Influence of prenatal EGCG treatment and Dyrk1a dosage reduction on craniofacial features associated with Down syndrome

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Abstract

Trisomy 21 (Ts21) affects craniofacial precursors in individuals with Down syndrome (DS). The resultant craniofacial features in all individuals with Ts21 may significantly affect breathing, eating and speaking. Using mouse models of DS, we have traced the origin of DS-associated craniofacial abnormalities to deficiencies in neural crest cell (NCC) craniofacial precursors early in development. Hypothetically, three copies of Dyrk1a (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A), a trisomic gene found in most humans with DS and mouse models of DS, may significantly affect craniofacial structure. We hypothesized that we could improve DS-related craniofacial abnormalities in mouse models using a Dyrk1a inhibitor or by normalizing Dyrk1a copy number. In vitro and in vivo treatment with Epigallocatechin-3-gallate (EGCG), a Dyrk1a inhibitor, modulated trisomic NCC deficiencies at embryonic time points. Furthermore, prenatal EGCG treatment normalized some craniofacial phenotypes, including cranial vault in adult Ts65Dn mice. Normalization of Dyrk1a copy number in an otherwise trisomic Ts65Dn mice normalized many dimensions of the cranial vault, but did not correct all craniofacial anatomy. These data underscore the complexity of the gene–phenotype relationship in trisomy and suggest that changes in Dyrk1a expression play an important role in morphogenesis and growth of the cranial vault. These results suggest that a temporally specific prenatal therapy may be an effective way to ameliorate some craniofacial anatomical changes associated with DS.

Introduction

Down syndrome (DS, OMIM 190685) affects 140 of 100 000 live births, and all humans with DS exhibit dysmorphic craniofacial phenotypes and impaired cognition. During prenatal morphogenesis and growth, individuals with Trisomy 21 (Ts21) develop craniofacial abnormalities including reduced maxillary length, altered frontomaxillary facial angles and short, broad heads (1–4). These developmental craniofacial differences preface those seen in newborn, children and adult humans with DS including midfacial hypoplasia, a small oral cavity, brachycephaly and a generalized reduction in facial dimensions due to decreased growth rates and increased fluctuating asymmetry of facial soft tissues associated with decreased developmental homeostasis (4–8). Ts21 results in altered craniofacial
morphogenesis and growth that may impact orofacial structure or function, potentially resulting in eating, breathing and speech impairments (9–11).

Ts65Dn mice are the most widely used model of DS, have three copies of orthologs to ~50% of the genes on human chromosome 21 (Hsa21) and exhibit phenotypes associated with DS including craniofacial dysmorphology, skeletal alterations and cognitive deficits (7,12,13). Newborn Ts65Dn mice exhibit reduced dimensions along the rostrocaudal and mediolateral axes of the face and palate, anterior and posterior neurocranium, and mandible (14). Adult Ts65Dn mice exhibit increased neurocranial widths (i.e. brachycephaly), a flattened occiput and an overall reduction in size of much of the craniofacial skeleton; including the midface, maxilla, mandible, facial height and interorbital breadth (7).

Neural crest cells (NCC) are an important component of craniofacial development and contribute to the majority of the bone, cartilage, connective tissue and peripheral nervous tissue in the craniofacial complex (15–17). Using the Ts65Dn DS mouse model, we provided the first experimental evidence that trisomy disrupts NCC that would lead to the craniofacial abnormalities associated with DS (18). We found that the trisomic mandibular precursor, or first pharyngeal arch (PA1), was smaller in size and had fewer NCC by embryonic day 9.5 (E9.5). Additional deficits were quantified in trisomic embryonic cranial NCC generation, migration and proliferation (18). In later development, Ts65Dn mice also displayed a smaller mandibular precursor than normal E13.5 embryos (19). These and other developmental changes result in modified craniofacial structures in newborn and adult Ts65Dn mice that are similar to those found in humans with DS (7,14).

It has been hypothesized that differential expression of Dyrk1a (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A), a gene found in three copies in humans with Ts21 as well as in Ts65Dn mice, significantly affects many DS traits including craniofacial and neurological malformations (20). DyrK1a is a serine–threonine kinase that regulates many downstream proteins and transcription factors (20,21) and affects cell proliferation, differentiation and survival (22–24). Transgenic mice with increased dosage of Dyrk1a show developmental delay, motor impairment, learning and memory deficiencies as well as reduced bone mass and altered bone structure (21,25–28) similar to developmental, cognitive and morphological phenotypes observed in Ts65Dn mice. We have shown that eliminating one copy of Dyrk1a in otherwise trisomic Ts65Dn mice normalizes the appendicular skeleton (29).

One of the many mechanisms regulated by Dyrk1a via protein phosphorylation is the nuclear localization and activity of nuclear factor of activated t-cells (Nfat) transcription factor. Dyrk1a may control bone development and homeostasis through the Nfat pathway (28). Nfat has not been widely studied in vivo in bone, but Nfatc2/2−/− mice exhibit craniofacial abnormalities (20), suggesting that the Nfat pathway, in addition to other pathways, may be involved in the morphogenesis, growth and maintenance of the craniofacial complex.

Epigallocatechin-3-gallate (EGCG) is a known inhibitor of Dyrk1a protein kinase activity. Dyrk1a is an excellent drug target because small molecules like EGCG may attach to the ATP binding site of the Dyrk1a protein kinase (30). EGCG crosses the placental and blood/brain barriers (31,32). Treatment with an EGCG-containing supplement (Mega Green Tea Extract—45% EGCG, 98% polyphenols) in adulthood transiently improved spatial learning, thigmotaxis and novel object cognitive deficits in Ts65Dn and Dyrk1a transgenic mice (33) and adult hippocampal neurogenesis in Dyrk1a transgenic mice. Treatment with a similar EGCG-containing supplement in humans with DS showed some transient improvements in episodic and working memory (33), and with additional cognitive training, improvement of some secondary cognitive measures of executive functioning and adaptive behavior in functional academic skills (34). We have used a pure EGCG treatment for 3 weeks in adolescent mice to improve appendicular bone mineral density and trabecular skeletal phenotypes in Ts65Dn DS mice (29). A perinatal pure EGCG treatment transiently improved hippocampal neurogenesis (35). Yet, the aforementioned therapies with EGCG or EGCG-containing supplements are not likely to produce a permanent change in either cellular structure of the brain or cognitive function. Conversely, EGCG treatment during development and maturation of critical cellular and organ systems has the potential to induce permanent changes in adult mice or humans.

