Cichlid fishes as a model to understand normal and clinical craniofacial variation

Kara E. Powder*, R. Craig Albertson*

Department of Biology, University of Massachusetts Amherst, 221 Morrill Science Center South, 611 North Pleasant Street, Amherst, MA 01003, USA

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**ABSTRACT**

We have made great strides towards understanding the etiology of craniofacial disorders, especially for ‘simple’ Mendelian traits. However, the facial skeleton is a complex trait, and the full spectrum of genetic, developmental, and environmental factors that contribute to its final geometry remain unresolved. Forward genetic screens are constrained with respect to complex traits due to the types of genes and alleles commonly identified, developmental pleiotropy, and limited information about the impact of environmental interactions. Here, we discuss how studies in an evolutionary model – African cichlid fishes – can complement traditional approaches to understand the genetic and developmental origins of complex shape. Cichlids exhibit an unparalleled range of natural craniofacial morphologies that model normal human variation, and in certain instances mimic human facial dysmorphologies. Moreover, the evolutionary history and genomic architecture of cichlids make them an ideal system to identify the genetic basis of these phenotypes via quantitative trait loci (QTL) mapping and population genomics. Given the molecular conservation of developmental genes and pathways, insights from cichlids are applicable to human facial variation and disease. We review recent work in this system, which has identified lbh as a novel regulator of neural crest cell migration, determined the Wnt and Hedgehog pathways mediate species-specific bone morphologies, and examined how plastic responses to diet modulate adult facial shapes. These studies have not only revealed new roles for existing pathways in craniofacial development, but have identified new genes and mechanisms involved in shaping the craniofacial skeleton. In all, we suggest that combining work in traditional laboratory and evolutionary models offers significant potential to provide a more complete and comprehensive picture of the myriad factors that are involved in the development of complex traits.

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1. Craniofacial development and disease: progress and challenges

Over 70% of all birth defects are associated with craniofacial malformations (Hall, 2009). Although significant advances have been made in understanding the etiology of many Mendelian craniofacial disorders (e.g., Treacher Collins Syndrome (Kadakia et al., 2014), Apert Syndrome (Wilkie et al., 1995), and Crouzon Syndrome (Reardon et al., 1994)), much less is known about the factors that contribute to complex diseases (e.g. non-syndromic cleft lip and/or palate) and normal facial variation, which result from the cumulative effects of many alleles and their interactions with the environment (Fish et al., 2014; Glazier et al., 2002; Hallgrimsson et al., 2009; Hallgrimsson et al., 2014; Hirschhorn and Daly, 2005; Hochheiser et al., 2011). Even for monogenic diseases in which the causative genes are known we still lack a comprehensive picture of the genetic, cellular, and environmental interactions that contribute to the severity of the disease (e.g., the impact of genetic background (Dixon and Dixon, 2004)). The National Institute of Dental and Craniofacial Research (NIDCR) iterative FaceBase, a consortium aimed at advancing research in craniofacial research, states that “much research is needed to achieve a molecular and cellular understanding of the mechanisms by which genes and gene products interact to generate complex phenotypes.” Thus, a significant challenge in craniofacial biology remains to uncover the genomic and developmental mechanisms that underlie the production of subtle and complex patterns of variation in craniofacial shape.

2. The limitations of induced mutations

Much of our knowledge of craniofacial development has been acquired from induced mutations and loss-of-function

* Corresponding authors.
E-mail addresses: keepower@bio.umass.edu (K.E. Powder), albertson@bio.umass.edu (R.C. Albertson).

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experiments in a small handful of model organisms, particularly *Mus musculus* (mouse), *Gallus gallus* (chicken), *Xenopus laevis* (African clawed frog), and *Danio rerio* (zebrafish). These studies have provided critical insights into the basis of early developmental patterning events and the etiology of craniofacial diseases, particularly “simple” Mendelian disorders. However, traditional forward genetics screens and the use of knockout mutants are not without limitations. We propose that embracing the natural variation present in “evolutionary mutant” models (Albertson et al., 2009), including cichlid fishes, can overcome some of these limitations and complement our current knowledge of the genetic and developmental basis of normal and extreme, clinical craniofacial variation. The crux of the argument is that all forward genetic analyses are, by definition, based on phenotype, and natural systems provide a vast reservoir of phenotypic variation. An understanding of this natural variation will supplement findings about phenotypes that have been artificially generated through mutagenesis. The limitations of traditional approaches and the complementary nature of evolutionary models are discussed in greater detail below.

