Using the avian mutant talpid² as a disease model for understanding the oral-facial phenotypes of Oral-facial-digital syndrome

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Keywords: Primary cilia, craniofacial, neural crest, talpid², ciliopathies, chicken, Oral-facial-digital syndrome

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ABSTRACT

Oral-facial-digital syndrome (OFD) is a ciliopathy characterized by oral-facial abnormalities including cleft lip/palate, broad nasal root, dental anomalies, micrognathia and glossal defects. In addition, these patients have several other characteristic abnormalities typical of a ciliopathy including polysyndactyly, polycystic kidneys and hypoplasia of the cerebellum. Recently, a subset of human OFD cases has been linked to mutations in the centriolar protein C2 calcium-dependent domain-containing 3 (C2CD3). Our previous work identified mutations in C2CD3 as the causal genetic lesion for the avian talpid² mutant. Based on this common genetic etiology, we re-examined the talpid² mutant biochemically and phenotypically for characteristics of OFD. We found that, as in OFD patients, protein-protein interactions between C2CD3 and OFD1 are reduced in talpid² cells. Furthermore, we found that all common phenotypes were conserved between human OFD patients and avian talpid² mutants. In light of these findings, we utilized the talpid² to examine the cellular basis for the oral-facial phenotypes present in OFD. Specifically, we examined the development and differentiation of cranial neural crest cells (CNCCs) when C2CD3-dependent ciliogenesis is impaired. Our studies suggest that, whereas disruptions of C2CD3-dependent ciliogenesis do not affect CNCC specification or proliferation, CNCC migration and differentiation are disrupted. Loss of C2CD3-dependent ciliogenesis affects the dispersion and directional persistence of migratory CNCCs. Furthermore, loss of C2CD3-dependent ciliogenesis results in dysmorphic and enlarged CNCC-derived facial cartilages. Thus, these findings suggest that aberrant CNCC migration and differentiation could contribute to the pathology of oral-facial defects in OFD.
INTRODUCTION

Primary cilia are non-motile, microtubule-based organelles that function as cellular antennas to coordinate the transduction of several signaling pathways. Ciliopathies are a growing class of diseases whose cellular etiology lies in either disrupted structure or function of the primary cilia. Common clinical features of ciliopathies are broad and include: renal cystic disease, polydactyly, situs inversus, retinitis pigmentosa, hepatic disease, and mental retardation (Baker and Beales, 2009). Of the known human ciliopathies, approximately 30% are primarily defined by their oral-facial phenotype (Zaghloul and Brugmann, 2011). The most well known examples of oral-facial ciliopathies are Bardet-Biedl syndrome, Joubert syndrome, Meckel-Gruber syndrome, and Oral-facial-digital syndrome (Beales et al., 1999; Ferrante et al., 2001; Fraser and Lytwyn, 1981; Lorda-Sanchez et al., 2001; Maria et al., 1999). For many of these syndromes, both the genetic basis and cellular processes affected remain unclear.

Cranial neural crest cells (CNCCs) make a large contribution to the developing oral-facial complex. The facial skeleton, melanocytes, glia, and smooth muscle are directly derived from the CNCCs (Le Douarin et al., 2004), while other oral-facial structures such as teeth and tongue require CNCC contributions (Chai et al., 2000). Before CNCCs can differentiate into their final lineage, they undergo several cellular processes. CNCCs are specified at the dorsal neural tube and migrate ventrolaterally into the developing facial primordia and brachial arches. CNCCs must then proliferate to give the developing facial prominences their mass and shape. Later, CNCCs differentiate into various cell types including those that make up the skeletal elements of the face (Noden and Trainor, 2005). Given the significant oral-facial phenotypes present in many ciliopathies, we wanted to explore the role of primary cilia during
CNCC development.

The *talpid* (ta²) is a naturally occurring avian mutant that is best characterized by severe polydactyly and its oral-facial phenotype (Abbott et al., 1959; Abbott et al., 1960; Dvorak and Fallon, 1991; Schneider et al., 1999). The faces of affected ta² embryos are characterized by dysmorphic frontonasal prominences, facial clefting, hypoplastic maxillary prominences, incomplete fusion of the primary palate and hypoglossia (Chang et al., 2014). While the ta² mutant itself was first identified in the 1960s, the cellular and genetic basis for this mutant remained unknown for decades.

Our recent work determined that the ta² mutation affects ciliogenesis via a deletion mutation in C2CD3 (Brugmann et al., 2010; Chang et al., 2014), a centriolar protein required for ciliogenesis (Hoover et al., 2008). Concurrently, mutations in C2CD3 were found in a subset of individuals with Oral-facial-digital syndrome (OFD) (Thauvin-Robinet et al., 2014). Herein, we utilize the avian ta² mutant to determine the cellular etiology of the common oral-facial phenotypes present in OFD patients. Specifically, we examine how C2CD3-dependent ciliogenesis affects the development of CNCCs. Together, these experiments shed light on the cellular mechanism by which ciliary dysfunction leads to oral-facial anomalies in OFD.

**RESULTS**

*talpid* is an avian model for human Oral-facial-digital syndrome

The ta² is a naturally occurring avian mutation in which homozygous embryos (ta²/ta²) exhibit a characteristic phenotype including oral-facial and limb defects (Abbott et al., 1959; Abbott et al., 1960; Chang et al., 2014; Harris et al., 2006; Schneider et al., 1999). Our previous work identified ta² as a ciliopathic mutant (Brugmann et al., 2010; Chang et al., 2014). Specifically, we identified the causal
genetic lesion as a 19-bp deletion at the 3’ end of C2CD3 (Chang et al., 2014), a gene important for centriole elongation and primary cilia formation (Hoover et al., 2008; Thauvin-Robinet et al., 2014; Ye et al., 2014). Recently, mutations in C2CD3 were also identified as the causal genetic lesion for a subset of patients with Oral-facial-digital syndrome (OFD) (Thauvin-Robinet et al., 2014). In light of these genetic findings, we re-examined ta<sup>2</sup> embryos both phenotypically and biochemically to determine if they could be classified as a model for OFD.

Phenotypically, we found a remarkable resemblance between ta<sup>2</sup> mutants and OFD patients. As previously shown, ta<sup>2</sup> embryos display a myriad of oral-facial defects including cleft lip/palate, ectopic archosaurian-like first generation teeth, hypo- or aglossia (Fig. 1A-F, data not shown) and polydactylous limbs (Fig. 1G, H) (Abbott et al., 1959; Abbott et al., 1960; Brugmann et al., 2010; Chang et al., 2014; Harris et al., 2006; Schneider et al., 1999). Additionally, whole mount and histological examination revealed that ta<sup>2</sup> kidneys were polycystic (Fig. 1I-L), consistent with frequently reported kidney defects in OFD patients (Gorlin et al., 1990). Finally, human OFD patients can present with cerebellar vermal hypoplasia or agenesis, and an enlarged fourth ventricle (Gorlin et al., 1990; Poretti et al., 2008; Thauvin-Robinet et al., 2014). Using micro-computed tomography (micro-CT), we analyzed gross morphology of the avian brain and detected analogous malformations in the cerebellum of ta<sup>2</sup> mutants. Specifically, mutants exhibited hypoplasia of the cerebellum with reduced number of folia, agenesis of the cerebellar vermis and an enlarged fourth ventricle (Fig. 1M, N; control n=3, ta<sup>2</sup> n=3). Taken together, the co-presentation of these symptoms supported our suggestion that ta<sup>2</sup> could be an animal model for OFD.

