to diagnose ALGS, there is little to no consensus on the specific morphological changes in the skull or alterations in the dentition.

Skeletal abnormalities in ALGS have been documented but relatively little is known about the craniofacial skeleton and dentition (9). A prior report showed that craniosynostosis was reported to be associated with ALGS in two patients (10). Butterfly vertebrae are the most common skeletal anomalies seen in approximately 50% of ALGS patients (7), likely due to the failure of the anterior vertebral arches to fuse (8). And to date, only a handful of case reports have presented ALGS dental defects, which can occur in deciduous and permanent teeth ranging from missing teeth, macrodontia, talon cusps, and enamel hypo-mineralization (11–15). Depending on the severity of the disease, ALGS may also affect the salivary glands, periodontium, and mucous

membranes (8). Liver dysfunction, such as cholestasis and hyperbilirubinemia has been shown to lead to variable greenish-brown pigmentation of primary and permanent dentition as bilirubin accumulates during tooth development (16–19).

To understand the changes in craniofacial and dental development in ALGS, we analyzed the skulls of *Jag1*$^{Ndr/Ndr}$ (also known as Ndr) mice, a model that closely phenocopied ALGS1 (1). *Jag1*$^{Ndr}$ is a missense mutation in the second EGF-like repeat (H268Q; Nodder) and results in a hypo-morphic JAG1 ligand that selectively binds NOTCH2 receptor, but not NOTCH1 and to a lesser degree NOTCH3 (1). However, it is currently unknown how post-translational modification of Notch receptors or ligands (e.g., Fringes) might impact specific receptor-JAG1$^{Ndr}$ interactions. Moreover, although Alagille syndrome was initially considered an autosomal dominant condition, some individuals carry mutations in JAG1 that do not result in classical Alagille syndrome. Kamath *et. al.* (2003) studied 53 family members carrying identical mutations in JAG1 (20). Twenty-one percent (21%) of mutation-positive relatives presented with clinical characteristics leading to ALGS diagnosis; 32% had mild features of ALGS; 47% of mutation-positive relatives did not meet the clinical criteria for an ALGS diagnosis, including 2 relatives who possessed none of the ALGS clinical characteristics. Therefore, these observations suggest that the inheritance pattern of Alagille syndrome is quite complex.

The Notch signaling pathway is highly conserved, cell-contact dependent, and comprises 4 transmembrane Notch receptors (Notch1-4) and 5 ligands (Jag1, Jag2, Dll1, Dll3, and Dll4). The roles of Bmp, Eda, Fgf, Shh, and Wnt signaling pathways in craniofacial and tooth development have been well documented (21,22), but surprisingly little is known about Notch signaling (23,24). The relative lack of studies and knowledge

regarding *in vivo* biological roles for Notch signaling appears largely due to premature lethality (before mineralization of bones and teeth) upon inactivation of Notch components during mouse development (25–31). Several prior studies have noted possible changes to mouse skulls with *Jag1* or *Jag2* inactivation. *Jag1*-null heterozygous mice exhibited eye dysmorphology (29); craniosynostosis was observed when *Jag1* was removed from the mesoderm (32)*; Jag2*-null homozygous mice died perinatally because of defects in craniofacial morphogenesis, including cleft palate and fusion of the tongue with the palatal shelves (27).

Analysis of *Jag1*$^{Ndr/Ndr}$ mice offers an unprecedented opportunity to not only advance our understanding of Alagille syndrome, but also to expand upon the roles of Notch signaling in the craniofacial skeleton and dentition.

## Materials and Methods

### *Animals*

All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at UCSF and the mice were handled in accordance with the principles and procedure of the Guide for the Care and Use of Laboratory Animals under the approved protocol AN084146. Briefly, mice with a missense mutation (H268Q) in *Jag1* (*Jag1*$^{Ndr\ /+}$ mice) were outbred to a C3H/C57bl6 background, as previously described (1). Male and female *Jag1*$^{Ndr/+}$ mice were mated to generate control (*Jag1*$^{+/+}$ and *Jag1*$^{Ndr/+}$) and mutant (*Jag1*$^{Ndr/Ndr}$) mice (1). Mutant *Jag1*$^{Ndr/Ndr}$ mice are also referred to as Ndr mice. Mice at the following stages were collected and analyzed - embryonic day (E) 14.5 and E16.5, postnatal day (P) 0 and P7, and 8 months old (adult). All collected specimens were fixed in 4% paraformaldehyde for 24-48h.