### 2.1. Screens commonly miss non-essential genes and subtle mutations

Classic forward genetic approaches screen for abnormal phenotypes in populations of mutagenized animals. These screens typically focus on early and often lethal phenotypes, and are well suited to identify genes that are essential for a particular function. They have a bias towards certain types of genes, such as those that regulate a critical developmental switch or act as integrators of multiple pathways. Many genes are routinely missed by such an approach, including those that have redundant function, act as a genetic modifier, or contribute to variation in a process but are not essential for trait development. While specialized (e.g., “enhancer”) screens (Kile and Hilton, 2005; Patton and Zon, 2001) can uncover some of these factors, these are labor intensive and require base knowledge or assumptions about gene action. In all, it is becoming increasingly appreciated (e.g., (Bolker, 2012)) that new approaches and new models are needed to identify the genes that may have been missed by classic forward genetic screens.

In addition to disproportionally targeting genes with early essential functions, screens also unequally identify certain types of alleles and mutations. Upwards of 75% of mutant alleles identified in standard laboratory screens are in coding regions and result in severe disruption or complete loss of gene function (Nguyen and Xu, 2008). This is perhaps unsurprising, as severe genetic lesions are likely to have more prominent phenotypic defects that are easier to identify by researchers, and speaks to the power and efficiency of forward genetic screens. However, complex traits such as normal facial variation and clinical conditions like non-syndromic cleft lip and/or palate predominantly do not result from null alleles with large effects, but rather from a compilation of small effects from many genes that act at multiple developmental stages or confer susceptibility to environmental effects (Fish et al., 2014; Glazier et al., 2002; Hallgrimsson et al., 2009, 2014; Hirschhorn and Daly, 2005; Hochheiser et al., 2011). In accordance with this, the overwhelming majority (> 80%) of variants implicated in complex traits and diseases by genome-wide association studies (GWAS) are predicted to be in non-coding regions (Hindorff et al., 2009; Khandelwal et al., 2013; Manolio, 2009). Further, genes with essential roles in early development are likely to face strong stabilizing selection, and thus may not contribute to normal patterns of variation. For instance, *Shh* is critical for proper facial morphogenesis (Belloni et al., 1996; Chiang et al., 1996; Hu and Helms, 1999; Jeong et al., 2004; Wada et al., 2005; Young et al., 2010). While subtle modulation of this pathway can produce a dose-dependent phenotypic continuum during embryonic development, the effect is non-linear such that the range of “normal” variation is quite narrow and even slight changes in *Shh* levels can result in major malformations such as clefting in adults (Young et al., 2010). This experiment may explain why this gene has yet to be identified in studies of normal facial variation (Bohringer et al., 2011; Liu et al., 2012; Paternoster et al., 2012; Sherwood et al., 2011). As pointed out by Hallgrimsson and colleagues (Hallgrimsson et al., 2014), it is quite possible (and even likely) that genes not identified by embryonic mutagenesis screens are contributing to the majority of normal facial variation in humans.

### 2.2. Developmental pleiotropy limits the assessment of later developmental stages

Mutagenesis screens are also constrained by developmental pleiotropy, wherein a single gene acts in multiple tissues and/or at multiple developmental stages. Defects at the earliest stage in which the gene has an essential function (e.g. neural crest cell migration) often preclude examination of phenotypes at later developmental stages (e.g. chondrogenesis and facial growth). This problem can be circumvented using conditional knockouts or targeted activation of alleles (Campbell et al., 2012; Chan et al., 2007; Patton and Zon, 2001), but this requires prior knowledge about the gene of interest. Pleiotropy is a critical concern for facial development, as defects in neural crest cell specification and migration have a higher likelihood of being lethal (Hall, 2009), and thus may be less applicable to normal human variation than alterations in later developmental stages such as osteogenesis. In order to understand the etiology of complex traits that manifest later in development, there is a need to supplement traditional approaches with those that specifically address later developmental stages, including adults (Albertson and Yelick, 2004).

