Title
Chapter Eleven Regulation of Jaw Length During Development, Disease, and Evolution

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Regulation of Jaw Length During Development, Disease, and Evolution

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ABSTRACT

Molecular and cellular mechanisms that control jaw length are becoming better understood. This is significant since the jaws are not only critical for species-specific adaptation and survival, but they are often affected by a variety of size-related anomalies including mandibular hypoplasia, retrognathia, asymmetry, and clefting. This chapter overviews how jaw length is established during the allocation, proliferation, differentiation, and growth of jaw precursor cells, which originate from neural crest mesenchyme (NCM). The focus is mainly on results from experiments transplanting NCM between quail and duck embryos. Quail have short jaws whereas those of duck are relatively long. Quail-duck chimeras reveal that the determinants of jaw length are NCM-mediated throughout development and include species-specific differences in jaw progenitor number, differential regulation of various signaling pathways, and the autonomous activation of programs for skeletal matrix deposition and resorption. Such insights help make the goal of devising new therapies for birth defects, diseases, and injuries to the jaw skeleton seem ever more likely.

"The most obvious differences between different animals are differences of size, but for some reason the zoologists have paid singularly little attention to them.…For every type of animal there is a most convenient size, and a large change in size inevitably carries with it a change of form." —J. B. S. Haldane, 1926

Introduction: On being the right jaw size during development and evolution

As so eloquently expressed by Haldane in his classic essay, "On Being the Right Size", every animal achieves its own individual size, which is closely tied to form, function, and fitness (Haldane, 1926). In this context, size must be precisely controlled throughout development in order for animals and their constituent parts to attain proper structural integration and adaptation. Nowhere is this notion truer than in the craniofacial complex, where size-related malformations are some of the most common human birth defects (Gorlin et al., 1990; Smith, 1997). The jaw skeleton, in particular, displays a range of anomalies in size including hypo- and hyperplasia, pro- and retrognathia, micro- and macrognathia, asymmetry, and clefting. Such variation in jaw length during development can often produce a spectrum of debilitating to life-threatening conditions. Nonetheless, variation in jaw length during evolution has also been essential for the adaptive radiation of vertebrates. Thus, identifying molecular and cellular mechanisms that both control jaw length and generate species-specific variation is critical to understanding disease and evolution.

The jaws are among the most precisely adapted and highly modified structures of vertebrates, which facilitates complex species-specific behaviors related to feeding, respiration, predation, vocalization, mating, and grooming. Such fundamental

connections between jaw size and jaw function provided a foundation for early theories of evolution by natural selection best exemplified by the beaks of Darwin's finches (Darwin, 1859). Since then, a wide array of genetic and embryological studies have shown that the establishment of jaw size is a complex process involving numerous gene regulatory networks, reciprocal signaling interactions, and hierarchical levels of control. Yet what has remained unclear are the particular determinants of jaw size that may play a role during the induction, allocation, proliferation, differentiation, and growth of neural crest mesenchyme (NCM), which serve as the progenitors of the jaw. For example, mandibular hypoplasia and cleft palate may have as part of their etiology disruptions to the rate of proliferation or timing of differentiation in NCM (Dudas et al., 2006; Dudas et al., 2004; Ito et al., 2003; Oka et al., 2007; Satokata and Maas, 1994; Sharpe and Ferguson, 1988). Identifying molecular and cellular mechanisms through which the jaw skeleton achieves its proper length is crucial for devising new and efficacious treatments that could ultimately prevent birth defects. This lack of knowledge is significant since a major clinical objective is to devise molecular and cell-based strategies to lengthen the jaw in cases of mandibular hypoplasia, asymmetry, or malocclusion.

Jaw length defects can arise from a wide-range of genetic or environmental perturbations. For example, disruptions to Sonic Hedgehog (SHH) pathway members including *Shh*, *Ptch1*, *Gas1*, *Gli2*, *Gli3*, and *Hhat* contribute to micrognathia associated with conditions such as holoprosencephaly (Allen et al., 2007; Dennis et al., 2012; Hui and Angers, 2011; Melnick et al., 2005; Mo et al., 1997; Pineda-Alvarez et al., 2012; Roessler and Muenke, 2010). Mutations in Fibroblast Growth Factor (FGF) pathway

members including *Fgf8*, *Fgfr1*, *Fgfr2*, and *Fgfr3* also cause jaw length defects, especially in Crouzon and Apert syndromes (Martinez-Abadias et al., 2013a; Martinez-Abadias et al., 2013b; Stanier and Pauws, 2012; Trumpp et al., 1999). Mutations in Bone Morphogenetic Protein (BMP) pathway members such as *Bmp4*, *Bmp7*, *Chordin*, *Noggin*, *Twist1*, and *Msx2* cause mandibular hypoplasia (Boell et al., 2013; Foerst-Potts and Sadler, 1997; Stottmann et al., 2001; Zhang et al., 2012; Zouvelou et al., 2009). Disruptions to the Transforming Growth Factor-Beta (TGFβ) signaling pathway also affect jaw length. Mutations in *TGFβ2* and *TGFBR1* cause microretrognathia in Loeys-Dietz Syndrome and when restricted to NCM (Loeys et al., 2005; Sanford et al., 1997; Zhao et al., 2008). Jaw length defects are also associated with mutations in *TGFBR2* and *Smad2*, and *Matrix metalloproteinase 2 (Mmp2)* in Torg-Winchester Syndrome, *Mmp13* in Spondyloepimetaphyseal dysplasia, and *Osteoprotegerin* (*Opg)* in Juvenile Paget's disease (Gorlin et al., 1990; Nomura and Li, 1998; Oka et al., 2008; Oka et al., 2007).