### *Image processing and mineral density analysis*

Micro-computed tomography (microCT) scans were performed on fixed P7, 6-week-old, and 8-month-old mouse heads using a Scanco Medical µCT50 at the Center of Musculoskeletal Biology and Medicine in the Skeletal Biology Core at UCSF. Specimens were scanned at 20-micron resolution (55kVP, 109µA, 6W, 0.5mm AI filter). Reconstructions were generated using Scanco Medical's integrated µCT Evaluation Program V6.5-3, then converted into 3D volumes using µCT Ray V4.0-4.

Reconstructed microCT scans were imported to Avizo software (Avizo 2019.4, Thermo Fisher Scientific, Waltham, MA, USA), where specimens' regions of interest (maxillary molars, mandibular molars, maxillary incisors, and mandibular incisors) were

segmented. After establishing a threshold grey value specific to enamel, region-specific enamel density values were extracted and normalized, with control specimens' enamel density as a baseline for comparison.

*Landmarking and geometric morphometric analysis (GMA)*

Coordinate locations of 44 landmarks on the cranium and midface, as well as 13 landmarks on each hemi-mandible (total 26 landmarks in the mandible) of 8-month-old control (N=4 males) and Ndr mutant (N=4 males) mice, were placed using Landmark software (Fig. 1). To determine the accuracy and reproducibility of landmark identification, the samples were landmarked twice by CPC one week apart using the 43 cranium and midface landmarks (data not shown). None of the 43 landmarks placed exceeded an arbitrary difference of 7 voxels (0.125mm) between the 2 sets of measurements (33).

Variations in skull shape were assessed using principal components analysis (PCA) using the residuals of multivariate regression of Procrustes coordinates on centroid size to analyze the shape only (without influence from size differences) (34,35). PCA of Procrustes coordinates was based on eigenvalue decomposition of a covariance matrix, which transforms the Procrustes coordinates into scores along with principal components (PCs). In most cases, the first few PCs described most of the variance in the dataset. Each observation was scored for each principal axis and the score for each observation along the principal axes map in the morphospace was defined by the principal component axes using MorphoJ software (36).

*Histology*

The skulls of E14.5, 16.5, P0, and P7 mice were demineralized in 0.5M EDTA for 1-7

days, dehydrated, embedded in paraffin wax in either frontal or sagittal orientations, and serially sectioned at 7 microns. Histological sections were stained with hematoxylin and eosin (H&E) following standard procedures. Brightfield images were taken using a DM5000B microscope with a DFC500 camera (Leica, Wetzlar, Germany).

*Scanning Electron Microscopy (SEM)*

Mouse skulls from 8-month-old control (N=3 males) and Ndr mutants (N=3 males) were dissected free of soft and connective tissue and fixed in 4% PFA in PBS overnight. The proceeding steps were described previously (37). Briefly, the hemi–mandibles were then embedded in epoxy resin, ground to the desired thickness, and polished. The exposed tissue was etched with 10% phosphoric acid for 30s, rinsed with water, and dried in a vacuum desiccator. Samples were mounted on SEM stubs and imaged in a Philips SEM instrument (XL30 ESEM, Philips, Andover, MA, USA).

*Statistical analysis*

For centroid sizes, the normal distribution of data was tested using the Kolmogorov-Smirnov non-parametric test. An independent *t*-test was employed to verify the existence of any significant differences in skull measurements between control and Ndr groups.