### 2.3. Gene x environment (GxE) interactions account for variation in both Mendelian diseases and complex traits

Genetic screens and genome-wide association studies (GWAS) have identified hundreds of loci that are associated with complex human traits and diseases. However, for most complex traits, these cumulatively have only been able to account for a small percentage of the heritability of phenotypes. Sources of such “missing heritability” are thought to be genetic modifiers (GxG or epistasis) and environmental interactions (GxE) (Eichler et al., 2010; Gibson, 2010; Manolio et al., 2009). Even for “simple” monogenetic disorders (e.g. cystic fibrosis), we are only beginning to understand the genetic modifiers and environmental interactions that impact disease severity and penetrance (Nadeau, 2001); in this way Mendelian phenotypes can act as complex traits (Dipple and McCabe, 2000). In addition, the influence of the genetic, cellular, and external environment can have significant impacts on craniofacial development. For instance, sex hormones have drastic effects on embryonic facial patterning (Cohen et al., 2014), juvenile development (Fujita et al., 2004; Marquez Hernandez et al., 2011; Verdonck et al., 1998), and bone remodeling at juvenile and adult stages (Frenkel et al., 2010; Nicks et al., 2010). The hardness of diet can also have a significant impact on craniofacial geometry (Genbrugge et al., 2011; Parsons et al., 2014; Swiderski and Zelditch, 2013). The role of such sex-specific and GxE interactions represent a long-standing gap in our knowledge of the etiology and pathophysiology of complex diseases that are unlikely to be answered with traditional mutagenesis screens.
3. Harnessing natural variation in evolutionary mutants to understand complex traits

3.1. Nature as a potent “mutagenizing” force

While Charles Darwin and other 19th century biologist embraced the astounding natural diversity of living things, the 20th century became the era of model organisms such as the fly and mouse (Davis, 2004). Technological advances in genome sequencing and molecular techniques have enabled modern biologists to once again turn to natural variation in non-model organisms (discussed in Albertson et al., 2009; Bolker (2012); Mahar (2009); Schartl (2014)), spurring on the current “golden age” of comparative and evolutionary genetics (Nadeau and Jiggins, 2010). We argue that these advances also open up opportunities to use evolutionary systems to better understand the human condition.

Nature is a source of vast biodiversity, which has accumulated over millions of years as populations are continually challenged by natural and/or sexual selection. For craniofacial structures, trophic adaptations have resulted in morphologies exquisitely matched to feeding niches. Sometimes these phenotypes are extreme and/or discontinuous (i.e., may be considered a phenotypic novelty), such as the snout of an ant eater or the cranio-dental skeleton of toothed whales. Alternatively, adaptive radiations can produce extensive continuous variation in craniofacial form. Textbook examples of this continuous variation include Darwin’s finches (Grant, 1986; Grant and Grant, 2014) and African cichlids (Kocher, 2004). Because the mutations that underlie these adaptive phenotypes are part of a well integrated genetic architecture (i.e., they are not deleterious), such systems offer great potential to reveal new insights into the genes and mechanisms that underlie this important trait. Moreover, given that changes in gene regulation are the primary source of both the development of complex traits in humans (Hindorff et al., 2009; Hirschhorn and Daly, 2005; Kandel et al., 2013; Korstanje and Paigen, 2002; Manolio, 2009) and morphological evolution (Carroll et al., 2005; Jacob, 1977; King and Wilson, 1975; Stern, 2000; Wray, 2007), the study of natural variants should more accurately reflect the number, effect, mode of action, interaction, and environmental sensitivity of genes that underlie complex traits compared to induced mutagenesis screens.

3.2. Craniofacial development is conserved among vertebrates

During craniofacial development, neural crest cells (NCCs) must coordinate a complex pattern of molecular and cellular signals from the ectoderm and the endoderm to properly induce, specify, migrate, proliferate, and differentiate into the bones and cartilages of the face (reviewed in (Brugmann et al., 2006; Santagati and Rijli, 2003; Sawa-Spangler and Bronner-Fraser, 2008)). Alterations in any of these processes can produce clinical malformations or variation on which natural selection can act. One of the major biological findings of the late 20th century was that all multicellular animals share a conserved set of genes and circuits that govern development (Carroll et al., 2005). Craniofacial development is no exception; despite dramatic differences in adult structures, the molecular and morphogenetic processes that mediate NCC and craniofacial development are highly conserved among vertebrates (Knight and Schilling, 2006; Medeiros and Crump, 2012; Schilling 1997). In other words, homology occurs at multiple levels, spanning adult skeletal elements, cellular mechanisms such as migration and fusion, and genes (Shubin et al., 2009; Wagner, 2014). This “deep homology” (Shubin, et al., 2009) allows insights into human facial development from non-mammalian systems, including both traditional models (e.g. zebrafish and chicken) and evolutionary models (e.g. cichlids). For example, mammalian and fish palates have distinct morphologies and are comprised of non-homologous elements (Dougherty et al., 2013; Murray and Schutte, 2004; Swartz et al., 2011). Despite this, their development requires the action of homologous cell- and tissue-level processes including migration of cranial neural crest cells, as well as the coordinated outgrowth and fusion of multiple processes and structures (Dougherty et al., 2013; Murray and Schutte, 2004). Further, homologous genes regulate these cellular mechanisms. For instance, loss of irf6 or the microRNA Mirn140 and Pdgf signaling network results in conspicuous orofacial clefting in both humans and zebrafish (Dougherty et al., 2013; Eberhart et al., 2008; Kondo et al., 2002; Li et al., 2010; Rattanapho et al., 2012). These insights confirm the deep homology of the vertebrate skull. While the component parts may vary across taxa, the underlying genetic and developmental logic that assemble these structures are highly conserved. This and other findings (see (Albertson et al., 2009)) support the idea that a common set of genes may underlie adaptive diversification in the wild and facial dysmorphologies in the clinic. Therefore, insights gleaned from studies in evolutionary models are directly relevant to human development and possibility even disease. In fact, as we illustrate below, the same extreme phenotypic state could even be considered a disease in one species/lineage and an adaptation in a second lineage (Fig. 1).

