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# Author's Accepted Manuscript

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**Utilizing the chicken as an animal model for human craniofacial ciliopathies**

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**Abstract**

The chicken has been a particularly useful model for the study of craniofacial development and disease for over a century due to their relatively large size, accessibility, and amenability for classical bead implantation and transplant experiments. Several naturally

occurring mutant lines with craniofacial anomalies also exist and have been heavily utilized by developmental biologist for several decades. Two of the most well known lines, *talpid<sup>2</sup>* (*ta<sup>2</sup>*) and *talpid<sup>3</sup>* (*ta<sup>3</sup>*), represent the first spontaneous mutants to have the causative genes identified. Despite having distinct genetic causes, both mutants have recently been identified as ciliopathic. Excitingly, both of these mutants have been classified as models for human craniofacial ciliopathies: Oral-facial-digital syndrome (*ta<sup>2</sup>*) and Joubert syndrome (*ta<sup>3</sup>*). Herein, we review and compare these two models of craniofacial disease and highlight what they have revealed about the molecular and cellular etiology of ciliopathies. Furthermore, we outline how applying classical avian experiments and new technological advances (transgenics and genome editing) with naturally occurring avian mutants can add a tremendous amount to what we currently know about craniofacial ciliopathies.

*“A box without hinges, key, or lid, yet golden treasure inside is hid.” - The Hobbit*

## Introduction

### *Avians as a model for craniofacial development and disease*

There has been a long-standing relationship between the study of craniofacial development/disease and the avian model system. The chicken has provided insights into much of what is known about craniofacial development. Several seminal experiments performed in avian models furthered our understanding of craniofacial growth and patterning. Cranial neural crest, the progenitors of anterior facial skeleton and connective tissue, were first observed in the chick embryo (His, 1868). In addition to fate mapping the cellular contributions to the craniofacial complex (Couly et al., 1993), chickens have been used to identify the extent of neural crest cell plasticity (Couly et al., 2002; Couly et al., 1996; Couly et al., 1998; Köntges and Lumsden, 1996; Le Douarin et al., 2004), explore the molecular and cellular basis for species-specific facial patterning (Schneider and Helms, 2003; Tucker and Lumsden, 2004) and determine the requirements for tissue-tissue interactions during craniofacial development (Chong et al., 2012; Creuzet et al., 2004; Creuzet et al., 2006; Etchevers et al., 1999; Hu and Marcucio, 2009a). Furthermore, several naturally occurring avian mutants have been utilized to understand pathological conditions (Robb et al., 2011). Here, we review and compare two of the most utilized avian models of craniofacial disease (*talpid*<sup>2</sup> and *talpid*<sup>3</sup>) and highlight what they have revealed about the molecular and cellular etiology of a disease class that has a profound affect on craniofacial development: ciliopathies.

The avian embryo has been highly relevant for human craniofacial development due to the highly conserved organization and growth of the facial prominences between avians and

mammalian species (mouse and human) during craniofacial development (Fig. 1). Facial development, regardless of species, begins with the formation and growth of five distinct facial prominences: a singular frontonasal prominence (FNP), paired maxillary prominences (MXP) and paired mandibular prominences (MNP) (Brugmann et al., 2006) (Fig. 1A-I). In humans, the FNP gives rise to midline features including the forehead, the middle of the nose, the philtrum of the upper lip, and the primary palate. In avians the FNP is also present and gives rise to the upper beak and primary palate (pre-maxilla). The other more lateral component of the frontonasal prominence, the lateral nasal prominences, contributes to the sides (alae) of the nose in humans and sides of the beak in avians. The MXP contributes to the upper lip, upper jaw and secondary palate in humans, and the lateral aspects of the upper beak and secondary palate in avians. In both humans and avians the MNP gives rise to the lower lip/beak, jaw, and the anterior two-thirds of the tongue (the 3<sup>rd</sup> and 4<sup>th</sup> branchial arches contribute to the posterior third and the intrinsic glossal musculature comes from the occipital somites (Parada et al., 2012)). The tissues that compose the facial prominences (surface ectoderm, neural crest, mesoderm and endoderm) are also highly conserved between avians and other mammalian species (mice and humans). This degree of morphological and cellular conservation has propelled the chicken into the forefront of animal models for human craniofacial disease, specifically craniofacial ciliopathies: a rapidly growing class of craniofacial anomalies caused by a defect in the structure or function of primary cilia.

### ***Craniofacial ciliopathies and the avian model system***

Primary cilia are microtubule-based organelles that dynamically extend from the cell surface (Fig. 1J, K). In the last decade, the cilium has gained increasing popularity due to their

nearly ubiquitous presence on various cell types, their role as regulators of developmental signaling pathways (e.g., Sonic Hedgehog) and association with a number of syndromes collectively known as ciliopathies (Badano et al., 2006; Eggenschwiler and Anderson, 2007). Currently there are over 100 conditions that are either known, or likely ciliopathies (Baker and Beales, 2009). When evaluating these disorders, approximately 30% of the conditions, including Oral-facial-digital syndrome, Joubert syndrome, Bardet-Biedl syndrome, Meckel-Gruber syndrome, Sensenbrenner Syndrome (Cranioectodermal dysplasia) and Ellis-van Creveld syndrome (Zaghloul and Brugmann, 2011) (Table 1), are primarily defined by their craniofacial phenotype. These craniofacial ciliopathies frequently present with some combination of craniosynostosis, micrognathia, midfacial dysplasia and/or cleft lip/palate, thus significantly impacting the development of all facial prominences (Fig. 1L). In recent years several animal models, from various species, have been identified for the study of craniofacial ciliopathies (Table 1). Perhaps one of the most exciting discoveries focused on the identification of two avian models for human craniofacial ciliopathies. In the next section we discuss these mutants and how the unique features of the avian model system have contributed to understanding the etiology of craniofacial ciliopathies.