Moreover, studies in birds have identified some factors that influence jaw size. For example, differential expression of *Bmp4* in jaw progenitor cells influences variation in jaw depth and width among birds including Darwin's finches, chicks, ducks, and cockatiels (Abzhanov et al., 2004; Wu et al., 2006; Wu et al., 2004) whereas jaw length appears to be regulated separately through a calmodulin-dependent pathway (Abzhanov et al., 2006; Schneider, 2007). Likewise, factors such as SHH, FGFs, WNTs, and BMPs that are secreted from adjacent epithelial tissues have also been implicated in mediating the shape and outgrowth of the jaw and facial skeletons (Abzhanov and Tabin, 2004; Ashique et al., 2002; Bhullar et al., 2015; Brugmann et al.,

2007; Brugmann et al., 2010; Doufexi and Mina, 2008; Foppiano et al., 2007; Grant et al., 2006; Havens et al., 2008; Hu and Marcucio, 2009, 2012; Hu et al., 2015a; Hu et al., 2015b; MacDonald et al., 2004; Mina et al., 2002; Richman et al., 1997; Rowe et al., 1992; Schneider et al., 1999; Schneider et al., 2001; Szabo-Rogers et al., 2008; Wu et al., 2006; Young et al., 2014). But precisely how these pathways are regulated by NCM and how alterations to their regulation affect jaw length still needs to be clarified. Thus, an important and clinically relevant research goal is to address the question of jaw length on multiple hierarchical levels, and to manipulate developmental programs in ways that test the potential efficacy of molecular strategies for modulating jaw length. Also, observing how embryos respond to these changes is critical to devising new treatments for craniofacial defects. Currently, invasive surgery is the only option and is often needed on several occasions during childhood (Albanese and Harrison, 1998; Cordero et al., 2002; Joshi et al., 2014).

Experiments in tissue regeneration and transplantation demonstrate that organs have an intrinsic capacity to "know" their proper size and to regulate growth accordingly (Leevers and McNeill, 2005). But how intrinsic molecular and cellular programs operate within the local environment to modulate growth, and how the range of normal to abnormal phenotypic variation in size arises, remain poorly understood. Also unclear are those mechanisms that serve as the targets of natural selection for evolutionary changes in organ size. To address the question of how the jaw skeleton achieves its proper size and shape during development, we have been using a unique avian chimeric transplantation system that exploits species-specific differences between Japanese quail and white Pekin duck (Ealba and Schneider, 2013; Fish and Schneider,

2014a; Jheon and Schneider, 2009; Lwigale and Schneider, 2008; Schneider, 2005, 2007; Schneider and Helms, 2003). In particular, we have been asking the question: how do quail and duck achieve their remarkably different jaw sizes? Quail have short jaws compared to those of duck, which are relatively long (Fig. 1A). We have focused on the lower jaw skeleton, which forms from the paired mandibular primordia. NCM that migrates out of the caudal midbrain and rostral hindbrain is the only source of skeletogenic mesenchyme within the mandibular primordia (Couly et al., 1993; Köntges and Lumsden, 1996; Le Lièvre and Le Douarin, 1975; Noden, 1978; Noden and Schneider, 2006b). Our work has revealed that the orchestration of developmental programs regulating jaw length is under the regulatory control of NCM (Eames and Schneider, 2008; Jheon and Schneider, 2009; Schneider, 2005; Schneider and Helms, 2003; Tokita and Schneider, 2009), but how NCM carries out this complicated task has remained unclear.

Our experimental approach is relatively straightforward: pre-migratory NCM is exchanged between quail and duck embryos at the level of the neural tube. Depending on the experimental design and desired outcome measures, we can transplant NCM unilaterally so that donor cells fill one side of the host jaw skeleton (Fig. 1B). This maintains the non-surgical side of the host embryo as an internal control, and allows us to compare donor- and host-derived tissues directly in the same chimeric embryo (Eames and Schneider, 2005, 2008; Fish and Schneider, 2014a; Lwigale and Schneider, 2008; Solem et al., 2011; Tokita and Schneider, 2009; Tucker and Lumsden, 2004). Quail embryos mature at a much quicker rate than do duck (17 versus 28 days from fertilization to hatching), which causes faster-developing quail cells and relatively

slower-maturing duck cells to interact with one another as they become progressively asynchronous. Such different developmental trajectories also provide a means to screen for the effects of donor cells on the host by looking for species-specific changes to the timing of gene expression, cell differentiation, and tissue formation. Additionally, we can use an anti-quail antibody (Q¢PN), which does not recognize duck cells, in order to distinguish donor from host contributions (Fig. 1C). We can also quantify the proportion of quail versus duck cells on the molecular level by applying a PCR-based strategy (Ealba and Schneider, 2013), which is particularly useful for gene expression studies (Ealba et al., 2015*;* Fish et al., 2014; Hall et al., 2014).

Once quail and duck cells are intertwined with one another, resulting chimeras become challenged to integrate two distinct morphogenetic programs for species-specific size and shape. This allows us to pinpoint mechanisms underlying the patterning of the jaw skeleton through an empirical strategy where we 1) characterize donor-mediated transformations to jaw size and shape; 2) look for changes to the timing and location of developmental events underlying skeletogenesis such as mesenchymal condensation and differentiation; 3) evaluate effects of NCM on host derivatives involved in skeletogenesis including epithelia, blood vessels, muscles, and osteoclasts; 4) assay for genes that become differentially expressed in chimeras; and 5) modulate the expression of these genes (*i.e.*, perform gain- and loss-of-function experiments) to test the extent to which they regulate skeletal pattern and account for the chimeric phenotype (Eames and Schneider, 2005, 2008; Hall et al., 2014; Merrill et al., 2008; Noden and Schneider, 2006b; Solem et al., 2011; Tokita and Schneider, 2009). Overall, a major strength of this chimeric system is its ability to reveal in a relatively

normal physiologic context those signals that mediate interactions between donor NCM and host tissues, and ultimately lead to the establishment of species-specific size and shape.