To further test our hypothesis that ta<sup>2</sup> is a bona fide animal model of OFD we
performed biochemical analyses. The majority of OFD cases have been linked to mutations in Oral-facial-digital syndrome 1 (OFD1), a distal centriolar protein that restricts centriole elongation (Ferrante et al., 2003; Ferrante et al., 2001; Ferrante et al., 2006; Singla et al., 2010). Previous studies have shown that protein-protein interactions between OFD1 and C2CD3 are impaired in OFD cases (Thauvin-Robinet et al., 2014). To test if the ta^2 C2CD3 (C2CD3^ta^2) was able to physically interact with OFD1, we cloned the avian ortholog of OFD1, transiently transfected constructs for both OFD1 and C2CD3^Ctrl or C2CD3^ta^2, and performed co-immunoprecipitation in chicken embryonic fibroblasts (CEFs) (Fig. 1O, P). Whereas avian OFD1 and C2CD3^Ctrl physically interact in CEFs, there is a significant reduction in the amount of co-precipitation with OFD1 and C2CD3^ta^2 (Fig. 1O, P; Supplemental material; Fig. S1). These results closely mimic what is observed in human patients. Thus, from genetic, phenotypic and biochemical evaluations, our data strongly suggest that the ta^2 is a bona fide animal model for human OFD. We next wanted to understand the cellular mechanism behind the oral-facial phenotypes of OFD.

**Primary cilia extend from cranial neural crest cells during all ontogenic phases**

Cranial neural crest cells (CNCCs) make substantial contributions to the oral-facial complex (Le Douarin et al., 2004; Le Douarin and Dupin, 1993), specifically the oral-facial regions affected in OFD patients. To determine if and when CNCCs extend primary cilia *in vivo*, we performed co-immunostaining for the ciliary marker glutamylated-tubulin and markers of CNCCs or their derivatives. To determine if primary cilia were extended during CNCC specification, we performed co-immunostaining for the CNCC marker, PAX7, and glutamylated-tubulin. At HH 8^+, multiple PAX7-positive cells extended a primary cilium (Fig. 2A-A’’; arrows). We next examined whether migrating CNCCs extended a primary cilium. At HH 10 we
detected HNK1-positive cells that extended a primary cilium (Fig. 2B-B’’; arrows). Next, we examined correlations between cell cycle stages and the presence of a cilium on CNCCs in HH 22 embryos. Consistent with other reports, we did not observe any ciliary extension during M-phase as marked by PHH3-staining (Supplemental material; Fig. S2), but detected extended cilia during G1, S-phase, and G2 as marked by co-staining of glutamylated-tubulin and PCNA (Fig. 2C-C’’; arrows). Finally, we examined primary cilia extension on differentiating CNCCs. While CNCCs can differentiate into a wide variety of cell types, we chose to use cartilage as our readout for differentiation since CNCC-derived cartilages make up a significant portion of the developing face. Co-immunostaining for COL2A1 and glutamylated-tubulin on HH 28 embryos showed robust extension of primary cilia in differentiating chondrocytes (Fig. 2D-D’’; arrows). It should be noted that because we performed staining on tissue sections, rather than synced cells in culture, we were only able to detect a percentage of cells extending primary cilia during these stages (Fig. 2E, F). In previous experiments we isolated and cultured facial mesenchyme under serum-starved conditions and observed the expected 65-75% ciliary extension (Chang et al., 2014). Taken together, these data strongly support that CNCCs are extending cilia at all developmental time points, except during active mitosis. Thus, we hypothesized that the oral-facial phenotypes present in OFD patients were due to CNCC ciliary-dependent defects. To test this hypothesis, we next examined if disruption of C2CD3-dependent ciliogenesis could affect CNCC progression through any of these developmental phases.

CNCC specification is not affected by loss of C2CD3-dependent ciliogenesis

Both OFD patients and ta2 mutants present with facial clefting. One possible cause of clefting is CNCC insufficiency (Dixon et al., 2006). To determine if CNCCs
were properly specified when C2CD3-dependent ciliogenesis was disrupted, we performed whole mount *in situ* hybridization for various neural crest specifier genes including *Sox10, Snai2* and *Pax7*. There was no change in the domains of expression between control and ta2 embryos for any of these genes (Supplemental material; Fig. S3A-H; *Sox10* control n= 6, ta2 n= 4; *Snai2* control n= 22, ta2 n= 5; *Pax7* control n=16, ta2 n=6). We sought to confirm this finding by performing PAX7 immunostaining on cranial cross sections of HH 8+ control and ta2 embryos. There was neither a significant expansion nor a reduction in the domain of PAX7 (Supplemental material; Fig. S3I-J; control n=4, ta2 n=3). Thus, we concluded that C2CD3-dependent ciliary function is not required for the generation or proper specification of CNCCs, and therefore is not a possible mechanism for the oral-facial phenotypes of OFD.

**Defects in C2CD3-dependent ciliogenesis increase dispersion and reduce directional persistence in migrating CNCCs**

For proper facial development, CNCCs must migrate from the dorsal neural tube into the developing facial prominences. Disrupted CNCC migration has previously been associated with facial clefting (He and Soriano, 2013; Vasudevan and Soriano, 2014). For CNCCs to migrate properly into the developing facial prominences, they must collectively move in streams, maintaining close contact between adjacent cells (Kuo and Erickson, 2010; Minoux and Rijli, 2010). Furthermore, it has been shown that these local and transient cell-cell contacts are required for proper CNCC migration (Mayor and Carmona-Fontaine, 2010). To test if CNCC migration was aberrant in cells lacking C2CD3-dependent ciliogenesis, we performed whole mount immunostaining for HNK1 in HH 10 control and ta2 embryos. While CNCCs were able to migrate in mutants, HNK1 immunostaining...
suggested that $ta^2$ CNCCs were more dispersed when compared to control CNCCs (Fig. 3A-B'; control n=6, $ta^2$ n=4).

To confirm and quantify the observation that the loss of C2CD3-dependent ciliogenesis increased the dispersion of migrating CNCCs, we performed Delaunay triangulation: a mathematical application used to determine the density of a set of cells, previously described in (Carmona-Fontaine et al., 2011). Migrating CNCC nuclei were marked (Fig. 3C, D; white dots) and images were bisected and imported into Image J for analysis (Fig. 3E, G). The Delaunay triangulation algorithm was used to determine the two closest neighbors of a given cell, resulting in the formation of triangles (Fig. 3F, H). The area of triangles was calculated as a measure of cell dispersion (Fig. 3I; control n=12, $ta^2$ n=10). The average area between triangulated CNCC nuclei was significantly increased in $ta^2$ embryos, suggesting that the loss of C2CD3-dependent ciliogenesis increases the dispersion of migrating CNCCs.