4. The evolutionary history of cichlids makes them an ideal system to study the genetic basis of craniofacial variation

Cichlids (family: Cichlidae) are tropical freshwater fish that have undergone an extraordinary adaptive radiation in terms of the pace, number of species, and degree of morphological diversity (Irryer and Iles, 1972; Kocher, 2004; Salzburger and Meyer, 2004). In all, cichlids account for about 8% of all fish species (Kuraku and Meyer, 2008), making them one of the most species-rich vertebrate families (Salzburger and Meyer, 2004). Their main radiation (~2000 endemic species) occurred in the East African rift lakes, namely Lakes Malawi, Tanganyika, and Victoria. Within Lake Malawi alone, there are upwards of 1000 species that have evolved within ~1–2 million years (Kornfield and Smith, 2000; Salzburger and Meyer, 2004; Sturmbauer et al., 2001; Turner et al., 2001), with speciation events having occurred as recently as 1000 years ago (Won et al., 2005).

4.1. Cichlids demonstrate unparalleled craniofacial variation, analogous to normal and clinical variation in humans

A hallmark of cichlid radiations is their incredible diversity in behavior, color, body shape, and, critical for this synopsis, facial morphologies (Fig. 1). Cichlids encompass an extensive variety of trophic strategies, including algae grazing, snail crushing, sand sifting, egg predation, scale eating, and piscivorous hunting, and each of these is associated with different morphological and functional demands (Fryer and Iles, 1972; Konings, 2001; Kornfield and Smith, 2000) (Fig. 1). Despite this wide range of phenotypes, the primary axis of facial variation among all African cichlids distinguishes two generalized feeding strategies – biters and suckers (Cooper et al., 2010). Fish with stout faces, small eyes, tricuspid teeth, and short, downturned jaws have adapted to bite, pick, or scrape attached algae or crush hard mollusks. On the other hand, fish with elongated faces, larger eyes, unicuspid or bicuspid teeth, and longer, isognathus jaws have adapted to suction other fish, small invertebrates, or algae from the water column (Albertson et al., 2005; Cooper et al., 2010; Konings, 2001; Streelman and Albertson, 2006). Notably, cichlid craniofacial variation has evolved along this same axis independently in the three African Great Lakes (Lakes Malawi, Tanganyika, and Victoria) (Cooper et al., 2013).
Extreme / clinical variation

Continuous / “normal” variation

Extreme / clinical variation

Fig. 1. Cichlids demonstrate extensive craniofacial variation analogous to normal and clinical variation in humans. Comparison of human (top) and cichlid (bottom) facial variation. Most cichlid facial variation is continuous across species adapted to bite or scrape food from the substrate (left), or to suck food from the water column (right). This is similar to the continuous spectrum of “normal” facial variation found among human populations. Notably, some cichlid species have evolved extreme morphologies that mimic pathological variation in humans such as micrognathia (far left) and midface hypoplasia (far right). Cichlid images courtesy of Ad Konings. All cichlids are from Lake Malawi. From left to right, including feeding mode (Konings 2001): Labeotropheus fuelleborni (biting; scraping connected algae), Cynotilapia zebroides (suctioning: plankton from water column or combing loose algae), Tyrannochromis nigrovittatus (suctioning: ambush predator of other fish), Aulonocara rostratum (sonar hunting of invertebrates buried in sand), and Caprichromis orthognathus (head-buts mouth brooding females, then eats released eggs and larvae). Illustrations of human skulls from different populations (from left to right: American Indian, European, and Sub-Saharan African) by Diana Marques, courtesy of the Smithsonian’s National Museum of Natural History.