### ***The talpids***

The *talpid* mutants (*talpid*, *talpid*<sup>2</sup>, *talpid*<sup>3</sup>) are three distinct, autosomal recessive avian mutants with the shared phenotypes of polydactyly and severe craniofacial malformations. These mutants received the name “*talpid*” due to their polydactylous phenotype, reminiscent of the forelimbs of moles, shrews, and desmans belonging to the Talpidae family. The original *talpid* mutant was identified by Randall Cole in 1942. *talpid* had severe craniofacial defects, including:

shortened inferior maxilla and general retarded growth of the facial structures (Cole, 1942). Sadly, before gene identification was possible, the original *talpid* went extinct. Years later, however, two separate avian lines, existing on different continents, with similar phenotypes to *talpid* would emerge. Aptly named *talpid*<sup>2</sup> (*ta*<sup>2</sup>) and *talpid*<sup>3</sup> (*ta*<sup>3</sup>), these two lines would serve as staples in the developmental biology communities for the study of limb and craniofacial development for the next half century. In recent years, with the advances in whole genome sequencing, the causative genetic mutations for both lines were identified, significantly increasing their value as animal models.

### ***The genetic, molecular and cellular etiology of *ta*<sup>2</sup>***

*ta*<sup>2</sup> was identified in 1953 by Isador Michael Lerner, and more completely described by Ursula Abbott, Lewis Taylor, and Hans Abplanalp in 1960 at the University of California, Davis. Like the original *talpid*, *ta*<sup>2</sup> was identified as an embryonic lethal, autosomal recessive mutation. The original phenotypic description of *ta*<sup>2</sup> noted that the mutants had shortened wing/leg bones accompanied by severe polysyndactyly, a shortened vertebral column, retarded feathering, and reduced MXPs. Additionally, Abbott noted an overall reduction in body size and a failure of the ventral body wall to completely close, causing subsequent protrusion of the internal viscera. With respect to craniofacial development, it was also noted that the position of the egg tooth was ventralized and that mutants often had crossed beaks (Abbott et al., 1960). Recent studies have provided a more detailed description of the craniofacial defects present in *ta*<sup>2</sup>. Combined, these studies found that *ta*<sup>2</sup> mutants have: a rounded head, shorter and broader FNP that eventually results in a shortened upper beak, bilateral clefting, hypoplastic MXPs, incomplete fusion of the primary palate, hypoglossia, and integumentary outgrowths on the developing jaw that resemble



tooth-like structures (Brugmann et al., 2010; Chang et al., 2014; Harris et al., 2006; Schneider et al., 1999). While no obvious morphological differences are observed at HH16, the oral cavity and MXPs of  $ta^2$  mutants are misshapen at HH21 (Fig. 2A, B, data not shown). This difference becomes more pronounced at HH25 along with differences in the morphology of the FNP (Fig. 2D, E). By day 7, the FNP and MXP have fused in control embryos and outgrowth of the upper beak has begun (Fig. 2G). In contrast, the FNP and MXP have yet to fuse in  $ta^2$  embryos and there is little outgrowth of the upper beak (Fig. 2H). Additionally, the tongue of  $ta^2$  mutants is smaller (hypoglossia; Fig. 2J, K). By day 10, the upper beak is significantly shorter and wider than that of controls, tooth-like structures are present on the upper and lower beaks and the palate remains patent (Fig. 2M, N, P, Q). The recent identification of the causative genetic mutation for the  $ta^2$  mutant has allowed us to begin to understand the molecular and cellular origins of the craniofacial defects present in this mutant.

Despite being a model for countless developmental studies of limb and facial development, there were limitations to the utility of the  $ta^2$  because the genetic cause was unknown. The advancement of genomic technology as well as improved coverage of the chicken genome alleviated these limitations. Using a 60K SNP array, whole genome sequencing, and cDNA sequencing, a telomeric region on GGA1q was identified to have at least a 3-fold higher ratio of  $ta^2$ -specific SNPs over any other interval. Subsequent analysis of the line confirmed a 19 bp deletion in the 3' end of exon 32 within *C2CD3* (C2 calcium-dependent domain containing 3) (Chang et al., 2014; Robb et al., 2011). *C2CD3* encodes a protein that is universally conserved in organisms that assemble centrioles or cilia. Whereas the function of *C2CD3* remains largely elusive, various studies indicate that the protein localizes near the distal tip of centrioles, physically interacts with other centriolar and IFT proteins, and is essential for ciliogenesis

(Hoover et al., 2008; Thauvin-Robinet et al., 2014; Ye et al., 2014). The deletion in *C2CD3* in *ta<sup>2</sup>* embryos correlated with a decrease in the number of cells extending a primary cilium. Both SEM and immunofluorescence confirm that approximately 20% of *ta<sup>2</sup>* cells extend a cilium, in contrast to approximately 70% in control embryos (Chang et al., 2014). Taken together, these results were the first to give a genetic and cellular etiology to the long utilized *ta<sup>2</sup>* mutant.

While various studies have reported aberrant *SHH* expression in the *ta<sup>2</sup>* mutant (Agarwala et al., 2005; Harris et al., 2006; Schneider et al., 1999), changes in *SHH* ligand expression, as well as expression of downstream targets, are not consistent across organ systems and appear to occur in a tissue-specific manner (Chang et al., 2014). For example, *SHH* ligand expression is expanded in the FNP, yet decreased in other prominences and cranial domains (Fig. 3). Despite increased *SHH* ligand expression, *PTCH1* expression is significantly reduced in the FNP. These data suggest that the loss of cilia in facial tissues causes tissue specific changes to *SHH* expression and canonical SHH pathway activity. Whereas these inconsistent expression changes were the source of much confusion during the early study of the mutant, classification of the *ta<sup>2</sup>* as ciliopathic has helped shed light on how such a result is possible.

The mechanisms through which the primary cilium affects Shh signal transduction has been the subject of extensive research over the last decade. In regard to the Shh pathway, functional primary cilia are required for the proper processing of the downstream transcription factors of the pathway, the GLI proteins. Full-length GLI proteins (GLIFL) are processed into either a full-length activator (GLIA) or a cleaved repressor (GLIR) (Haycraft et al., 2005), and the ratio of GLIA to GLIR determines net Shh pathway activity. Evaluation of the *ta<sup>2</sup>* facial prominences and limb buds revealed significant and consistent increases in the levels of full-length GLI3A, thus skewing the GLIA to GLIR ratio and supporting the hypothesis that increased GLI3A

activity is the cause of the  $ta^2$  facial phenotypes. These findings were in line with what was known about the molecular etiology of ciliopathies, as several ciliary mutants exhibit disruptions in GLI processing and skewed GLIA to GLIR ratios (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005).

