EVOLUTION AND DEVELOPMENT OF VERTEBRATE AIR-FILLED ORGANS

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The presence of an air-filled organ, either lungs or a swimbladder, is a defining character of the Osteichthyes (bony vertebrates, including tetrapods). It has long been hypothesized that lungs and swimbladders are transformational homologs, with lungs being ancestral for the Osteichthyes. This homology is supported by many structural, functional and developmental similarities, as well as the developmental genetic data presented in this dissertation. Chapter one examines the swimbladder expression of a core set of developmental regulatory genes previously believed to only be co-expressed in the tetrapod lung, and compares the timing and location of expression in the tetrapod and fish model systems (mouse and zebrafish). While the order of expression initiation appears to be conserved across taxa, tetrapod *Nkx2.1* is the earliest known marker of the lung primordium, where in zebrafish its homologs (*Nkx2.1a* and *Nkx2.1b*) are not expressed until two days after bud formation. Because the mouse *Nkx2.1* null lacks tracheal septation and branching morphogenesis (like a swimbladder) I hypothesize that this delay in expression relative to morphological developmental time-points could be responsible for the transformation of an ancestral lung to a more swimbladder-like morphology. Though a lack of tracheal septation and branching morphogenesis are often cited as characters that differentiate lungs and swimbladders, these characteristics are subject to variation and convergence and the fundamental difference is the point of evagination from the posterior pharynx: lungs always evaginate ventrally and swimbladders always evaginate dorsally. This difference has been cited by many as evidence that lungs and swimbladders are not homologous, as there is no evidence for the traditionally cited mechanism of transformation, a gradual migration through laterally budding intermediates. Chapter two takes our knowledge of dorso-ventral patterning in the mouse lung bud and examines the expression of two critical patterning genes (*Nkx2.1b* and *Sox2*) in the developing swimbladder. I show that the expression pattern of the zebrafish homologs of these patterning genes are reversed, with *Nkx2.1b* expression restricted dorsally at the point of swimbladder evagination, and *Sox2* expression restricted ventrally. This is only the second known case of dorso-ventral inversion of structure and underlying patterning gene expression.

BIOGRAPHICAL SKETCH

PART 1: On the Origin of Mandy

CHAPTER 1: Variation under domestication

I grew up on an island in Maine, a very unusual island. On this island scientists immigrate in unusual numbers, drawn by a rich scientific community and spectacular surroundings, gradually supplanting and out-competing the traditional island population of old-time lobstermen and blueberry pickers. Three quarters of the high school students are first cousins, and one quarter are products of the emerging immigrant community adding a limited amount of variability. The definition of domestication is the continued genetic inbreeding of a specific group to amplify certain desirable traits. By this definition, I am the product of variation under domestication. My parents were both members of the later group: immigrants and scientists, drawn by a thriving scientific community and the idyllic setting.

CHAPTER 2: Variation in nature

Though I was the product of two scientists grew up in in the midst of a natural park, I tried my best to be something other than a scientist. When I was young, my parents both worked as scientists and sought out the best early education possible. So I spent my days at Montessori preschool, where I had my choice of educational activities cleverly disguised as games. The best way for a child to learn is to give them a choice, and trick them into thinking they're playing. I was never one for math, but became a voracious reader kept meticulous track of the plastic horse and dinosaur

collection, calling each by its name and demanding any lost souls be found and put in order before the end of play time.

CHAPTER 3: The struggle for existence

When I started public school, my mom tried to explain to the teachers that I probably didn't need kindergarten; after all, I was older than most of my classmates, taller, and ahead in most subjects. But skipping wasn't allowed, until I reached first grade, when the teacher had me read to the class while she prepared lesson plans. First grade lasted two weeks, until the "no skipping grades" rule was abandoned and I was promoted. So for the rest of my school years, I was the youngest. Being too tall too young led to an inevitable lack of coordination that made gym class and organized sports one of the deeper rings of my personal hell. So I buried myself in books and became the yearbook's inevitable most studious and most likely to succeed, thus persisting and thriving despite occupation of marginal habitat.

CHAPTER 4: Natural selection

Natural selection leads to the persistence of certain genetic variants under a given set of conditions. I was a unique variant, living a mile down a dirt road on an island in Maine, spending a large percentage of my time lost in books. The neighbors and we free to break fresh trails, build fairy houses from moss, precarious tree houses from scrap wood, name unnamed landmarks and generally get lost in the woods. We came covered in the home covered with sun burns, bug bites, scraped knees and a Rorschach collection of rocks, sticks, animal bones and other priceless bits of nature that were

surely our abstract art. It was the opposite of the standard suburban childhood, we simply didn't do pavement, and our phenotype excelled. Transposed to another selective environment this phenotype may have failed miserably, but in this niche it thrived.

CHAPTER 5: Laws of variation

High school brought a broader adaptive landscape. I initially headed towards a scientific adaptive peak, but my biology teacher fell victim to the all too common "open inquiry" method, which left me feeling unsatisfied and uninterested in science. My once boundless enthusiasm for the natural world evaporated. But, through a series of excellent teachers I discovered an interest in politics and started up the base of a different peak on my way Mount Holyoke College. MHC, like all good liberal arts schools requires a diversity of core courses and a minor. No matter which adaptive peaks are summited, valleys must be crossed and all students are canalized to develop into a well-rounded liberal arts student. After breezing through first-year biology and being hired as a department tutor, as a sophomore I hit the evolutionary biology portion of the core curriculum. It was the perfect melding of my interests in history and biology—is evolution not the history of life? And the cast of characters that have shaped evolutionary biology over the years are every bit as critical to understanding the development of evolutionary theory as the textbook versions of the theories themselves. Luckily, I discovered an advisor who loved both biology and history as much as I did, and encouraged me to meld my interests. I had found my calling, my strange little niche, the spandrel filling the gap between evolutionary biology and the

history of science. Because of my love of diversity, and an arbitrary preference for vertebrates I settled on a research project in the evolution of flatfishes, bizarre creatures who will always have special a place in my heart.

CHAPTER 6: Difficulties on the theory

While I was quite comfortable buried in the library stacks, I realized that a career in science required bench experience, so I applied for several summer internships. I accepted an internship at the National Museum of Natural History. What possessed them to accept such an odd academic amalgamation as myself I will never know. When I arrived, it was learning by immersion, sink or swim (appropriate for the Division of Fishes). I learned the minutia of fish anatomy from some of the best fish anatomists in the world. I learned about museum collections, fish diversity and morphology. I asked a lot of questions, but didn't get a lot of answers: if each of five gill arches has the same feature, does that count as five characters or one? If two larval fish look the same but the adults look different, which is more informative? So I came back to school with more questions than answers, and found myself confronted with the same problems Darwin faced regarding development and evolution, that Gould so aptly addressed in his post-modern synthesis work and that modern evo-devo was now brought back to the forefront with a whole new quiver of molecular toys. So my thesis became a combination of my morphology work, modern molecular systematics, the history of evolutionary thought and the integration of developmental biology and fish evolution. My thesis was awarded *summa cum laude*, the department

award for best thesis and the Phi Beta Kappa Award. That was a good day. I thought I knew it all, but like anyone who thinks this, I was naïve.

Part 2: On the Descent of Mandy

From Mount Holyoke I planned to head to graduate school and had to make the decision between an advanced and established evo-devo program at the University of Chicago and a budding opportunity at Cornell. Knowing that my phenotype was pre-adapted for a more rural environment and the desire to develop an independent program, I chose Cornell by way of a detour to Australia. My original Australian Fulbright Fellowship project proved impossible, so I ended up working on alpha taxonomy of several local species and being quite bored with it. This is not how I had imagined my research career. I wanted to unlock the secrets of diversity and the big patterns in morphological evolution, not count myomeres and melanophores. It was a learning experience, but mostly learning what I did not want.

When I returned home I headed to Cornell to join the McCune lab, an eclectic array of brilliant and disparate researchers, amongst whom I felt totally out of my depth. I understood fish morphology and development, I was well read in the classic works of evolutionary biology, but there was no one in my department integrating development and evolution. How was I, a lowly first year graduate student, to connect my knowledge to understanding the fundamental basis of diversity? I lacked a system, I lacked funding and I lacked wet-lab. My advisor provided a system, one with a blessedly long history of study that I could easily and comfortably delve into: the

evolution of the fish swimbladder. A major morphological novelty! Nearly untouched by modern biology in a hundred years! Discussed by every vertebrate morphologist since Darwin! I could not believe my luck. So I did what I knew how to do: I hit the stacks, and a brick wall. What could I add to this? I knew very little about the wet-work of developmental biology, and it seemed that the next step would be to look for lung development genes in swimbladder development and apply those fancy new tools to this fantastically classic system.

With another stroke of luck, and an odd reversal of traditional roles, the Cornell graduate student joined an Ithaca College lab to learn to be a molecular biologist. The goal was to do look for transcripts of lung development genes in swimbladders, which required a whole lot of luck and a little fairy dust. After two years of heart-breaking and motivation-sapping failures, but extensive experience learning how to persevere, troubleshoot and try again, finally some progress: a working *in situ* hybridization. Bands! Staining! Results! Just in time for my Ithaca College mentor to move to Washington State and send me back to Cornell with a handful of lab equipment to start from scratch.

So, with a space that previously housed a rock-saw and a lot of hand-me-down equipment I built a molecular biology lab at Cornell. Working with RNA is notoriously painful. Human skin secretes the dreaded “fingerases”, the RNA degrading molecules that literally seep from every pore, and without constant vigilance will destroy any RNA based project. So up went the aggressive signage to always wear gloves, don’t touch anything and don’t contaminate solutions (a difficult transition for a morphology and paleontology lab). From there, we described the

expression of important developmental regulatory genes, and even a potential mechanism for the origin of the dorsal swimbladder by inversion of the ventral lung developmental program. We were in the business of describing the genetic mechanism that eluded so many eminent biologists before us!

Because of the lack of evolutionary developmental biologists at Cornell, we developed collaborations with a variety of researchers in molecular biology and development, neurobiology and behavior and biomedical sciences to provide assistance and support. We also participated in a recurring course on Development and Evolution, where I coincidently met my now husband.

Once things were rolling along nicely with zebrafish, we branched out into other species where the real roots of diversity lie. The bowfin is what is known as a living fossil, not having accumulated much morphological change since the late Cretaceous, and they live in our proverbial back yard, Oneida Lake. After several years of trolling Oneida Lake like some sort of bizarre New York gondoliers collecting information regarding bowfin natural history, gobs of bowfin eggs and fry, and fin clips from unfortunate daddy bowfin guarding their nests we have learned more about the natural history of this taxonomically critical species than anyone in the last hundred years, and begun to develop the tools to conduct molecular developmental biology studies on this species as well.

The McCune lab and I are now equipped to charge forward and contemplate Darwin's entangled bank at a deeper level than ever. We are equipped to examine the differential expression of homologous genes between distantly related species and correlate these differences with phenotypes, thus describing the ultimate mechanism

for morphological innovation. Without this innovation and novelty, there would be no entangled bank, for variation is as necessary as natural selection for evolution to occur. There is indeed grandeur in this view of life… and how all organisms [from phyla to individuals such as myself] have been and are being evolved.

ACKNOWLEDGMENTS

As the saying goes, life is what happens while you are making other plans. This is never truer than in graduate school. When I applied to PhD programs the best advice I received was to pick somewhere you wanted to be, because graduate school is not what you do before life starts, it is life. This piece of advice led me to choose Cornell, to spend several years in a beautiful place and to interact with some truly exceptional people. I have faced many challenges over the years, and this place and these people have allowed me to keep going when things seemed hopeless, find resources in unusual places and keep moving towards my goals (even if they always seemed to be two weeks away).

First and foremost, I have to thank my advisor Amy McCune, for putting up with my odd sense of humor, my frustrating inability to admit when a project is complete and allowing me a great deal of freedom to develop my research. While this freedom produced frustration, it taught me to be an independent scientist and be that much more gratified when things finally worked out (which I'm sure they always will, in about two weeks).