The use of the quail-duck chimeric system has led us to postulate that NCM employs a variety of very precise mechanisms to govern jaw length through three principal phases of development. Initially, during migration and allocation of NCM, quail and duck have distinct numbers of progenitors destined to form the jaw skeleton, with duck having significantly more cells (Fish et al., 2014). Then, as these populations expand, there is species-specific regulation of, and response to, various signaling pathways (Eames and Schneider, 2008; Hall et al., 2014; Merrill et al., 2008). Finally, when these progenitors begin to differentiate into the cartilages and bones of the jaw skeleton, they execute autonomous molecular and cellular programs for matrix deposition and resorption through patterns and processes that are intrinsic to each species (Ealba et al., 2015*;* Eames and Schneider, 2008; Hall et al., 2014; Merrill et al., 2008; Mitgutsch et al., 2011). A long-term goal is to understand the way these mechanisms affect jaw length, how they are regulated, and the extent to which they can be targeted. Much work points to the SHH, FGF, BMP, and TGFβ pathways as crucial players, and numerous pathway members and targets become altered in our quail-duck chimeras. We are finding that NCM differentially regulates and responds to SHH, FGF, BMP, and TGFβ signaling in a species-specific manner, which likely modulates the proliferation, differentiation, and growth of jaw progenitors, and generates variation in jaw length. This provides us with insight into how these pathways empower NCM with its regulatory abilities during development, disease, and evolution. Our expectation is

that using highly divergent bird species to illuminate the determinants of jaw length will provide enough resolution to detect equivalent but likely much more subtle mechanisms generating normal and abnormal variation in humans. In this framework, we have been striving to define developmental periods when cells and tissues are responsive to inductive signals, which we hope will eventually help move the standard of care towards treating craniofacial defects *in utero*.

Part 1: Early determinants of jaw length

The generation of NCM involves multiple and sequential developmental events, starting with induction at the boundary between neural and non-neural ectoderm, regional specification along the dorsal neural tube, maintenance of multi-potency and cell cycle control, transition from epithelium to mesenchyme (EMT), and migration (Betancur et al., 2010; Nikitina et al., 2008). NCM that emigrates from the midbrain through the first and second rhombomeres of the hindbrain populates the mandibular primordia (Couly et al., 1993; Köntges and Lumsden, 1996; Le Lièvre and Le Douarin, 1975; Noden, 1978). Much has been written about the ways in which the gene regulatory networks and developmental programs that control these events have remained highly conserved across vertebrates, and especially function as a mechanism for the elaboration of the vertebrate head (Bronner-Fraser, 2008; Depew and Olsson, 2008; Nikitina et al., 2008; Northcutt, 2005). Yet there is very little known about how changes to these programs can occur in ways that account for the evolution of species-specific morphology.

In this context, we asked when, where, and how do duck embryos generate their long bills compared to quail embryos who make short beaks. We started with the simple analogy that building a bigger structure such as a wall might involve using more bricks, as opposed to bigger bricks (Fish and Schneider, 2014c). Therefore, we concentrated on determining the number of jaw precursor cells, which are the NCM that migrate into the mandibular primordia. We began by counting NCM at key embryonic stages (Fish et al., 2014). At an early stage, when NCM is specified at the level of the neural folds, quail and duck appear to have equivalent amounts of NCM. However, shortly thereafter, when NCM accumulates along the dorsal neural tube, duck have approximately 15% more NCM in the midbrain and rostral hindbrain, which is the population destined to migrate into the presumptive jaw region (Fig. 2B, C). Moreover, slightly thereafter, the jaw primordia of duck contain twice as many cells as do quail. To explain how an initial 15% difference could allow the population to double, we assayed for specific-specific variation in cell proliferation and cell cycle length (Fig. 2D, E). We found that while duck have a longer cell cycle, once embryonic stage is taken into account over absolute time, then duck cells actually proliferate more than those of quail, and in so doing provide duck with a cellular mechanism to increase their jaw length progressively throughout development.

To search for molecular mechanisms through which duck might possibly generate more midbrain NCM that can migrate into the jaw primordia, we assayed for species-specific differences in the expression of genes known to be involved in the regionalization of the brain. We looked at *Pax6* expression in the forebrain, *Otx2* in the forebrain and midbrain, *Fgf8* at the midbrain-hindbrain boundary, and *Krox20* in

rhombomeres three and five of the hindbrain (Fig. 2A). We compared duck and quail embryos at the time of neurulation and identified species-specific differences in brain shape and spatial domains of gene expression. In particular, we observed that the midbrain of duck is shorter and broader, which is also evidenced by a distinct pattern of *Otx2* expression. Ostensibly, this broader midbrain of duck enables more NCM to aggregate in the region that will ultimately populate the jaw region. Surprisingly, we also detected differences in the *Otx2* expression domain between duck and quail embryos even before neurulation, indicating that essential species-specific patterning mechanisms that affect jaw size may operate at the earliest developmental stages. Overall, our results demonstrate precisely where and when changes to early developmental programs underlying the allocation and proliferation of NCM have likely played a role in the evolution of jaw size.