## Results

*Morphologic changes in the skull of Ndr mutant mice*

To evaluate changes in adult skull morphology using GMA, we identified and plotted landmarks on the skull, which was divided into two groups - cranium/midface and hemi-mandible (Fig. 1). The mandible was segmented out to ensure that all landmarks could be placed on the surface, particularly in the condylar area. Centroid size analysis showed some differences in size (Fig. S1), so we used the residuals of multivariate regression of Procrustes coordinates to eliminate the influence of size and to analyze the shape only.

*Cranium/midface*

GMA of the cranium/midface showed shape differences between male control and Ndr mutant mice (Fig. 2). Control and Ndr cranium/midface shapes were grouped into distinct, non-overlapping clusters (Fig. 2A) with PC1 and PC2 accounting for 30.9% and 24.9% of the total variation, respectively (Fig. 2B). Wider variability was observed within the Ndr specimens relative to controls, especially on the x-axis or PC2 (Fig. 2A). Hence, control and Ndr cranium/midface mainly differed in PC1 with Ndr mice showing lower values. Lower PC1 values were associated with Ndr mutants possessing less convex and vertically shorter crania (landmark #3), longer snouts (landmark #1), decreased transverse widths (landmark # 12-15), and the intersection between parietal, occipital, and squamosal bones (landmark #8-9) along with the joint of the squamosal body to the zygomatic process (landmark #10-11) were located more posteriorly in Ndr mutants (Fig. 2C). Little to no differences were observed in PC2 (Fig. 2D). Interestingly, we noted the presence of a bony extension in Ndr skulls in 2 out of the 4 specimens analyzed (Fig. 2E).

The extra bone appears to be an extension of the joint of the squamosal body to the zygomatic process (landmark #10-11).

*Hemi-mandible*

Control and Ndr right hemi-mandibles showed some shape differences with PC1 and PC2 accounting for 44.6% and 21.6%, respectively, of the total variation. Similar to the analysis of the cranium/midface, Ndr hemi-mandibles showed wider shape variability compared to controls, especially on the x-axis or PC2. Ndr hemi-mandibles trended towards lower values of PC1, which was associated with a more posterior mandibular or gonial angle (landmarks #10,11) and a decrease in ramus height (landmarks #7,8) due to changes in the condyle (Fig. 3C). Little to no differences were observed in PC2 (Fig. 3D). Notably, we saw similar results for the left hemi-mandibles (data not shown).

*MicroCT analysis of teeth*

MicroCT analysis of adult control and Ndr skulls showed some qualitative differences in the dentition (Fig. 4). Ndr maxillary and mandibular molars possessed smoother occlusal surfaces with less defined, less distinct cusps compared to controls (Fig. 4A-I). Control and Ndr mandibular incisors looked similar except that the occlusal plane, which contacts and occludes with the upper incisor, appeared to encompass a smaller area (Fig. 4K,L).

From the microCT data, we determined enamel densities of control and Ndr incisors and molars (Fig. 4E,J,O). Ndr molars tended to be slightly denser compared to controls with maxillary and mandibular molars showing $p=0.04$ and $p=0.06$, respectively. Control and Ndr mandibular incisors did not show differences in enamel densities.

*Tooth development*

To determine whether the changes in dentition were due to defects in tooth development, we sectioned control and Ndr hemi-mandibles at E14.5, E16.5, P0, and P7 (Fig. 5). We analyzed all teeth but did not always present both maxillary and mandibular teeth since we did not observe any differences (e.g., between maxillary and mandibular incisors). No histological differences were detected between control and Ndr teeth at E14.5 and E16.5 (Fig. 5A-H). In Ndr mice at P0, we noted separation between the ameloblasts and the enamel matrix in the maxillary molars and mandibular incisors (Fig. 5I-L''). In Ndr mice at P7, the detachment of the ameloblasts from the enamel matrix was more obvious (Fig. 5M-P''). Furthermore, the stratum intermedium (SI) appeared to be relatively disorganized in Ndr dentition compared to controls (Fig. 5M'',N'',O'',P''). And in other sections, the detachment of the ameloblasts to the SI was more pronounced (Fig. 5R,S).