Second, I would like to thank all of the researchers who allowed me to work and learn in their labs. First and foremost, I would like to thank Marc Servetnick, for teaching me the basics of molecular biology and *in situ* hybridization. The time I spent in his lab at Ithaca College was absolutely invaluable (and a lot of fun). I would also like to thank Tim O'Brien, Ian Welsh and Drew Noden from Cornell's Department of Biomedical Sciences for picking up where Marc left off, facilitating my *in situs* at Cornell. Additionally, I would like to thank Joe Fetcho (and the members of his lab) for always sharing their wealth of knowledge about the zebrafish model system.

Of course no acknowledgements section is complete without noting the organizations that funded this research. I wrote many grants while at Cornell, and was fortunate enough to be funded by a variety of organizations: The National Science Foundation, The Society for Integrative and Comparative Biology, Sigma Xi, The American Society of Icthyologists and Herpetologists, The Mount Holyoke College Alumnae Association, The Cornell Center for Vertebrate Genomics and the Cornell Department of Ecology and Evolutionary Biology.

Third, I could not have completed this degree without the personal support of my lab-mates and my committee members. The McCune is exceptionally diverse, and being a part of it has made me a significantly more well-rounded biologist, as well as giving me the confidence to express my opinions in the face of intimidatingly smart colleagues. My committee members, Amy, Willy and Mariana have also done a great deal to develop my confidence, academic breadth and ability to communicate science. I have known Willy for over a decade, and his boundless enthusiasm, personal empathy and belief in my abilities was essential to making me the scientist I am today.

Finally, I have to thank my parents and my in-laws for supporting me through my graduate career. Though it would have been easy to question why I would spend so many years studying something as esoteric as fish developmental genetics, they have been constantly supportive and never critical, even when I wasn't sure if I made the right choice. Finally, thank you to my husband Brian, for staying in Ithaca for all these years while I finished my degree and taking care of me when things were not going smoothly (and I was intensely self-critical and generally unpleasant to be around). I could not have made it to the end without your love and support.

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LIST OF ABREVIATIONS

AO: air-filled organ

ChIN: character identity network

SB: swimbladder

aSB: anterior swimbladder

pSB: posterior swimbladder

PD: pneumatic duct

Nkx2.1: NK2 homeobox 1

FoxA2: forkhead box protein A2

Wnt7b: wingless-type MMTV integration site family, member 7B

Sox2: sex determining region Y box 2

Cdx2: Caudal type homeobox 2

Cdx1b: Caudal type homeobox 1b

A-P: anterior-posterior

D-V: dorso-ventral

CHAPTER 1

INTRODUCTION

I. INTRODUCTION: HOMOLOGY, TRANSFORMATION & NOVELTY

Understanding the process by which complex features arise and evolve is central to understanding the origin and evolution of life. Evolutionary developmental biology has yielded great insight into the genetic and developmental changes that occurred to produce many major morphological innovations. Of particular interest are features that resulted in major ecological innovations (such as jaws or wings) or that characterize major extant clades (such as feathers and teeth). These innovations are often referred to as novelties, but a phylogenetic and developmental perspective shows that novelties are almost always exaptations (1) or co-options (2) and subsequent re-specializations of other structures, which were once "novel" themselves (3): jaws evolved from anterior gill arches (4), tetrapod limbs evolved from fish fins (5) and even things that seem completely novel have anatomical precursors, such as the evolution of feathers from scales (6).

Novelty & Transformational Homology

Each novelty defines a monophyletic taxonomic group containing all species with that character and their most recent common ancestor. As such novelty and synapomorphy can usually be used interchangeably (7, 8), though the emphasis on novelty is what is *different* in descendent taxa, where the emphasis on synapomorphy is what is *the same*. The cases that are most interesting to biologists are generally those in which the

difference is emphasized, because the descendent structure is so profoundly changed from its antecedent as to be nearly unrecognizable, and these are generally the cases referred to as novel instead of simply modified or adapted. These cases are known as transformational homologies (8, 9), and are recognized as a subclass of all other more easily identified taxic homologies (synapomorphies, or unique derived traits that characterize clades). Transformational homologies are often so obscured by secondary modification that it is difficult to determine what the preceding structure was; such as the transformation of bones involved in jaw joint articulation of amphibians and reptiles to the inner ear bones of mammals (10). In these cases, homology is often established by examination of taxonomically intervening taxa with intermediate morphological conditions, or by studying the ontogenetic development of the organ, as structures that appear very different in adults often arise from similar tissues and locations relative to other developing structures during early development. This similarity of developmental origin and relation to other structures was first proposed as the principle of connections in the mid 19th century (11) and remains one of the fundamental tests of structural (12, 13) and now molecular homology (14).

Modern Genetics & Biological Homology

Homology is an ancient concept first applied to biology by Richard Owen (15, 16). Owen did not have any concept of the mechanistic processes that produce different phenotypes or even attribute similarity to common ancestry. Because the concept of homology was formulated without understanding its mechanistic basis, the findings of modern molecular genetics are not always in line with the predictions based on

comparative anatomical data and not genetics.

Historically, a biological definition of homology has been invoked to address this apparent conflict. Biological homology emphasizes the historical continuity of information underlying the development of a structure (14, 17, 18). In modern language, this can imply the shared expression of genes or gene regulatory networks, shared developmental programs or shared tissue origins. Though biological homology is theoretically appealing because it seems logical that homologous structures would be developed by homologous developmental programs, modern developmental biology has shown that homologous structures often employ different genes during their ontogeny (19, 20) and that homologous genes are often involved in the development of non-homologous structures (14, 21). Thus, biological homology, while intuitive, has encountered increasing resistance and restructuring in the post-modern synthesis era of evolutionary biology.

Molecular homology: co-option, convergence & conservation

At its heart, any comparative study is an exercise in identifying similarities and differences, and similarities are always attributable to conservation, co-option or convergence. When a conserved genetic program is expressed in clear structural homologs, both the network and the structure are synapomorphies for the group containing all taxa with both the structure and genetic program, and their last common ancestor. These are the most straightforward cases, and either a biological or taxic definition of homology is easily applied as the similarity of structure is due to a

conserved genetic mechanism. When a genetic or developmental characteristic is shared due to co-option, the mechanism generating a morphological character is a synapomorphy (and thus homologous) at some phylogenetic level but the developing structure may or may not be (as in the case of vertebrate and insect eyes both expressing *Pax6*, or limbs and butterfly wing spots both expressing *Dlx* (3). Here traditional biological homology is problematic, because the genetic information is conserved, but the structures are clearly convergent, and the presence of conserved information is the biological criterion for structural homology.

These problems with traditional biological homology have led to its transformation from simply the continuity of information, to continuity of a very specific type of information, and the reformulation of biological homology into the modern notions of Character identity networks or ChINs (14) and to some extent, deep homology (21). These ideas, while quite different, are designed to dispense with the problem of discontinuity between genetic developmental mechanisms and structural homology. Most will not consider deep homology to be a case of biological homology, as it simply uncouples the homology of structure and function rather than using one to support the other. Deep homology states that a genetic or developmental mechanism may be homologous at a deeper level on the tree than a structure, and may be repeatedly involved in the evolution of non-homologous structures at more shallow points in the phylogeny (19, 20). This allows the genetic mechanism to be its own taxic homology preceding the evolution of structures which may or may not be homologous themselves. Those who cling to a more traditional definition of

biological homology, where the genetic and structural homologies remain perfectly linked, prefer the concept of Character Identity Networks (ChINs). The concept of a ChIN is that some kernel of molecular developmental machinery is always involved in the development of homologous structures, that this kernel gives the structure its identity, and that both the kernel and the structure are homologous between ancestral and derived taxa. The ChIN addresses the problems of convergence and co-option, but is sufficiently vague in its requirements that it is unclear how useful it is in the determination of structural homology. To address convergence, the ChIN borrows the traditional wisdom of morphological systematics that complex characters are less likely to be due to convergence than simple characters, and the principle of connections which emphasizes the conserved connection between individual homologous elements (11) and thus requires that the ChIN be a gene regulatory network, and not simply a single identifying gene. Co-option is addressed by not allowing the ChIN to be expressed anywhere but the structural homologs, which again is requires a gene regulatory network as nearly all genes are expressed in multiple places and times during development. However, beyond that, the number of genes, their interactions and other features of a ChIN can vary making it an easily applied concept, but somewhat circular and of unclear usefulness.

Regardless of the difficulties with biological homology, developmental mechanisms can often provide compelling evidence regarding structural homology and the mechanism of evolutionary transformation of homologous structures. This is particularly true in the case of transformational homologies. Transformational

homologies are (by definition) difficult to identify, and shared developmental mechanisms, provided those mechanisms are chosen carefully to avoid the problems of convergence and co-option, can be powerful evidence for or against structural homology.

II. BACKGROUND: VERTEBRATE AIR-FILLED ORGANS

Defining swimbladders & lungs

The presence of an air-filled organ (AO) is a taxic homology (synapomorphy) characterizing the bony fishes including tetrapods (Osteichthyes *sensu* Rosen et al. 1981). Though counter-intuitive to some, the ancestral state of the Osteichthyan AO is the presence of lungs (22, 23); AOs that evaginate ventrally from the posterior pharynx during development. This ancestral state is supported by the presence of lungs in the most basal extant lineage of ray-finned fishes (Actinopterygii *sensu* Nelson 2006) the Polypteriformes (25). The swimbladder is a taxic homolog of the Acinopteri *and* transformational homolog of lungs.

A structure is primarily described as a swimbladder if it is unpaired, occupies the dorsal portion of the body cavity and functions primarily in buoyancy control. However this is by far an over-simplification, as both lungs and swimbladders are very structurally and functionally diverse. Structurally, both lungs and swimbladders can be paired (26) or unpaired (27), respiratory (28) or not respiratory (29). In addition to buoyancy control, the swimbladder has repeatedly evolved functions in sound production (26, 30), sound amplification (31–33) and gas-exchange (28), and lungs

have been repeatedly co-opted for buoyancy control (29). The structural diversity of the swimbladder is relatively poorly documented, but it is clear that great variation occurs there as well, with swimbladders having multiple compartments along their anterior-posterior and dorso-ventral axes, presence or absence of a connection with the gut, and often complex and unique internal structures (28). The only descriptive morphological characteristic of swimbladders that is not subject to repeated convergence and modification is the point of evagination from the gut: all members of the Actinopteri have a air-filled organ (generally called a swimbladder) which evaginates dorsally from the posterior pharynx during development, while all other Osteichthyes have ventrally evaginating lungs.

The Remaining Controversy

Though the homology of swimbladders and lungs was first proposed by Owen (1846) and has been addressed by many great comparative anatomists since (23, 34–36), some have remained agnostic about the proposed homology, and some studies still propose that swimbladders and lungs are instead independent derivations of the posterior pharynx (37–40). The point of contention for those who do not accept that swimbladders are modified lungs is often that lungs evaginate ventrally from the gut and swimbladders evolve dorsally, and to date there has been no data regarding a mechanism for this transposition. Traditionally, it has been hypothesized that swimbladders evolved from lungs by the gradual migration of the point of evagination from ventral through several lateral intermediates (41–43), but this hypothesis is problematic. To date, there have been no data generated to support this hypothesis,

and the taxa cited as potential examples of laterally budding morphological intermediates are not phylogenetic intermediates, but rather well nested within the Sarcopterygii (44) or Actinopteri (45). Only recently have molecular developmental similarities between lungs and swimbladders begun to emerge (46–48) and add evidence to this historically controversial topic.

III. CHAPTER 2: MOLECULAR HOMOLOGY & AIR-FILLED ORGANS

The mouse lung is an important model for studies of human lung disease and development, and thus its morphology and developmental genetics have been extensively described and manipulated. As such, dozens of genes have been implicated in its normal development (49–51). This dissertation uses a candidate gene approach obtain a better understanding of swimbladder development in the zebrafish (*Danio rerio*) by applying our knowledge of tetrapod lung development. Candidate genes were chosen specifically to address the question of structural homology, target similarities due to conservation and avoid detecting similarities due to co-option or convergence.