et al., 2010; Fryer and Iles, 1972). Despite their independent evolution over markedly different time scales, cichlids from each lake demonstrate remarkable convergence in craniofacial morphologies (Albertson et al., 2003a; Cooper et al., 2010; Fryer and Iles, 1972). These “experimental replicates” provide a powerful opportunity to determine whether convergent morphologies result from alterations of the same genes and pathways, or whether there are multiple genetic and cellular mechanisms that result in similar phenotypes. Not only is this an important question in evolutionary theory, but such insights into genetic architecture would also provide a better understanding of which aspects of craniofacial anatomy are developmentally plastic and which are inflexible (i.e. canalized).

Craniofacial variation among most cichlid species is continuous between alternate short versus long face morphologies, and in this way analogous to “normal” facial variation among human populations (Fig. 1). However, certain species have evolved extreme morphologies adapted to specialized feeding methods. In these instances, craniofacial morphologies between cichlid lineages are more discontinuous, mimicking pathological variation in the human facial skeleton such as micrognathia, midface hypoplasia, and facial asymmetries (Fig. 1, Albertson et al., 2009)). As with human dysmorphologies, craniofacial differences among cichlids can be detected as early as NCC migration (Albertson and Kocher, 2006; Powder et al., 2014), with additional shape differences accumulating into larval and juvenile stages (Parsons et al., 2014; Powder et al., 2015). Cichlids are thus an ideal evolutionary system to study the genetic and developmental origins of both subtle and extreme facial variation due to their (1) extensive morphological variation, (2) mimicking of variation found in humans, and (3) similarities to mammals at the molecular and tissue level.

4.2. The genomic architecture of Lake Malawi cichlids is ideal for association and QTL mapping studies

Despite their remarkable phenotypic diversity, cichlids exhibit a relatively high degree of genomic homogeneity due to their rapid evolution (~1–2 million years for Lake Malawi (Stumbauer et al., 2001)) and ongoing hybridization. Five complete cichlid genomes are now available (Brawand et al., 2014), and among the major insights from this work is that approximately half of the polymorphisms identified among species (which shared a common ancestor ~10 million years ago) exhibit incomplete lineage sorting. In other words, a significant proportion of ancestral polymorphisms are still segregating between species via genetic recombination and hybridization (Brawand et al., 2014; Loh et al., 2013). The result is a young species flock characterized by relatively low levels of nucleotide diversity (i.e. lower than that between different laboratory strains of zebrafish, (Loh et al., 2008)) and high levels of shared polymorphisms. Notably, these properties mean that genetic/genomic comparisons between cichlid species (or genera) are analogous to those between human populations.

A combination of high phenotypic variation and low genotypic variation make cichlids an ideal system for population genomics. Specifically, given the high degree of shared polymorphisms (Brawand et al., 2014; Loh et al., 2013), global genetic differentiation is low among Lake Malawi cichlids (average $F_{ST} \approx 0.2$ (Loh et al., 2008)). Thus outlier loci that exhibit signatures of divergence (e.g. high $F_{ST}$ values) are de facto candidates for trait differences between species or genera. Essentially, neutral polymorphisms have not had time to become fixed between species, which means that outlier loci are likely to exist because they mediate a phenotype that is under divergent selection. For instance, a SNP near the neural patterning gene irx1 was found to exhibit an outlier $F_{ST}$ value in a genome-wide comparison between cichlid lineages that have distinct brain morphologies (Loh et al., 2008). This observation suggested that irx1 may mediate differences in brain development between these fishes. Subsequent functional studies supported this hypothesis by showing that differential expression of irx1 is associated with changes in brain patterning that result in differences in the relative size of the telencephalon versus the thalamus in cichlids (Sylvester et al., 2010).