We hypothesized that increased Dyrk1a activity during embryonic trisomic development, through interactive effects including changes in Nfat nuclear localization, significantly contributes to DS craniofacial dysmorphology in Ts65Dn mice and individuals with Ts21. By altering Dyrk1a expression, either by inhibiting activity or by lowering gene dosage during embryonic development, we hypothesized that craniofacial skeletal dysmorphology would be permanently improved in the Ts65Dn DS mouse model. Our work delineating the time and tissue specificity of the NCC deficit related to DS led us to propose that prenatal treatment with EGCG during embryonic stages, when trisomy disrupts the NCC population, would result in improved craniofacial structure in both embryonic and adult stages and may correct gene expression changes caused by trisomy. We further hypothesized that elimination of one copy of Dyrk1a in an otherwise trisomic animal would result in improved craniofacial anatomy in Ts65Dn mice. We report herein the effect of EGCG treatment and reduction of Dyrk1a copy number on the trisomic craniofacial phenotypes associated with DS.

Results

Expression of Dyrk1a and Rcan1 RNA is dysregulated in the Ts65Dn E9.25 and E9.5 PA1 and neural tube

Dysregulation of Dyrk1a and Regulator of Calcineurin 1 (Rcan1) have been hypothesized to influence craniofacial phenotypes associated with DS (20), and we hypothesized that these trisomic genes may be involved in the NCC deficit of E9.5 Ts65Dn embryos (18). Before the NCC deficit is established in trisomic embryos at E9.25 (15–19 somites), trisomic embryos displayed decreased expression of Dyrk1a RNA (relative Ts65Dn to euploid expression) and increased Rcan1 RNA expression in both the E9.25 PA1 and neural tube (NT) (Fig. 1A). At E9.5 (21–24 somites), when the NCC deficit is apparent, Dyrk1a RNA expression was upregulated and Rcan1 RNA expression was downregulated in the E9.5 PA1 and NT (Fig. 1B).

Nfat localization not altered in E9.5 or E13.5 PA1

To determine the effects of increased copy number of Dyrk1a and Rcan1 on the cellular localization of Nfatc1 protein in trisomic craniofacial precursors, we conducted immunohistochemistry on the PA1 of E9.5 and E13.5 Ts65Dn and euploid embryo sections. Ts65Dn embryos exhibited a similar amount of nuclear Nfatc1 protein in the PA1 cells when compared with euploid littermates at E9.5 (Supplementary Material, Fig. S1; P = 0.22). These results suggest that the increased Dyrk1a RNA expression and reduced
Figure 1. Dysregulation of RNA expression from Hsa21 homologous genes found in three copies in Ts65Dn mice occurs in the PA1 and NT early in development. (A) Dyrk1a RNA expression was significantly downregulated in the E9.25 PA1 and NT of trisomic embryos, whereas Rcan1 RNA expression was significantly upregulated in both the E9.25 PA1 and NT in trisomic embryos (N = 5 euploid, 5 Ts65Dn PA1; 5 euploid, 5 Ts65Dn NT). Conversely, Dyrk1a RNA expression was upregulated in the E9.5 PA1 and NT in trisomic embryos relative to euploid littermates, whereas Rcan1 RNA expression was downregulated in these tissues (N = 7 euploid, 7 Ts65Dn PA1; 5 euploid, 5 Ts65Dn NT). For statistical analysis, two-tailed Student’s t-tests were performed. Statistical significance is annotated as * for $P \leq 0.05$. Error bars indicate standard error of the mean.

Figure 2. Ts65Dn PA1 cells displayed a proliferation deficit which can be overcome with EGCG treatment. Cells from the PA1 of E9.5 Ts65Dn embryos displayed impaired proliferation compared with euploid cells ($P < 0.01$) (N = 8 euploid, 7 Ts65Dn). A significant cellular proliferation occurred with 25 µM EGCG treatment for 4 h ($P < 0.001$) (N = 10 euploid, 10 Ts65Dn). Addition of 100 µM EGCG led to an increase in proliferation above euploid levels ($P < 0.001$). Cells derived from the NT displayed no deficit in proliferation, but were still affected by EGCG treatment. A dose of 10 µM (N = 10 euploid, 10 Ts65Dn) appeared to adversely affect proliferation of cells from NT ($P < 0.01$), whereas 25 µM EGCG produced no change from untreated trisomic cells ($P = 0.89$). A significant increase in cells was seen, however, in cells treated with 100 µM EGCG (N = 10 euploid, 10 Ts65Dn) to above euploid levels ($P < 0.001$). For statistical analysis, a two-tailed Student’s t-test was performed. Statistical significance is annotated as * for $P \leq 0.05$ and ** for $P \leq 0.001$ with respect to the untreated cells by tissue type. Error bars indicate standard error of the mean.

Rcan1 RNA expression in the E9.5 PA1 of Ts65Dn as compared with euploid embryos do not affect Nfatc1 nuclear localization in the PA1 cells at this time point. Similar to what was observed in the E9.5 PA1, no significant differences in Nfatc1 nuclear localization were found between the E13.5 mandibular precursor cells of Ts65Dn and euploid embryos, when we have shown a reduced expression of Dyrk1a RNA and increased expression of Rcan1 RNA in the trisomic mandibular precursor (19) [Supplementary Material, Fig. S2; $P = 0.37$]. Nfatc2 is involved in cartilage development (36) and may be an alternative pathway contributing to the Ts65Dn mandibular phenotype. Nuclear localized Nfatc2 protein was also not significantly different in the E13.5 mandibular precursor cells between euploid and trisomic embryos (data not shown). Together these data suggest that trisomic Dyrk1a- and Rcan1-mediated regulation of the Nfat nuclear protein localization in cells of the mandibular precursors is likely not a factor in the altered development of the Ts65Dn mandibular phenotype at these two developmental time points.

EGCG ameliorates Ts65Dn PA1 cellular proliferation and migration deficits in vitro

Due to the aberrant cellular phenotype in the trisomic PA1, we looked at the biological activity of cells that comprise this structure. Cells isolated from trisomic E9.5 embryonic PA1 showed significantly less proliferation than those from euploid PA1 in vitro (Fig. 2). Treatment of E9.5 PA1 cells with 10 µM EGCG in vitro led to not only a significant increase in proliferation of trisomic cells from the PA1 but also a significant decrease in the proliferation of trisomic cells from the NT. Treatment with 25 µM EGCG was sufficient to ameliorate the trisomic E9.5 PA1 proliferation deficit without altering NT proliferation significantly. In addition, treatment with 100 µM EGCG led to significantly higher proliferation in both E9.5 PA1 and NT cells than those of euploid animals.

Ts65Dn and euploid E9.5 PA1 and NT cellular migration was assessed using the scratch assay, in conjunction with dimethyl sulfoxide (DMSO) or EGCG treatment for 4 h. Ts65Dn E9.5 PA1 cells treated with 10 µM EGCG showed a significant increase in migration (average number of cells entering the open space) over euploid levels compared with DMSO treatment over the 72 h of the assay (Fig. 3A). This concentration of EGCG treatment led to an increase in migration of Ts65Dn NT cells that nearly reached euploid levels after 72 h in culture (Fig. 3B). The ratio of trisomic to euploid migration of E9.5 PA1 and NT cells was normalized with 10 or 25 µM EGCG treatment (Fig. 3A and B).