Chapter two of this dissertation describes the expression of a lung-specific cassette of developmental regulatory genes (ChIN *sensu* Wagner (14)), including transcription factors (*Nkx2.1* and *FoxA2*), secreted signaling molecules (*Wnt7b*) and proteins important in lung function (SP-A and SP-B). The interactions between these gene products are well described in the tetrapod lung, and these genes are not co-expressed in any organ other than the lung (or any non-osteichthyan taxon) minimizing the

possibility of similarity due to co-option or convergence. I employed reverse-transcriptase PCR, RNA *in situ* hybridization and whole mount immunohistochemistry to show that all candidate genes are expressed in the adult and developing zebrafish swimbladder, with some differences in the spatial and temporal expression relative to the mouse lung. This conservation of gene expression is strong evidence that lungs and swimbladders are indeed homologous, and the differences in time and space provide promising insight into the mechanisms generating morphological differences between swimbladders and lungs. Notable, is the relatively delayed expression of zebrafish *Nkx2.1a* and *Nkx2.1b* relative to AO developmental stage and the mouse homolog, *Nkx2.1*. In mouse, *Nkx2.1* is the first known marker of the lung primordium (52), where in zebrafish its homologs are not expressed until well after bud formation (53). This result is particularly interesting given the phenotype of the mouse *Nkx2.1* null, which lacks tracheal septation and branching morphogenesis (54), both of which are reminiscent of normal swimbladder morphology. It is tempting to hypothesize that the delay in *Nkx2.1a* and *Nkx2.1b* expression in zebrafish is at least partially responsible for this proposed phenocopy, and further functional studies of the role of *Nkx2.1a* and *Nkx2.1b* in swimbladder development will be needed to fully elucidate their roles.

IV. CHAPTER 3: FROM HOMOLOGY TO TRANSFORMATION

After establishing that a core suite of developmental regulatory genes are shared between lungs and swimbladders, I again used a candidate gene approach to address a hypothesized developmental evolutionary mechanism of transformation from an

ancestral lung to a swimbladder. Though lungs and swimbladders are structurally and functionally diverse, the one phenotypic characteristic that consistently defines the two morphologies is that lungs evaginate from the ventral endoderm during development while swimbladders evaginate dorsally. The transformation from a ventral to dorsal evagination point has traditionally been difficult for comparative anatomists to reconcile with the traditional gradualist paradigm (37, 39, 41–43). It has been hypothesized that either swimbladders and lungs were independent derivatives of the posterior pharynx (with lungs ancestrally budding ventrally and swimbladders ancestrally budding dorsally), or that a gradual change occurred through a series of morphological intermediates such as the condition seen in *Neoceratodus* and *Erythrinus* (42, 44, 45). However, these morphological intermediates are not also phylogenetic intermediates, so to date no substantial evidence exists to support a mechanism of transformation from ventral to dorsal.

The location of the mouse lung bud is specified by the mutually antagonistic expression of two transcription factors: *Nkx2.1* and *Sox2* (55). As previously described, *Nkx2.1* marks the point where the lung bud will emerge, and *Sox2* is expressed in the dorsal region of the endoderm from the anterior endoderm through the mid-stomach region. I asked whether the expression pattern of these two lung-bud specifying genes was inverted in the zebrafish relative to expression in mouse. I found that *Nkx2.1b* was expressed dorsally at the point of evagination, and *Sox2* was expressed throughout the ventral foregut in a reciprocal fashion. My data are only the second known case of correlated genetic and morphological inversion, after the

inversion of the expression pattern of early patterning molecules to form the dorsal neurectoderm of chordates (56). However, it was initially unclear whether this was a causal or connection between gene expression and structural position, or perhaps a correlation due to the inversion of another upstream patterning mechanism. To address this question, I determined which of the two genes was most critical for organ specification. With the help of John Olthoff, we conducted functional experiments to determine the causal link between gene expression and structural position.

Though it is clear that the actions of *Sox2* and *Nkx2.1* specify the lung bud location (55), it appears that it is actually *Sox2* expression that antagonizes the formation of the bud, rather than *Nkx2.1* expression allowing for its formation. This theory is supported by the morphology of the mouse *Nkx2.1* null. Even when *Nkx2.1* is directly (54) or indirectly (55) knocked down, the lung bud still forms in the appropriate (ventral) location. For this reason, it is likely that the absence of *Sox2* not the presence of *Nkx2.1* in the posterior pharynx specifies of the lung bud location. To test this hypothesis, we injected single cell zebrafish embryos with a *Sox2* morpholino (a modified oligonucleotide that binds to the *Sox2* mRNA and represses its translation) to determine the effect of knocking down *Sox2* expression on swimbladder development. What we found is that swimbladder development is profoundly effected by a reduction in *Sox2* expression, and that the results are consistently abnormal and often results in a ventrally budding swimbladder; or rather, a lung. Though the morphology (as illustrated by confocal microscopy and nano-CT) is compelling, morpholinos are notorious for causing abnormal development not directly correlated with the reduction

of a specific gene product. We are currently conducting the necessary controls to support our hypothesis that in the absence of functional *Sox2*, the zebrafish swimbladder reverts to its ancestrally ventral state.

IIV. FROM *DANIO* AND TO…

The data collected for this dissertation add a long-overdue dimension to our understanding of the molecular development of fish swimbladders, the connection between structural and genetic homologies and non-homologies and the developmental-genetic changes required to generate a "novel" morphology. I have shown that a unique and conserved suite of key lung developmental regulatory genes is also expressed in the proposed lung homolog, the swimbladder. Though traditionally this would be a case of biological homology, because of my careful gene choice this suite of genes fits the criteria for the more modern and less problematic concepts of deep homology and character identity networks. Though I did not describe the roles of these genes in swimbladder development, functional studies of their roles in mouse lung development suggest that these genes are excellent candidates for future studies elucidating the developmental mechanism for the morphological differences between lungs and swimbladders. In particular, the delayed timing of *Nkx2.1a* and *Nkx2.1b* expression in in zebrafish and the morphology of the mouse *Nkx2.1* null mutant suggests that these genes may have a role in determining the presence or absence of tracheal septation and branching morphogenesis. The zebrafish expression pattern of *Nkx2.1b* also suggested that this gene could have a role in the transposition of an ancestrally ventral lung to a dorsal swimbladder via an

inversion of dorso-ventral patterning gene expression. During my candidate gene study it was observed that a homolog of mouse *Nkx2.1* (*Danio Nkx2.1b*) was expressed dorsally where its mouse homolog was expressed ventrally. Given that *Nkx2.1* and its antagonist *Sox2* have been strongly implicated in the specification of the lung bud location in mouse, I examined the expression of *Danio Sox2* was in the developing endoderm. What I found was that not only was the expression of *Nkx2.1b* inverted relative to its mouse homolog, but also that *Danio Sox2* was also inverted and expressed only in the ventral portion of the pharynx, thus providing us evidence not only of structural homology but of the mechanism of structural transposition.

Taken as a whole, these data are strong evidence both of the structural homology of lungs and swimbladders, and provide a starting point for elucidating the developmental mechanisms that generated the morphological differences between lungs and swimbladders.

REFERENCES

1. Gould SJ, Vrba ES (1982) Exaptation-A Missing Term in the Science of Form. *Paleobiology* 8:4–15.

2. Sanetra M, Begemann G, Becker M, Meyer A (2005) Conservation and co-option in developmental programmes : the importance of homology relationships. 17:1–17.

3. Carroll S, Grenier J, Weatherbee S (2004) *From DNA to Diversity* (Wiley-Blackwell, New York)Second.

4. Kuratani S (2004) Evolution of the vertebrate jaw : comparative embryology and molecular developmental biology reveal the factors behind evolutionary novelty. *J Anat*:335–347.

5. Davis MC, Dahn RD, Shubin NH (2007) An autopodial-like pattern of Hox expression in the fins of a basal actinopterygian fish. *Nature* 447.

6. Prum RO (1999) Development and Evolutionary Origin of Feathers. *J Exp Zool* 306:291–306.

7. Mccune AR, Schimenti JC (2012) Using Genetic Networks and Homology to Understand the Evolution of Phenotypic Traits. *Curr Genomics* 13:74–84.

8. Patterson C (1982) in *Problems of Phylogenetic Reconstruction*, eds Joysey KA, Friday AE (London), pp 21–74.

9. Eldridge N (1979) in *Phylogenetic analysis and paleontology*, eds Cracraft J, Eldridge N (Columbia University Press, New York), pp 165–198.

10. Allin EF (1975) Evolution of the Mammalian Middle Ear. *J Morphol* 147:403–437.

11. Geoffroy Saint-Hilaire É (1830) *Principes de philosophie zoologique, discutée en mars 1830, au sein de l'Académie Royale des Sciences* (Pichon et Didier, Paris).

12. Appel TA (1987) *The Cuvier-Geoffroy Debate: French Biology in the Decades before Darwin* (Oxford University Press, Oxford).

13. Shubin NH, Alberch P (1986) in *Evolutionary Biology*, eds Hecht MK, Wallace B, Prance GI (Plenum Press, New York), pp 319–287.

14. Wagner G (2007) The Developmental Genetics of Homology. *Nat Rev Genet* 8:473–479.

15. Owen R (1843) *Lectures on the Comparative Anatomy and Physiology of the Invertebrate Animals, Delivered at the Royal College of Surgeons in 1843* (Longman, Brown, Green, and Longmans, London).

16. Owen R (1846) *Lectures on the comparative anatomy and physiology of the vertebrate animals* (Longman, Brown, Green, and Longmans, London).

17. Roth VL (1984) On homology. *Biol J Linn Soc* 22:13–29.

18. Vanvalen L (1982) Homology and Causes. *J Morphol* 312:305–312.

19. Wray GA, Abouheif E (1998) When is homology not homology? *Curr Opin Genet Dev* 8:675–680.

20. Wray GA (1999) Evolutionary dissociations between homologous genes and homologous structures. *Novartis Found Symp* 222:189–203; discussion 203–6.

21. Shubin, N. Tabin, C. Carroll S (2009) Deep homology and the origins of evolutionary novelty. *Nature* 457:818–823.

22. Rosen DE, Forey PL, Gardiner BG, Patterson. C (1981) Lungfishes, tetrapods, paleontology, and plesiomorphy. *Bull Am Museum Nat Hist* 167:163–275.

23. Lauder GV, Liem KF (1983) The evolution and interrelationships of the actinopterygian fishes. *Bull Museum Comp Zool* 150:95–197. Available at: http://biostor.org/reference/636.

24. Nelson JS (2006) *Fishes of the World* (Wiley). 4th Ed.

25. Budgett JS (1901) On some points in the anatomy of Polypterus. *Trans Zool Soc London* 15:323–338.

26. Rice AN, Bass AH (2009) Novel vocal repertoire and paired swimbladders of the three-spined toadfish, Batrachomoeus trispinosus: insights into the diversity of the Batrachoididae. *J Exp Biol* 212:1377–1391.

27. Greene HW (2000) *Snakes: The Evolution of Mystery in Nature* (University of California Press).

28. Graham JB (1997) *Air-Breathing Fishes: Evolution, Diversity and Adaptation* ed Graham J (Academic Press, San Diego, CA).

29. Thompson KS (1992) *Living fossil: the story of the coelacanth* (W.W. Norton & Company).

30. Veerappan N, Pandi V, Balasubramanian T (2009) Sound Production Behaviour in a Marine Croaker Fish, Kathala axillaris (Cuvier). *World J Fish Mar Sci* 1:206–211.

31. Webb JF, Smith WL (2000) The laterophysic connection in chaetodontid butterfly fish : morphological variation and speculations on sensory function. 1125–1129.