Cell behaviors and processes are also disrupted in  $ta^2$  mutants. Several ciliary mutants exhibit aberrant cell migration (Osborn et al., 2014; Tabler et al., 2013; Tobin et al., 2008). In  $ta^2$  mutants, cranial neural crest cells (CNCCs) fail to migrate properly. The dispersion of CNCCs is significantly increased, and they exhibit decreased directional persistence (Schock et al., 2015). Furthermore  $ta^2$  mutants have cell differentiation defects. There is an increase in cartilage both in the face and in the limbs of  $ta^2$  mutants (Schock et al., 2015). The precise molecular mechanisms causing these migration and differentiation defects remain unknown. Continued study of  $ta^2$  mutants will undoubtedly elucidate the intricacies of how cilia function during these cellular processes.

### ***The genetic, molecular and cellular etiology of $ta^3$***

$ta^3$  arose as a spontaneous, recessive, embryonic lethal mutation in a chicken flock kept at Wye College, Kent, UK. As part of an undergraduate study, Donald Ede, a limb developmental biologist who had trained under the famous developmental biologist C.H. Waddington, noted that there was reduced hatchability in an avian flock at the college. Ede and his student, W.A. Kelly, determined that the reduced hatchability was due to the widespread presences of a recessive, lethal allele in the flock. Further examination of this mutant revealed a phenotype similar to that of Cole's original *talpid*, particularly the formation of large polydactylous paddle shaped limbs, thus, the mutant was named *talpid*<sup>3</sup>. Recent studies have provided a more detailed

description of the developmental anomalies present in the  $ta^3$ . Despite the situs of the viscera and turning/patterning of the heart tube being normal (Stephen et al., 2014), the anatomy of the  $ta^3$  body is severely dysmorphic. The  $ta^3$  body axis is shortened (Stephen et al., 2015), the lungs are hypoplastic and the liver is fibrotic and cholestatic (Davey et al., 2014). Skeletogenesis is aberrant (Macrae et al., 2010), embryos exhibit unusual vascular abnormalities (Davey et al., 2007) and development and differentiation of the central nervous system is impaired (Buxton et al., 2004; Stephen et al., 2013). With respect to craniofacial abnormalities,  $ta^3$  embryos exhibit a holoprosencephalic-like phenotype: apposition of the eyes at the ventral midline (hypotelorism) with the reduction and anterior displacement of the FNP (Ede and Kelly, 1964a; Ede and Kelly, 1964b). By HH21, there already are significant morphological changes in the developing face of  $ta^3$  mutants. The FNP is shifted anteriorly and MXPs reside near the midline (Fig. 2A, C). At HH25, the MXPs have fused with each other and the FNP remains anteriorly displaced and hypoplastic (Fig. 2D, F). At day 7, the MXP and MNP, which are also reduced at the midline, are displaced posteriorly below the eyes, and MXPs fuse medially (Fig. 2G, I). Furthermore, complete loss of the tongue (aglossia) is also apparent at this stage (Fig. 2J, L). By day 10, the anteriorly displaced remnant of the upper beak fails to project, while the lower jaw is narrow and described as ‘peg-like’ (Fig. 2M, O). Ectopic lenses derived from, or connected to, the hypophyseal duct also frequently form at the facial midline (Fig. 2O) and the medial fusion of the MXPs splits the oral cavity in two (Fig. 2P, R).

Similar to the  $ta^2$ , the  $ta^3$  has been a heavily utilized model within the developmental biology community despite the genetic cause of the mutation remaining a mystery. Linkage analysis identified a candidate region on GGA5, and subsequent sequencing of cDNA clones identified an insertional mutation of a single thymine residue, resulting in frame-shift and

premature stop codon in a previously uncharacterized gene, *KIAA0586* (Davey et al., 2006). *KIAA0586*, which encodes a 1533aa protein containing several coiled-coil regions and a proline-rich region, was not otherwise homologous to any other gene or gene family. The *KIAA0586* protein, eventually named TALPID3, was isolated in an early centrosomal proteome analysis (Andersen et al., 2003) and was confirmed to be a centrosomal protein (Yin et al., 2009) that normally localized in a ring at the distal end of the basal body (Kobayashi et al., 2014). The TALPID3 protein was shown to be required for docking of the basal body to the cell surface (Yin et al., 2009) prior to ciliogenesis (Kobayashi et al., 2014) and has also been implicated in centrosome migration (Stephen et al., 2013), control of centrosome length (Stephen et al., 2015) and formation of centriolar satellites (Kobayashi et al., 2014). Loss of the TALPID3 protein, in various model species, resulted in a loss of cilia (Bangs et al., 2011; Ben et al., 2011; Yin et al., 2009), both non-motile and motile (Stephen et al., 2013).

Molecularly, most of what is known about the  $ta^3$  has been gained from studies in the limb and neural tube. Despite normal expression of *SHH* in the limb, expression of down-stream targets including, *PTCH1*, *PTCH2* and *GLI1*, are all down-regulated (Lewis et al., 1999). In contrast, there is a complete loss of pathway activity in the neural tube, with expression of both *SHH* and its targets being down-regulated (Fig. 3). The developing craniofacial complex more closely resembles the expression patterns seen in the neural tube. In the head there is a loss of *SHH* and *PTCH1* expression in the ventral forebrain and developing face (Buxton et al., 2004; Davey et al., 2006).

As previously mentioned, loss of primary cilia frequently results in altered ratios of GLIA to GLIR. Western blot analysis revealed that post-translational processing of GLI3 was indeed disrupted in the  $ta^3$  (Davey et al., 2006). In both the limb and the head there was a significant

increase in nuclear GLI3A, but no change in GLI3R. Thus, as was reported in the  $ta^2$ , these data suggest that the primary molecular defect in the  $ta^3$  was aberrant Shh pathway activity resulting from a failure of modification/processing of the GLI3 protein.