Harmine ameliorates Ts65Dn PA1 proliferation deficits in vitro

Similar to EGCG, harmine binds the ATP-binding pocket of Dyrk1a and inhibits its activity (37). Harmine was also assessed
in in vivo EGC2 treatment alters gene expression in pathways implicated in trisomic craniofacial development

Relative RNA expression (to that of untreated euploid embryos) of Dyrk1a, Rcan1, Shh (sonic hedgehog), Gli1 (glioma-associated oncogene family zinc finger 1), Ptc1 (patched 1) and Ets2 (V-ets avian erythroblastosis virus E26 oncogene homolog 2), all of which have been implicated in DS craniofacial development or developmental pathways (20,38,39), was examined in the PA1 of E9.5 embryos obtained from mothers receiving EGC2 or PBS on E7 and E8. Significant decreases in Ptc1 and Ets2 RNA expression and significant increases in Rcan1 and Shh RNA expression to euploid levels were observed in the EGC2-treated trisomic PA1 relative to original trisomic baseline expression values in untreated embryos (Fig. 5).

Elongated treatment of lower dose EGC2 has little effect on trisomic embryos

We hypothesized that treatment of pregnant Ts65Dn mothers with a lower dose of EGC2 from the start of pregnancy to E9.5 might more comprehensively correct the cellular and volumetric deficits of developing trisomic PA1 and embryos to resemble measurements in euploid littermates. When pregnant Ts65Dn mice were given 0.124 mg/ml EGC2 from E0 to E9, females ingested ~12 mg/kg/day EGC2 (taking into account degradation of EGC2) (40). There was no difference in the amount of EGC2 or water (control) that the pregnant females drank. There was a significant increase in PA1 volume in euploid E9.5 embryos from trisomic mothers [Eu/(Ts)] compared with untreated embryos of the same genotype (P = 0.046) (Fig. 6A). This elongated treatment of a lower dosage of EGC2, however, did not lead to significant improvements in the number of NCC in the PA1 (Fig. 6B) nor total embryo volume of E9.5 trisomic embryos compared with those treated with water (Fig. 6C).
Effects of EGCG treatment or Dyrk1a copy number on postnatal craniofacial structure

We tested whether prenatal EGCG treatment or a reduction in Dyrk1a dosage would have a permanent effect on postnatal craniofacial structure in trisomic and euploid offspring at 6 weeks of age. Craniofacial structure from trisomic and euploid offspring that received EGCG treatment at E7–E8 or offspring from Ts65Dn × Dyrk1a+/− matings was examined at 6 weeks of age. Using Euclidian distance matrix analysis (EDMA) (7,41,42), a total of 231 unique linear distance measures were evaluated from each mouse skull, and separate two-sample analyses of the cranial vault, facial skeleton, cranial base and mandible were carried out. EDMA confidence interval tests (α = 0.10) revealed morphological patterns of variation unique to each comparison of mouse samples.

When Ts65Dn and euploid offspring that received PBS from E7–E8 were compared, differences in the percentage of significant linear distances were notable in the cranial base, cranial face, mandible and cranial vault (Table 1). The great majority of significant differences across the craniofacial complex was larger in euploid as compared with Ts65Dn mice. No significant differences were seen across all dimensions of craniofacial regions when euploid + EGCG were compared with euploid + PBS mice (Table 1). The great majority of significant differences was larger in the euploid + EGCG mice as compared with euploid + PBS mice; however, four measures localized to the anterior and posterior cranial vault including mediolateral measures of the frontal and zygomatic bones and an anterior-posterior measure of the interparietal bone were significantly smaller in the euploid + EGCG relative to euploid + PBS mice (Fig. 7D).

Fewer significant differences were seen when one copy of Dyrk1a was removed from otherwise trisomic Ts65Dn mice (Ts65Dn,Dyrk1a+/−—two copies of Dyrk1a) or euploid mice (euploid,Dyrk1a+/−—one copy of Dyrk1a) (Table 1). When euploid and Ts65Dn mice from a (Ts65Dn × Dyrk1a+/−) mating were compared, relatively few significant differences were found across the face, cranial base and mandible, but numerous significant differences occurred across the cranial vault (Table 1 and Fig. 7E). Unexpectedly, almost all significant differences were smaller in the euploid as compared with Ts65Dn mice. No significant differences were seen in the cranial base, and few facial, mandibular or cranial vault differences were found when comparing Ts65Dn,Dyrk1a−/− and euploid mice (Fig. 7E). In Ts65Dn,Dyrk1a−/− mice, differences in the cranial vault were smaller in the euploid relative to the Ts65Dn + EGCG mice (Fig. 7B). Very few differences were seen when Ts65Dn mice that received EGCG were compared with Ts65Dn mice that received PBS (Fig. 7C). The most significant differences were seen across all dimensions of craniofacial regions when euploid + EGCG were compared with euploid + PBS mice (Table 1). The great majority of significant differences was larger in the euploid + EGCG mice as compared with euploid + PBS mice; however, four measures localized to the anterior and posterior cranial vault including mediolateral measures of the frontal and zygomatic bones and an anterior-posterior measure of the interparietal bone were significantly smaller in the euploid + EGCG relative to euploid + PBS mice (Fig. 7D).
Effects of EGCG treatment or Dyrk1a copy number on postnatal mass and airway volume

Similar to previous studies (43), Ts65Dn mice had a significantly smaller mass than euploid 6-week-old littermates when treated with PBS or when compared with littermates with Dyrk1a copy number variants (Table 2). Treatment with EGCG significantly reduced the mass of Ts65Dn and euploid littermates. Consistent with previous results (29,44), Ts65Dn/Dyrk1a+/- mice exhibited significantly increased mass when compared with Ts65Dn littermates, and euploid/Dyrk1a+/- mice were significantly smaller than euploid littermates. Nasopharyngeal airway volume was not significantly different between Ts65Dn and euploid mice or between Ts65Dn and Ts65Dn mice receiving EGCG. There were no significant differences in nasopharyngeal airway volume between Ts65Dn and Ts65Dn/Dyrk1a+/- littermates, but euploid/Dyrk1a+/- mice had a significantly smaller nasopharyngeal airway volume than euploid mice (Table 2).

Discussion

Expression of Dyrk1a RNA was decreased in the PA1 and NT of Ts65Dn as compared with euploid embryos at E9.25 but significantly increased in the same structures at E9.5. Rcan1 RNA expression exhibited the opposite effect at these time points. We have previously shown that Dyrk1a RNA expression was significantly decreased and Rcan1 expression increased in the E10 PA1 of Ts65Dn relative to euploid embryos (45) and both Dyrk1a and Rcan1 exhibited significantly increased RNA expression in the Ts65Dn as compared with euploid E13.5 mandibular precursor (19). Taken together, these data indicate that expression of trisomic Dyrk1a and Rcan1 RNA are not consistently increased or decreased in the mandibular precursor of developing Ts65Dn embryos. These data may help clarify why reducing Dyrk1a copy number in Ts65Dn mice throughout development does not seem to significantly normalize the entire mandibular structure in 6-week-old Ts65Dn mice. Changes in RNA expression of Dyrk1a and Rcan1 in trisomic embryos appear to be temporally specific in the developing mandible. Furthermore, the dysregulation of Dyrk1a and Rcan1, both hypothesized to be linked to altered Nfatc expression in trisomic animals (20), do not seem to affect Nfatc nuclear localization in mandibular precursor cells at either E9.5 or E13.5. It may be that Nfatc cellular localization or expression is dysregulated at other time points during mandibular development or not altered by trisomic genes. We have previously shown that Nfatc nuclear localization is not altered in trisomic bone development at the time of the cartilage anlagen (E13.5) (46).