32. Grande T, Pinna M De (2004) in *Mesozoic Fishes 3 – Systematics, Paleoenvironments and Biodiversity*, eds Arratia G, Tintori A (Springer Verlag, München, Germany), pp 429–448.

33. Schulz-Mirbach T, Heß M, Metscher BD, Ladich F (2013) A unique swim bladder-inner ear connection in a teleost fish revealed by a combined high-resolution microtomographic and three-dimensional histological study. *BMC Biol* 11:75–88.

34. Darwin C (1859) *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life.* (John Murray, Oxford). 1st Ed.

35. Romer AS (1970) *The vertebrate body* (Saunders, Philadelphia). 6th Ed.

36. Goodrich ES (1930) *Studies on the structure & development of vertebrates* (Macmillan and Co. Ltd, London).

37. Wassnetzov W (1932) Über die Morphologie der Schwimmblase. *Zool Jahrbücher Abteilung Anat und Ontog der Tiere* 56:1–36.

38. Denison RH (1941) The soft anatomy of Bothreolepis. *J Paleontol* 15:553–561.

39. Perry SF, Wilson RJ, Straus C, Harris MB, Remmers JE (2001) Which came first, the lung or the breath? *Comp Biochem Physiol A Mol Integr Physiol* 129:37–47.

40. Sullivan LC, Daniels CB, Phillips ID, Orgeig S, Whitsett JA (1998) Conservation of surfactant protein A: evidence for a single origin for vertebrate pulmonary surfactant. *J Mol Evol* 46:131–138.

41. Sagemehl M (1885) Beitrage zur vergleichenden anatomie der fische III. Das cranium der characiniden nebst allgemeinen bermerkungen uber die mit einen

Weber'schen apparat versehenen physostomen familien. *Morphol Jahrb* 10:1–119.

42. Wilder BG (1877) Gar-pikes old and young,. *Pop Sci Mon* 11:1–12.

43. Dean B (1895) *Fishes Living and Fossil* (Macmillan and Co. Ltd, New York).

44. Grigg GC (1965) Studies on the Queensland lungfish, Neoceratodus forsteri (Krefft). III Aerial respiration in relation to habits. *Aust J Zool* 13:413–421.

45. Rowntree WS (1903) On some points in the visceral anatomy of the Characinidae, with an enquiry into the relations of the ductus pneumaticus in Physostomi generally. *Trans Linn Soc London, Second Ser Zool* 9:8–83.

46. Winata CL et al. (2009) Development of zebrafish swimbladder: The requirement of Hedgehog signaling in specification and organization of the three tissue layers. *Dev Biol* 331:222–236.

47. Teoh P-H, Shu-Chien AC, Chan W-K (2010) Pbx1 is essential for growth of zebrafish swim bladder. *Dev Dyn* 239:865–874.

48. Yin A, Korzh S, Winata CL, Korzh V, Gong Z (2011) Wnt signaling is required for early development of zebrafish swimbladder. *PLoS One* 6:e18431.

49. Chuang PT, McMahon AP (2003) Branching morphogenesis of the lung: new molecular insights into an old problem. *Trends Cell Biol* 13:86–91.

50. Cardoso WV, Lü JN (2006) Regulation of early lung morphogenesis: questions, facts and controversies. *Development* 133:1611–1624.

51. Morrisey EE, Hogan BLM (2010) Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell* 18:8–23.

52. Lazzaro D, Price M, De Felice M, Di Lauro R (1991) The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* 113:1093–1104.

53. Cass AN, Servetnick MD, McCune AR (2013) Expression of a lung developmental cassette in the adult and developing zebrafish swimbladder. *Evol Dev* 15:119–132.

54. Minoo P, Su G, Hong D, Bringas P, Kimura S (1999) Defects in Tracheoesophageal and Lung Morphogenesis in Nkx2.1(−/−) Mouse Embryos. *Dev Biol* 209:60–71.

55. Domyan ET et al. (2011) Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Dev Cambridge Engl* 138:971–981.

56. De Robertis EM, Sasai Y (1996) A common groundplan for dorsoventral patterning in Bilateria. *Nature* 380:37–40.

57. Smith AB, Peterson KJ, Wray G, Littlewood DTJ (2004) in *Assembling The Tree of Life*, eds Cracraft J, Donoghue M (Oxford University Press, New York), pp 365–383.

58. Swalla B (2006) Building divergent body plans with similar genetic pathways. *Heredity (Edinb)* 97:235–243.

59. Morris VB (2007) Origins of radial symmetry identified in an echinoderm during adult development and the inferred axes of ancestral bilateral symmetry. *Proc R Soc London B* 274:1511–1516.

60. Dahn RD, Davis MC, Pappano WN, Shubin NH (2007) Sonic hedgehog function in chondrichthyan fins and the evolution of appendage patterning. *Nature* 445:311–314.

61. Gillis JA, Dahn RD, Shubin NH (2009) Shared developmental mechanisms pattern the vertebrate gill arch and paired fin skeletons. *Proc Natl Acad Sci* 106:5720–5724.

62. Kimmel CB, Miller CT, Keynes RJ (2001) Neural crest patterning and the evolution of the jaw. *J Anat* 199:105–120.

63. Cohn M (2002) Lamprey Hox genes and the origin of jaws. *Nature* 416:386–387.

64. Kuratani S (2004) Evolution of the vertebrate jaw: comparative embryology and molecular developmental biology reveal the factors behind evolutionary novelty. *J Anat* 205:335–347.

65. Hurley IA et al. (2007) A new time-scale for ray-finned fish evolution. *Proc R Soc London B* 274:489–498.

66. Stiassny MLJ, Wiley EO, Johnson GD, de Carvalho M. (2004) in *Assembling The Tree of Life*, eds Cracraft J, Donoghue MJ (Oxford University Press, New York), pp 410–429.

67. McCune AR, Carlson RL (2004) Twenty ways to lose your bladder: common natural mutants in zebrafish and widespread convergence of swim bladder loss among teleost fishes. *Evol Dev* 6:246–259.

68. McMahon B (1969) A functional analysis of the aquatic and aerial respiratory movements of an African lungfish, Protopterus aethiopicus, with reference to the evolution of the lung-ventilation mechanism in vertebrates. *J Exp Biol* 51:407–430.

69. Kerr J (1907) in *The work of John Samuel Budgett*, ed Kerr J (Cambridge University Press, Cambridge), pp 195–290.

70. Liem KF (1988) Form and function of lungs: the evolution of air breathing mechanisms. *Am Zool* 28:739–759.

71. Janvier P, Desbiens S, Willet JA (2007) New evidence for the controversial "lungs" of the late devonian antiarch Bothreolepis canadensis (Whiteaves, 1880) (Placodermi: Antiarcha). *J Vertebr Paleontol* 27:709–710.

72. Cardoso W (2001) Molecular regulation of lung development. *Annu Rev Physiol* 63:471–494.

73. Zeng X, Yutzey KE, Whitsett JA (1998) Thyroid transcription factor-1, hepatocyte nuclear factor-3β and surfactant protein A and B in the developing chick lung. *J Anat* 193:399–408.

74. Sakiyama J, Yamagishi A, Kuroiwa A (2003) Tbx4-Fgf10 system controls lung bud formation during chicken embryonic development. *Development*:1225–1234.

75. Field HA, Ober EA, Roeser T, Stainier DYR (2003) Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev Biol* 253:279–290.

76. Yee N.S., Lorent K, Pack M (2005) Exocrine pancreas development in zebrafish. *Dev Biol* 284:84–101.

77. Hollemann T, Pieler T (2000) Xnkx-2.1: A homeobox gene expressed during early forebrain, lung and thyroid development in Xenopus laevis. *Dev Genes Evol* 210:579–581.

78. Small EM, Vokes SA, Garriock RJ, Li DL, Krieg P. (2000) Developmental expression of the Xenopus Nkx2-1 and Nkx2-4 genes. *Mech Dev* 96:259–262.

79. Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH (2004) Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* 132:35–47.

80. Goss A.M. et al. (2009) Wnt2/2b and b-Catenin Signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev Cell* 17:290–298.

81. Meyer A, Schartl M (1999) Gene and genome duplications in verterbrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* 11:699–704.

82. Hoegg S, Brinkmann H, Taylor JS, Meyer A (2004) Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J Mol Evol* 59:190–203.

83. Rohr KB, Barth KA, Varga ZM, Wilson SW (2001) The nodal pathway acts upstream of hedgehog signaling to specify ventral telencephalic identity. *Neuron* 29:341–351.

84. Weidenfeld J, Shu W, Zhang L, Millar SE, Morrisey EE (2002) The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. *J Biol Chem* 277:21061– 21070.

85. Oguchi H, Kimura S (1998) Multiple transcripts encoded by the thyroid-specific enhancer-binding protein (T/EBP)/thyroid-specific transcription factor-1 (TTF-1) gene: evidence of autoregulation. *Endocrinology* 139:1999–2006.

86. Boggaram V (2009) Thyroid transcription factor-1 (TTF-1/Nkx2.1/T1F1) gene regulation in the lung. *Clin Sci* 116:27–35.

87. Boggaram V (2003) Regulation of lung surfactant protein gene expression. *Front Biosci* 8:751–764.

88. Bohinski RJ, Di Lauro R, Whitsett JA (1994) . The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol Cell Biol* 14:5671–5681.

89. Bruno M, Bohinski R, Huelsman K, Whitsett J, Korfhagen T (1995) Lung cell specific expression of the murine surfactant protein A (SP-A) gene is mediated by interaction between the SP-A promoter and thyroid transcription factor-1. *J Biol Chem* 270:6531–6536.

90. Daniels CB et al. (2004) The origin and evolution of the surfactant system in fish: insights into the evolution of lungs and swim bladders. *Physiol Biochem*

Zool PBZ 77:732–749. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15547792.

91. Zwerger P., Nimeth K, Würtz J, Salvenmoser W, Pelster B (2002) Development of the swimbladder in the European eel (Anguilla anguilla). *Cell Tissue Res* 307:155–164.

92. Marty, G.D., Hinton DE, Summerfelt RC (1995) Histopathology of swimbladder noninflation in walleye (Stizostedion vitreum) larvae: role of development and inflammation. *Aquaculture* 138:35–48.

93. Prem, C., Salvenmoser W, Wurtz J, Pelster B (2000) Swim bladder gas gland cells produce surfactant: in vivo and in culture. *Am J Physiol Regul Integr Comp Physiol* 279:R2336–R2343.

94. Nüsslein-Volhard C, Dahm R (2002) *Zebrafish: a practical approach* (Oxford University Press, New York).

95. Wilkinson DG (1992) in *In In Situ Hybridization: A Practical Approach*, ed Wilkinson DG (IRL Press, Oxford), pp 75–83.

96. Wallace KN, Pack M (2003) Unique and conserved aspects of gut development in zebrafish. *Dev Biol* 255:12–29. Available at: http://cat.inist.fr/?aModele=afficheN&cpsidt=14649401 [Accessed April 9, 2013].

97. Cheng PY et al. (2008) Zebrafish cdx1b regulates expression of downstream factors of Nodal signaling during early endoderm formation. *Development* 135:941–952.

98. Carl M, Bianco IH, Bajoghli B, Aghaallaei N, Czerny T (2007) Wnt/Axin1/β-Catenin Signaling Regulates Asymmetric Nodal Activation, Elaboration, and Concordance of CNS Asymmetries. *Neuron* 55:393–405.

99. Cai KQ, Capo-Chichi CD, Rula ME, Yang DH, Xu XX (2008) Dynamic GATA6 expression in primitive endoderm formation and maturation in early mouse embryogenesis. *Dev Dyn* 237:2820–2829.

100. Morrisey EE et al. (1998) GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev* 12:3579–3590.

101. Cohen E.D. et al. (2009) Wnt signaling regulates smooth muscle precursor development in the mouse lung via a tenascin C/PDGFR pathway. *J Clin Invest* 9:2538–2549.