With accumulating genomic resources it is now relatively easy to identify divergent loci among cichlids, however linking these loci to a specific trait is not always straightforward because species/lineages usually differ with respect to multiple traits. Pedigree mapping of quantitative traits (e.g., QTL mapping) is a better method with which to link variation in genotype with variation in a specific phenotype. Given their recent divergence, most Lake Malawi cichlid species (and many genera) produce fertile hybrids, enabling the generation of large pedigrees for QTL mapping (e.g.
craniofacial traits in (Albertson et al., 2003b; Albertson et al., 2005; Cooper et al., 2011; Hu and Albertson, 2014; Parsons et al., 2012; Parsons et al., 2015; Stewart and Albertson, 2010; Streelman and Albertson, 2006). A key limitation of QTL mapping however is that the identification of causative genes and/or polymorphisms for a QTL can be challenging and expensive due the time and labor involved in generating sufficient numbers of recombinant animals for fine mapping. Thus, population genomics can efficiently identify divergent loci that are under selection, but cannot link geno-type to a specific phenotype. Alternatively, QTL mapping can statistically associate genetic intervals to specific phenotypes, but often lacks the resolution to pinpoint specific causative genes and nucleotides.

Recently, investigators have begun to address these methodo-logical limitations by combining both population genomics and QTL mapping into a single experiment, thereby leveraging the advantages while mitigating the disadvantages of each approach (Albertson et al., 2014; Barrett and Hoekstra, 2011; Parsons and Albertson, 2013). For example, we previously used this approach to implicate pax3a in cichlid coloration (Albertson et al., 2014). We first used QTL mapping to associate a 28 cM region with levels of red/yellow pigmentation. This region corresponds to approximately 11 Mb of physical sequence and contains ~315 genes, making it daunting to identify candidate genes. Population geno-mic analysis of this region identified 366 genetic polymorphisms between cichlid species that also differ with respect to pigmen-tation. However, of these, just 17 exhibited high levels of differ-entiation between the species, suggesting they may be driving the phenotypic differences. One was in the 5'UTR of pax3a, which has been previously associated with specification of pigment cells (Minchin and Hughes, 2008). This example demonstrates how population genomic analysis, which takes advantage of recombination in the wild, can be used to effectively narrow the list of candidate genes that underlie a QTL.

5. Cichlid studies can complement traditional approaches in model organisms

Given their morphological diversity and genomic architecture, cichlids offer a powerful evolutionary system to complement tradi-tional approaches in model organisms such as Mus musculus and Gallus gallus. With the vast number of genetic tools available in laboratory models (e.g., genome editing), the validation of candi-date genes identified in cichlids may require experiments in model systems. That said, functional validation of candidate genes (or at least pathways) can also be performed in the cichlid system. The choice of whether to examine the mechanism of gene action within cichlids themselves or within traditional model organisms is dependent on the particulars of the candidate gene or pathway. Cichlids are readily susceptible to pharmacological manipulations through addition of small molecules to their water (Blooomquist et al., 2015; Fraser et al., 2008, 2013; Hu and Albertson, 2014; Parsons and Albertson, 2013; Parsons et al., 2014; Powder et al., 2015; Roberts et al., 2011; Sylvester et al., 2010, 2013). These chemicals have varying degrees of target specificity (i.e. may an-tagonize the entire Wnt pathway versus a specific ligand), though, and result in embryo-wide changes in gene expression. For candi-dates that do not have pharmacological agents or require in-creased specificity, mechanism of gene action can be determined using the battery of experimental methods available in model organisms such as zebrafish and Xenopus, including transgenics, injections (morpholinos and/or overexpression), and transplanta-tions (Albertson et al., 2005; Powder et al., 2014). There is also much promise for bringing such experiments from traditional model organisms into the cichlid system. For instance, cichlids are amenable to transgenesis (Fujimura and Kocher, 2011; Juntti et al., 2013), and CRISPR/Cas9-mediated genome editing (Sternberg and Doudna, 2015) is a powerful new tool that has shown great pro-mise in non-model organisms, including cichlids (Li et al., 2014). Below we briefly illustrate how cichlids, in conjunction with such mechanistic analyses, have yielded novel insights into craniofacial development.

5.1. Lbh is a novel regulator of neural crest cell migration

Neurocristopathies (Bolande, 1974) are syndromes resulting from abnormal neural crest cell (NCC) development, commonly NCC migration or proliferation (Hall, 2009). Given the wide variety of NCC derivatives (Hall, 2009; Santagati and Rijli, 2003; Sauka-Spengler and Bronner-Fraser, 2008), neurocristopathies often present with a suite of defects in craniofacial, neural, otic, cardiac, and pigment structures or tissues. We recently identified a novel regulator of neural crest cell development in cichlids. We started with a QTL on linkage group 19 that contributed to variation in the relative length of the mandible (Albertson et al., 2005; Cooper et al., 2011). Notably, one of the cichlid species used to generate this cross exhibits an extremely short jaw (“evolved micrognathia”) (Fig. 1, far left). Using population genomics, we were able to narrow this region and identify limb bud and heart homolog (Lbh) as a candidate gene (Powder et al., 2014). Lbh is a largely un-characterized putative transcriptional regulator (Ai et al., 2008) expressed during vertebrate limb and heart development (Briegel and Jöynner, 2001), but previously not known to regulate facial development.