Although we find that early differences in NCM number appear to be important for establishing species-specific jaw length, we also discovered that if we reduce or augment the amount of jaw progenitors (up to 25%), we do not observe a significant effect on jaw length prior to hatching (Fish et al., 2014). Our results support other observations that the jaw can revert to its normal length after neural fold extirpation (Couly et al., 1996; Hunt et al., 1995; Scherson et al., 1993; Sechrist et al., 1995). In these previous reports, however, normal jaw length was argued to result from regeneration of NCM at the neural tube, either by re-specification of the residual dorsal neuroepithelium (Hunt et al., 1995; Sechrist et al., 1995), or by an expansion of NCM produced by adjacent neural folds (Couly et al., 1996; Scherson et al., 1993). In contrast, we find that NCM does not regenerate at the level of the neural tube and thus,

the return to normal jaw length requires some other compensatory mechanism likely involving signaling interactions with adjacent epithelia. In other words, normal jaw length may be achieved by local regulation of proliferation within the post-migratory environment of the jaw primordia. Importantly, such regulative development in the local environment allows for compensation of deficiencies in NCM up to some pre-specified species-specific population size, a capacity that could potentially be harnessed to supplement NCM number and restore normal jaw length in cases of human disease or injury.

Part 2: Determinates of jaw length during skeletal differentiation

The relationship between size and shape has long been a focus of developmental and evolutionary biology. Early size and shape studies focused principally on proportional scaling or "allometry" of anatomical structures that occurs ontogenetically during growth or phylogenetically across species (Huxley, 1932; Thompson, 1917). This type of research led to the field of geometric morphometrics, which has combined multivariate methods and computer-based algorithms to quantify and display ontogenetic and phylogenetic differences in size and shape (Benson et al., 1982; Bookstein, 1978, 1990; Hu et al., 2015b; Marcucio et al., 2011; Siegel and Benson, 1982; Smith et al., 2015; Young et al., 2010; Young et al., 2014). Often morphometric data have been contextualized with quantitative genetics or evolutionary developmental theories like heterochrony, as a way to explain changes in size and shape during ontogeny and phylogeny (Alberch et al., 1979; Atchley, 1981; Atchley and Hall, 1991; Gould, 1966; Lande, 1979; McKinney, 1988). We have combined the quail-

duck chimeric system with morphometric and molecular analyses to study the development of Meckel's cartilage in the lower jaw skeleton (Fig. 1D), and in so doing have found that NCM controls both stage-specific and species-specific size and shape (Eames and Schneider, 2008).

The foundation for our work is built upon many other studies of size and shape in the vertebrate skull (de Beer, 1937; Hanken and Hall, 1993), primarily in relation to genetic specification of skeletal element identity (Balling et al., 1989; Creuzet et al., 2002; Depew et al., 2002; Gendron-Maguire et al., 1993; Grammatopoulos et al., 2000; Hunt et al., 1998; Kimmel et al., 2005; Lufkin et al., 1992; Pasqualetti et al., 2000; Qiu et al., 1997; Rijli et al., 1993; Schilling, 1997; Smith and Schneider, 1998), epithelial-mesenchymal signaling interactions that are essential for the differentiation of cartilage and bone (Bee and Thorogood, 1980; Couly et al., 2002; Dunlop and Hall, 1995; Ferguson et al., 2000; Francis-West et al., 2003; Hall, 1980, 1982, 1987; Richman and Tickle, 1989; Richman and Tickle, 1992; Schowing, 1968; Shigetani et al., 2000; Thorogood, 1987; Thorogood et al., 1986; Tyler, 1978, 1983), secreted molecules that regulate skeletal polarity and dimensional growth (Abzhanov et al., 2004; Abzhanov and Tabin, 2004; Barlow and Francis-West, 1997; Crump et al., 2004; Francis-West et al., 1998; Hu et al., 2003; Liu et al., 2005; Marcucio et al., 2005; Schneider et al., 2001; Wilson and Tucker, 2004; Wu et al., 2006; Wu et al., 2004), and mesenchymal control of species-specific skeletal morphology (Andres, 1949; Mitsiadis et al., 2006; Noden, 1983; Schneider and Helms, 2003; Tucker and Lumsden, 2004; Wagner, 1959).

Historically, the ability of NCM to convey species-specific pattern has been revealed mostly through inter-specific grafting experiments (Lwigale and Schneider,

2008; Noden and Schneider, 2006a). Employing quail-duck chimeras has been a powerful means to understand how bones and cartilages in the face and jaws acquire their species-specific pattern (Jheon and Schneider, 2009; Schneider, 2005; Schneider and Helms, 2003; Tucker and Lumsden, 2004). Chimeric “quck” embryos, which are duck hosts with quail donor cells, possess quail-like beaks and jaw joints, whereas chimeric “duail” exhibit duck-derived morphology in quail hosts (Fig. 1F). We have spent the past decade or so trying to pin down the precise molecular mechanisms through which NCM accomplishes this complex task, and we have found most strikingly that donor NCM controls its own gene expression, cell cycle, and differentiation, as well as regulates certain aspects of the developmental programs of adjacent host tissues such as epithelia and muscles (Ealba and Schneider, 2013; Eames and Schneider, 2005, 2008; Fish and Schneider, 2014b; Fish et al., 2014; Hall et al., 2014; Merrill et al., 2008; Schneider, 2005, 2007; Schneider and Helms, 2003; Solem et al., 2011; Tokita and Schneider, 2009).