*Enamel structure*

Adult mouse mandibular incisors and molars were analyzed using SEM (Fig. 6). No obvious differences were observed with the enamel rods from control and Ndr incisors (Fig. 6A-B') and molars (Fig. 6C-D'). However, some differences appeared to be present at or near the dentin-enamel junction (DEJ). The DEJ region in Ndr incisors and molars appeared to show less organization and fewer enamel rod extensions from the DEJ. Further investigation will be required to quantify these changes.

## Discussion

Early lethality at embryonic and perinatal stages with inactivation of Notch signaling factors in knockout mouse models have posed challenges in studying Notch signaling during craniofacial and dental development. Early embryonic deaths were observed with inactivation of *Notch1* (28), *Notch2* (26), *Jag1* (29), and *Dll1* (31), whereas *Jag2*-null mice exhibited perinatal lethality (27). *Jag1*$^{Ndr/Ndr}$ (also known as Ndr) mice with a *Jag1* missense (H268Q) mutation are viable and survive into adulthood. Analysis of Ndr mice demonstrated that Notch signaling is important in the determination of craniofacial and dental morphology and tooth development.

Even though genetic mutations causing ALGS have been studied extensively, ALGS is considered a highly variable, multi-organ disease with no clear genotype-phenotype correlation. Because ALGS subjects are often dealing with severe systemic manifestations that can be life-threatening such as chronic cholestasis, the associated craniofacial and dental effects have been less studied. Despite this prioritization, dysmorphic facial features are considered to be one of the best diagnostic characteristics of ALGS (38). Data points from our shape analysis or GMA of the skull revealed that Ndr skulls were widely variable compared to controls (Fig. 2A,3A). Ndr cranium/midface tended to be less convex with vertically shorter crania, longer snouts, decreased transverse widths, and the intersection between parietal, occipital, and squamosal bones, along with the joint of the squamosal body to the zygomatic process, were located more posteriorly compared to control skulls (Fig. 2C). The joint of the squamosal body also appeared to be extended in 50% of our 4 Ndr specimens (Fig. 2E). Ndr hemi-mandibles were associated with a more posteriorly displaced mandibular angle and a decrease in

ramus height with possible changes in the condyle (Fig. 3C).

Our analysis suggested that the sutures between parietal and occipital bones, as well as parietal and squamosal bones were most affected. The anomalies in suture development can influence surrounding structures, as well as the entire skull. Subjects with ALGS have presented with unilateral coronal craniosynostosis (10) so we postulate that there may be multiple sutures that are affected by ALGS. Premature fusion of cranial sutures can lead to abnormal skull shape, as seen in other diseases associated with craniosynostosis, such as Crouzon and Pfeiffer syndromes (39). GMA also showed that Ndr mice were associated with slightly longer snouts (Fig. 2C). Two previous studies showed slightly reduced snout lengths in Alagille mouse models but both experiments used linear measurements without taking into account the three-dimensional shape, whereas GMA does take this into account (1,40). We hypothesize that there may be curvature to the Ndr snout that was not captured by linear measurements. Moreover, neither decrease in snout length was deemed to be significant (e.g., $p>0.05$). But considering that there is variable penetrance of the Jag1(H268Q) allele and unclear genotype-phenotype expression in ALGS, it is unsurprising to observe such variability in skull shape. Interestingly, patients with the same mutations, including the ones from the same family, presented with clinical variability in disease phenotypes.

MicroCT images of control and Ndr adult dentition suggested some defects attributed to *Jag1*(H268Q) (Fig. 4). Ndr molars appeared to be smoother and less defined than control molars suggesting that the cusps were eroded away due to systemic effects and/or decreased mineral accretion. Surprisingly, the enamel mineral density of Ndr molars, measured from microCT data, was slightly higher than controls (Fig. 4). We did not detect much difference in shape or mineral density between control and Ndr incisors,

but this observation may be due to the continuous renewal of mouse incisors vs. rooted molars (i.e., different cellular and molecular mechanisms).