To exclude the possibility that the observed decrease in density was due to fewer cells, increased apoptosis or reduced proliferation of CNCCs, we counted HNK-positive cells, and performed Sox10 qRT-PCR, TUNEL, and PHH3 immunostaining on both whole mount and cranial sections of HH 10 embryos (Supplemental material; Fig. S4). Neither the number of migrating CNCCs (Supplemental material; Fig. S4A; control n=11, $ta^2$ n=10) nor the amount of Sox10 expression (Supplemental material; Fig. S4B; control n=16, $ta^2$ n=9) was significantly altered in $ta^2$ mutants. Furthermore, there was no change in the amount of apoptosis (Supplemental material; Fig. S4C-F; control n=13, $ta^2$ n=6) or proliferation (Supplemental material; Fig. S4G, H; control n=6, $ta^2$ n=2) of CNCCs between control and $ta^2$ embryos. Given these data, we concluded that the observed increase in dispersion was not a consequence of reduced number of CNCCs. We next tested if
this increased dispersion correlated with disruptions in migratory behaviors.

To functionally test if loss of C2CD3-dependent ciliogenesis affects the ability of CNCC-derived mesenchyme to migrate, we performed three separate assays: a scratch assay, a transwell assay and time-lapse imaging of CNCCs migrating from dorsal neural tube explants. First, we isolated CEFs from the developing face of control and ta^2 embryos, plated cells to confluency and scratched through the cellular monolayer. CEF migration into the scratched area was measured at 0, 6, 12 and 24 hours (Fig. 4A-F; data not shown). The ability of ta^2 CEFs to fill the scratched area was significantly reduced relative to control CEFs after 6 hours (Fig. 4C, D, G). At 12 hours, 50% of control CEFs had completely filled the scratched area, while none of the ta^2 CEFs did (Fig. 4E, F, G; control n=6, ta^2 n=6). By 24 hours, both control and ta^2 CEFs were able to completely fill the scratched area (data not shown). To further assess the migratory ability of cells lacking C2CD3-dependent ciliogenesis, we performed transwell assays. Control and ta^2 CEFs were placed in the upper compartment of the transwell chamber and challenged to migrate through the pores of the membrane into the lower compartment (Fig. 4H). While over 40% of control CEFs migrated into the lower compartment of the transwell chamber, only 30% of ta^2 CEFs migrated into the lower compartment (Fig. 4I; control n=18,320 cells, ta^2 n=18,494 cells). Lastly, to confirm our migratory data from CEFs in CNCCs, we performed time-lapse imaging of dorsal neural tube explants (Fig. 4J-M). Imaris software was used to track both the speed and path of CNCCs migrating away from the explant (Fig. 4N-O). Examination of individual cell tracks for 40 minutes (control n=14, ta^2 n=14) revealed that despite traveling at the same speed (Fig. 4N), control CNCCs moved in a more consistent direction (Fig. 4O). Further analysis determined that control CNCCs maintain a significantly higher level of directional persistence.
relative to \( ta^2 \) CNCCs (Fig. 4P). Thus, taken together, our *in vivo, in vitro* and *ex vivo* studies suggest that loss of C2CD3-dependent ciliogenesis negatively impacts the directional migration and persistence of CNCCs.

**C2CD3-dependent ciliogenesis affects CNCC migration in a cell-autonomous manner**

CNCC migration requires both intrinsic signaling between CNCCs and signals from adjacent tissues (e.g., surface ectoderm and mesoderm). Since all tissues in the \( ta^2 \) mutant exhibit a loss of C2CD3-dependent ciliogenesis, we sought to determine if aberrant CNCC migration was cell-autonomous or non cell-autonomous. We performed dorsal neural tube transplants with control and \( ta^2 \) embryos (Fig. 5A, B, D, E). We first validated our experimental technique of transplantation using a control GFP-labeled explant (Supplemental material; Fig. S5). To test if there were any cell-autonomous mechanisms influencing aberrant CNCC migration, we generated sham and \( ta^2 \) chimeras and quantified the percentage of chimeras in which donor CNCC migrated away from the neural tube (Fig. 5A-A’). In sham chimeras, donor CNCCs migrated completely away from the dorsal midline in 63% of cases (Fig. 5A’, C; \( n=19/30 \)). In contrast, only 28.5% (\( n=2/7 \)) of \( ta^2 \) chimeras had CNCCs that migrated completely away from the dorsal midline, with the majority of cases (57%, \( n=4/7 \)) having CNCCs remaining at the dorsal midline (Fig. 5B’, C). To test if there were any non cell-autonomous mechanisms influencing aberrant CNCC migration in \( ta^2 \) embryos, we performed the reciprocal transplant experiments (Fig. 5D-F). Control CNCCs migrated equivalently in both control and \( ta^2 \) hosts (Fig. 5F). Together, these data suggest that loss of C2CD3-dependent ciliogenesis negatively affects CNCC migration in a cell-autonomous manner.
C2CD3-dependent ciliogenesis does not affect CNCC proliferation

Proliferation is a key event in CNCC development (Brugmann et al., 2007), as appropriate amounts of proliferation determine the shape and mass of the developing prominences of the oral-facial complex. To test if proliferation was altered when C2CD3-dependent ciliogenesis was lost, we examined control and \( ta^2 \) embryos and CEFs. Sections from the frontonasal prominence (FNP) of HH 22 control and \( ta^2 \) embryos were immunostained for PHH3, and the number of positive cells was quantified. No significant change in PHH3 positive cells was detected within developing FNPs (Fig. 6A-C; control n=17, \( ta^2 \) n=14). To confirm this finding, we analyzed control and \( ta^2 \) CEFs harvested from the facial mesenchyme. CEFs were cultured, pulsed with 5-ethynyl-2'-deoxyuridine (EdU) for 1 hour and immunostained (Fig. 6D). There was no significant change in EdU incorporation between control and \( ta^2 \) CEFs (Fig. 6E; control n=11,548 cells, \( ta^2 \) n=9,792 cells). Together, these analyses suggest that loss of C2CD3-dependent ciliogenesis does not cause oral-facial defects via disrupted rates of CNCC proliferation.

Defects in C2CD3-dependent ciliogenesis affect cartilage differentiation

OFD patients and \( ta^2 \) mutants both have significant skeletal defects (Abbott et al., 1959; Abbott et al., 1960; Gorlin et al., 1990). CNCCs give rise to a significant portion of the skeletal structures in the skull (Noden and Trainor, 2005). We examined formation of CNCC-derived cartilages in the developing faces of control and \( ta^2 \) embryos to determine if C2CD3-dependent ciliogenesis was important for skeletal development. We first examined cranial cartilage through whole mount alcian blue staining at HH 28. We observed a statistically significant increase in the width of the maxillopalatine process in the \( ta^2 \) mutant (Fig. 7A, B; compare length of black bars; control n=5, \( ta^2 \) n=5; Supplemental material; Fig. S6A). Additionally, Meckel’s
cartilage was thickened in $ta^2$ mandibles (Fig. 7C, D). We confirmed the expansion of facial cartilages in $ta^2$ embryos by examining COL2A1 expression in control and $ta^2$ cranial sections. In addition to observing an expansion of the maxillopalatine process (Fig. 7E, F), we also observed an increase in the size of the prenasal cartilage (Fig. 7G, H). Furthermore, we examined the expression of Sox9, a master transcriptional regulator of chondrogenesis (Bi et al., 1999), and found expanded expression throughout $ta^2$ facial prominences (Fig. 7I, J). Together, these results suggest that aberrant C2CD3-dependent ciliogenesis results in dysmorphic oral-facial cartilages.