To identify developmental mechanisms that generate skeletal size and shape, we focused on the differentiation and growth of Meckel’s cartilage (Eames and Schneider, 2008). Meckel’s cartilage of quail is substantially smaller than that of stage-matched duck and becomes distinctly shaped over time. Again, because quail embryos develop at a faster rate than do duck embryos, chimeras reveal those aspects of size and shape regulation that are NCM-dependent. We have found that NCM establishes both stage-specific and species-specific size and shape, and does so by exerting spatiotemporal control over molecular and cellular programs for chondrogenesis. NCM on the quail donor side of quck mandibles differentiated into chondrocytes on the timeframe of quail

controls as opposed to that observed on the contralateral duck host side. Donor-mediated shifts in cartilage differentiation were observed from the earliest stage of chondrogenesis. Both *Sox9*, which is the earliest known molecular marker of chondrogenic condensations (Eames et al., 2003; Eames et al., 2004; Healy et al., 1996; Zhao et al., 1997), and *Col2a1*, which is regulated directly by *Sox9* (Bell et al., 1997), were expressed prematurely by quail donor NCM relative to duck host NCM on the contralateral side. Additionally, we determined that FGF signaling, which functions upstream of *Sox9* and is essential for chondrogenesis (Bobick et al., 2007; de Crombrugghe et al., 2000; Eames et al., 2004; Govindarajan and Overbeek, 2006; Healy et al., 1999; Murakami et al., 2000; Petiot et al., 2002), is also regulated by NCM. While the secreted ligands *Fgf4* and *Fgf8* were expressed continuously by duck host epithelium prior to and during chondrogenesis, the receptor *Fgfr2* was expressed prematurely only by quail donor NCM relative to duck host NCM on the contralateral side. When we inhibited FGF signaling during this brief window of receptor activation, we blocked the formation of Meckel's cartilage. Therefore, by controlling the timing of FGF signaling as well as the expression of *Sox9* and *Col2a1*, NCM most likely conveys information for stage-specific and species-specific size and shape to Meckel's cartilage.

In terms of evolutionary developmental biology, one exciting aspect of this work is the insight about how NCM keeps track of both stage-specific and species-specific size and shape simultaneously. Seemingly, quail NCM makes a smaller jaw skeleton by shifting the timing of developmental events in the duck to resemble that found in the quail. This is because quail NCM orchestrates its spatiotemporal programs for chondrogenesis autonomously and in so doing provides size and shape information

across embryonic stages and between species in parallel. Ultimately, this reveals that the developmental programs under the regulatory control of NCM link ontogeny to phylogeny mechanistically, and likely play a generative role in morphological evolution, which is a concept central to the field of evolutionary developmental biology (Alberch, 1980, 1982; Alberch et al., 1979; Eames and Schneider, 2008; Gould, 1966, 1977; Hall and Olson, 2003; Schneider, 2005, 2007).

Similarly, for bone formation in the lower jaw, we have found that quail NCM, when transplanted into duck, maintains its faster timetable for development, and autonomously executes molecular and cellular programs for osteogenesis, including expression of essential transcription factors such as *Runx2* (Ealba and Schneider, 2013; Eames and Schneider, 2008; Hall et al., 2014; Merrill et al., 2008). Our experiments show that NCM establishes the timing of bone formation in the jaw skeleton by regulating cell cycle progression in a stage- and species-specific manner. Such work has led us to propose that NCM controls the timing of osteogenic induction, proliferation, differentiation, and matrix deposition through targets of TGFβ and BMP signaling, especially *Runx2*. We have found that quail NCM, when transplanted into duck, maintains its faster timetable for development and autonomously executes molecular and cellular programs for osteogenesis, including premature expression of matrix-producing genes such as *Col1a1*. In contrast, the duck host systemic environment appears to be relatively permissive and supports osteogenesis independently by providing circulating minerals and a vascular network. Taken together, our studies have revealed that NCM dictates when bone forms by controlling the timing of cell cycle progression and mediating the transition from cell proliferation to

differentiation. Transiently altering the cell cycle during early development can mimic chimeras by accelerating expression of *Runx2* and *Col1a1* (Hall et al., 2014). We also serendipitously discovered that *Runx2* expression might relate to jaw size in quail versus duck, since we observed higher endogenous expression of *Runx2* in quail coincident with their smaller head skeletons. By the time the jaw is becoming mineralized, *Runx2* levels in quail rise to more than double those of duck. By experimentally increasing the levels of *Runx2* we were able to decrease the size of the beak skeleton, and in effect mirror the relationship between species-specific beak size and endogenous *Runx2* levels. Other studies have also made a connection between expected *Runx2* expression levels (based on numbers of tandem repeats) and facial length such as in adult dogs and other mammals (Fondon and Garner, 2004; Pointer et al., 2012; Sears et al., 2007). These observations specifically point to precise control over the levels of key transcription factors and the timing of skeletal cell differentiation as a potential developmental mechanism through which NCM can affect jaw length during development, disease, and evolution.

Part 3: Determinates of jaw length during late-stage growth

While much of our work demonstrates that NCM conveys species-specific jaw size and shape by regulating the molecular and cellular programs that underlie the induction and deposition of cartilage and bone, we have also discovered that a previously unrecognized but equivalently important mechanism for regulating jaw length is the ability of NCM to mediate the process of bone resorption (Ealba et al., 2015). In adults, bone resorption is linked to bone deposition as a mechanism for maintaining

homeostasis throughout the skeleton (Buckwalter et al., 1996; Filvaroff and Derynck, 1998; Hall, 2005; Nguyen et al., 2013; O'Brien et al., 2008; Teitelbaum, 2000; Teitelbaum et al., 1997). Yet the role and regulation of bone resorption during formation of the embryonic skeleton are less well understood.