This study shows that a temporally specific prenatal EGCG treatment improves some craniofacial dysmorphology associated with DS in Ts65Dn embryos and mice. EGCG and harmine, both inhibitors of Dyrk1a activity (47), effectively improve NCC-related deficits in proliferation and migration in vitro in PA1 cells from Ts65Dn E9.5 embryos. In vivo treatment with EGCG at E7 and E8, around the time of the developing NCC deficit, appears to improve some of the NCC embryonic dysmorphology, especially in PA1 volume and NCC number in Ts65Dn E9.5 embryos. Gene expression was also altered by EGCG treatment and essentially normalized Rcan1, Shh, Ptc1, and Ets2 RNA expression—all of which are genes hypothesized to be related to some DS craniofacial deficits (18,38,39,48). EGCG treatment and Dyrk1a activity have been shown to be connected to and modulate the Shh pathway (49–51). However, a longer lasting EGCG treatment at a lower dose (E0–E9) did not have the same corrective effect on the trisomic embryonic structure at E9.5. These results suggest that timing and dosage of EGCG treatment are important in treating DS-related phenotypes.

The embryonic changes caused by prenatal EGCG treatment at E7–E8 appear to improve some craniofacial structures, particularly the cranial vault, in adult trisomic mice. The morphometric analysis used in this study (i.e. EDMA) assessed local form differences by comparing all unique linear measures across the cranial vault, face, base and mandible. Because of the potential for a large number of linear measures to significantly differ, it is useful to consider patterns of significant differences across craniofacial structures and to calculate summary statistics based on significant results. Here, we counted the number of significant linear distances in a particular region and divided this value by the total number of unique linear distances for that particular region (i.e. the number of linear distances that could have potentially been significantly different) to determine the percentage of significant differences for each craniofacial region (Table 1). This summary statistic is useful for comparing global results on a region by region basis from multiple two-sample EDMA analyses (4,52), although specific dimensions of interest for each region should be explored using local results (Fig. 7). EGCG treatment of Ts65Dn embryos reduced the percentage of significant differences from 19% to 14% in the cranial base, from 32% to 26% in

Figure 5. EGCG exposure leads to alterations of RNA expression from genes involved in pathways impacting craniofacial development E9.5 PA1. Baseline expression of RNA encoded by Dyrk1a, Rcan1, Shh, Gi1, Ptc1 and Ets2 quantified as cDNA by qRT-PCR revealed alterations in expression from euploid values (equivalent to a relative expression value of 1). RNA expression from these genes was altered in several cases in E9.5 trisomic PA1 receiving EGCG treatment in utero with most achieving near euploid values of relative expression. For statistical analysis, two-tailed Student’s t-tests were performed. Statistical significance is annotated as * for P ≤ 0.05 for Ts65Dn + PBS versus Ts65Dn + EGCG, †P ≤ 0.05 for Ts65Dn + PBS untreated versus euploid + EGCG and #P ≤ 0.05 for Ts65Dn + EGCG versus euploid + EGCG. Error bars indicate standard error of the mean.

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the face and from 22% to 5% in the cranial vault in adult mice. This suggests that EGCG treatment slightly normalized cranial base and face morphology and considerably improved cranial vault morphology. In contrast to the results from our embryonic study, EGCG treatment did not significantly improve the overall mandibular dysmorphology in 6-week trisomic animals. It may be that prenatal treatment with EGCG has a larger effect on trisomic NCC precursors that are components of other parts of the craniofacial skeleton and therefore developmentally integrated, whereas the mandible is developmentally modular and therefore less affected by treatments influencing development of other craniofacial structures such as the cranial vault, face, and base. Overall the results imply that EGCG treatment at E7–E8 improves some craniofacial morphology in Ts65Dn mice, especially for the cranial vault, but does not entirely correct craniofacial structural abnormalities attributed to trisomy.

Normalization of Dyrk1a to two copies in otherwise trisomic Ts65Dn mice did not fully correct craniofacial dysmorphology associated with DS. Yet, our data indicate that trisomic Dyrk1a may be important for morphogenetic events that give rise to the shape and size of the cranial vault. Comparisons of euploid mice with Ts65Dn (three copies Dyrk1a) and Ts65Dn,Dyrk1a\(^{+/+}\) (two copies Dyrk1a) (Table 1 and Fig. 7A and B) unexpectedly revealed numerous smaller dimensions in euploid cranial vaults relative to Ts65Dn mice, but removal of one copy of Dyrk1a in Ts65Dn mice improved cranial vault morphology as evidenced by the reduction in significant cranial vault differences shown in Figure 7F relative to Figure 7E. The comparison of Ts65Dn,Dyrk1a\(^{+/+}\) and Ts65Dn mice (Fig. 7G) yielded numerous significant cranial vault differences, but significant differences were smaller in Ts65Dn mice with only two copies of Dyrk1a. Furthermore, EGCG treatment at E7 and E8 corrected a large number of significant differences in the cranial vault of 6-week-old Ts65Dn mice as compared with euploid littermates given PBS (Table 1 and Fig. 7A and B). In agreement with these results, we previously found that trisomic Dyrk1a has a major influence on the appendicular skeleton, and that reduction of Dyrk1a copy number in Ts65Dn,Dyrk1a\(^{+/+}\) mice did not restore bone mineral density in the skull and mandible to euploid levels (29). Additionally, both of the airways of Ts65Dn and euploid mice from (Ts65Dn \(\times\) Dyrk1a\(^{+/+}\)) matings not carrying the null Dyrk1a allele were larger in magnitude in comparison with those mice with EGCG/PBS treatment. Taken together, these results underscore the complexity of the gene–phenotype analyses, and suggest that changes in trisomic Dyrk1a expression, either via EGCG treatment or copy number reduction, play an important role in morphogenesis and growth of the cranial vault, and perhaps to a lesser degree, other craniofacial structures. It seems likely that major primary changes in cranial vault morphogenesis and growth could produce minor changes in the face, cranial base and mandible through complex regulatory and pleiotropic interactions and integrated functional craniofacial requirements related to organ protection and mastication, but additional investigations are necessary to verify this hypothesis. A limitation of our study is that we have not shown how EGCG treatment affects Dyrk1a activity in craniofacial precursors or adult structures.