102. Rajagopal J. et al. (2008) Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. *Development* 135:1625–1634.

103. Shu W, Jiang YQ, Lu MM, Morrisey EE (2002) Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* 129:4831–4842.

104. DeFelice M et al. (2003) TTF-1 phosphorylation is required for peripheral lung morphogenesis, prenatal survival and tissue-specific gene expression. *J Biol Chem* 278:35574–35583.

105. Rubio S et al. (1995) Pulmonary Surfactant Protein A (SP-A) is expressed by epithelial cells of the small and large intestine. *J Biol Chem* 270:12162–12169.

106. Sofi MH, Bhatnagar A, Sapra S, Mahmood A, Majumdar S (2010) Influence of intestinal sufactant like particles on differential activation of secondary signaling molecules during Salmonella typhimurium infection. Journal. *J Food Saf* 30:455–469.

107. Finney JL, Robertson GN, McGee CA, Smith FM, Croll RP (2006) Structure and autonomic innervation of the swim bladder in the zebrafish (Danio rerio). *J Comp Neurol* 495:587–606.

108. Mendelson C (2000) Role of transcription factors in fetal lung development and surfactant protein gene expression. *Annu Rev Physiol* 62:875–915.

109. Wright JR (2004) Host defense functions of pulmonary surfactant. *Biol Neonate* 85:75–83.

110. Abouheif E (1997) Developmental genetics and homology: a hierarchical approach. *TRENDS Ecol Evol* 12:405–408.

111. Grande L (2010) An Empirical Synthetic Pattern Study of Gars (Lepisosteiformes) and Closely Related Species, Based Mostly on Skeletal Anatomy. The Resurrections of Holostei. *Am Soc Ichthyol Herpetol Spec Publ* 6:1–871; i–x.

112. Pough FH, Janis CM, Heiser JB (2008) *Vertebrate Life* (Benjamin Cummings). 8th Ed.

113. Ikeda K, Shaw-White JR, Wert S.E., Whitsett J (1996) Hepatocyte nuclear factor 3 activates transcription of thyroid transcription factor 1 in respiratory epithelial cells. *Mol Cell Biol* 16:3626–3636.

114. Perea-Gomez A., Shawlot W, Sasaki H, Behringer RR, Ang S (1999) HNF3β and Lim1 interact in the visceral endoderm to regulate primitive streak formation and anterior-posterior polarity in the mouse embryo. *Development* 126:4499–4511.

115. Zhou L, Lim L, Costa RH, Whitsett JA (1996) Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *J Histochem Cytochem* 44:1183–1193.

CHAPTER 2

EXPRESSION OF A LUNG DEVELOPMENTAL CASSETTE IN THE ADULT AND DEVELOPING ZEBRAFISH SWIMBLADDER[1]

Summary

The presence of an air-filled organ (AO), either lungs or a swimbladder, is a defining character of the Osteichthyes (bony vertebrates, including tetrapods). Despite the functional and structural diversity of air-filled organs, it was not previously known whether the same group of developmental regulatory genes are involved in the early development of both lungs and swimbladders. This study demonstrates that a suite of genes (*Nkx2.1*, *FoxA2*, *Wnt7b*, *GATA6*), previously reported to be co-expressed only in the tetrapod lung, is also co-expressed in the zebrafish swimbladder. We document the expression pattern of these genes in the adult and developing zebrafish swimbladder and compare the expression patterns to those in the mouse lung. Early-acting genes involved in endoderm specification are expressed in the same relative location and stage of AO development in both taxa (*FoxA2* and *GATA6*), but the order of onset and location of expression are not completely conserved for the later acting genes (*Nkx2.1* and *Wnt7b*). Co-expression of this suite of genes in both tetrapod lungs and swimbladders of ray-finned fishes is more likely due to common ancestry than independent co-option, because these genes are not known to be co-expressed anywhere except in the air-filled organs of Osteichthyes. Any conserved gene product interactions may comprise a character identity network (ChIN) for the osteichthyan air-filled organ.

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Introduction

The study of gene expression patterns has given us new insight into the evolution and development of morphological novelty. There has been great interest in the features that characterize large clades such as pentaradial symmetry of echinoderms (57–59), limbs of tetrapods (5, 60, 61) or jaws of gnathostomes (62–64). One important feature, or synapomorphy, of Osteichthyes (bony vertebrates, including tetrapods; *sensu* (22, 23, 65, 66) is the presence of an air-filled organ (AO) in the form of lungs or a swimbladder (22, 23). The best-studied AO is the lung, but equally common among the nearly 55,000 living species (24) of Osteichthyes, particularly among the ray-finned fishes, is the swimbladder, which primarily functions in buoyancy regulation (67).

Lungs and swimbladders have many functional, structural, and topographic similarities and have long been considered to be homologous (16, 34, 35). An AO is clearly a lung if it evaginates from the ventral portion of the posterior pharynx, is bilaterally paired and serves a primarily respiratory function. An AO is clearly a swimbladder if it evaginates from the dorsal portion of the posterior pharynx, is bilaterally unpaired and functions primarily in buoyancy regulation. This is, however, a simplification, as both swimbladders and lungs are extremely diverse in both structure and function and there are many examples of AOs that fit neither of these descriptions perfectly. For example, snakes have only one lung (27) many ray-finned fishes have respiratory swimbladders (28) and some ray-finned fishes even have paired swimbladders (26). Given the diversity of form and function in the air-filled

organs, we suggest that the critical distinction between lungs and swimbladders is that lungs develop as a ventral evagination of the gut whereas swimbladders develop as a dorsal evagination. Thus defined, all living fleshy-finned fishes (Sarcopterygii), including tetrapods, coelacanths (29), and lungfishes (Ceratodontidae: (44); Lepidosirenidae: (68)) have lungs or modified lungs and swimbladders are found only in a subgroup of ray-finned fishes, the Actinopteri (Figure 1). Because all sarcopterygians and the most basal lineage of actinopterygians (Polypteriformes) have paired ventral lungs (69), this is widely considered to be the ancestral condition for the Osteichthyes (22, 28, 70). Though the extant sister group to the Osteichthyes, the Chondrichthyes (sharks, skates, rays and chimeras) shows no evidence of having or having had an AO, there is some fossil evidence that one member of an exinct basal lineage of jawed vertebrates (the placoderm fish, *Bothreolepis canadensis)* may have possessed diverticula of the pharynx with notable similarities to lungs (38, 71). Due to the presence of these structures in only a single placoderm and their complete absence in the Chondrichthyes, the "lungs" of *B. canadensis* are likely due to convergence. However, if these structures are in fact homologous to the osteichthian AO, it would have no impact on the proposed ancestral AO state or the homology of lungs and swimbladders, except to make the presence of an AO a synapomorphy for a more inclusive clade and suggest its loss in the Chondrichthyes.

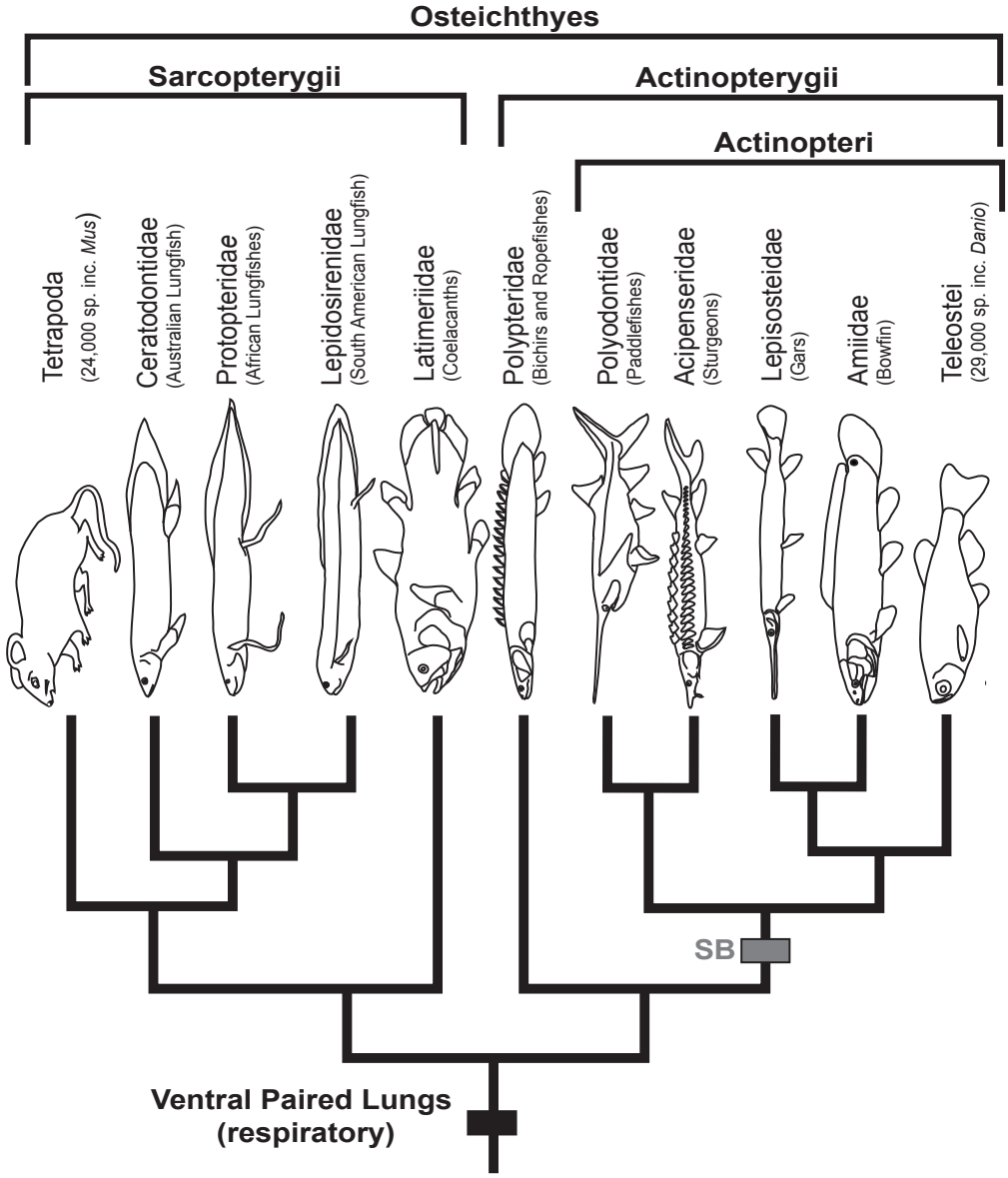


Figure 1: Phylogeny of living groups of the Osteichthyes based on Stiassny et al. (2004) and Grande (2010). Paired lungs have long been interpreted as a synapomorphy of the Osteichthyes (22, 23, 35, 112). Swimbladders (SB) are a synapomorphy for the Actinopteri, the group of ray-finned fishes which includes teleosts but excludes *Polypterus*, the African bichir (as shown).

Despite the immense structural variation of AOs across taxa, most developmental genetic studies of AOs focus on the lungs of *Mus musculus*; the laboratory mouse, hereafter referred to as simply "mouse" (reviewed by (49, 51, 72)), with occasional forays into other tetrapod taxa (chicken: 73, 74). Relatively little is known about developmental genetics of swimbladders, even in the zebrafish, *Danio rerio*, except for cases in which gene expression in the swimbladder has been mentioned in passing by a study focused on another organ system (e.g. 75, 76). Several recent studies have directly examined the genetic basis of swimbladder development (46–48, 67). McCune and Carlson (67) found that wild zebrafish populations harbor dozens of different recessive alleles producing swimbladderless phenotypes, but did not identify the specific genes involved. Winata et al. (46) documented a critical role for hedgehog signaling in swimbladder development by examining the expression of several hedgehog signaling molecules and their receptors, as well as conducting functional studies to elucidate the mechanism of its involvement. Teoh et al. (47) determined that *Pbx1*, a homeodomain transcription factor, is expressed in the developing zebrafish swimbladder mesoderm and is critical for swimbladder development, and Yin et al. (48) documented the importance of *Wnt* signaling in swimbladder development. These studies serve as important contributions to understanding the roles of specific genes and signaling cascades in the developing *Danio* swimbladder.