Using loss-of-function analyses in zebrafish and Xenopus, we demonstrated that Lbh is necessary in neural crest cells for proper migration (Powder et al., 2014). Severe depletion of Lbh resulted in dramatic inhibition of migration and complete loss of NCC-derived facial structures, resulting in lethality. Cichlids with alternate short and long jaw morphologies have distinct SNPs around Lbh, in-cluding a non-synonymous R→Q mutation in the evolutionarily derived fish with a short mandible. We showed that this single amino acid change resulted in discrete shifts in neural crest cell migration patterns. Whereas the “long jaw” variant causes more NCCs to migrate to the mandibular arch, the “short jaw” form results in fewer NCCs migrating anteriorly; this depletion of the anterior pool of NCCs may result in the diminished mandible derived from this population of cells. Even though Lbh plays a critical role in NCC migration and contributes to quantitative variation in craniofacial structures, this gene had not been previously identi-fied in traditional screens for craniofacial regulators, perhaps because severe abrogation of Lbh results in lethality (Powder et al., 2014).

5.2. Wnt and Hedgehog pathways regulate species-specific bone development

Contrary to our extensive understanding of how early develop-mental events and genetic mechanisms regulate neural crest cell induction, migration, and patterning (Brugmann et al., 2006; Santagati and Rijli, 2003; Sauka-Spengler and Bronner-Fraser, 2008), comparatively little is known about the mechanisms that determine the shape of bones. One challenge to understanding skeletal morphologies is the re-deployment of signaling pathways during multiple stages of development. For example, the Wnt signaling network plays successive critical roles during NCC in-duction (reviewed in (Sauka-Spengler and Bronner-Fraser, 2008)), proliferation and outgrowth of facial mesenchyme (Brugmann et al., 2007, 2010), fusion of facial prominences (Han et al., 2006; Song et al., 2009), and osteogenesis (reviewed in (Hartmann, 2006; Long, 2012)). Likewise, the Hedgehog developmental
pathway is necessary for NCC survival (Ahlgren and Bronner-Fraser, 1999; Billmyre and Klingensmith, 2015), facial patterning and outgrowth at multiple stages (Cordero et al., 2004; Heyne et al., 2015; Hu et al., 2003; Jeong et al., 2004), and chondrogenesis and osteogenesis (Mo et al., 1997; Wada et al., 2005; Yang et al., 2015). This developmental pleiotropy, the iterative usage of a common pathway in multiple tissues or stages, can limit the assessment of later developmental events. For example, conditional loss of a central mediator of Wnt signaling, β-catenin, in neural crest cells results in early loss of these cells (Brault et al., 2001), precluding assessment of how changes in expression levels, timing, and pattern can result in quantitative differences in bone shape.

Recently work has combined QTL mapping, population genomics, and experimental manipulations in cichlids to associate the Hedgehog and Wnt pathways with subtle, quantitative variation in bone shape (Hu and Albertson, 2014; Loh et al., 2008; Parsons et al., 2014; Roberts et al., 2011). In one study, we mapped QTL for the shape of the interopercle bone and length of the retroarticular process of the mandible to the same genetic interval (Hu and Albertson, 2014; Roberts et al., 2011). Population genomics identified a SNP in this region that is highly divergent in cichlids with alternate morphologies, and resides upstream of the Hedgehog receptor $p_tch1$. The evolutionarily derived $p_tch1$ allele is associated with decreased expression levels of this gene and thus decreased bone deposition, ultimately resulting in a shorter retroarticular process and narrower interopercle bone. These shape changes have important functional consequences, wherein the derived allele confers decreased bite forces but faster jaw movements to fish that eat by suctioning food from the water column (Hu and Albertson, 2014; Roberts et al., 2011). Population genomics also identified a highly divergent SNP in β-catenin between cichlids with alternate craniofacial shapes (Loh et al., 2008). This SNP is associated with increased levels of Wnt signaling and accelerated rates of bone deposition in the cichlid face (Parsons et al., 2014). The accelerated bone development prevents outgrowth of the preorbital region, effectively locking into place an early larval phenotype (i.e., paedomorphosis) (Parsons et al., 2014; Powder et al., 2015). This ultimately results in a steeper craniofacial slope more resistant to strong bite forces in an animal that eats by shearing algae off of rocks (Cooper et al., 2011; Parsons et al., 2014). These studies illustrate how common developmental pathways, often studied at early developmental stages, are iteratively deployed over extended periods of development to produce complex morphologies.