Whereas CNCCs have not been specifically examined in the  $ta^3$  as in  $ta^2$ , the limb provided a basis for understanding various cell behaviors, including migration and differentiation. Several groups have used the  $ta^3$  limb to determine that there was a decrease in cell death, changes in cell proliferation, abnormal cell adhesiveness and migration during limb development (Ede and Agerbak, 1968; Francis-West et al., 1995; Hinchliffe and Ede, 1967; Hinchliffe and Thorogood, 1974; Izpisua-Belmonte et al., 1992). Furthermore, there was a general loss of polarity in both the cell and developing tissues (Stephen et al., 2015). Cell differentiation was also affected, particularly during skeletal development. Endochondral cartilages failed to ossify; however, membranous bone of the clavicle and head underwent ossification (Macrae et al., 2010).

### ***Comparison of the $ta^2$ and $ta^3$ avian ciliopathic mutants***

While the  $ta^2$  and  $ta^3$  have been thought of together because of their nomenclature and polydactylous phenotypes, no direct comparison has ever been made between the two. Here we highlight that although these mutants have similar limb phenotypes, their craniofacial defects are surprisingly different.  $ta^2$  has a relatively mild craniofacial phenotype when compared directly to  $ta^3$  (Fig. 2). The FNP and MXP of  $ta^2$  are dysmorphic and fail to fuse properly, resulting in a cleft primary palate. In addition, primitive teeth-like structures are present on the FNP, and whereas the MNP is relatively normal, the tongue is underdeveloped. Conversely, in  $ta^3$  the midline is collapsed due to an underdevelopment and anterior shifting of the FNP. This shift

prevents the FNP and MXP from ever fusing and results in a direct fusion of the MXPs across the midline. With respect to the MNP,  $ta^3$  mutants have a narrowed and “peg-like” lower beak and aglossia. Thus, the highly distinctive phenotypes between these two mutants allow for the differential exploration of various cellular, molecular and ciliary-based processes during craniofacial development.

Despite the causative genes being different between  $ta^2$  and  $ta^3$  the mechanism by which ciliogenesis is impaired is surprisingly similar. The  $ta^2$  mutation is caused by a premature truncation of C2CD3, a ciliary protein that localizes to the distal centriole and is known to associate with OFD1, IFT88 and other transition zone proteins (Schock et al., 2015; Thauvin-Robinet et al., 2014; Ye et al., 2014). In these mutants, the mother centriole fails to dock to the ciliary vesicle and cell membrane, preventing ciliogenesis (Chang et al., 2014). Similar to C2CD3, TALPID3 localizes to the extreme distal end of the centriole and loss of function prevents centriole docking to the ciliary vesicle and cell membrane (Yin et al., 2009). TALPID3, however, is known to localize in a ring-like structure at the distal centriole associated with the CP110-containing protein complex (Kobayashi et al., 2014). Given the similarities between ciliary localization and the cellular mechanisms for loss of ciliogenesis in these two mutants, it is surprising that the two craniofacial phenotypes differ so greatly. This phenotypic variation could be due to differential spatiotemporal expression or specific functional roles of these ciliary genes during signal transduction.

On a molecular level, both  $ta^2$  and  $ta^3$  have aberrant Shh pathway activity (Fig. 3). This is not surprising given the relationship between the Shh pathway and primary cilia; however, there are pronounced differences between the two mutants with respect to pathway activity and associated craniofacial phenotype. In the  $ta^2$ , the FNP is reduced in length, yet significantly

wider. An expanded midline is classically associated with a gain of Shh function (Hu and Helms, 1999); and thus, it was not surprising to see an increase in *SHH* expression (Fig. 3A, B) and increase in *GLI3A* in the developing FNP of  $ta^2$  mutants (Chang et al., 2014). Interestingly, *PTCH1* expression is decreased in the FNP, despite the increase in *SHH* expression. This suggests that there is an uncoupling of the Shh pathway whereby the adjacent cells cannot properly interpret the initial ligand signal. Conversely, there is a collapse of the facial midline (reduced FNP) in the  $ta^3$ . A reduced midline is classically associated with a loss of Shh function (Cordero et al., 2004). Therefore, reduced expression of *SHH* (Fig. 3A, C) and its targets in the developing head is not surprising, but increase in *GLI3A* in the  $ta^3$  is. It is not clear what causes the dichotomy in *SHH* activity between these two mutants, especially given the similar cellular ciliary phenotype. Gaining a greater understanding of the roles of *C2CD3* and *TALPID3* during Shh signal transduction will likely improve our understanding of these data.

Examining distinct domains of *SHH* expression in both  $ta^2$  and  $ta^3$  mutants could also help to elucidate the role of primary cilia during Shh signal transduction. Typically *SHH* is expressed in both ‘primary’ (notochord) and ‘secondary’ domains (FEZ, zona limitans intrathalamica (ZLI) and the ventral floor plate). Expression in secondary domains requires induction by a primary source of SHH (e.g., expression in the telencephalon induces expression in the FNP) (Marcucio et al., 2005). While expression in primary regions remains relatively normal, induction of *SHH* expression in some secondary regions is disrupted in both the  $ta^2$  and  $ta^3$  mutants (Buxton et al., 2004; Chang et al., 2014) (Fig. 3D-I), suggesting a role for cilia in induction of a secondary signaling domain. Given the ease of embryonic manipulation and ability to perform tissue transplants, the *talpids* are perhaps an ideal model to further examine topics such as this. It should be noted; however, that while there are many alterations in Shh



pathway activity, it is unlikely that the Shh pathway is solely responsible for the entirety of phenotypes in these two mutants. Other signaling pathways such as Wnt, Notch, and PDGF-A have been associated with primary cilia and are known to play key roles during craniofacial development (Brugmann et al., 2007; Corbit et al., 2008; He and Soriano, 2013; Jiang et al., 1998; Schneider et al., 2005; Stasiulewicz et al., 2015). Further examination of both  $ta^2$  and  $ta^3$  is necessary to determine how these pathways are affected in these mutants.