Dysmorphic oral-facial cartilages have been reported in both murine and teleost models of OFD (Ferrante et al., 2009; Ferrante et al., 2006). The murine model of OFD, as well as another ciliopathic mutant with dysmorphic facial cartilages (Fuz$^{-/-}$), has increased Fgf8 expression within the developing facial prominences (Tabler et al., 2013). Based on these data we sought to determine if Fgf8 expression was altered in $ta^2$ embryos. Via both in situ hybridization and RNA-seq analyses we detected increased Fgf8 expression within the facial prominences (Fig. 7K-M). Furthermore, expression of Pea3, a key transcriptional target of FGF signaling was also increased in facial prominences (Fig. 7M). These data support a mechanism by which increased Fgf8 expression could account for increased chondrogenesis in the developing facial prominences of $ta^2$ mutants.

**DISCUSSION**

Oral-facial-digital syndrome (OFD) is a ciliopathy that affects development of the face, oral cavity, brain, kidneys and limbs (Anneren et al., 1984; Baker and Beales, 2009; Zaghoul and Brugmann, 2011). Herein we proposed the avian $ta^2$ mutant as an animal model for OFD based on genetic (Chang et al., 2014),
biochemical and phenotypic evidence (Fig.1). We examined the development of CNCCs in these mutants in order to understand the basis for oral-facial anomalies in human OFD patients. Our findings suggest that disruptions in C2CD3-dependent ciliogenesis do not affect the specification or proliferation of CNCCs, but do cause aberrant CNCC migration and differentiation. Taken together, these data propose a novel animal model that can be utilized to uncover the cellular and molecular etiology of OFD, as well as a system to test possible therapeutic treatments.

There are currently 13 different subtypes of OFD, classified by subtleties of phenotypic presentation. The phenotypic features of the various subtypes overlap significantly and some subtypes are not well defined. Although there is variability in the phenotypic presentation of OFD, most forms of this disorder involve anomalies of the oral cavity, facial features, digits and developing brain. C2CD3-dependent OFD represents the 14th OFD subtype (OMIM 615948) (Thauvin-Robinet et al., 2014), strongly suggesting that the *ta*2 mutant, previously shown to be caused by a mutation in C2CD3, could be classified as a novel generalized model for OFD, particularly relevant to OFD14.

**Biochemical mechanism of C2CD3-dependent OFD is conserved between species**

Until recently, only one gene had been exclusively linked to Oral-facial-digital syndrome. The *OFD1* gene encodes a ciliary protein that localizes to distal centrioles (Ferrante et al., 2006; Romio et al., 2004; Thauvin-Robinet et al., 2006). Most *OFD1* mutations identified in patients resulted in a truncated, non-functional protein (Thauvin-Robinet et al., 2006). Recent analysis of OFD patients found that a subset of cases were linked to mutations in C2CD3, a distal centriolar protein that promotes centriole elongation and recruitment of other distal centriolar proteins, including
OFD1 (Thauvin-Robinet et al., 2014; Ye et al., 2014). Data from human and mouse show that C2CD3 and OFD1 physically interact to mediate centriolar lengthening and ciliogenesis (Thauvin-Robinet et al., 2014). We found that this interaction was conserved in chicken (Fig. 1). Co-IP experiments show that avian C2CD3 and OFD1 physically interact, and that this interaction is negatively affected in ta2 mutants that produce a truncated form of the C2CD3 protein. Thus, it stands to reason that mutations in C2CD3 and OFD1 generate very similar phenotypes and result in OFD because they comprise a core protein complex at the distal centriole in avian, murine and human cells.

**The role of primary cilia in cell migration, a C2CD3-dependent mechanism**

Several ciliary mutants exhibit aberrant cell migration (Osborn et al., 2014; Tabler et al., 2013; Tobin et al., 2008). Fibroblasts from ciliopathy patients and fibroblasts and NCCs from ciliopathic animal models fail to migrate normally, exhibiting disruptions in actin cytoskeletal architecture, decreased velocity and decreased directional persistence (Hernandez-Hernandez et al., 2013; Madhivanan et al., 2012; Tobin et al., 2008). The mechanism by which primary cilia contribute to cell migration, however, remains nebulous. One potential mechanism is that C2CD3-dependent cell migration involves the localization/activation of the GTPases, RhoA and Rac1. RhoA controls many aspects of adhesion and cytoskeletal organization, promoting protrusion collapse. Rac1 is important for lamellipodia stabilization and cell repolarization (Ridley, 2011). RhoA and Rac1 have a mutually antagonistic relationship where activated Rac1 is localized to the migration front, promoting actin polymerization and driving protrusions; and activated RhoA is localized to the trailing edge, driving contraction. Various studies have shown that ciliopathies such as Lowe,
Joubert, and Bardet-Beidl syndromes have defects in RhoA or Rac1 localization or activity (Hernandez-Hernandez et al., 2013; Madhivanan et al., 2012; Valente et al., 2010). We analyzed Rac1 activation in control and ta<sup>2</sup> CEFS and did not detect any significant difference in levels of total or activated Rac1 between control and ta<sup>2</sup> cells (Supplemental material; Fig. S7, data not shown). We have yet to analyze RhoA activity in ta<sup>2</sup> cells. In light of the fact that RhoA can localize to the centriole/basal body (Valente et al., 2010), it remains possible that RhoA localization/activity is perturbed in ta<sup>2</sup> cells due to disruptions in protein complex formation at the distal centriole. Further investigation of RhoA localization and activity in ta<sup>2</sup> cells is a focus of our future work.

A second mechanism for aberrant cell migration focuses on the cilium functioning as an antenna to detect a chemoattractant gradient. This hypothesis has been supported by the fact that: 1) transduction of chemoattractant signals occurs through the primary cilium, 2) primary cilia are required for directed migration of fibroblasts towards a chemoattractant source and 3) receptors for chemoattractants, such as PDGFRα, localize to the axoneme (Schneider et al., 2010; Schneider et al., 2005). Specifically, data regarding PDGFRα provide strong support of this mechanism, as activation of PDGFRα plays dual roles in NCC migration by stimulating chemotaxis and regulating cell motility (He and Soriano, 2013; Vasudevan and Soriano, 2014). Loss of PDGFRα on NCCs produces a strikingly similar NCC migration phenotype to that of ta<sup>2</sup> mutants. NCCs that are unable to respond to PDGFA have migratory defects, a reduced ability to recover from scratch assays and lack directional persistence (He and Soriano, 2013; Vasudevan and Soriano, 2014). Testing the ability of ta<sup>2</sup> NCCs to respond to chemotactic gradients is a focus of our future work.
The role of C2CD3-dependent ciliogenesis in CNCC differentiation

CNCCs give rise to a wide variety of cell types; however, within the developing face their major contribution is to the facial skeleton. In $ta^2$ mutants, cartilaginous elements within the oral-facial complex are enlarged and dysmorphic (Fig. 7). Both patients with ciliopathies and numerous ciliopathic animal models have dysmorphic and/or ectopic facial skeletal elements (Brugmann et al., 2010; Ferrante et al., 2009; Ferrante et al., 2006; Kjaer et al., 1999; Lunt et al., 2009; Tobin et al., 2008; Weatherbee et al., 2009; Zhang et al., 2011). These data strongly indicate that primary cilia play a role in CNCC differentiation. The mechanism of how primary cilia contribute to this process, however, remains unclear.