5.3. Plasticity: remodeling and GxE interactions

A critical, but under-studied, aspect of craniofacial biology is the extent to which the environment can influence the genotype-phenotype (G–P) map and thus determine how the craniofacial skeleton develops and evolves. “Environment” is a broad term that could refer to either an organism’s internal or external environment. The internal environment includes such factors as endocrine signaling and the biophysical interactions among cells or tissues. The external environment is the physical (or physiological) conditions in which the organism develops, including light, temperature, and mechanical stresses. Both types of environments will act to filter the set of genetic variants that contribute to phenotypic variation (Janmczyk et al., 2010). Environmental effects cannot be accounted for in GWAS experiments and are thought to be an important source of the “missing” heritability in complex traits, including the craniofacial skeleton (Eichler et al., 2010; Gibson, 2010; Manolio et al., 2009).

In the interest of space we limit our discussion to the external forces that influence the shape of the craniofacial skeleton, mainly foraging environment that can impose distinct kinematic demands on the feeding apparatus. Bone is a dynamic tissue that can sense and respond to its mechanical environment (reviewed in (Alaeej et al., 2006; Nguyen and Jacobs, 2013; Papachroni et al., 2009; Robling and Turner, 2009)). Consistent with this, genes that participate in bone cell development and matrix deposition were found to be up or down regulated during remodeling of the cichlid pharyngeal jaw in response to diet changes (Gunter et al., 2013; Schneider et al., 2014). However, despite an emerging understanding of plasticity at the transcript level, we lack a comprehensive picture of the genetic architecture of this important trait. For instance, how many genes contribute to a plastic response? Do different sets of genes underlie trait development in different environments? Or is plasticity regulated by the same genes acting across environments but in which alleles are differentially sensitive to environmental conditions? Genetic mapping experiments performed in different environments can help to address these outstanding questions (e.g., (Kutnter et al., 2014)), and we have recently performed such studies in cichlids (Parsons, et al., in preparation). Specifically, $F_2$ hybrid families were split and reared for 6 months in alternate foraging environments that imposed distinct kinematic demands on the feeding apparatus, but were otherwise nutritionally identical. The mapping results demonstrate that the foraging environment strongly influences the G–P map. Of 22 identified QTL for a variety of traits, only 1 was significant in both environments; in other words, distinct loci regulate the response to distinct environments. This observation suggests that cryptic genetic variation, a pool of genetic variation that does not result in phenotypic variation under “normal” conditions (Gibson and Dworkin, 2004; Paaby and Rockman, 2014), is prominent for the craniofacial skeleton. We further detected instances of allele sensitivity to foraging conditions in that one haplotype showed a plastic response while the alternate haplotype did not (Parsons, et al., in preparation). While much work is needed to determine the mechanistic basis of such GxE interactions, studies like this confirm that the foraging environment is a potent force that can influence how genetic variation translates to morphology. Given differences in food items between distinct human populations (e.g., hunter-gatherer to agrarian transitions in early humans as well as modern geographical differences in diet), these results are directly relative to human craniofacial biology. More generally, they support a shift to an eco–devo approach (Abouheif et al., 2014; Gilbert and Epel, 2008), wherein the environment is explicitly incorporated into genetic mapping and developmental studies.

6. Conclusions

Despite our extensive knowledge of early craniofacial patterning, we are relatively ignorant of the genetic, cellular, and environmental mechanisms that combine over extended periods of development to produce “normal” variation in humans, and underlie many complex craniofacial malformations. We propose that studying natural variation in non-model animals will be an effective and fruitful approach to determine the types of genes, mutations, and environmental influences that ultimately result in the extraordinary range of human faces. The “evolutionary mutant” cichlid system is ideal for this approach, given their unparalleled array of facial variation and powerful genetic tools. Experiments described here complement traditional approaches, and will advance our understanding of the genomic and developmental origins of complex morphologies.
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