Figure 6. Effects of EGCG treatment given at E0–E9.5 on E9.5 PA1 volume, NCC number and embryo volume. Ts/(Ts), trisomic embryos from Ts65Dn mothers; Eu/(Ts), euploid embryos from Ts65Dn mothers; Eu/(Eu), euploid embryos from euploid B6C3F1 mothers. Ts/(Ts) + H2O: n = 9; Ts/(Ts) + EGCG: N = 5; Eu/(Ts) + H2O: N = 6; Eu/(Ts) + EGCG: N = 9; Eu/(Eu) + H2O: N = 8; Eu/(Eu) + EGCG: N = 7. (A) No significant differences were seen in the PA1 volume of EGCG-treated trisomic Ts/(Ts) or euploid Eu/(Eu) embryos compared with untreated embryos (P = 0.058). PA1 volume was significantly increased in EGCG-treated euploid embryos [Eu/(Ts)] compared with untreated embryos [Eu/(Ts)]. (B) EGCG treatment from E0 to E9.5 at −12 mg/kg/day did not significantly alter the number of NCC in any treatment groups compared with untreated embryos. The slight but non-significant decrease in NCC in EGCG-treated Ts/(Ts) embryos was likely due to a lower average somite number in this group compared with water treated embryos (P = 0.0613). (C) No differences were found between treated and untreated embryo volume of any trisomic or euploid embryos. Statistical significance is annotated as * for P ≤ 0.05. Error bars indicate standard error of the mean.
Differences in craniofacial and airway measurements between trisomic and euploid mice from the pharmacological and genetic alterations were not expected. Ts65Dn and euploid mice from either control (PBS) treatment or that did not carry the Dyrk1a null allele exhibited similar overall weights at 6 weeks of age (Table 2). Yet, the craniofacial measurements in trisomic offspring (without a null Dyrk1a gene) from (Ts65Dn × Dyrk1a−/−) matings were generally larger than euploid littermates (Fig. 7E and F). To generate the Dyrk1a−/− mice used in the breeding scheme of this study, C57BL/6-129/Ola-mixed genetic background Dyrk1a−/− mice carrying the null Dyrk1a allele (44,53) were bred onto a B6C3 advanced intercross background for seven or more generations. This advanced intercross background was intended to be similar to the approximate 50% B6 and 50% C3H genetic background of Ts65Dn mice. It may be that residual background 129 alleles on the normal mouse chromosome 16 (not carrying the null Dyrk1a allele) cause the disparate craniofacial and airway volume phenotypes observed in the trisomic and euploid offspring from the (Ts65Dn × Dyrk1a−/−) matings. A possible explanation for the larger Ts65Dn cranial vault in the Ts65Dn versus euploid and Ts65Dn versus Ts65Dn,Dyrk1a−/− comparisons is that a residual background 129 allele on chromosome 16 is interacting with B6 or C3H alleles possibly indicating the interactive effects of heterotrisomy (54). Alternatively, an unknown epigenetic influence from the Dyrk1a−/− fathers may also have caused the differences in airway volume and craniofacial abnormalities seen between Ts65Dn and euploid mice from the (Ts65Dn × Dyrk1a−/−) matings and mice treated with PBS. These hypotheses should be tested in offspring from additional matings of (Ts65Dn × Dyrk1a−/−) mice with a similar genetic background.

Supplements containing EGCG have been shown to improve some cognitive and skeletal DS phenotypes in adult mice (33, 46). As the development of the face and brain is highly related (55–57), embryonic EGCG treatment may also alter brain morphology, which influences overall cranial vault shape during morphogenesis and growth (57). The reduction in neurocranial shape in Ts65Dn mice has been shown to correlate with the characteristic flattened occiput found in humans with DS. This region of the neurocranium overlies the cerebellum, which has reduced volume in both Ts65Dn mice and individuals with DS and mandibular bones. (E) Euploid compared with Ts65Dn: significant differences were present along the rostrocaudal and mediolateral axes of the face including the malar process and the nasal, premaxillae and maxillae bones. Numerous significant differences were present across the cranial vault including the nasal, premaxillae and maxillae bones. Many anterioposterior, mediolateral and superioinferior differences were found across the face that include the nasal, premaxillae and maxillae bones. Many anterioposterior, mediolateral and superioinferior differences of the mandible significantly differed. Several anterioposterior, mediolateral and superioinferior measures of the cranial vault were also significant. (F) Euploid compared with Ts65Dn + EGCG: significant cranial base differences were found that include anterioposterior dimensions of the basisphenoid and occipital bones. Numerous rostrocaudal, mediolateral and superioinferior differences were found across the face that include the nasal, premaxillae and maxillae bones. Many height, width and length measurements of the mandible significantly differed. Several anterioposterior, mediolateral and superioinferior measures of the cranial vault were also significant. (G) Euploid compared with Ts65Dn + PBS: significant superioinferior and mediolateral facial differences including the premaxillae and nasal bones and significant cranial vault width differences localized to the interparietal bone were smaller, whereas a mandibular measure of incisor height was larger in Ts65Dn + EGCG relative to Ts65Dn + PBS mice. (H) Euploid + EGCG compared with euploid + PBS: differences were found across craniofacial skeleton and localized to the nasal, premaxillae, maxillae, frontal, parietal, interparietal, occipital, basisphenoid and mandibular bones. (E) Euploid compared with Ts65Dn: significant differences were present along the rostrocaudal and mediolateral axes of the face including the malar process and the nasal, premaxillae and maxillae bones. Numerous significant differences were present across the cranial vault including the nasal, premaxillae and maxillae bones. Many anterioposterior, mediolateral and superioinferior differences of the mandible significantly differed. Several anterioposterior, mediolateral and superioinferior measures of the cranial vault were also significant. (F) Euploid compared with Ts65Dn,Dyrk1a−/−: significant facial aspects along the superioinferior–rostrocaudal dimensions including the maxillae bones were larger in euploid mice, whereas significant cranial vault dimensions along the anterioposterior and mediolateral axes including the parietal and interparietal bones and a significant mandibular measure of incisor height were smaller in euploid relative to Ts65Dn,Dyrk1a−/− mice. (G) Ts65Dn,Dyrk1a−/− compared with Ts65Dn: significant mediolateral differences in palatine bone width were found. One superioinferior–rostrocaudal facial measure of the maxillary bone significantly differed between samples. A measure of incisor width was significant. There were numerous significant differences in mediolateral and anterioposterior cranial vault dimensions crossing the zygomatic, frontal, parietal, interparietal and squamosal bones. (H) Euploid compared with euploid,Dyrk1a−/−: significant facial differences along the superioinferior and rostrocaudal dimensions including the premaxillae and maxillae bones were larger, whereas a mandibular measure of incisor width was smaller in euploid relative to euploid,Dyrk1a−/− mice.