### ***Using the talpids as models for human craniofacial ciliopathies***

While it was purely serendipitous that both existing *talpid* models were determined to be ciliopathic in nature, it was almost kismet that shortly after the causal genes were identified in both mutants, mutations in their human orthologs were shown to be linked to human ciliopathies (Roosing et al., 2015; Thauvin-Robinet et al., 2014). The benefit to having an avian model for a ciliopathy is significant. The ubiquitous nature of the primary cilium make many ciliopathies early embryonic lethal. Mutations of this nature are difficult to study as they are frequently resorbed before the embryo can be harvested or studied. The external, *in ovo* development of chicken embryos, rather than the *in utero* development of mammals, make chickens one of the most informative models for severe congenital anomalies. Mutant chicken embryos remain in the egg, and this allows for an assessment the embryonic phenotype at anytime. Thus, the discovery of the *talpids* as two distinct models for craniofacial ciliopathies is a promising step in determining the molecular and cellular etiology of these syndromes, as well as exploring possible avenues for therapeutic treatment.

*ta<sup>2</sup> as a model for human Oral-facial-digital syndrome (OFD)*

Very recently *ta<sup>2</sup>* was identified as a possible disease model for Oral-facial-digital syndrome (OFD) (Schock et al., 2015). 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. There are 14 different subtypes of OFD. While each subtype has phenotypes that are unique, all subtypes share the common core phenotypic characteristics.

To date, there are six different genes and one open reading frame that have been identified in OFD patients: *OFD1*, *C2CD3*, *TCTN3*, *DDX59*, *SCLT1*, *TBC1D32*, and *C5orf42*. These genes have been implicated in regulating the Shh pathway and/or ciliogenesis (Hoover et al., 2008; Ko et al., 2010; Reiter and Skarnes, 2006; Shamseldin et al., 2013; Singla et al., 2010; Tanos et al., 2013). As discussed previously, the causal mutation for *ta<sup>2</sup>* is a 19bp deletion in *C2CD3*, one of the genes identified in OFD patients (Hoover et al., 2008; Thauvin-Robinet et al., 2014; Ye et al., 2014). The phenotypic presentation of the *ta<sup>2</sup>* encompasses the core phenotypes (craniofacial and other) present in human OFD patients, including cleft lip/palate, ectopic teeth, hypoglossia, polydactyly, polycystic kidneys, and a hypoplastic cerebellum. Biochemically, it has been shown that *C2CD3* and *OFD1* co-localize to the same region of the primary cilium (distal centriole) and are able to physically interact in mammalian cells. This physical interaction is conserved in chicken (Schock et al., 2015). The truncation of *C2CD3* in the *ta<sup>2</sup>* impedes the interaction between *C2CD3* and *OFD1*. Given these striking similarities between *ta<sup>2</sup>* mutants and OFD patients, the *ta<sup>2</sup>* can now be classified as a bona fide disease model for OFD. As such, *ta<sup>2</sup>* can be used to explore the molecular and cellular origins of OFD. Additionally, avian model

systems provide a unique opportunity to test potential therapies, as the embryos is easily accessible and susceptible to drug treatment and modification of gene expression (via electroporation, virus exposure or bead implantation).

### *ta<sup>3</sup> as a model for human Joubert syndrome (JS)*

Mutations in the human ortholog of TALPID3 have recently been associated with the ciliopathy Joubert Syndrome (JS) (Bachmann-Gagescu et al., 2015; Roosing et al., 2015; Stephen et al., 2015) which is characterized by the presentation of core symptoms including molar tooth sign of the midbrain-hindbrain junction, hypotonia, ataxia and intellectual disabilities. Craniofacially, these patients frequently present with a prominent forehead, high rounded eyebrows, epicanthal folds, ptosis, upturned nose with evident nostrils, hypotelorism and an open mouth with protruding tongue (Baala et al., 2007; Delous et al., 2007; Maria et al., 1999; Zaghloul and Katsanis, 2010). Currently, there are over twenty different subtypes of JS, defined by distinct genetic mutations and the presence of the core symptoms. To date, over twenty different genes have been associated with JS, and most, if not all of them, have been implicated in the ciliogenesis and or regulating the Shh pathway. For patients with TALPID3 mutations, most have been identified to have biallelic mutations, which frequently include one ‘common’ rare variant (c.428delG). It is currently unclear if the patients have a total loss of TALPID3 function (as in *ta<sup>3</sup>* mutants) or if they are hypomorphic.

The defining symptom of JS is a failure of axonal projections from the cerebellum. Whether this is due to a failure of cell polarity, tissue specific requirement for TALPID3 or aberrant Shh signaling remains to be understood. With the new knowledge of the *ta<sup>3</sup>* as a bona fide model for JS, we can now examine JS phenotypes and their underlying cellular and

molecular etiology. Currently CRISPR/Cas9 technology is being utilized to uncover additional information regarding the function of TALPID3 in JS.

### ***Other avian models with craniofacial phenotypes***

While the *talpids* have been the most well studied of the avian mutants, it should not be overlooked that several additional mutant lines with craniofacial phenotypes, yet unknown genetic causes, exist. It should be noted that the number of facilities maintaining these valuable resources are few in number. Currently, only nine academic institutions actively maintain established genetic lines in the United States (Delany, 2004), while in Europe four additional institutions, The Roslin Institute, Pirbright Institute, Uppsala University and INRA, maintain lines of developmental significance. Here we highlight three mutant lines with craniofacial phenotypes similar to that of  $ta^2$  and  $ta^3$ ; *diplopoda-4* (Taylor and Gunns, 1947), *coloboma* (Abbott et al., 1970) and *cleft primary palate* (Abbott and MacCabe, 1966) .