Mouse models for OFD, as well as mutants that have similar phenotypes to OFD ($Fuz^{-/-}$), have expanded facial cartilages (as determined by increased $Sox9$, $Col2a1$ and alcian blue staining) concordant with increases in $Fgf8$ expression (Tabler et al., 2013; Zhang et al., 2011). The FGF pathway has previously been implicated in inducing chondrogenesis in the developing facial prominences (Abzhanov and Tabin, 2004). Thus, a possible mechanism explaining enlarged facial cartilages in $ta^2$ mutants (and possibly OFD patients) is a gain of FGF8 activity. We favor this hypothesis due to the previously established relationship between $Fgf8$ and $Shh$. The growth and development of craniofacial cartilages in avian embryos has been shown to be positively regulated through synergistic actions of $Fgf8$ and $Shh$ (Abzhanov and Tabin, 2004). We previously reported that Shh activity is increased in $ta^2$ embryos via increased production of GLI3 activator (GLI3A) and reduced production of GLI3 repressor (GLI3R) (Chang et al., 2014). In non-pathological conditions, GLI3R suppresses $Fgf8$ expression in the face (Aoto et al., 2002). Thus, in ciliary mutants
increased GLI3A or attenuated GLI3R production could alleviate transcriptional inhibition of Fgf8 and promote chondrogenesis.

Another hypothesis to explain the production of enlarged cartilages focuses on the role of primary cilia in responding to a range of stimuli used to coordinate and regulate cell fate decisions (Irigoin and Badano, 2011). Primary cilia extend from human embryonic stem cells (Kiprilov et al., 2008), and there is precedence for the loss of cilia inherently changing the potentiality of stem cells and other multipotent cell types (Huang et al., 2014; Hunkapiller et al., 2011). Lineage experiments on cephalic neural crest stem cells suggests a hierarchical model in which lineage decisions are generated through progressive restrictions in the potentialities of a highly multipotent progenitor CNCC that can give rise to specific percentages of neural and mesenchymal (i.e., cartilage) derivatives (Le Douarin et al., 2008). In the absence of Shh, the majority of derivatives from clonal culture were ‘neural only’, with a smaller population giving rise to ‘mesenchymal or neural’ derivatives, and an even smaller population giving rise to ‘mesenchymal only’ derivatives. Treatment with Shh increased the percentage of clones exhibiting “mesenchymal or neural” and “mesenchymal only” potentialities while decreasing the frequency of the “neural only” colonies (Le Douarin et al., 2012). Thus, a second possible mechanism to explain enlarged cartilages in ta2 mutants, and possibly OFD patients, is that the C2CD3-dependent loss of cilia leads to altered CNCC progenitor potential through increased responsiveness to Shh. Understanding how loss of cilia affects the potentialities of CNCCs is the focus of our ongoing and future work.

In summary, genetic findings from our previous work (Chang et al., 2014) coupled with phenotypic and biochemical analyses performed herein, provide the first direct evidence that the ta2 mutant can serve as a bona fide model for a human OFD.
In light of these findings, we have used the ta² to examine the possible cellular etiology of the oral-facial phenotypes for human OFD. We focused specifically on CNCC development in this disease process because these cells make essential contributions to the majority of the oral-facial domains affected in OFD. We confirmed that CNCCs extended primary cilia during all ontogenic phases and that loss of C2CD3-dependent ciliogenesis affected CNCC migration and differentiation, but not specification or proliferation. Based on these observations, we speculate that disrupted migration, aberrant molecular signaling, and/or altered CNCC potentialities could contribute to the oral-facial phenotypes in human OFD patients. With the knowledge of specific C2CD3 mutations in human patients (Thauvin-Robinet et al., 2014) and the understanding of the role of C2CD3 in the primary cilia (Ye et al., 2014), our future work will focus on understanding the molecular mechanisms of C2CD3-mediated CNCC migration and differentiation, in hopes of uncovering avenues for therapeutic intervention.

MATERIALS AND METHODS

**Avian embryo preparation:** Control and ta² eggs were incubated at 39 °C until they reached the desired Hamburger and Hamilton stage (Hamburger and Hamilton, 1951) and then were harvested for analysis.

**Embryo Genotyping:** Embryos younger than HH 25 were genotyped as previously described (Chang et al., 2014).

**Hematoxylin and Eosin Staining:** Sections were deparaffinized, rehydrated, and nuclei were stained with hematoxylin (Polysciences, Inc., Warrington, PA, USA). Sections were rinsed in water then placed briefly in Eosin Y (Sigma-Aldrich, St.
Louis, MO, USA). Sections were dehydrated and mounted using Permount (Fisher Scientific, Waltham, MA, USA).

**Micro-computed tomography:** Day 13 embryos were harvested and fixed overnight in 4% PFA, washed in PBS and placed in 50% Lugol solution (L6146-1L, Sigma, St. Louis, MO, USA) for 2 weeks, with frequent refreshing. Heads were scanned using MicroCAT II v. 1.9d (Imtek, Inc.) with COBRA v. 7.4 (Exxim Computing Corporation) software used for image reconstruction. OsiriX was used for image display and analysis.

**Chicken embryonic fibroblast (CEF) isolation:** Facial prominences and limb buds were collected from HH 25 control or *ta<sup>2</sup>* embryos, and digested in PBS (pH 7.4) with 1 mg/ml collagenase (Roche, Indianapolis, IN, USA) at 37º C for 30 minutes. Cells were dissociated by gentle pipetting and collected by centrifugation. The medium contained DMEM (Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (Fisher, Waltham, MA, USA), and 50 U/mL Penicillin-Streptomycin (Life Technologies, Grand Island, NY, USA). CEFs from passage five to fifteen were used for experiments.

**CEF Transfection:** 90% confluent CEFs were transfected with p3xFLAG-C2cd3<sup>Ctrl</sup>, p3xFLAG-C2cd3<sup>ta2</sup> and pcDNA3.1-*Ofd1*-myc (2.5 μg per 60 mm dish) and harvested after 24 hours for co-IP experiments using transfection reagent (X-tremeGENE, Roche, Indianapolis, IN, USA).

**Co-immunoprecipitation and Western Blot:** Lysates were prepared from transfected CEFs and incubated with monoclonal anti-FLAG M2 antibody (1:1000; F1804, Sigma, St. Louis, MO, USA). Dynabeads Protein G was added to pull down
3xFLAG-C2cd3 protein. Beads were washed and boiled in 1x Laemmlie sample buffer for 3 minutes. Proteins were separated on 8% SDS-PAGE (for Ofd1-myc) or 6% SDS-PAGE (for 3xFLAG-C2cd3Ctrl or 3xFLAG-C2cd3ta2) and transferred to PVDF membrane. The membranes were blocked in 6% non-fat milk in 1x TBST for 20 minutes at 4°C. Western Blot was used to analyze immunoprecipitated proteins by using monoclonal anti-FLAG or anti-myc (9B11, Cell Signaling, Beverly, MA, USA) as primary antibodies. Horseradish peroxidase (HRP)-conjugated (goat) anti-mouse (Santa Cruz, Santa Cruz, CA, USA) was used as the secondary antibody. ECL assay (ECL prime, Amersham, Pittsburg, PA, USA) was performed to develop the chemiluminescence signals. Image J software was used to quantify the signals on the X-films (CL-XPosure film, Thermo Scientific, Waltham, MA, USA) as follows: peak percentage of Ofd1 proteins (co-IP) were normalized by C2CD3 proteins (IP), and then the relative OFD1 precipitation was calculated by the ratio of OFD1/C2CD3ta2 to Ofd1/C2CD3Ctrl.