Our study begins to address the degree of conservation of developmental regulatory mechanisms between swimbladders and lungs. Though previously documented similarities in single, widely expressed genes (such as *Shh* and *Pbx1*) hint at the

possibility of common evolutionary origin of AOs, these individual similarities could alternatively be due to the common developmental origin of lungs and swimbladders as endodermal derivatives, to convergence, or even to repeated co-option of individual network components into their developmental program. To distinguish similarity due to common ancestry from other possibilities (common developmental origin, convergence, and co-option), we investigated whether a suite of developmental regulatory genes, with known lung-specific network interactions, is expressed during swimbladder development. Though functional studies are the only definitive test of conserved network relationships, the expression in the zebrafish swimbladder of a suite of gene products with known lung-specific network interactions is a necessary first step towards characterizing an AO-specific developmental cassette.

Wagner (14) coined the term Character Identity Network (ChIN) to describe such organ-specific gene regulatory networks. He proposed that underlying the development of homologous morphological characters is some subset of a gene regulatory network, with both conserved gene products and their interactions, which is also homologous and defines the "character identity" (e.g., lungness) of a particular morphology. The presence of a conserved suite of interacting genes in the mouse lung and zebrafish swimbladder, but not in the endoderm from which they are developmentally derived, would be strong evidence that this suite constitutes a starting point for the identification of an AO-specific ChIN

Dozens of genes have been implicated in early mouse lung development (reviewed in

refs 49–51, 72). We chose our candidate genes from this pool, based on two key criteria. First, we sought genes with limited spatial expression outside of the lung endoderm. Second, we chose genes involved in very early lung development (before mouse E9.5); at this stage, the developing swimbladder and lung appear most similar. Mouse *Nkx2.1* (also known as *TTF-1* or *T/ebp*), is a homeodomain transcription factor that fits both criteria. *Nkx2.1* is expressed in only two vertebrate organs in addition to the lung: the thyroid and telencephalon (52, 77, 78). Since these three organs are regionally disjunct, it is easy to differentiate *Nkx2.1* expression in the AO from its expression in either thyroid or brain. *Nkx2.1* is also the earliest known marker of the lung anlage in mouse (52). Upstream signaling molecules that affect *Nkx2.1* transcription are expressed widely, and many are secreted from the surrounding mesoderm but not expressed in the lung primordium (46, 79, 80). Though there is only one copy of *Nkx2.1* in tetrapods, two paralogs have been identified in zebrafish (*Nkx2.1a* and *Nkx2.1b*), due to a whole-genome duplication in the lineage leading to teleost fishes (81, 82). Because the sequence of both zebrafish paralogs has diverged (83), and the possibility of subfunctionalization, we examined the expression of both copies.

We also include, in our comparison with mouse, new data regarding the expression of zebrafish *Wnt7b*, as well as data collected by previous studies on *FoxA2* and *GATA6*, bringing the total to five zebrafish genes. The mouse orthologs of these genes exhibit lung-specific interactions with *Nkx2.1*. *Nkx2.1* is known to upregulate *Wnt7b* expression (84), and is known to be upregulated by the mouse ortholog of *FoxA2*, also

known as *HNF3β* and *GATA6* (85) as well as to be subject to positive auto-regulation (85) and affected by the combinatorial or cooperative actions of these factors (86) (Figure 2).

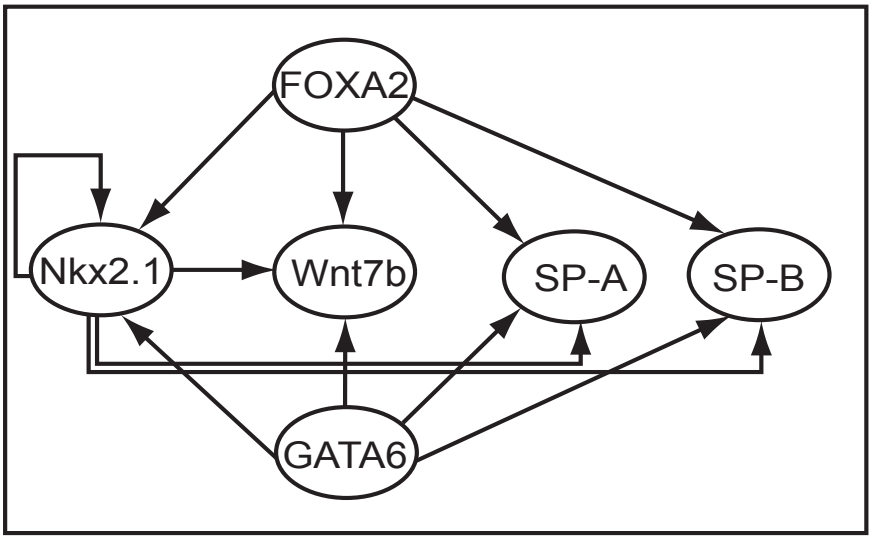


Figure 2: Simplified schematic of an early early lung-budding network in mouse based on results in the literature (84, 87–89, 113). Arrows indicate that there is positive regulation of one factor by another (not that factors are necessary for expression). No temporal information is implied by this figure (for order of expression, see Figure 7). For a recent review of the expression and regulation of these genes see (51).

In mouse, this network is known to be upstream of surfactant proteins, A, B and C, coded by *SP-A, SP-B* and *SP-C* (87–89) (see Figure 2). Presence of surfactant proteins A and B has been demonstrated in the adult swimbladders of a taxonomically diverse array of fishes, including a close relative of zebrafish, the goldfish, *Carassius auratus* (39, 40, 90). A BLAST search of the *Danio rerio* transcriptome returns no matches at either the RNA or protein level for surfactant protein C, so we have concluded that this gene is not present in the zebrafish genome and therefore have not attempted to include it here. Other studies have found that surfactant proteins A and B are present

in the swimbladder during development, and are important for swimbladder inflation in some taxa (*Anguilla rostrata* (91) and *Stizostedion vitreum* (92)). Though initially it was unclear if this surfactant was expressed in swimbladder tissue or diffused from another structure. Prem et al. (93) showed that these surfactant proteins are in fact secreted from the gas gland cells of the adult swimbladder, in eels (*Anguilla rostrata)* and perch (*Perca fluviatilis)*. To date, there are no data regarding the expression of these proteins in the adult zebrafish or larval fishes of any kind. We examined the expression of surfactant proteins A and B in developing zebrafish via immunohistochemical staining, and documented their expression patterns through our developmental window of interest. This increases the size of our presumptive network to seven zebrafish genes (including both *Nkx2.1a* and *Nkx2.1b*, only one of which is present in mouse). In mouse, the component genes of this network are co-expressed only in the lung endoderm, and the network interactions are well documented in this tissue, making it a unique and lung-specific network.

Materials and Methods

Zebrafish culture

Adult and larval wild-type zebrafish were maintained on a 14:10hr light:dark cycle at 26°C in Aquatic Ecosystems re-circulating rack systems. Adults were fed twice daily on cultured brine shrimp and bred according to standard conditions (94). Eggs were collected at approximately one-hour post fertilization (hpf) and placed directly into mesh tubes in the re-circulating system. Embryos and larvae were collected in subsequent 24-hour intervals and staged in days post fertilization (dpf).

Reverse transcriptase (RT)-PCR

Gene specific PCR primers were designed based on published *Danio rerio* gene sequences (Table 1). Primers specific for *Nkx2.1a* and *Nkx2.1b* were located in regions of divergent sequences between the two paralogs.

TABLE 1: PCR Primers

Gene Name	Accession Number	Forward Primer Sequence (3'-5')	Reverse Primer Sequence (3'-5')	Product Size
Nkx2.1a	AF253054	CCGGGAATGGACGCCAG	GTTCTGCCGTACAGCAGGTT	461bp
Nkx2.1b	AF321112	TTGGTAAAGGCATGGGTCC	GGAACCATTGTCTTGTTGC	222bp
FoxA2	BC086703	GCTACACTCATGCCAAGCCCCC	CCCGGCTTATCCGGAGAGCGCGG	222bp
Wnt7b	XM_686786	ATCCCCGGCCTGGCCCCC	GTCTCTGGCTCATGCACCAC	512bp

Primers were tested and optimized on cDNA from whole 1dpf larvae, because all genes of interest are expressed in other organs at this stage (*FoxA2* and *Nkx2.1a*: Wendl et al. 2007; *Nkx2.1a* and *Nkx2.1b*: Tessmar-Raible et al. 2007; *Wnt7b*: Viktorin et al. 2009). cDNA was prepared using Superscript III Reverse Transcriptase (Invitrogen) according to the supplier's protocol, from Trizol (Invitrogen)/Chloroform (Sigma-Aldrich) extracted total larval RNA. Before reverse transcription, each RNA sample was treated with RQ1 DNAse (Promega) to eliminate genomic DNA contamination. Controls lacking reverse-transcriptase verified that samples contained no genomic DNA (Figure 3B). Gel electrophoresis was used to verify that PCR products of the correct size were produced. These products were extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen) and sequenced by the Cornell University Core Life Sciences Center.

Preliminary RT-PCR was conducted on whole adult swimbladder samples. We found that all candidate genes were expressed in this sample, as confirmed by gel extraction and sequencing. To determine more precisely the distribution of gene transcripts in the adult *Danio* swimbladder, tissue samples were taken from three regions of the swimbladder: anterior lobe, posterior lobe and pneumatic duct (Figure 3). RNA from whole brains was used as a positive control and tail epaxial muscle was sampled as a negative control. Each sample was removed using a new, sterile surgical scalpel (to prevent cross-contamination of swimbladder regions) rinsed in deionized water (to remove any blood, peritoneal membrane or other contamination), flash frozen and stored at -80°C until Trizol extraction. RNA concentration was standardized at 500ng per reverse-transcription reaction and PCR controls using previously developed zebrafish *β-actin* primers were conducted on templates to confirm that samples were of the same approximate cDNA concentration (Figure 3A).

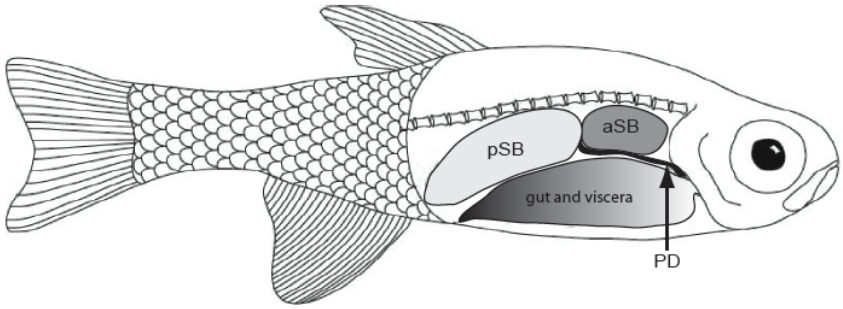


Figure 3: Three morphological regions of the adult zebrafish swimbladder. The anterior lobe (aSB), posterior lobe (pSB) and pneumatic duct (PD) of the swimbladder were sampled and analyzed independently for gene expression by RT-PCR.