*diplopodia-4 (dp-4), coloboma (co) and cleft primary palate (cpp)*

*diplopodia-4 (dp-4)* is one of five mutants that have all been grouped together under the “*diplopodia*” title due to similar limb and craniofacial phenotypes (Robb et al., 2011). Two of the *diplopodia* lines, *dp-2* and *dp-5*, are extinct, but the remaining three lines continue to be used for study. *dp-4* mutants are characterized by truncated extremities, exposed viscera, short stature, preaxial polydactyly, a shortened upper beak, and mild to severe cleft palate. The causative gene is unknown, but the inheritance is sex-linked recessive and mapping has identified a region on the p-arm of the Z chromosome (Robb et al., 2011). Phenotypically *dp-4* has craniofacial characteristics similar to both  $ta^2$  and  $ta^3$ . Similar to  $ta^2$ , early *dp-4* embryos can be identified by non-polarized limb buds (an early indication of polydactyly), a narrowed stomodeum and

dysmorphic MXPs (Fig. 4A, F). At day 7, the FNP and MXPs in the *dp-4* have yet to fuse and the MNP is narrowed (Fig. 4B, G). By day 10, *dp-4* mutants have a shortening of the upper beak with cleft primary palate, similar to *ta*<sup>2</sup>, their lower beak is narrowed and ‘peg-like’ and they exhibit hypo/aglossia, similar to *ta*<sup>2</sup> and *ta*<sup>3</sup> (Fig. 4C-E, H-J).

The *coloboma* mutant (*co*) was first described by Ursula Abbott in 1970. *co* is an embryonic lethal, sex-linked recessive mutant with limb and craniofacial defects. While the causative gene has yet to be identified, the *co* trait has been mapped to the p-arm of the Z chromosome (Robb et al., 2011). The craniofacial phenotype in *co* mutants can range from mild to severe cleft palate and manifests early in craniofacial development. There are early morphological changes in all facial prominences of *co* mutants. At HH25 the FNP is reduced and dysmorphic (Fig. 4A, K). At day 7, the FNP and MXP failed to fuse and the FNP is severely hypoplastic and displaced slightly anteriorly, similar to what is observed in *ta*<sup>3</sup> (Fig. 4B, L). At day 10, the cleft primary palate is obvious and the hypoplastic FNP fails to occlude with the MNP, leading to skewed MNP outgrowth (Fig. 4C, D, M, N). Hypoglossia is also evident in these mutants (Fig. 4E, O).

*cleft primary palate (cpp)* mutants were first found among the stocks of *scaleless* mutants at UC Davis in 1966. Despite the dramatic phenotype, there has been surprisingly little study of this mutant, save the original characterization by Ursula Abbott and one paper examining mechanism of beak outgrowth (MacDonald et al., 2004; Yee and Abbott, 1978). While a gene has yet to be identified for *cpp*, the mode of inheritance is autosomal recessive, with embryonic lethality, similar to that of *ta*<sup>2</sup> and *ta*<sup>3</sup>. These mutants have a striking craniofacial phenotype characterized by a recessed and upturned FNP. At HH21, *cpp* mutants are phenotypically indistinguishable from control embryos (data not shown); however, by HH25 there are subtle

changes in the morphology of the FNP of *cpp* mutants that resemble  $ta^3$  (Fig. 4A, P). At day 7, the FNP remains hypoplastic and broad, and does not fuse with the MXPs (Fig. 4B, Q). By day 10, the upper beak is anteriorly displaced and highly dysmorphic, lacking any form of normal structure (Fig. 4C, D, R, S). Interestingly, the MNP remains unaffected in this mutant, as both the mandible and tongue are comparable to that of control embryos (Fig. 4E, T).

Together, these mutants have unique and severe craniofacial phenotypes, partially similar to what is observed in the ciliopathic mutants,  $ta^2$  and  $ta^3$ . It is possible that one or more of these avian mutants may be yet another model for a human ciliopathic condition. Alternatively, they could be models for other human diseases. Given what we have learned about craniofacial development and human ciliopathies from the  $ta^2$  and  $ta^3$ , further investigation into the genetic, cellular, and molecular etiologies of these avian mutants will likely provide valuable insights into craniofacial development and human disease in general.

***The future of avian based craniofacial studies: combining classical techniques with modern technology***

Although the chicken model system has many experimental advantages, its usefulness has previously been limited by the lack of genetics. Compared to mouse, which has undergone eight genome assemblies, the chicken has only undergone four. Thus, the chicken genome has several gaps making whole genome and whole exome sequencing difficult. The lack of full genome coverage also means the identification of causative mutations will take a significantly longer time in chicken than in models that have more thoroughly covered genomes. Furthermore, there are severe limitations with respect to animal husbandry. Individual labs are most likely unable to dedicate the space, personnel, and resources required to maintain a flock of transgenic chickens. Despite these limitations, improvements in technology are allowing for increased use of the chicken in a genetic context. Recent high-density SNP-typing efforts have improved linkage map resolution (Groenen et al., 2009) and currently a 60K SNP chip is in use that allows for fine-scale linkage and association mapping (Dodgson et al., 2011).

Even with the genomic limitations, chickens have tremendous experimental utility. Chicken embryos have been classically utilized in “cut and paste” transplant experiments, in which specific donor tissues are transplanted into a host environment in order to test their developmental potential or function. While many tissues can be transplanted, ones especially relevant for craniofacial development are the dorsal neural tube (Fig. 5A), the frontonasal ectodermal zone (FEZ), and endoderm (Couly et al., 2002; Hu and Marcucio, 2009b; Schneider and Helms, 2003). Dorsal neural tube explants between control and *talpid*<sup>2</sup> embryos have been particularly useful in the pursuit of understanding tissue-specific requirements of primary cilia

during neural crest migration (Schock et al., 2015). Furthermore, transplant experiments of this nature could also be useful for understanding the role of primary cilia in tissue-tissue interactions.