Bone resorption occurs following the actions of two cell types that are distinguished by their different embryological lineages and morphology. Osteoclasts, which are derived from the mesodermal hematopoietic lineage (Jotereau and Le Douarin, 1978; Kahn et al., 2009), have historically been considered the predominant bone-resorbing cells (Boyle et al., 2003; Filvaroff and Derynck, 1998; Hancox, 1949; Martin and Ng, 1994; Teitelbaum, 2000; Teitelbaum et al., 1997). Osteoclasts are multinucleated cells with ruffled borders and large and irregular morphology. In our quail-duck chimeras, osteoclasts are derived solely from host mesoderm. However osteocytes, which in the skeleton of the jaws and face arise entirely from NCM (Helms and Schneider, 2003; Le Lièvre, 1978; Noden, 1978), also resorb bone (Belanger, 1969; O'Brien et al., 2008; Qing et al., 2012; Tang et al., 2012; Xiong and O'Brien, 2012; Xiong et al., 2014). Osteocytes typically are small, star-shaped cells with long cytoplasmic extensions. When osteoclasts and osteocytes resorb bone they both secrete tartrate-resistant acid phosphatase (TRAP) (Minkin, 1982; Qing et al., 2012; Tang et al., 2012). Additionally, each express distinct molecular markers such as *Mmp9*, which is found in osteoclasts (Engsig et al., 2000; Reponen et al., 1994), and *Mmp13*, which is detected in osteocytes (Behonick et al., 2007; Johansson et al., 1997; Sasano et al., 2002). When cartilage is replaced by bone during endochondral ossification, *Mmp9* and *Mmp13* also become expressed by hypertrophic chondrocytes

(Colnot and Helms, 2001). However, there is essentially no endochondral ossification in the lower jaw of birds since Meckel's cartilage persists as a permanent cartilage (de Beer, 1937; Eames et al., 2004; Ekanayake and Hall, 1994; Kavumpurath and Hall, 1990). The only replacement of cartilage by bone in birds occurs in the proximal-most region within the articular cartilage beginning shortly before hatching (Mitgutsch et al., 2011; Starck, 1989). The remaining bone in the lower jaw differentiates directly from NCM through intramembranous ossification (Helms and Schneider, 2003; Noden and Schneider, 2006a; Noden, 1978, 1982; Noden and Trainor, 2005). Thus, within the lower jaw of chimeric quck following transplant of NCM, *Mmp9* would be almost entirely expressed by duck host-derived osteoclasts and *Mmp13* by quail donor-derived osteocytes.

When we compare the process of bone resorption in short-beaked quail versus long-billed duck we find that quail have dramatically higher levels of TRAP (Fig. 2H, I), *Mmp9*, and *Mmp13*. Similarly, our chimeric quck develop quail-like jaw skeletons coincident with higher quail-like levels of TRAP, *Mmp9*, and *Mmp13.* This means that in chimeric quck, quail donor NCM not only continues to act out its own intrinsic species-specific program for bone resorption via higher *Mmp13* expression and TRAP activity, but also up-regulates the expression of *Mmp9* in duck host osteoclasts. This reveals an unexpected NCM-mediated mechanism through which quail and chimeric quck acquire their shorter jaws. In other words, the amount of bone resorption in birds appears to be inversely proportional to jaw length. This conclusion is substantiated by the fact that either blocking or activating bone resorption with drugs (*e.g.*, bisphosphonates),

recombinant proteins (*e.g.,* rOPG or rRANKL), or small molecule inhibitors, can significantly lengthen or shorten the jaw.

Thus, quail and duck express species-specific molecular programs underlying bone resorption, and these programs are governed by NCM. Such experiments point to a novel function for bone resorption, which is to help establish species-specific jaw length, and they build upon prior work on Darwin's finches and other species, which contend that a critical regulator of beak length is the calcium binding protein, *calmodulin* (Abzhanov et al., 2006; Gunter et al., 2014; Schneider, 2007). *Calmodulin* has been shown to control osteocytes and osteoclasts locally (Choi et al., 2013a; Choi et al., 2013b; Seales et al., 2006; Zayzafoon, 2006). In this regard, calcium signaling and its effects on bone resorption (Hwang and Putney, 2011; Kajiya, 2012; Xia and Ferrier, 1996; Xiong et al., 2014), may function as a developmental mechanism that facilitates the evolvability of the avian beak more generally (Kirschner and Gerhart, 1998), and dictates jaw length more specifically (Gunter et al., 2014; Parsons and Albertson, 2009). Furthermore, taken together these studies suggest that bone resorption may function like a rheostat during jaw length evolution, and one that is particularly sensitive to the availability of dietary calcium in varying ecological niches, the endocrine effects of calcium-dependent hormones, and the temporal and spatial modulation of calcium signaling within the primordia of the developing jaw (Schneider, 2007).

Such conclusions are in agreement with previous work postulating that differential fields of deposition and resorption lead to changes in size and shape during growth of the jaw skeleton in humans (Enlow et al., 1975; Moore, 1981; Radlanski and Klarkowski, 2001; Radlanski et al., 2004). These findings also help explain the basis for

abnormal snouts in mice with mutations in genes known to affect resorption such as *Mmp2* (Egeblad et al., 2007), and they provide insights into the etiologies of jaw length defects in humans with conditions such as Spondyloepimetaphyseal dysplasia (*i.e.*, Mmp13), Juvenile Paget's disease (*i.e.,* Opg), and after treatments with high doses of bisphosphonates such as zoledronic acid, which inhibit bone resorption (Gorlin et al., 1990; Lezot et al., 2014). Based on these types of experiments, we have become increasingly optimistic that precise pharmacological strategies can be devised to target and carefully modulate bone resorption as a non-invasive, non-surgical means for treating human defects in jaw length such as malocclusion or even mandibular hypoplasia. Overall, the extraordinary ability of NCM to exert spatiotemporal control over the induction, differentiation, deposition, mineralization, and resorption of bone (Eames and Schneider, 2008; Hall et al., 2014; Merrill et al., 2008; Schneider and Helms, 2003) is what integrates the molecular and cellular determinants of jaw length throughout embryonic development (Fig. 2J), and is what endows NCM with its unique ability to generate variation in jaw length during disease and evolution.