Whole-mount *in situ* hybridization

RNA probes were synthesized by *in vitro* transcription (Ambion Maxiscript), containing digoxygenin-labelled UTP (Roche), from a plasmid template containing greater than 500 bases of each gene of interest via suppliers protocols. *GATA6* expression was not examined because its expression has previously been documented in zebrafish at 52 and 72hpf throughout the swimbladder (76). *In situ* hybridization was conducted using the InSituProVS *in situ* hybridization robot (Intavis) using a standard mouse *in situ* hybridization protocol (95) with the following adaptations: Embryos were digested with Proteinase K (Invitrogen) at a concentration of 10mg/ml for 15 minutes at 37°C and additionally permeablized with a 20 minute incubation in RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma-Aldrich). Before the addition of probe, specimens were pre-hybridized with hybridization buffer containing 100ug/ml yeast tRNA (Sigma-Aldrich) for 3 hours at 68°C. Probe solution contained ~10ug probe in 500ul hybridization buffer with 100ug/ml tRNA and an additional 500ug/ml salmon sperm DNA. Specimens were then treated with RNase cocktail (Invitrogen) and blocked in 5% Blocking Solution (Roche) in 1x maleic acid buffer (MAB). Anti-digoxigenin-AP Fab fragments (Roche) were applied at a concentration of 1:5000 in 5% Roche Blocking Solution in MAB, washes were conducted in .2X SSC and stained at room temperature on a nutator using the BM Purple (Roche) alkaline phosphatase substrate for 24-96 hours, until expression domains were apparent but background remained low. Negative controls with a sense-transcribed version of each probe template were also conducted to determine the level of

background staining in the absence of an anti-sense probe (results not shown). Two rounds of *in situ* hybridization with ten replicates of each probe and stage combination were run with consistent results.

Whole-mount *in situ* hybridizations were photographed, then two representatives of each probe and stage were embedded in low-melting point agar for sectioning. Sections were cut on a Micro-Cut H1200 vibrating microtome, one specimen at 50μm and one at 100μm. Both section widths illustrated the same distribution of staining, but the 100μm were more easily interpretable and are therefore shown here (Figure 5).

Immunohistochemistry

Expression of surfactant proteins A and B was detected by immunohistochemistry instead of *in situ* hybridization, as previous studies have confirmed cross-reactivity of mammal derived polyclonal antibodies with surfactant proteins of fishes (Sullivan et al. 1997; Daniels et al. 2004). Primary rabbit polyclonal antibodies to mature SP-A and SP-B were obtained from AbCam (ab40876 and ab87674). Embryos were fixed as described for *in situ* hybridization, permeabilized with a .5% trypsin solution (in saturated sodium borate) followed by 20 minutes in acetone at -20°C then blocked and hybridized according to the Vector Labs ABC Elite Kit (PK-6100) and stained with Vectastain DAB peroxidase staining solution (SK-4100) with nickel enhancement until completely developed. Two runs of ten individuals per primary antibody and stage combination were conducted to ensure reproducibility of results.

Interpretation of *in situ* hybridization results

Comparing expression of genes in developing mouse lungs and the zebrafish swimbladder poses two challenges: the comparison of location in two morphologically very different structures, and the comparison of developmental timing in two organisms that develop at very different rates. Though zebrafish swimbladder and tetrapod lung do not much resemble each other in adults, in their earliest stages of development they are more similar: a median tube connected to either a single lobed sac (in zebrafish) or two continuous lobes (in mouse). In mouse, after the initial budding event, a septum forms between the trachea and esophagus. Though there is no comparable event in swimbladder development (46), the overall structure of a simple tube and sac is the same in mouse and zebrafish.

The second difficulty in comparing mouse and zebrafish morphology is in determining the posterior extent of expression of surfactant protein. Though our other genes of interest are expressed only in the swimbladder bud and forward, both SP-A and SP-B were also expressed posterior to the location of the swimbladder bud. Because the zebrafish lacks a stomach (96), it is difficult to define comparable locations along the endodermal axis between mouse and zebrafish. Therefore, for the purposes of this study we have reported expression as either being anterior to the pneumatic duct, posterior to the pneumatic duct or in the swimbladder proper, without reference to the exact posterior extent of expression in the intestine.

With regard to timing, we mapped the timing of gene expression onto a timeline of

AO developmental milestones that are common to all bony vertebrates: molecular specification of the AO endodermal domain (Mouse E9.0; Zebrafish >48hpf), formation of the AO bud (Mouse E9.5; Zebrafish 48-52hpf), AO elongation (Zebrafish 3-4dpf, Mouse E9.5-10.5) and adult.

Results

RT-PCR of adult tissues Reverse-transcriptase PCR analysis indicated that transcripts of *Nkx2.1a*, *Nkx2.1b*, *Wnt7b* and *FoxA2* are present in whole adult zebrafish swimbladders (results not shown). Examination of dissected regions of swimbladder – anterior lobe, posterior lobe, and pneumatic duct (Figure 3) – further indicated that these genes were expressed in all three morphological regions (Figure 4).

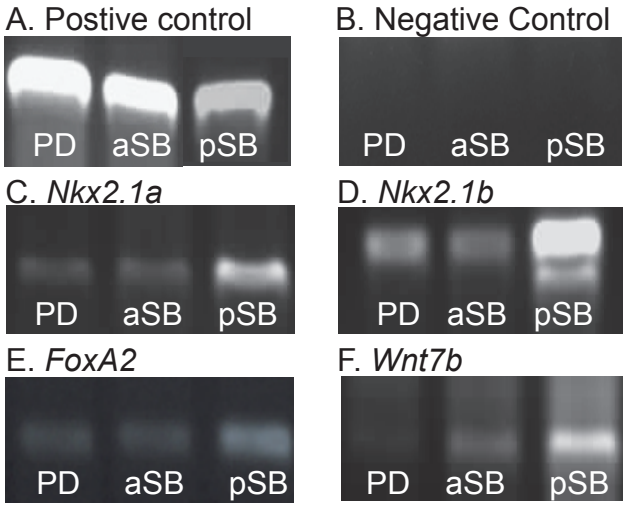


Figure 4: RT-PCR analysis of *Nkx2.1a*, *Nkx2.1b*, *FoxA2* and *Wnt7b* expression in the three morphological regions of the adult zebrafish swimbladder. cDNA concentrations were standardized using a *β-actin* control (A), and absence of genomic DNA contamination was confirmed with a minus reverse-transcriptase control (B). All four of these genes are expressed in varying intensities across the three swimbladder regions (aSB, anterior swimbladder lobe; pSB, posterior swimbladder lobe; PD, pneumatic duct).

Whole-mount *in situ* hybridization of larval stages

In situ hybridization of larvae at 3dpf and 4dpf was used to characterize the onset and location of expression throughout early swimbladder development (Figure 5). We began our developmental series at 3dpf, the stage at which the swimbladder bud begins to evaginate and stopped at 4dpf because *in situ* hybridization of older embryos is problematic, due to difficulty permeablizing the tissue without loss of integrity. Additionally, in these larvae it is difficult to conclude whether lack of staining in these older embryos indicates lack of expression or inadequate digestion and reagent penetration.

The earliest detected gene product was *FoxA2*, which was already strongly expressed in the brain and throughout the anterior pharynx at 3dpf (Figure 5). This result was expected, based on the reports of Field et al. (75), that *FoxA2* is expressed in the developing anterior pharynx spanning the region that forms the AO bud from as early as 24hpf, and Cheng et al. (97), who showed that it is expressed in the primitive endoderm from 8hpf. In our experiments, *FoxA2* expression did not change between 3dpf and 4dpf except to expand into newly developed tissue, including the emerging swimbladder and liver as the endodermal organs matured (Figure 5).

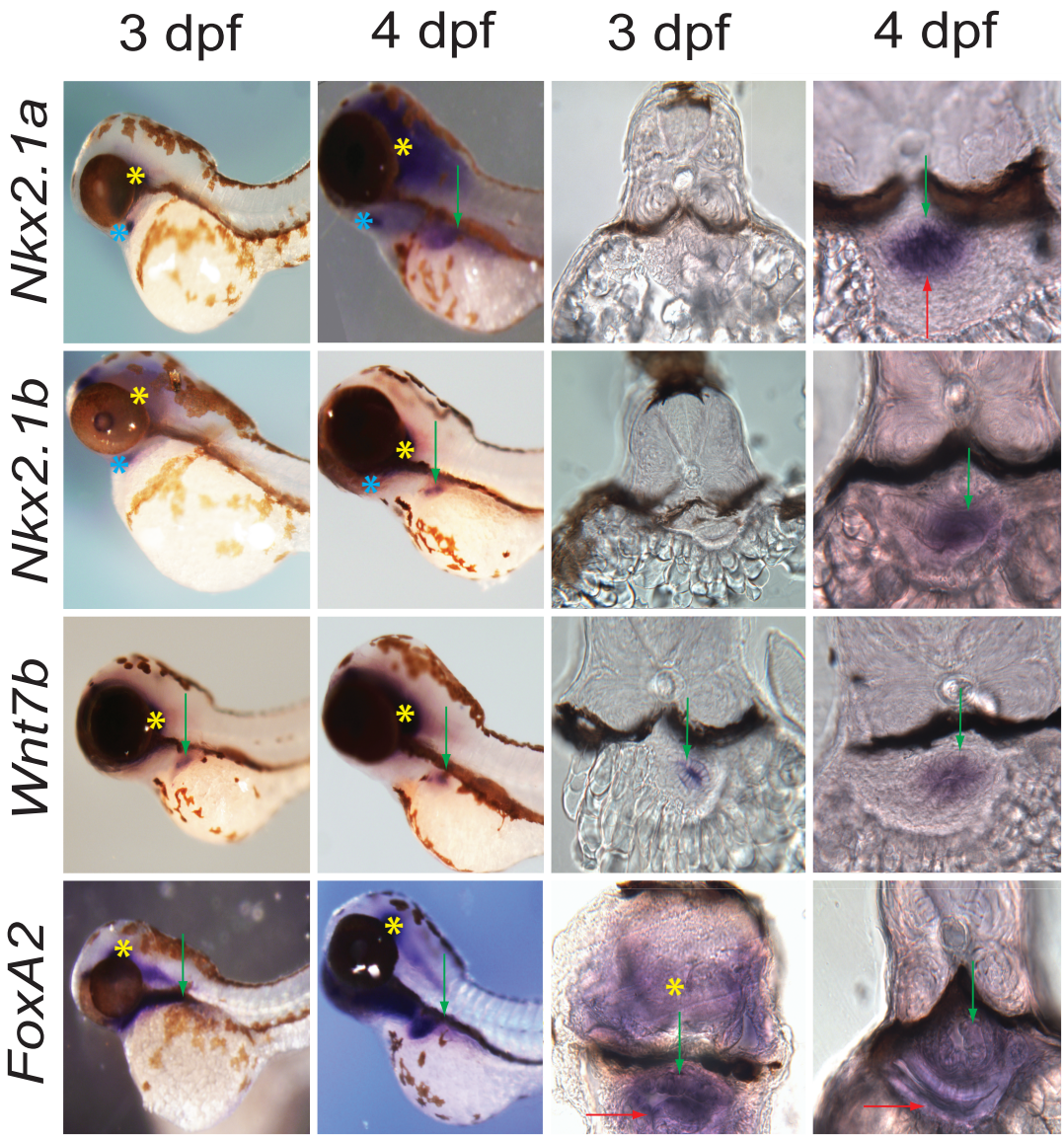


Figure 5: *In situ* hybridization results for *Nkx2.1a*, *Nkx2.1b*, *FoxA2* and *Wnt7b* at 3 and 4dpf. Gene names are indicated in rows, with developmental time points in columns. The intersection of a given row and column shows expression of a single gene at a single time. Results are shown both as whole mounts with anterior facing left (left two panes) and in transverse section (right two panes) through the evaginating swimbladder (100μm sections taken between 500 and 600 μm from the anterior of the specimen). Green arrows indicate expression in the swimbladder bud. Red arrows indicate expression in the posterior pharynx surrounding the swimbladder bud. Yellow asterisks indicate expression in regions of the brain, which serves as a positive control for all genes of interest. Blue asterisks indicate expression of *Nkx2.1a* and *Nkx2.1b* in the developing thyroid. Note that there is no expression of *Nkx2.1a* or *Nkx2.1b* at 3dpf, but both are expressed by 4dpf. *Wnt7b* and *FoxA2* are already expressed at 3dpf, when the swimbladder first begins to develop.