Chicken embryos have also been widely and successfully used for gene over-expression and knockdown studies. There are several methods used to alter levels of gene expression in avians. The most common transient over-expression method particular to the avian model system has been the viral Replication-Competent ASLV long terminal repeat with a Splice acceptor (RCAS) system (Hughes, 2004), although the pCAGGS vector has also been widely successful, particularly in studies of the neural tube and limb. Likewise, transient transfections with siRNA expressing constructs (Das et al., 2006) and morpholinos, via *in ovo* electroporation, have also been used in chick to knockdown a gene in both a spatially and temporally restricted manner (Nakamura et al., 2004) (Fig. 5B). Another commonly applied technique to the chick model system is the application of Heparin acrylic or Affi-gel blue beads soaked with growth factors (i.e. FGFs, BMPs, or SHH). These beads can be placed at specific locations on the embryo to locally manipulate signaling pathways (Fig. 5C). These techniques have been frequently applied to discern the molecular mechanisms driving craniofacial morphogenesis (Abzhanov and Tabin, 2004; Hu et al., 2003; Lee et al., 2001; Marcucio et al., 2005; Schneider and Helms, 2003). Techniques such as these could serve particularly useful when exploring how loss of primary cilia affects signaling pathways. Applying beads soaked in SHH protein (Shh-N) or small molecules that block the SHH pathway (cyclopamine) could shed light on how transduction of the SHH is altered in Oral-facial-digital syndrome (*talpid*<sup>2</sup>) or Joubert syndrome (*talpid*<sup>3</sup>). Using locally applied exogenous factors to manipulate signaling pathways within mutant cells would not be feasible in other species.



Perhaps the most exciting technological advancement affecting the use and potential of the avian model system is the advent of transgenic chickens. Stable germ-line transgenic chicken flocks expressing eGFP, mCherry and other fluorescent markers, either ubiquitously (McGrew et al., 2004) or under tissue-specific reporters (Balic et al., 2014), are available. Specific flocks, such as the Roslin Green eGFP line (also known as Glo-chicks (Fig. 5A), available in the US through Susan Chapman at Clemson University), H2B-YFP, Tie1-GFP and PGK-mCherry (available through the Ozark egg company) or Tg(PGK1:H2B-chFP) quails (Huss et al., 2015) can be widely used by developmental biologists, and make it possible to generate chimeras between fluorescent and non-fluorescent embryos (McGrew et al., 2008) (Fig. 5A). Furthermore, current advances in the field of avian transgenesis, such as the development of a Cre-inducible stable transgenic line (Freem et al., in preparation), are now set to again revolutionize classic avian approaches. For example, application/co-application of Cre-protein on beads or via transient transfection of cells could soon be used to specifically knock-out ciliary genes with temporal and spatial precision. Finally, chickens are not far behind on the genome-editing front. By targeting chicken primordial germ cells *in vitro* (Macdonald et al., 2012), the chicken genome can now be specifically targeted via TALENS or CRISPR/Cas9 technology to edit various ciliary genes and their specific function during craniofacial development.

### ***Conclusions***

The chicken has long been an important model organism for developmental biology, particularly the study of craniofacial development. The chicken embryo has been an especially useful vertebrate system for developmental biologists owing to experimental advantages of *in ovo* embryogenesis (Brown et al., 2003), as well as its place in the evolutionary gap between

mammals and non-mammalian vertebrates. The recent success in cloning the genes responsible for the *talpid* mutants, together with the discovery that mutations in these genes are responsible for human craniofacial ciliopathies, serve as promising examples for the hundreds of well-characterized mutant stocks whose genetic basis remains unknown (Pisenti et al., 2001). The possibility of examining of these mutants with a combination of classical and modern techniques, suggests a bright future for the use of chicken and other avian models in the study of development and disease.

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**Figure Legends:**

**Figure 1. Craniofacial development and primary cilia.** (A-I) Development of the embryonic face in chick, mouse, and human. Note that following prominence fusion, species-specific differences become clear (e.g., beak in chicken, snout in mouse). (J) TEM of a primary cilium. Axoneme (yellow), basal body (green), IFT cargo (red), transition zone (blue). (K) Cross section of a primary cilium showing 9+0 microtubule arrangement. (L) Table of common phenotypes seen in ciliopathic patients arranged by facial prominence of origin.

**Figure 2. The craniofacial phenotype in  $ta^2$  and  $ta^3$  embryos.** Frontal views of control,  $ta^2$ , and  $ta^3$  embryos at (A, B, C) HH21, (D, E, F) HH25, (G, H, I) day 7, and (M, N, O) day 10. (J, K, L) Dorsal view of developing tongue and mandible in control,  $ta^2$ , and  $ta^3$  embryos at day 7. (P, Q, R) Palatal views of control  $ta^2$ , and  $ta^3$  embryos at day 10. Dotted white lines outline affected facial prominences in (A-F) and the tongue in (J, K), asterisk denotes lack of tongue in (L). Black arrows in (H) denote lack of fusion between FNP and MXP; black arrow in (I) denotes anteriorly shifted FNP; black arrows in (N) denote tooth-like structures; white arrows in (O) denotes ectopic lens. FNP, frontonasal prominence; MXP, maxillary prominence, MNP, mandibular prominence; t, tongue; 1°, primary palate; 2°, secondary palate. Scale bars: (A-F) 1.15 mm; (G, H, I) 1.5 mm; (J, K, L) 1 mm; (M, N, O) 2.25 mm; (P, Q, R) 2.5 mm.

**Figure 3. Shh expressing domains in  $ta^2$  and  $ta^3$  embryos.** Schematic representation of *SHH* expression in control,  $ta^2$  and  $ta^3$  embryos. (A, B, C) *SHH* expression in the frontonasal prominence (FNP), (D, E, F) notochord and floor plate, (G, H, I), zona limitans intrathalamica (ZLI) of control,  $ta^2$ , and  $ta^3$  embryos. Primary domains of *SHH* expression (notochord) are unchanged in  $ta^2$  and  $ta^3$  mutants. Secondary domains of *SHH* expression (FEZ/oral ectoderm, floor plate and ZLI) are all disrupted in  $ta^3$  mutants. *SHH* expression in the floor plate is maintained in  $ta^2$  embryos, but *SHH* expression at the other secondary domains (FEZ and ZLI) is disrupted.