Figure 7. Craniofacial measurements of 6-week-old Ts65Dn and euploid mice with or without EGCG treatment or Dyrk1a genetic reduction. Linear distances that significantly differ by confidence interval testing (α = 0.10) from two-sample EDMA comparisons of mouse cohorts are shown on lateral, superior and inferior views of the mouse cranium and superior and lateral views of the mandible. Linear distances that were significantly smaller or larger are depicted for each comparison as dashed or solid lines. (A) Euploid vs PBS compared with Ts65Dn + PBS: significant cranial base differences were found that include anterioposterior dimensions of the palatine, basisphenoid and occipital bones. Numerous rostrocaudal, mediolateral and superioinferior differences were found across the face that include the nasal, premaxillae and maxillae bones. Many height, width and length measurements of the mandible significantly differed. Several anterioposterior, mediolateral and superioinferior measures of the cranial vault were also significant. (B) Euploid + PBS compared with Ts65Dn + EGCG: significant cranial base differences were found that include anterioposterior dimensions of the basisphenoid and occipital bones. Numerous length, width and height significant differences were found across the face that include the nasal, premaxillae and maxillae bones. Many anterioposterior, mediolateral and superioinferior differences of the mandible significantly differed. Mediolateral differences in skull width were localized to the anterior portion of the cranial vault including the zygomatic arches and frontal bone and to the posterior cranial vault including the parietal bones. (C) Ts65Dn + EGCG compared with Ts65Dn + PBS: significant superioinferior and mediolateral facial differences including the premaxillae and nasal bones and significant cranial vault width differences localized to the interparietal bone were smaller, whereas a mandibular measure of incisor height was larger in Ts65Dn + EGCG relative to Ts65Dn + PBS mice. (D) Euploid + EGCG compared with euploid + PBS: differences were found across craniofacial skeleton and localized to the nasal, premaxillae, maxillae, frontal, parietal, interparietal, occipital, basisphenoid

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Table 1. Percentage of significant differences in cranial structures of 6-week-old Ts65Dn and euploid mice with or without EGCG treatment or Dyrk1a dosage reduction

| Comparison | Cranial base | Cranial face | Mandible | Cranial vault |
|------------|--------------|--------------|-----------|---------------|
| Ts65Dn + PBS versus euploid + PBS | 19 (4/21) | 32 (21/66) | 52 (34/66) | 22 (17/78) |
| Ts65Dn + EGCG versus euploid + PBS | 14 (3/21) | 26 (17/66) | 53 (35/66) | 5 (4/78) |
| Ts65Dn + EGCG versus Ts65Dn + PBS | 0 (0/21) | 5 (3/66) | 3 (2/66) | 3 (2/78) |
| Euploid + EGCG versus euploid + PBS | 67 (14/21) | 80 (53/66) | 79 (52/66) | 32 (25/78) |
| Ts65Dn versus euploid | 0 (0/21) | 8 (5/66) | 3 (2/66) | 17 (13/78) |
| Ts65Dn,Dyrk1a+/− versus euploid | 10 (2/21) | 3 (2/66) | 5 (2/66) | 12 (9/78) |
| Euploid,Dyrk1a+/− versus euploid | 0 (0/21) | 6 (4/66) | 2 (1/66) | 0 (0/78) |

Table 2. Body weights and nasopharyngeal airway volumes of 6-week-old Ts65Dn and euploid mice with or without EGCG treatment or Dyrk1a dosage reduction

| Genotype/treatment | Mean 6-week weight in grams (SEM) [N] | Mean airway volume in mm$^3$ (SEM) [N] |
|--------------------|----------------------------------------|----------------------------------------|
| Ts65Dn + PBS       | 20.4 (0.85)$^{a,b}$ [6]                  | 8.7 (1.76) [5]                          |
| Ts65Dn + EGCG      | 18.1 (0.73) [5]                          | 7.8 (0.93) [7]                          |
| Euploid + PBS      | 23.3 (1.08)$^c$ [5]                     | 7.3 (1.31) [5]                          |
| Euploid + EGCG     | 20.5 (0.57) [7]                          | 8.7 (1.99) [7]                          |
| Ts65Dn             | 19.7 (0.85)$^{a,d}$ [7]                  | 11.4 (0.78)$^d$ [8]                    |
| Ts65Dn,Dyrk1a+/−   | 21.3 (0.45) [12]                        | 9.2 (0.94) [12]                        |
| Euploid            | 24.7 (0.37)$^e$ [14]                    | 13.0 (1.04)$^h$ [13]                   |
| Euploid,Dyrk1a+/−  | 20.2 (1.57) [3]                          | 8.9 (2.53) [7]                          |

Data reported as mean ± (SEM).

$^aP < 0.05$ when compared with euploid + PBS.

$^bP < 0.001$ when compared with Ts65Dn + EGCG.

$^cP < 0.05$ when compared with euploid + EGCG.

$^dP < 0.05$ when compared with euploid.

$^eP < 0.05$ when compared with Ts65Dn,Dyrk1a+/−.

$^fP < 0.06$ when compared with Ts65Dn,Dyrk1a+/−.

$^gP < 0.001$ when compared with euploid,Dyrk1a+/−.

$^hP < 0.05$ when compared with euploid,Dyrk1a+/−.

(7). Future studies will need to test the hypothesis that mice with normalized cranial vault and other cranial structures may also demonstrate a correlation in improved cognitive function.

Previous research has shown that relative to euploid mice, Ts65Dn mice exhibit numerous differences across the craniofacial complex including increased cranial vault width (i.e. brachycephaly) and a generalized reduction in linear dimensions across much of the skull including the face, mandible and dimensions of the cranial vault length (7). Previous investigations of adult Ts65Dn mice aged 4–7 months found brachycephaly in the anterior and middle portions (near bregma) of the cranial vault (frontal and parietal bones) (7), but the current investigation of 6-week-old Ts65Dn mice only found increased width for some measures of the anterior vault (frontal and zygomatic bones). Brachycephaly was not reported in P0 Ts65Dn mice (14); thus, it seems likely that brachycephaly is only beginning to develop in 6-week-old mice and becomes fully developed in older Ts65Dn adult mice.

Euploid mice prenatally treated with EGCG exhibited numerous craniofacial differences affecting practically all craniofacial bones relative to euploid mice receiving PBS. The great majority of significant differences was larger in the euploid + EGCG mice, suggesting that EGCG treatment increased overall skull size relative to untreated euploid mice. This result also underscores the important, but complicated role Dyrk1a plays in early osteomorphogenetic developmental events and subsequent craniofacial growth. We have shown that the uterine environment of the trisomic mother does not influence gross differences in non-trisomic offspring (58) and therefore these results may have implications in treating non-trisomic offspring with EGCG.