Immunohistochemistry: Immunostaining was performed according to standard protocols. Briefly, embryos were fixed in DENTS or 4% PFA. Sections were incubated in primary antibody for 1 hour at room temperature or overnight at 4°C. Secondary antibodies with fluorescent tags were then applied at 1:1000 and incubated at room temperature for 1 hour. Slides were stained with 4’, 6-diamino-2-phenylindone (DAPI; 1:10,000; Life Technologies, Grand Island, NY, USA) and mounted with mounting media (ProLong Gold, Life Technologies, Grand Island, NY, USA). For whole mount immunostaining, embryos were blocked overnight at 4°C then incubated in primary antibody overnight. Embryos were incubated in secondary antibodies (1:500) overnight at 4°C and DAPI (1:10,000). Embryos were cleared with 50% glycerol and then mounted in 70% glycerol. Antibodies used in this study
included rabbit anti-Glutamylated-tubulin (1:500; AB3201, Millipore, Billerica, MA, USA), PAX7 (1:20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), HNK1 hybridoma (1:20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), mouse anti-PHH3 (1:1000; 05-1336, Millipore, Billerica, MA, USA); mouse anti-PCNA (1:1000; 2586, Cell Signaling Technology, Danvers, MA, USA), and mouse anti-COL2A1 (1:500; MAB8887, Millipore, Billerica, MA, USA).

**Delaunay triangulation:** HNK1 stained sections were imaged using Leica DM5000 B, and files were analyzed in Photoshop. Nuclei of HNK1 positive cells (migrating NCCs) were marked and spatial distribution was analyzed bilaterally using the Delaunay /Voronoi algorithm (ImageJ). The coordinates of all line segments were exported for further analysis. Using MATLAB, vertices of triangles created by the Delaunay/Voronoi algorithm were identified. Coordinates were used to determine the area of each triangle.

**Quantitative RT-PCR:** HH 10 embryos were harvested and the anterior portion of the embryos were retained for Sox10 qRT-PCR. RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and cDNA was made using SuperScript III (Life Technologies, Grand Island, NY, USA). SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) were used to perform quantitative real-time PCR. Sox10 expression was normalized to GAPDH expression. Student's *t*-test was used for statistical analysis. Primers: GAPDH-F: CAACATCAAATGGGCAGATG; GAPDH-R: AGCTGAGGGAGCTGAGATGA; Sox10-F: AACGCCTTCATGGTCTGG; Sox10-R: GGGACGCTTATCACCTTTCA.
**TUNEL staining:** TUNEL assay was performed using the *In Situ* Cell Death Detection Kit, Fluorescein according to manufacture’s protocol (Ref: 11 684 795 910, Roche, Indianapolis, IN, USA). For whole mount TUNEL staining, anti-fluorescein antibody, Fab fragment from sheep, conjugated with alkaline phosphatase (Ref: 11 772 457 001, Roche, Indianapolis, IN, USA) was applied and embryos were developed in NBT/BCIP (Amresco, Solon, OH, USA).

**Scratch assay:** Control and *ta*² CEFs were seeded in 12 well plates and allowed to recover overnight in DMEM GlutaMAX media (Life Technologies, Grand Island, NY, USA) with 10% Sheep serum (Fisher, Waltham, MA, USA). Once confluent, a pipette tip was used to scratch through the cellular monolayer. Wells were scratched twice so that a cross was formed in order to create a point of reference for imaging. Cells were briefly washed to remove debris and then immediately imaged. Plates were returned to the incubator and imaged at 6, 12, and 24 hours following injury.

**Transwell assay:** 2 X 10⁴ control or *ta*² CEFs were seeded onto FluoroBlok cell culture inserts with 8 μm pores (351157, Corning, Tewksbury, MA, USA) and left to migrate overnight (16 hours). Inserts were fixed and stained with Hoechst. Six representative fields from the top and bottom of the membrane were imaged. Nuclei were counted and statistical analysis performed.

**Time lapse:** Dorsal neural tubes were harvested from HH 8⁺/⁹ control and *ta*² embryos and cultured in neural crest cell medium (Bajpai et al., 2010) in a μ-slide 8 well (80821, Ibidi, Verona, WI, USA) coated with fibronectin. The remaining portion of the embryo was used for genotyping. Explants were incubated at 37° C for 5-6 hours. Approximately 30 minutes before imaging, neural crest cell medium was
replaced with 3 μM Di-8-Anneps (gift of B. Sirosky) in order to fluorescently label cell membranes. Explants were imaged for 12 hours at 2 minute intervals using Nikon A1Rsi inverted confocal microscope. Videos were analyzed using Imaris software.

**Transplant assay:** Control and \( ta^2 \) eggs were windowed at HH 8-9. The vitelline membrane was removed near the anterior portion of the host embryo using tungsten needles. Tyrode’s solution (calcium-magnesium free) was added to the egg and the right side of the dorsal neural tube from the mesencephalon to the second rhombomere was removed. The host egg was briefly set aside as the donor was prepared. HH 8\(^+\) control or \( ta^2 \) embryos were harvested and placed in Tyrode’s solution. An equivalent portion of the right dorsal neural tube was removed from the donor and transferred to the host egg. The donor explant was placed into the ablation site. Transplanted embryos recovered at room temperature for 10 minutes, sealed with tape and returned to the incubator. The remaining portion of the donor embryos was used for genotyping. Following overnight incubation, the transplanted embryos were examined for a heartbeat and embryos that survived were harvested and fixed in 4% PFA for analysis.

**In situ hybridization:** Patterns of gene expression in control and \( ta^2 \) embryos were analyzed via whole mount *in situ* hybridization using digoxigenin-labeled riboprobes as described on the Gallus Expression *in situ* hybridization Analysis site (GEISHA) (Bell et al., 2004; Darnell et al., 2007). Probes for Pax7, Sox10, Snai2, Sox9 and Fgf8 were designed according to sequences listed on GEISHA (Darnell et al., 2007).

**EdU staining:** 1 X \( 10^4 \) control and \( ta^2 \) CEFs were plated on fibronectin (BD Biosciences, San Jose, CA, USA) coated coverslips and left to recover overnight.
CEFs were pulsed with 10 μm EdU for one hour and then fixed for 15 minutes with 3.7% formaldehyde. EdU staining was carried out according to the manufacture’s protocol (Click-IT EdU Alexa Flour 488 Imaging Kit; Life Technologies, Eugene, OR).

**Alcian blue:** Embryos were fixed in Bouin’s overnight and the washed with 70% EtOH, 0.1% NH₄OH solution, equilibrated in 5% acetic acid and stained with 0.05% alcian blue 8GX (Fisher Scientific, Waltham, MA, USA) in 5% acetic acid for 4 hours. Embryos were washed with 5% acetic acid followed by 100% MeOH. Benzyl alcohol/benzylbenzoate (1:2) was used to clear the embryos.