FIGURE LEGENDS

Figure 1. The quail-duck chimeric system for investigating the origins of species-specific jaw length. (A) Lower jaw skeletons of adult Japanese quail (*Coturnix coturnix japonica*) and white Pekin duck (*Anas platyrhyncos*). **(B)** Schematic of rostral neural tube at embryonic stage (HH) 9.5, depicting the levels of neural crest mesenchyme (NCM) destined for the jaw primordia and grafted from quail (red) to duck (blue). **(C)** Horizontal section through the mandibular primordium of a HH29 chimeric quck embryo (rostral at top), which will give rise to the lower jaw skeleton. Quail donor mesenchyme (black), stained with a quail-specific antibody (Q¢PN), is distributed throughout the transplanted side, while only a few quail cells are found on the contralateral duck host side. **(D)** Schematic of a lower jaw skeleton in a chimeric quck embryo at HH38, showing the contributions of transplanted quail donor NCM (red) to cartilage and bone. **(E)** The lower jaw skeletons of quail and duck display species-specific differences in size and shape with duck being longer and more curved. Meckel's cartilage is stained with Alcian blue and the bones are stained with Alizarin red. **(F)** In quck mandibles, the quail donor-derived jaw skeleton is shorter and straighter than that observed for the contralateral duck host-derived jaw skeleton, which is longer and curved. Panels A–E modified from Eames and Schneider (2008); F modified from Fish and Schneider (2014a).

Figure 2. Molecular and cellular mechanisms regulating jaw length. (A) Quail and duck have distinct head shapes and species-specific regionalization of the neural tube at embryonic stage (HH) 10. Duck have a foreshortened and mediolaterally broader midbrain (mesencephalon). Genes including *Foxg1*, *Pax6*, *Otx2*, *Fgf8*, and *Krox20* are expressed in domains at HH10 with each being shifted more anteriorly in duck versus quail. **(B, C)** Differences in the allocation of NCM to the maxillary (mx) and mandibular (ma) primordia of the presumptive jaw region can be seen following *in situ* hybridization for *Dlx2* at HH13 in quail versus duck. The *Dlx2*-positive NCM domain and stomodeum (st) demonstrate that duck (blue) have a larger population of NCM relative to quail (red). **(D, E)** Phosphohistone H3 (PH3) identifies mitotic cells at HH16. While duck develop at a slower rate (taking about 45 hours to progress from HH13 to HH20 versus 32 hours in quail), duck NCM completes roughly 3.3 cycles during this developmental window compared to an average of 2.9 cycles for quail. Thus, the rate of proliferation relative to the rate of development is faster in duck. **(F, G)** As a result by HH20, the jaw of duck is approximately twice the size of that of quail. **(H, I)** Staining for tartrate-resistant acid phosphatase (TRAP) in the jaw skeleton reveals less bone resorption in duck versus quail at HH37. **(J)** Multiple developmental events regulate jaw length. Specification at the neural plate establishes a shorter and wider midbrain in duck. This difference, evidenced by distinct *Otx2* expression domains, is evident by HH6 and leads to a larger allocation of NCM to the jaw primordia by HH13. Duck NCM have a higher proliferation rate due to differences in developmental rate (time arrows on side). Finally, lower amounts of bone resorption result in differential growth and elongation of the duck jaw skeleton. Panels A–G, and J from Fish et al. (2014); H, I from Ealba et al. (*2015*).