To gauge whether our tooth phenotype was due to developmental defects, we performed histology at various stages of tooth development (Fig. 5). We identified histological changes in the ameloblast-enamel matrix and ameloblast-SI interfaces in Ndr mutants between P0 and P7 stages (Fig. 5). We observed separation between the enamel matrix and ameloblasts, as well as disorganization in the SI and/or separation of the SI from ameloblasts. Similar detachment of the ameloblast-SI interface in adult mouse incisors was previously reported when Notch signaling was disrupted using injected inhibitory antibodies against Notch1, Notch2, Jag1, and/or Jag2 (41). Hence, we conclude that Notch signaling is critical in tooth development, especially to maintain the integrity of the ameloblast-SI interface. And *Jag1*(H268Q) may be essential for the integrity of the ameloblast-enamel matrix interface based on this and prior studies (41).

Enamel is composed of mineralized rods that extend from the DEJ to the enamel surface. These highly organized enamel rods run parallel in the same direction from the DEJ to the enamel surface. With antibody inhibition of Notch components, the mouse incisor enamel rods appeared to be smaller and rounder compared to controls (41). To evaluate the enamel rods in Ndr mice, we performed SEM on adult hemi-mandibles. We did not detect any obvious differences within the enamel (Fig. 6). However, we noted potential differences in the DEJ of control and Ndr dentition, specifically that the mineralized rods extending out to the enamel surface appeared to be fewer and less organized. Further experiments will be required to test this hypothesis.

We noted that Ndr mice were maintained on C3H/C57bl6 background as previously described (1). In a pure C3H background, homozygous Ndr mice are

embryonic lethal at ~E12.5 (42). In the mixed C3H/C57bl6 background, using F1-F3 generations, there is increased mortality of homozygous mice, which reflects Alagille syndrome variable penetrance and increased mortality in patients. Kamath *et. al.* (2003) showed that liver and cardiac defects had highly variable penetrance, whereas craniofacial dysmorphology had the highest penetrance (94% in probands and relatives). As such, it is possible that the surviving homozygous mice present with milder cardiac or liver phenotypes, but we expect the craniofacial dysmorphology to be more penetrant, even in surviving (possibly more mildly affected) homozygous mice.

In summary, analyses of *Jag1*$^{Ndr/Ndr}$ mice highlighted the importance of Notch signaling in craniofacial and dental development. *Jag1*(H268Q) disrupted Notch signaling, leading to specific changes in the cranium/midface and defective tooth development due in part, to the detachment of the ameloblast-enamel matrix and ameloblast-SI interfaces between stages P0 to P7. However, it remains unclear to what extent systemic factors, if any, play a role in the changes that we observed in Ndr skulls. The next steps will be to understand the cellular and molecular mechanism of Notch signaling in craniofacial and dental development, and to understand the variability associated with Alagille syndrome.

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## Figure Legends

**Fig. 1.** Mouse skull and landmarking. **(A)** The skull was segmented into the cranium/midface and hemi-mandible. *Red dots* indicate the position of landmarks. Cranium/midface in dorsal, ventral, lateral, and frontal views. Hemi-mandible in lateral and medial views. **(B)** Landmark descriptions of the cranium/midface. **(C)** Landmark descriptions of the hemi-mandible.

**Fig. 2.** Morphological changes in adult mouse control and Ndr cranium/midface. **(A)** PCA of cranium/midface shape. Wide variability in shape was observed amongst Ndr specimens. The two distinct clusters for control and Ndr mice represent the confidence range of the means of each group. **(B)** Graphs showing the variability associated with each PC (to PC7). PC1 and PC2 comprised 55.8% of the total shape variability identified. **(C)** Wireframe illustrations demonstrate shape changes associated with minimum (MIN, red lines) and maximum (MAX, blue lines) PC1 values. Gray lines represent the average shape. Cranium/midface in dorsal, ventral, lateral, and frontal views, same as in Fig. 1A. **(D)** Wireframe illustrations demonstrate shape changes associated with MIN (red lines) and MAX (blue lines) PC2 values. Gray lines represent the average shape. **(E)** Two out of 4 Ndr specimens analyzed by GMA possessed an extra bone or bony extension not observed in controls (arrows). Cranium/midface are in dorsal and lateral views.