**RNA-seq:** RNA-seq and data analysis was performed as previously described in (Chang et al., 2014).

**Rac1 activity:** Rac1 levels and activity were determined using the Active Rac1 Detection Kit (8851, Cell Signaling, Beverly, MA, USA) as per manufacture’s protocol.

**ACKNOWLEDGEMENTS**

We would like to thank the UC Davis Avian Facility and Jackie Pisenti for husbandry of the ta³ line. We would also like to acknowledge and thank Dr. Matt Kofron for microscopy assistance and discussion of the research project, John Pearce from the Imaging Research Center, CCHMC for assistance with micro-CT, and Dr. Paul Kulesa and Dr. Kenneth Campbell for helpful discussions. The PAX7 supernatant and HNK1 hybridoma cell lines were developed by A. Kawakami and W. Halfter obtained through the Developmental Studies Hybridoma Bank, developed auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City.
COMPETING INTERESTS STATEMENT: We have no financial or competing interests.

AUTHOR CONTRIBUTION: E.N.S performed most experiments including all immunostaining, *in situ* hybridization, cell proliferation assays, histological analyses, transplantation studies. C-F.C developed genotyping methods, cloned *C2CD3* and generated CEFs. J.S performed transwell assays. J.C performed *in silico* analyses of cell migration assays Y-T.C cloned *OFD1* and performed Co-IP experiments. M.E.D maintained *ta²* lines and supplied *ta²* eggs. S.A.B conceived the project, analyzed data and wrote the manuscript with input from E.N.S.

FUNDING: This work was funded by National Institutes of Health grant R00-DE01985 (S.A.B) and C.T.O funds from the Cincinnati Children’s Research Foundation (S.A.B).

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Figure 1. The avian mutant ta2 phenocopies human OFD. (A-H) Whole mount images of day 10 control and ta2 embryos. (A, B) Lateral view of control and ta2 face with cleft lip (B) denoted by dotted white line. (C, D) Ventral view of control and ta2 palate with cleft primary palate (D) denoted by white arrows. Compare width of naturally occurring cleft of the secondary palate in (C) and pathological cleft in (D); dotted white lines. (E, F) Dorsal view of control and ta2 mandible. (F) ta2 embryos exhibit hypoglossia (compare dotted white lines in (E) and (F)). (G, H) Lateral view of control and ta2 forelimb. (H) ta2 embryos exhibit severe polydactyly. (I, J) Whole mount image of day 13 control and ta2 kidneys with polycystic kidneys denoted by black asterisks (J). (K, L) H&E staining on day 13 control and ta2 kidneys with cysts marked with black asterisks (L). (M, N) Micro-CT images of day 13 control and ta2 cerebella with folia numbered. (O, P) Co-immunoprecipitation (Co-IP) of 3xFLAG-C2CD3Ctrl and 3xFLAG-C2CD3ta2 with OFD1-myc. (P) Quantification of Co-IP. 3xFLAG-C2CD3ta2 has a significantly reduced ability to bind to OFD1-myc, *p<0.01. Fourth ventricle (fv) Scale bars: (A, B) 750 μm; (C, D) 1000 μm; (E, F) 1150 μm; (G, H) 2000 μm; (I, J) 700 μm; (K, L) 175 μm (M, N) 325 mm.
Figure 2. Primary cilia extend from CNCCs during all ontogenic phases. (A-D’’)
Cranial sections of control embryos co-immunostained for glutamylated-tubulin (green) and CNCCs (red) at different ontogenic stages. (A-A’”) HH 8+ (specification; PAX7), (B-B’”) HH 10 (migration; HNK1), (C-C’”) HH 22 (G1, S-phase, G2; PCNA), and (D-D’”) HH 28 (differentiation; COL2A1). Arrows indicate cells positive for both glutamylated-tubulin and CNCCs markers. (E, F) Quantification of in vivo primary cilia extension on CNCCs. Scale bars: (A-B) 100 μm; (C-D) 1000 μm; (A’-D’) 20 μm; (A’’- D’’) 10 μm.
Figure 3. Loss of C2CD3-dependent ciliogenesis increases CNCC dispersion during migration. (A-B’) Whole mount immunostaining for HNK1 on (A, A’) control and (B, B’) ta^2 embryos at HH 10. (C, D) HNK1 immunostaining on cranial cross-sections of HH11 control and ta^2 embryos. Nuclei of HNK1-positive cells are marked with a white dot. (E-H) CNCC nuclei dispersion and Delaunay triangulation. (E, G) Unilateral representation of migrating CNCC nuclei from embryos in C and D, respectively. (F, H) Delaunay triangulation of migrating CNCC nuclei. ta^2 CNCCs show enhanced dispersion (compare size of triangles in F and H). (I) Quantification of triangulated areas from migrating CNCCs in control and ta^2 embryos show that migrating ta^2 CNCCs are significantly more disperse, *P<0.05. Neural tube (nt), Scale bars: (A-B’) 100 μm; (C, D) 400 μm.
Figure 4. **Loss of C2CD3-dependent ciliogenesis affects cell migration by reducing directional persistence.** (A-F) Scratch assay with control and tα2 CEFs. (G) Quantification of scratch assays, 0 hours: *P* =0.8; 6 hours: *P*<0.05; 12 hours: **P**<0.01. (H) Schematic of transwell assay. (I) Quantification of results from the transwell assay, *P*<0.05. (J-M) Time-lapse analysis of CNCC migration from dorsal neural tube explants. Still images from time-lapse analysis of (J, L) control and (K, M) tα2 explants labeled with di-8-ANEPPS (red; blue dots mark migrating CNCCs). (N) Quantification of average speed of migration of control and tα2 CNCCs, *P* =0.43. (O) Individual cell migration tracks (different colors) from control and tα2 CNCCs over a 40-minute period. (P) Quantification of directional persistence (cell displacement/total distance travelled) for control and tα2 CNCCs, *P*<0.05. Scale bars= (A-F) 100μm; (J-M) 100 μm.
Figure 5. C2CD3-dependent ciliogenesis affects CNCC migration in a cell-autonomous manner. (A) Schematic illustration of sham cell-autonomous dorsal neural tube transplant. (A’) In situ hybridization for Sox10 on HH12 sham chimera. Final location of Sox10 positive transplanted cells is indicated by black arrow. (B) Schematic illustration of ta2 cell-autonomous dorsal neural tube transplant. (B’) In situ hybridization for Sox10 on HH12 ta2 chimera (ta2 donor in a control host). Sox10-positive transplanted cells remain at original transplant site, rather than migrating laterally (black asterisk). (C) Quantification of cell-autonomous transplant results. Transplants were categorized in three classes: no migration with non-migrative Sox10 positive cells (*P<0.01); migration with non-migrative Sox10 positive cells (P=0.51);
migrative ($P=0.10$). (D) Schematic illustration of sham non cell-autonomous dorsal neural tube transplant. (D’) In situ hybridization for Sox10 on HH12 sham chimera. Final location of Sox10 positive transplanted cells is indicated by black arrow. (E) Schematic illustration of $ta^2$ non cell-autonomous dorsal neural tube transplant. (E’) In situ hybridization for Sox10 on HH12 $ta^2$ chimera (control donor in a $ta^2$ host). Final location of Sox10 positive transplanted cells is indicated by black arrow. (F) Quantification of non cell-autonomous transplant results. Transplants were categorized in three classes: no migration with non-migrative Sox10 positive cells ($P=0.41$); migration with non-migrative Sox10 positive cells ($P=0.73$); migrative ($P=0.34$). (A’, B’, D’, E’) Original transplant site is outlined (dotted black line). Neural tube (nt), Scale bars: (A’, B’, D’, E’) 150 μm.
Figure 6. C2CD3-dependent ciliogenesis does not affect CNCC proliferation. (A, B) PHH3 immunostaining on frontal FNP sections of HH 22 control and ta² embryos. (C) Quantification of PHH3-positive cells normalized over the sample area, $P=0.48$. (D) EdU staining of control CEFs. (E) Quantification of EdU incorporation in control and ta² CEFs. There is no significant difference in EdU incorporation in ta² CEFs, $P=0.14$. Nasal pit (np), Scale bars: (A, B) 200 μm; (D) 100 μm.
Figure 7. C2CD3-dependent ciliogenesis affects development of CNCC-derived cartilages. (A-D) Whole mount alcian blue staining of (A, C) control and (B, D) ta^2 HH 28 embryos. (A, B) Ventral view of control and ta^2 maxillopalatine processes, black bars represent location of width measurements. (C, D) Dorsal view of control and ta^2 mandibular prominences. (E-H) COL2A1 (red) immunostaining in (E, G) control and (F, H) ta^2 embryos (plane of section indicated by dotted white lines in (A, B). (I-L) Whole mount *in situ* hybridization for (I, J) Sox9 and (K, L) Fgf8 on HH 25 control and ta^2 embryos. (M) Heatmap of Fgf8 and Pea3 expression from RNA-seq analysis of HH 25 control and ta^2 facial prominences. Frontonasal prominence (fnp); maxillary prominence (mxp); mandibular prominence (mnp); maxillopalatine process (mpp); prenasal cartilage (pnc). Scale bars: (A, B, E-H) 500 μm; (C, D) 1000 μm; (I, J) 250 μm; (K, L) 200μm.
TRANSLATIONAL IMPACT