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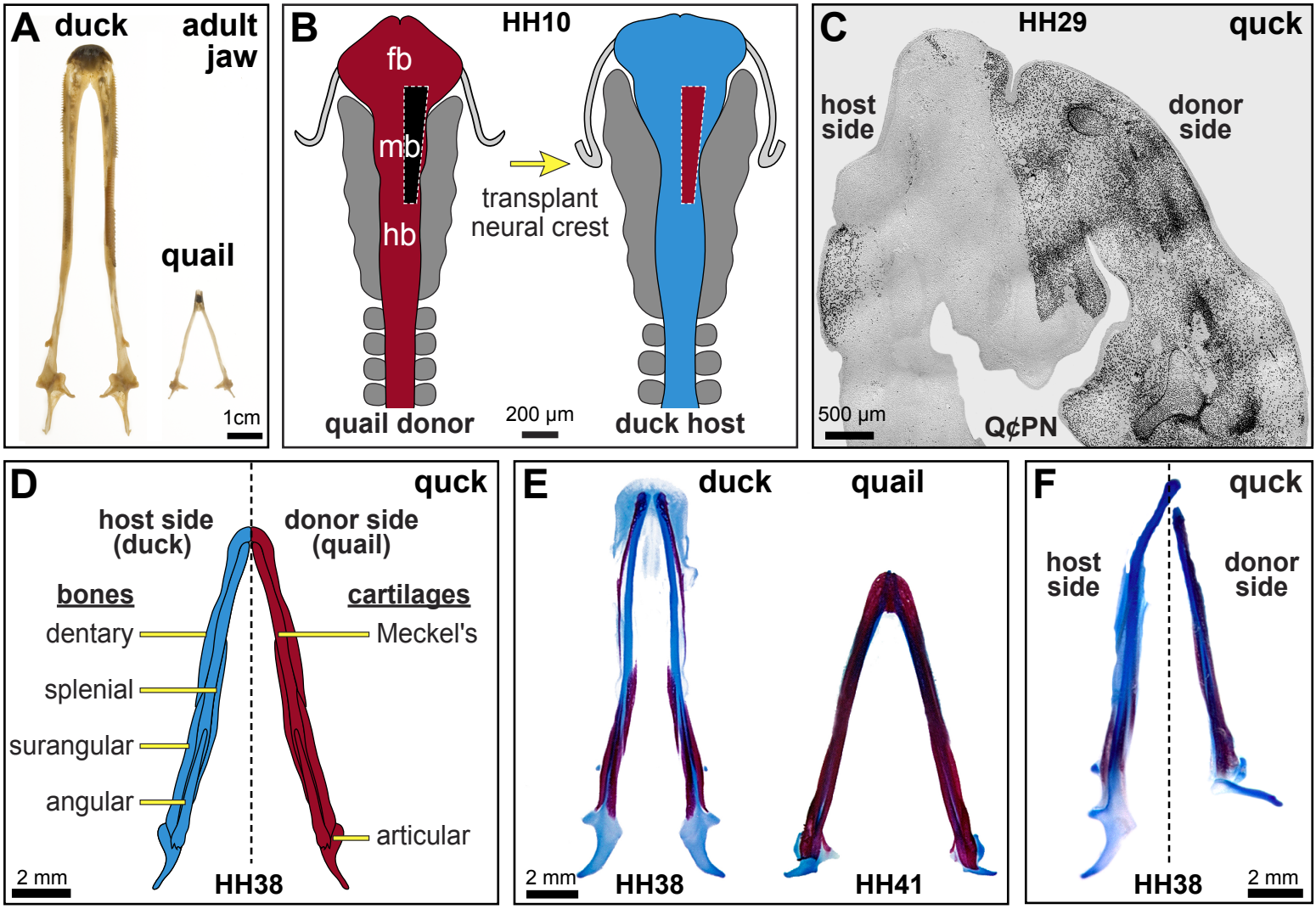
A
duck
adult jaw
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200 µm
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bones
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surangular
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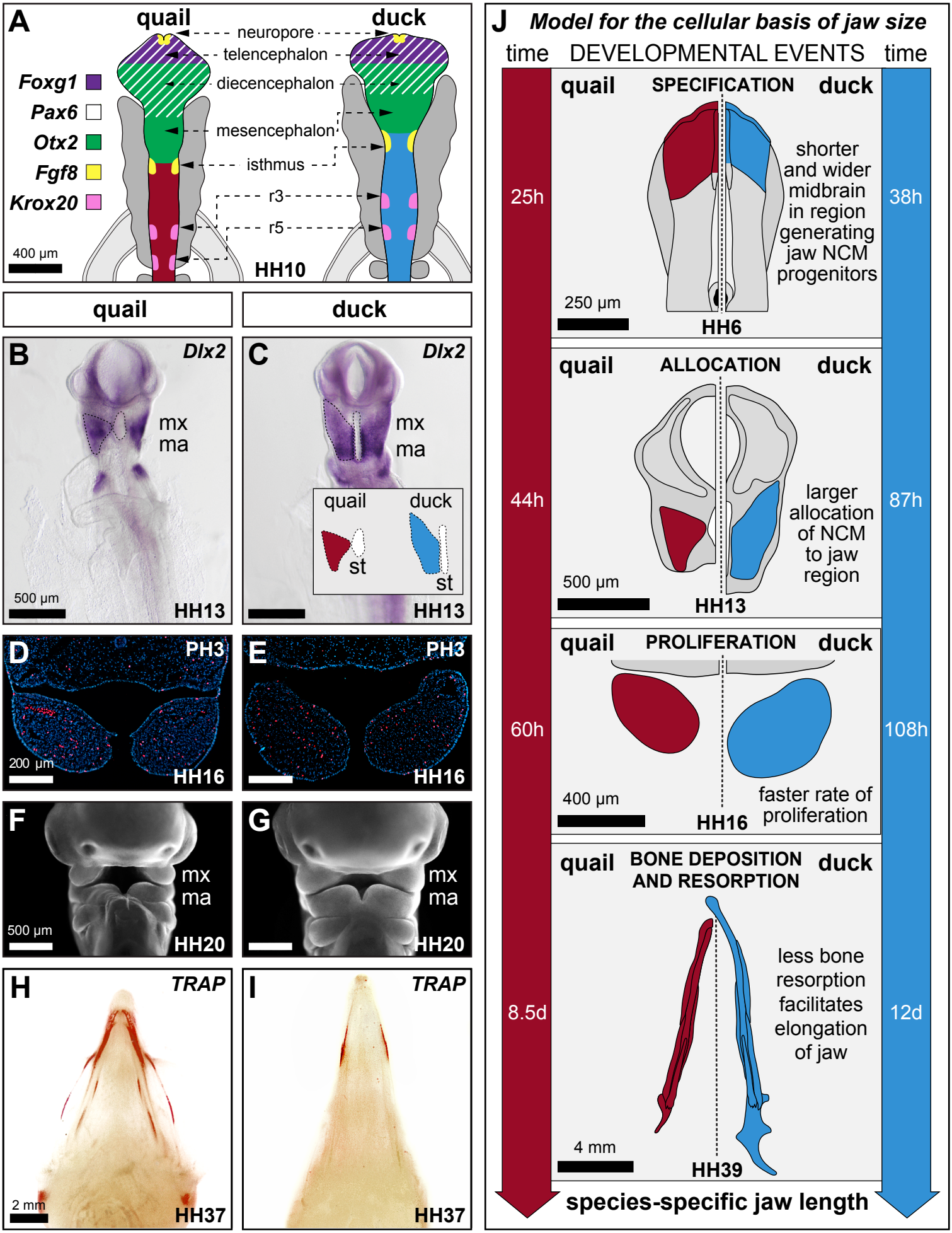
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Krox20
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TRAP
2 mm
HH37
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J
Model for the cellular basis of jaw size
time
DEVELOPMENTAL EVENTS
time
25h
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quail
SPECIFICATION
duck
shorter and wider midbrain in region generating jaw NCM progenitors
250 µm
HH6
44h
87h
quail
ALLOCATION
duck
larger allocation of NCM to jaw region
500 µm
HH13
60h
108h
quail
PROLIFERATION
duck
faster rate of proliferation
400 µm
HH16
8.5d
12d
quail
BONE DEPOSITION AND RESORPTION
duck
less bone resorption facilitates elongation of jaw
4 mm
HH39
species-specific jaw length