It is possible that trisomic Dyrk1a expression only alters craniofacial and other precursors to specific DS-related phenotypes in a temporally and spatially specific manner. Our data suggest that an acute treatment of EGCG around the time of the NCC deficit in the trisomic PA1 affects embryonic craniofacial
precursors and structure of the adult cranial vault. Reducing tri-
somic Dyrk1a copy number did not correct embryonic Ts65Dn
appendicular skeletal development (46), but did significantly
correct adult skeletal phenotypes in the femur (29). In a similar
manner, upregulating the Shh pathway throughout develop-
ment did not correct Ts65Dn craniofacial abnormalities but did
have some effect on cognitive phenotypes (59,60). Reduction of
the trisomic gene Ets2 to normal copy number in an otherwise
trisomic mouse also had minimal effect on correcting DS-
related craniofacial abnormalities (38). Temporally specific reg-
ulation of Dyrk1a or other genetic pathways may be important
to correct craniofacial and other DS-related phenotypes.

Because Ts21 can be detected prenatally, it is possible that
prenatal treatments could lead to corrections of DS phenotypes
(61,62). Many DS craniofacial and neurological processes are ini-
tiated or completed before birth, and a window for prenatal
treatment and permanent correction of DS-related phenotypes
may exist. A number of studies have examined prenatal treat-
ments of DS phenotypes and have mostly concentrated on cog-
titive impairments [reviewed in (62)]. The use of prenatal
supplements to correct DS phenotypes needs to be further stud-
ied in animal models, including investigations of treatment tim-
ing and dosage, and precise quantification of phenotypes before
clinical trials in humans are proposed.

Materials and Methods

Generation and genotyping of mice and embryos
Female B6Ec3 Sn a/A-Ts(17;16)65Dn (Ts65Dn), B6CBA-Tg(Wnt1-
lacZ)206Amc/J (Wnt1-LacZ) and male B6C3F1 mice were
obtained from The Jackson Laboratory (Bar Harbor, ME). CS7BL/
6-129/Ola Dyrk1a heterozygous mutant mice (Dyrk1a+/−) were
obtained from Dr Mariona Arbones (Institut de Recerca
Oncologica, Barcelona, Spain) (44,53). CS7BL/6-129/Ola Dyrk1a−/−
mice were backcrossed to B6C3F1 mice for ≥7 generations to
parallel the genetic background of Ts65Dn mice. All mice were
bred at Indiana University–Purdue University Indianapolis
(IUPUI) to produce the offspring needed for this study. Female
Ts65Dn mice were bred with Wnt1-LacZ or B6C3F1 males for
embryonic studies. Females were checked for vaginal plugs the
morning after mating to ascertain copulation. Noon on the day
of the plug was established as embryonic day 0.5 (E0.5) for de-
veloping embryos. Embryos were dissected from pregnant fe-
males and were assessed for developmental stage by somite
number. For E9.5 embryos (21–24 somites), pregnant mothers
were euthanized between 10 am and 12 pm 9 days after the
plug was visualized. For E9.25 embryos (15–18 somites), embryos
were dissected at 6 am 9 days after the plug was observed. For
E13.5 embryos, embryos were dissected at noon 13 days after
the plug was observed. Dissected E9.25, E9.5 and E13.5 embryos
were then fixed for histological analysis or PA1 and NT tissues
were isolated for further investigation. Mice and embryos uti-
lized for this study were genotyped by PCR (44,63) or fluores-
cence in situ hybridization (FISH) (64) as described. All animal
use and protocols were approved by the IUPUI School of Science
Institutional Animal Care and Use Committee (IACUC).

RNA isolation
The PA1 and NT of E9.5 or E9.25 embryos were dissected from
the embryos in an RNase free environment using 30 gauge ne-
eddles. Structures dissected were specified by a cut matching the
line of the body for the PA1 and above the otic vesicle but below
the midbrain for the NT. RNA was extracted using the PureLink
RNA Micro Kit (Invitrogen, Carlsbad, CA) according to the manu-
facturer, and RNA concentration was assessed using the
Nanodrop ND-1000 (Thermo Scientific, Waltham, MA).

Immunohistochemistry for Nfat expression
E9.5 and E13.5 embryos for immunohistochemistry were fixed
in 4% paraformaldehyde, 5% sucrose in 0.1x phosphate buffer
pH 7.4 for 4 h and infiltrated overnight with 20% sucrose in
phosphate buffer for cryo-embedding. Sections of E9.5 and E13.5
Ts65Dn and euploid embryos were permeabilized in 1x PBS
with 0.5% triton X-100, washed in 1x PBS with 5% SDS for anti-
gen retrieval, blocked with 10% donkey serum in PBS with 0.2%
Triton X-100 for 1 h, and treated with rabbit polyclonal Nfatc1
(sc-13033, dilution 1:20, SCBT) or goat polyclonal Nfatc2 (CSC-
1151, dilution 1:20, SCBT) diluted in blocking buffer overnight in
a humidified chamber at 4°C. Sections were washed and in-
cubated with secondary antibody [Alexaflour 594 donkey anti-
rabbit IgG (Invitrogen A21207), 1:750 and Alexafluor 488 donkey
anti-goat IgG (Invitrogen A-11055), 1:750] for 1 h at room tem-
perature and treated with Prolong gold DAPI antifade
(Invitrogen, P36935), coverslipped and sealed with nail polish.
Sections were examined for the nuclear localization of Nfatc1
(Nfatc1nuc) in the PA1 (E9.5) or Nfatc1 and Nfatc2 in Meckel’s car-
tilage (E13.5) using an Olympus FV-111-MPE confocal multipho-
ton microscope (Olympus, Center Valley, PA). Colocalization of
Nfatc1/2 and DAPI in the nuclei was analyzed using Image J soft-
ware (National Institute of Health, Bethesda, MD) utilizing
quantitative co-localization methodology (46,65).

Cell culture of trisomic and euploid embryonic cells
For cell proliferation and migration, the E9.5 PA1 and NT were
removed as described above and placed into 0.025% trypsin/
0.1% collagenase/Dulbecco’s phosphate-buffered saline (DPBS)
for 4 min at 37°C. Cells were triturated ~10 times per sample
after incubation and then spun down at 9,280 g for 6 min.
The supernatant was extracted from each tube and cells were
resuspended in 50 μl of MCDM (66). Resuspended cells were
plated on fibronectin-coated 96-well plates (Becton, Dickinson
and Company, Franklin Lakes, NJ) (proliferation assays) or
fibronectin-coated coverslips (migration assays). Cells were
incubated at 5% CO2 and 37°C in MCDM until confluence (~12–
24 h, depending on genotype and tissue). During migration
assays, media changes occurred daily until assays were
completed.

Proliferation assay for trisomic and euploid craniofacial precursors
Cells derived from E9.5 PA1 and NT tissues were treated with
collagenase/DPBS and grown on the 48-well plate for 24 h, then were quantified for cell titer using a hemacytometer.
For proliferation assays, cells were grown to confluence on 96-
well plates, transferred to 48-well plates, quantified, and
replated on new wells at a density of ~1 x 10^4. From each sam-
ple consisting of suspended cells and 200 μl MCDM, 10 μl was
removed for quantification. For each cell titer, three counts were
taken on a hemacytometer and averaged to obtain a cell titer
per 4 x 10^-3 mm^2.