Clinical Issue

Ciliopathies are a class of diseases caused by defects in primary cilia. Individuals with ciliopathies present with a wide range of phenotypes and often multiple organ systems are affected. Oral-facial-digital syndrome (OFD) is a ciliopathy primarily characterized by severe oral-facial and digit defects, such as polysyndactyly. To date, only two genes have been identified as solely causative for OFD: OFD1 and C2CD3. Both genes give rise to proteins that are important for ciliogenesis. Individuals with OFD are treated for their symptoms and undergo multiple surgeries to correct malformations. Additionally, individuals with OFD may require speech therapy and special education. While OFD can be diagnosed \textit{in utero}, there are limitations to understanding the cellular and molecular basis for this syndrome. Animal models are essential to provide valuable insights into understanding the etiology of the disease phenotype in OFD.

Results

This study utilizes a naturally occurring avian mutant known as \textit{talpid}^2 to determine the cellular basis for the oral-facial phenotypes present in OFD. Like a subset of patients with OFD, \textit{talpid}^2 mutants have mutations in the ciliary gene \textit{C2CD3}. \textit{talpid}^2 mutants display strikingly similar phenotypes to individuals with OFD including: cleft lip/palate, ectopic teeth, glossal defects, polydactyly, polycystic kidneys, and brain defects. To better understand the cellular etiology for the oral-facial defects in OFD patients and \textit{talpid}^2 mutants, we examined a population of cells called cranial neural crest cells (CNCCs), which give rise to a significant portion of the oral-facial complex affected in OFD. Our results indicate that while CNCC specification and proliferation were unaltered in \textit{talpid}^2 mutants, migration and differentiation were aberrant.
Implications and Future Directions

This work suggests that the oral-facial defects in OFD patients may result due to disruptions in CNCC migration and differentiation. These findings highlight two cellular processes during development that may be critical for the onset of this disease. Understanding when and how potential therapeutic agents could have an impact on OFD is an essential step toward more effective treatment options for patients. Our future work will focus on understanding the mechanism responsible for altered migration and differentiation in CNCCs that lack C2CD3-dependent ciliogenesis.
**Fig. S1.** Quantification of C2CD3 and OFD1 co-immunoprecipitation.

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**Fig. S2.** CNCCs do not extend cilia during mitosis. (A, A’) Cranial sections of control HH 22 embryos co-immunostained for glutamylated tubulin (green) and Phospho-histone H3 (PHH3; red). Mitotic cells do not extend a primary cilium. (B) Quantification of *in vivo* primary cilia extension on PHH3-positive CNCCs. Scale bar: (A) 1000 μm, (A’) 10 μm.
Fig. S3. Loss of C2CD3-dependent ciliogenesis does not affect expression of CNCC specifier genes. (A-H) Whole mount in situ hybridization on (A, C, E, G) control and (B, D, F, H) ta^2 embryos at HH 8-9 for (A, B) Sox10, (C, D) Snai2, and (E-H) Pax7. (I, J) Immunostaining for PAX7 (green) on cranial cross sections of control and ta^2 embryos at HH 8+. Scale bars: (A-H) 250 μm; (I-J) 100 μm.
Fig. S4. C2CD3-dependent ciliogenesis does not alter CNCC number, apoptosis or proliferation. (A) Quantitative analysis of the number of HNK1-positive cells/section in control and \(\text{ta}^2\) embryos, \(P=0.99\). (B) qRT-PCR analysis for \(\text{Sox10}\) expression in control and \(\text{ta}^2\) embryos \(P=0.17\). (C, D) Whole mount and (E, F) sectioned TUNEL staining on HH 10 control and \(\text{ta}^2\) embryos. (G, H) Whole mount PHH3 immunostaining (red) on control and \(\text{ta}^2\) embryos. Scale bar: (C, D) 400 \(\mu\)m; (E, F) 250 \(\mu\)m; (G, H) 100 \(\mu\)m.
**Fig. S5. Dorsal neural tube transplants proof of concept.** (A-A’’) Sham transplant with a control, GFP-positive donor explant implanted into a control HH 9 host embryo. (A) Dorsal view of sham chimera at the time of transplant. (A’) Dorsal view of sham chimera 24 hours after transplant. Donor GFP-positive cells have migrated laterally from the dorsal neural tube. (A’’) Dorsal view of *in situ* hybridization for Sox10 on sham chimera. Donor GFP-positive cells are Sox10-positive CNCCs (black arrow). (B-C’) Bright-field (B, C) and GFP (B’-C’’) images for non cell-autonomous transplant experiments from Fig. 5 D’, E’. (D) Transplants were categorized in three classes: no migration (black asterisk) with non-migrative Sox10 positive cells (white arrow); migration (black arrow) with non-migrative Sox10 positive cells (white arrow); migrative (black arrow). Scale bars: (A) 200 μm; (A’) 175 μm; (A’’, B-C’’) 200 μm, (D) 275 μm.
**Fig. S6. Alcian blue staining measurements.** (A) Quantification of width of the maxillopalatine cartilage, *P*<0.05.

**Fig. S7. Rac1 activity is not altered due to loss of C2CD3-dependent ciliogenesis.**

Western blot for active Rac1, total Rac1, and GAPDH in control and *ta²* CEFs.