**Figure 4. The craniofacial phenotype in *dp-4*, *co*, and *cpp* embryos.** Frontal views of control *dp-4*, *co*, and *cpp* embryos at (A, F, K, P) HH25, (B, G, L, Q) day 7, and (C, H, M, R) day 10. (D, I, N, S) Palatal views of control, *dp-4*, *co*, and *cpp* embryos at day 10. (E, J, O, T) Dorsal view of developing tongue and mandible in control, *dp-4*, *co*, and *cpp* embryos at day 10. Dotted white lines outline affected facial prominences in (A, F, K, P) and the tongue in (E, J, O, T). Asterisk in (J) denotes lack of tongue. Black arrows in (G, L, Q) denote lack of fusion between FNP and MXP. FNP, frontonasal prominence; MXP, maxillary prominence, MNP, mandibular prominence; t, tongue; 1°, primary palate; 2°, secondary palate. Scale bars: (A, F, K, P) 1.15 mm; (B, D, G, I, L, N, Q, S) 1.5 mm; (C, H, M, R) 2.25 mm; (E, J, O, T) 2.5 mm.

**Figure 5. Schematic representations of common experimental techniques used for craniofacial studies in chicken embryos.** (A) Dorsal neural tube transplants between control host and GFP-positive (Glo) donor chick embryos. A portion of the dorsal neural tube is removed from an HH8<sup>+/9</sup> control host and replaced with an equivalent portion from a Glo donor embryo. Donor Glo neural crest cells migrate toward the facial prominences in chimeras. (B) Schematic of experimental design for *in ovo* electroporation. DNA construct is injected into the neural tube and electroporated into the tissue. Cells that successfully incorporated the DNA express GFP. Examples of electroporated embryos at 24 and 48 hours post electroporation are shown. (C) Affi-Gel Blue beads (red dashed circles) grafted into the facial prominences (FNP, MXP, or MNP) of HH25 chick embryos. Hematoxylin and eosin staining of sections of above chick embryos with implanted Affi-Gel Blue beads (blue dashed circles). nt, neural tube; FNP, frontonasal prominence; MXP, maxillary prominence, MNP, mandibular prominence. Scale bars: (A) 250  $\mu$ m, (B) 1 mm and 150  $\mu$ m, (C) 700  $\mu$ m and 650  $\mu$ m.

**Table 1. Available animal models for human craniofacial ciliopathies.** Human craniofacial ciliopathies and their associated phenotypes are listed. In addition, the causal gene and relevant animal models (chicken in red, mouse in blue) for each disorder are listed.

Ciliopathy	Craniofacial Phenotype	Gene with Animal Model	Animal Model References
Oral-facial-digital syndrome (OFD)	facial asymmetry; hypertelorism; micrognathia; broadened nasal ridge; hypoplasia of the malar bones and nasal alar cartilages; frontal bossing; pseudocleft; cleft palate; hamartomas of the tongue; bifid tongue; hyperplastic oral frenula; up-slanting palpebral fissures	<i>C2CD3 - talpid<sup>2</sup> (ta<sup>2</sup>); C2CD3-Hearty; C2CD3 - C2cd3<sup>GT</sup>; OFD1 - Ofd1<sup>-/-</sup></i>	Schock et al., 2015; Chang et al., 2014; Hoover et al., 2008; Ferrante et al., 2003
Joubert syndrome (JS)	prominent forehead; high rounded eyebrows; epicanthal folds; ptosis; upturned nose with evident nostrils; hypertelorism; open mouth and tongue protrusion with rhythmic tongue motions	<i>KIAA0586- talpid<sup>3</sup> (ta<sup>3</sup>); CEP290 - Cep290<sup>LacZ/LacZ</sup>; JBTS17 - Hug; RPGRIP1L- Rpgrip1/Ftm</i>	Damerla et al., 2015; Hynes et al., 2014; Delous et al., 2007; Sayer et al., 2006; Davey et al., 2006
Bardet-Biedl syndrome (BBS)	Mid-face shortening and flattening; nasal bridge hypoplasia; reduced	<i>BBS4 - Bbs4<sup>-/-</sup>; BBS6 - Bbs6<sup>-/-</sup></i>	Tobin et al., 2008

Meckel-Gruber syndrome	length/bulbosity of the nasal tip; mild retrognathia  encephalocele; cleft lip and palate	<i>MKS1</i> - <i>Mks1</i> <sup>krc</sup> ; <i>MKS1</i> - <i>Mks</i> <sup>del64-323</sup> ; <i>MKS1</i> - <i>Mks1</i> <sup>tm1a(EUCOMM)Wtsi</sup> ; <i>TMEM67</i> - <i>Tmem67</i> <sup>tm1Dgen/H1</sup>	Abdelhamed et al., 2015; Wheway et al., 2013; Cui et al., 2011; Weatherbee et al., 2009
Cranioectodermal dysplasia/Sensenbrenner syndrome	sagittal craniosynostosis; epicanthal folds; hypodontia or microdontia; everted lip; multiple oral frenula; high arched palate; skeletal and ectodermal anomalies	<i>IFT122</i> - <i>ift122</i> <sup>-/-</sup>	Walczak-Sztulpa et al., 2010
Ellis-van Creveld syndrome	hypertrophy labiogingival frenulum; upper lip abnormalities; presence of teeth at birth; microdontic teeth	<i>EVC1</i> - <i>Evc</i> <sup>-/-</sup> ; <i>EVC2</i> - <i>Evc2</i> <sup>-/-</sup>	Nakatomi et al., 2013; Caparrós-Martín et al., 2013; Ruiz-Perez et al., 2007

**Highlights**

- Chickens are useful models for studying craniofacial development and disease.
- The *talpid<sup>2</sup>* and *talpid<sup>3</sup>* are avian models for ciliopathies that represent the first spontaneous mutants to have their causative genes identified.
- *talpid<sup>2</sup>* can be used as an animal model for the human ciliopathy Oral-facial-digital syndrome (OFD)
- *talpid<sup>3</sup>* can be used as an animal model for the human ciliopathy Joubert Syndrome (JS).

Accepted manuscript