Cells were allowed to grow on the plate for 8 h and then were
treated with DMSO (Fair Lawn, NJ), Epigallocatechin-3-gallate
Migration assay of trisomic and euploid embryonic cells

Cells were placed on fibronectin-coated coverslips and incubated at 5% CO2 and 37 °C for 24 h in MCDM or until confluent, and then the scratch assay was performed as described (67) by creating a diagonal scratch across the cell layer on the coverslip. Media was changed to remove debris from the scratch, and cells were treated for 4 h with DMSO, ECGG in DMSO or harmine in DMSO at concentrations described above. Migration was quantified 0, 24, 48 and 72 h after the scratch was made. MCDM was changed daily with care taken to avoid disturbing the cells. Using ImageJ, the number of cells in the scratch was quantified and recorded as follows: each scratch was outlined to ensure conformity among scratch width before proceeding to quantification. Any images with inconsistent scratch widths were not utilized in this analysis. Using the counting tool, the number of cells within each scratch not touching the borders outlining the scratch was quantified for three images per culture. An average of these three numbers was then used as a migration count for that sample. Migration rates were established as the number of cells fully migrated into the scratch divided by the length of time since the scratch test was initiated in hours. Trisomic migration rates were then compared with euploid migration rates of the same tissue and culture group.

Quantitative PCR to assess differential RNA expression in E9.5 PA1

RNA was converted to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). Using this cDNA, master mixes were created for the reference gene (actin, Ev1) and target genes using TaqMan Gene Expression Master Mix (Applied Biosystems). Samples were analyzed in duplicate using the AB 7300 Real Time PCR System (Applied Biosystems). Crossing point (Cp) values from each duplicate trisomic sample were then averaged and divided by the average Cp value of the two euploid samples as performed by the 7300 System Software (Applied Biosystems). To determine a fold change, Cp values were subjected to the 2^-ΔΔCt formula as described (68). Relative quantification was performed on Tis65Dn and euploid littermate PA1 from both treated and untreated embryos. Expression was quantified for Ev1 (actin, housekeeping gene), Dyrk1a, Ranl1, Shh, Gli1, Pch1 and Ets2 probes (Applied Biosystems).

In vivo assessment of ECGG treatment during embryonic stages

For mice treated on embryonic (gestational) days 7 and 8 (E7 and E8), plugged female mice (Tis65Dn x Wnt1LacZ matings) were given 200 mg/kg ECGG dissolved in PBS or PBS alone twice daily at least 6 h apart by oral gavage using a 22 gauge feeding needle (69). 200 mg/kg ECGG oral gavage using a 15 mg/ml solution in a 30 g mouse results in the delivery of ~13 μmol ECGG. Mice were given water ad libitum before and after the oral gavage. For mice treated from E0 to E9 (Tis65Dn or euploid x B6C3F1 matings), pregnant females were given water or 0.124 g/ml ECGG in 25 ml water in feeding tubes after the plug was detected at E0.5, without other liquid, and allowed to drink ad libitum. ECGG or water volume was measured and changed every 48 h. Mice were strictly monitored for general health, assessed by daily weight changes, locomotor activity in the cage, and response to handling over the 2-day period of treatment before euthanizing. On day 9 after the plug was found, females were euthanized and postmortem embryos dissected. Embryos were processed, fixed and sectioned as previously described (18).

Stereological analysis of craniofacial precursors

Unbiased stereology (70) was used to quantify embryo and PA1 size and cell number. Systematic random sampling was used to quantify the number of cells in the PA1 and Calvalieri-point counting was utilized from volumetric measurements. Embryonic volume was assessed on every fourth section and PA1 volume and cell number were assessed sampling every third section containing PA1. Embryo volume was assessed with a frame area of 25 μm^2, 10 μm frame height, 2 μm guard height, 200 μm from spacing, 8000 μm^2 area per point and sampling from the top of the section. PA1 quantification was performed using disectors spaced at intervals of 60 μm with dimensions of 150 μm^2 area and 8 μm depth with a 2 μm guard height. Average coefficient of error for all volumes and cell counts was <0.10. Statistical differences were determined using a one-tailed Student’s t-test.

Assessment of embryonic ECGG treatment or Dyrk1a reduction on adult mice

Pregnant Tis65Dn mice were given 200 mg/kg ECGG twice daily by gavage on E7 and E8 as described above. Mice were housed until parturition, genotyped and offspring weaned after 21 days. Four types of mice were analyzed: Tis65Dn (n = 6), Tis65Dn + ECGG (n = 7), euploid (n = 5) and euploid + ECGG (n = 7). Tis65Dn mice were bred to Dyrk1a heterozygous knockout mice (44). Offspring from the (Tis65Dn x Dyrk1a^+/−) cross were genotyped for trisomy as well as the Dyrk1a knockout as described (44,63). Four types of mice from this breeding scheme were analyzed: Tis65Dn (n = 8), Tis65Dn,Dyrk1a^+/− (n = 13), euploid (n = 14) and euploid,Dyrk1a^+/− (n = 7). At the age of 6 weeks, multiple skulls/heads from these offspring were imaged at the same time using high resolution micro-computed tomography (μCT; 35 μm resolution) at the Indiana University School of Medicine. The images were segmented into separate heads and 3D bony isosurfaces of μCT image data were visualized and measured using Dolphin software (v11.5; Chatsworth, CA). Anatomical landmark coordinates from the craniofacial skeleton (n = 44) were identified (Supplementary Material, Fig. S7) and their x, y and z coordinates recorded for morphometric analysis. Additionally, nasopharynx airway volumes (mm^3) were measured from μCT images using Dolphin software (Supplementary Material, Fig. S8). Anatomical landmark coordinate values and nasopharynx airway volumes were measured on two separate occasions with at least 24 h in between measurement sessions.
to avoid memory bias, inspected for gross errors and then averaged to minimize potential effects of measurement error.

Differences in skull form among samples were analyzed using 3D landmark coordinate data and Euclidean distance matrix analysis (EDMA) as previously reported (52). EDMA converts 3D landmark coordinate data into an equivalent description of form called a form matrix, which consists of all unique linear distances among landmarks. Morphological differences between samples were statistically compared using a nonparametric bootstrap (10,000 resamples) and confidence interval testing ($\alpha = 0.10$) to determine local differences in craniofacial form. The null hypothesis was that average linear distance measures between the samples are the same (41,42). The percentage of significant differences in summary statistics was calculated by counting the number of significant linear distances in a particular craniofacial region (e.g. cranial vault, face, base or mandible) and dividing it by the total number of unique linear distances for that particular region (i.e. the number of landmarks measured in the region of interest) (44) to assess global region by region differences in patterns of variation across multiple two-sample EDMA form comparisons (45,52). Nasopharyngeal airway volumetric values were compared between samples using independent $t$-tests.

## Supplementary Material

Supplementary Material is available at HMG online.

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## Conflict of Interest statement

None declared.

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