**Fig. 3.** Morphological changes in adult mouse control and Ndr hemi-mandible. **(A)** PCA of hemi-mandible shape. Wide variability in shape was observed amongst Ndr specimens. The two distinct clusters for control and Ndr mice represent the confidence range of the means of each group. **(B)** Graphs showing the variability associated with each PC. PC1 and PC2 made up 66.2% of the total shape variability. **(C)** Wireframe illustrations demonstrate shape changes associated with minimum (MIN, red lines) and maximum (MAX, blue lines) PC1 values. Gray lines represent the average shape. **(D)** Wireframe illustrations demonstrate shape changes associated with MIN (red lines) and MAX (blue lines) PC2 values. Gray lines represent the average shape.

**Fig. 4.** MicroCT analysis of teeth. **(A-E)** Maxillary (MX) molars in occlusal (A,B) and oblique (C,D) views. Ndr MX molars showed smoothened and less defined cusps compared to controls (arrows). M1, M2, M3 indicate the 1st, 2nd, and 3rd molars respectively. Enamel mineral density was slightly higher in Ndr MX molars relative to control (E). **(F-J)** Mandibular (MN) molars in occlusal (F,G) and oblique (H,I) views. Ndr MN molars showed smoothened and less defined cusps compared to controls (arrows). Enamel mineral density trended to be slightly higher in Ndr MN molars relative to control (J). **(K-O)** MN incisors in occlusal and lateral views. Little differences were observed in

control and Ndr MN incisors with the exception of the occlusal table or the area that the MN incisor occludes with the MX incisor appeared to be smaller relative to control (white lines). No difference in enamel mineral density was detected.

**Fig. 5.** Histology of tooth development. **(A-S)** H&E staining of frontal (E14.5, E16.5) and sagittal (P0, P7) sections of maxillary (MX) and mandibular (MN) molars and/or incisors. **(A-D)** Control (Con) and Ndr molars in frontal view at E14.5 (cap stage) showed no obvious differences. **(E-H)** Con and Ndr molars and mandibular incisors (dotted lines) in frontal view at E16.5 (bell stage) showed no obvious differences. **(I-L'')** Con and Ndr molars and mandibular incisors at P0 in sagittal views. Initial defects at the junction between the enamel matrix and ameloblast were observed (arrows). Ndr teeth showed slight but consistent separation between the enamel matrix and ameloblast (arrows). **(M-S)** Con and Ndr MX molars and MN incisors at P7 in sagittal views. Ndr dentition showed complete separation between the enamel matrix and ameloblast (black arrows; M',N',O',P'). Defects in the ameloblast-stratum intermedium (SI) interface in Ndr teeth are also visible (white arrows; M''N'',O'',P''), where the SI appears to be less organized in Ndr teeth compared to controls. A second representative image of MN incisors (R,S) clearly shows detachment in the ameloblast-SI interface (white arrows) with smaller detachment spaces between the enamel matrix and ameloblast (black arrows). Am, ameloblasts; Od, odontoblasts; stratum intermedium (SI); enamel (En).

**Fig. 6.** Scanning electron microscopy (SEM) of adult mouse control and Ndr mandibular incisor and molar enamel. **(A–B')** Control (Con) and Ndr mandibular molar enamel in sagittal views. De, dentin; DEJ, dentin-enamel junction; En, enamel. Mineralized rods enamel rods extend from the DEJ to the enamel surface. Ndr incisors seemed to have fewer, less distinct enamel rods extending from the DEJ (area denoted by black dotted lines). **(C-D')** Con and Ndr mandibular incisor enamel in sagittal views. De, dentin; DEJ, dentin-enamel junction; En, enamel. Ndr molars appeared to possess fewer, less distinct enamel rods extending from the DEJ (area denoted by black dotted lines). **(A'-D')** Magnified views of white-boxed regions in **A-D**.