In zebrafish, other studies have shown that *Wnt7b* expression in regions of the brain begins around 1dpf (98). Other regions of *Wnt7b* expression in zebrafish have not been previously described. We identified an additional *Wnt7b* expression domain in the developing swimbladder and the adjoining pharyngeal endoderm at 3dpf persisting through 4dpf (Figure 5), and spreading distally to the emerging swimbladder some time between 5dpf and adulthood (Figure 4B).

Nkx2.1a expression begins at 4dpf throughout the developing AO and the pharyngeal endoderm surrounding the region of evagination (Figure 5). *Nkx2.1b* was also expressed at 4dpf but, like early *Wnt7b* expression, it was restricted to the evaginating swimbladder and was not expressed throughout the pharyngeal endoderm (Figure 5).

Immunohistochemistry of larval stages

Expression of surfactant proteins A and B was characterized in both the gut and swimbladder from 3 to 7dpf. Though RNA *in situ* hybridization was not consistent after 4dpf, IHC staining remained reliable through 7dpf; for this reason and because of the anticipated late onset of swimbladder surfactant protein expression, we extended our window of interest for SP-A and SP-B expression. Both proteins were expressed in the gut immediately posterior to the pharynx, and to variable extents in the foregut and midgut from prior to 3dpf. SP-A was strongly expressed in the gut adjacent and posterior to the swimbladder bud from 3dpf (Figure 6), but only present transiently in the swimbladder itself. SP-A was present at low-levels in the swimbladder before 3dpf, absent at 4dpf and weakly present at 7dpf (Figure 6) raising the possibility that

its presence is due to diffusion from the gut, rather than expression in swimbladder tissue (see discussion). SP-B was also expressed strongly in the gut starting 3dpf, with continued though restricted expression in the foregut through 7dpf, but not in the swimbladder at least as late as 7dpf. Given that both SP-A and SP-B are universally present in the adults of a taxonomically comprehensive sampling of fishes (40, 90) it is assumed that both are present in the adult zebrafish swimbladder, but our data show that there is only light and/or transient expression before 7dpf.

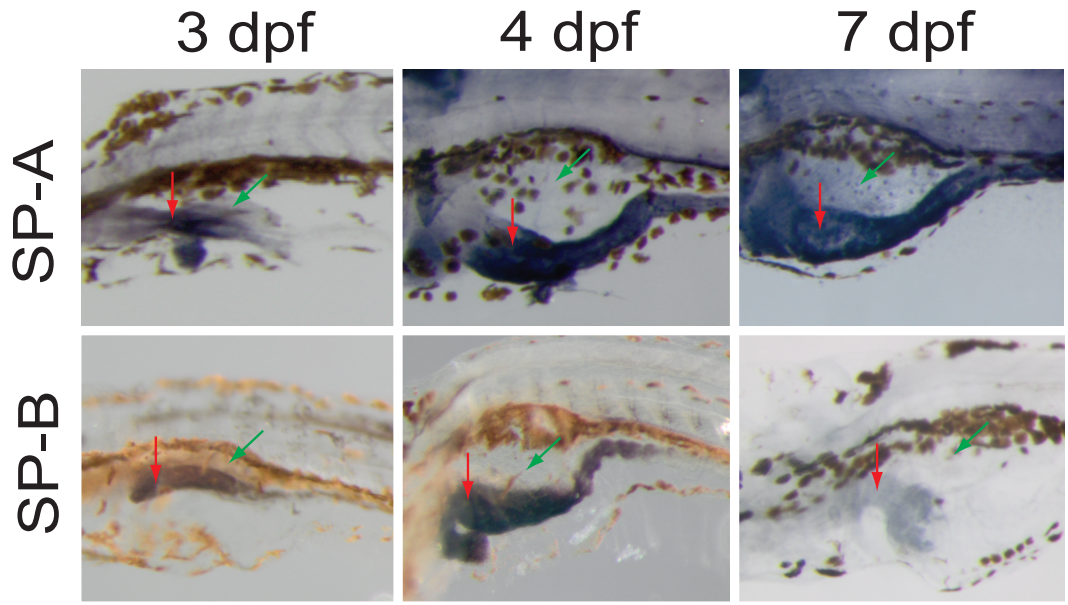


Figure 6: Immunohistochemistry results for SP-A and SP-B from 3 to 7dpf. Results are shown as whole mounts, with anterior at left and posterior at right. Location of the gut is indicated with red arrows, where the swimbladder is indicated with green arrows. Note that SP-A is expressed in the gut surrounding the swimbladder bud at 3dpf and expands posteriorly thereafter with light and transient presence in the swimbladder. SP-B is also expressed in the gut surrounding the swimbladder at 3dpf and extends posteriorly later in development, but is never detected in the swimbladder proper.

Discussion

All early lung-budding genes studied were expressed throughout the adult swimbladder, but their times of expression initiation varied, and their spatial

expression patterns changed through the course of ontogeny. The expression patterns of early acting (and upstream, at least in mouse) gene products, *FoxA2* and *GATA6*, were identical during the developmental window of interest for the lung in mouse and the swimbladder in zebrafish. Later-acting (and downstream, at least in mouse) gene products, *Nkx2.1a*, *Nkx2.1b* and *Wnt7b*, showed marked differences in both the timing and location of expression between these two taxa. Expression of SP-A and SP-B appears to be consistent with data from mouse, though further analysis of older larvae will be necessary to pinpoint the time of expression onset in the swimbladder. We interpret all of these observations as being consistent with a common origin for lungs and swimbladders, in which early steps are conserved in time and space, while later steps – although still conserved – reflect divergence in the pattern and timing of gene expression.

Comparison of zebrafish and mouse gene expression data

Conservation of *FoxA2* and *GATA6* Expression

Our *FoxA2* expression data are consistent with both the literature on mouse lung development and a previous report of *FoxA2* expression in the swimbladder (75). *FoxA2* expression was observed from the earliest zebrafish stage we examined (3dpf) in the brain and pharynx and continued throughout the foregut endoderm and in the proximal and distal regions of the swimbladder as they developed. It is also expressed throughout the adult swimbladder (Figure 4E), which is similar to its reported expression pattern during mouse lung development (79) (see Figure 7).

Based on previously published literature, the pattern of *GATA6* expression also

appears to be conserved between mouse and zebrafish. *GATA6* is expressed in the primitive endoderm of both mouse (99) and zebrafish (73) embryos, and expands into the growing AO shortly after budding; zebrafish at 52hpf (76) and mouse E12.5 (100). A low level of expression in the lung endoderm is also present in the adult.

This conservation is not unexpected given the critical roles of these genes in early endoderm specification and their expression in many endodermal derivatives, but is also important given the lung-specific interactions of *FoxA2* and *GATA6* with our other candidate genes. It is the expression of all candidate genes, taken together, in the developing swimbladder that suggests these lung-specific network interactions are conserved in zebrafish.

Divergence of *Wnt7b* and *Nkx2.1* Expression

The spatial and temporal expression patterns of *Wnt7b* and *Nkx2.1* in the developing AO are clearly different between mouse and zebrafish. The onset of mouse lung *Wnt7b* expression has not been published, but its null phenotype is apparent at least by E10.5 (101). At E11.5 *Wnt7b* is expressed throughout the lung endoderm, becoming more highly expressed in the distal tips and eventually becoming distally restricted to the points of highest cell proliferation in the post-natal lung (84). Consistent with this restricted expression pattern, *Wnt7b* has been shown to function primarily in stimulating the proliferation of both lung epithelium and mesenchyme, with null mutants showing normally patterned but markedly hypomorphic lungs (102). In zebrafish, *Wnt7b* expression begins by 3dpf, where it is expressed throughout the

pharyngeal endoderm at the point of swimbladder budding. By 4dpf the expression becomes localized to the evaginating swimbladder bud (Figure 5) and it is expressed throughout the adult swimbladder, never becoming restricted (Figure 4). It is worth noting that a similar expression pattern was noted in the homogeneous expression of *Shha* in the developing swimbladder relative to the distally restricted pattern of its mouse homolog, S*hh*(46). As both genes are expressed in the lung epithelium and critical for mesenchymal proliferation in lung(103), their coordinated shift in expression in swimbladder relative to that in lung may indicate that the gene regulatory network containing both of these gene products and underlying lung mesenchymal proliferation is also conserved between mouse lung and the zebrafish swimbladder.

In mouse, *Nkx2.1* is the earliest known genetic marker of the region of the primitive endoderm that will form the lung. In the zebrafish swimbladder this is not the case. Neither *Nkx2.1a* nor *Nkx2.1b* is expressed before the initial swimbladder budding event. Further, like *Wnt7b*, neither copy of *Nkx2.1* becomes distally restricted like its mouse homolog. Rather, all three genes remain expressed throughout the adult swimbladder.

Expression of both *Nkx2.1a* and *Nkx2.1b* in the swimbladder is likely due to the expression of an *Nkx2.1* homolog in the AO of the common ancestor of mouse and zebrafish and subsequent duplication of *Nkx2.1* during the whole genome duplication that occurred in the ray-finned fish lineage leading to teleosts (including zebrafish).

Though *Nkx2.1* is expressed very early in mouse lung development, its role in early development is poorly understood and it is apparently not required for the early events in lung bud specification. *Nkx2.1* null mice do develop lungs, but these lungs are rudimentary and suffer three primary defects(54). First, they fail to develop a trachea separate from the esophagus (a condition termed tracheoesophageal fistula). Second, lung branching morphogenesis is greatly reduced. Third, the lungs of *Nkx2.1* null mice do not express surfactant proteins. The first two of these deficiencies are apparent relatively early in mouse lung development (between E10 and E11), and are reminiscent of the normal condition during zebrafish development—zebrafish neither develop a trachea nor do they undergo branching morphogenesis. *Nkx2.1a* and *Nkx2.1b* are not expressed during the period of swimbladder development that corresponds to E10-E11 (approximately 3-4dpf), and this delayed expression may be at least partially responsible for the similarity between the mouse-null and the zebrafish wild-type phenotype. The third deficiency seen in the mouse *Nkx2.1* null is the lack of surfactant expression, but this does not become evident until E20.

In addition to differences in the timing of *Nkx2.1* expression in the AO of zebrafish and mouse, the spatial expression is also divergent between species and between paralogs. *Nkx2.1a* is expressed throughout the pharyngeal endoderm at the point of swimbladder evagination from its time of initiation. *Nkx2.1b* is expressed only in the evaginating bud and not in the surrounding pharyngeal endoderm. Whether this is indicative of some sub- or neo-functionalization of these gene copies or simply change allowed by redundancy remains to be determined.

In mouse, *Nkx2.1* is expressed before *Wnt7b* during lung budding, and *Nkx2.1* has been shown to activate *Wnt7b* expression *in vitro* (84). However, we detected expression of *Wnt7b* before we detected expression of either copy of *Nkx2.1* in the developing zebrafish swimbladder. It is possible that during early swimbladder development *Nkx2.1* is expressed at a level that is undetectable by whole mount *in situ* hybridization but sufficient to activate *Wnt7b* transcription. It is also possible that *Wnt7b* expression is initiated before *Nkx2.1*, and therefore activated by another upstream factor.

Several other factors, including GATA6 and FoxA2, have been shown to bind independently to, and activate, the *Wnt7b* promoter in mouse (84). Both *FoxA2* and *GATA6* are expressed in the zebrafish swimbladder before *Nkx2.1* (see Figure 6) making them candidates for regulating the AO expression of *GATA6* in this species (see Figure 3).

Surfactant protein expression

Expression of SP-A and SP-B were examined from 3 to 7dpf, and neither was strongly expressed in the developing zebrafish swimbladder at any time during this window. Some SP-A may be present in the swimbladder, but it was minimal and/or transient (Figure 6). However, both SP-A and SP-B were detected to varying extents in the developing gut from 3 to 7dpf. This is consistent with expression in the developing mouse lung, where surfactant proteins are not expressed until very late (E20) in

development (104) and where surfactant-like particles in the gut contain both SP-A and SP-B (105, 106).

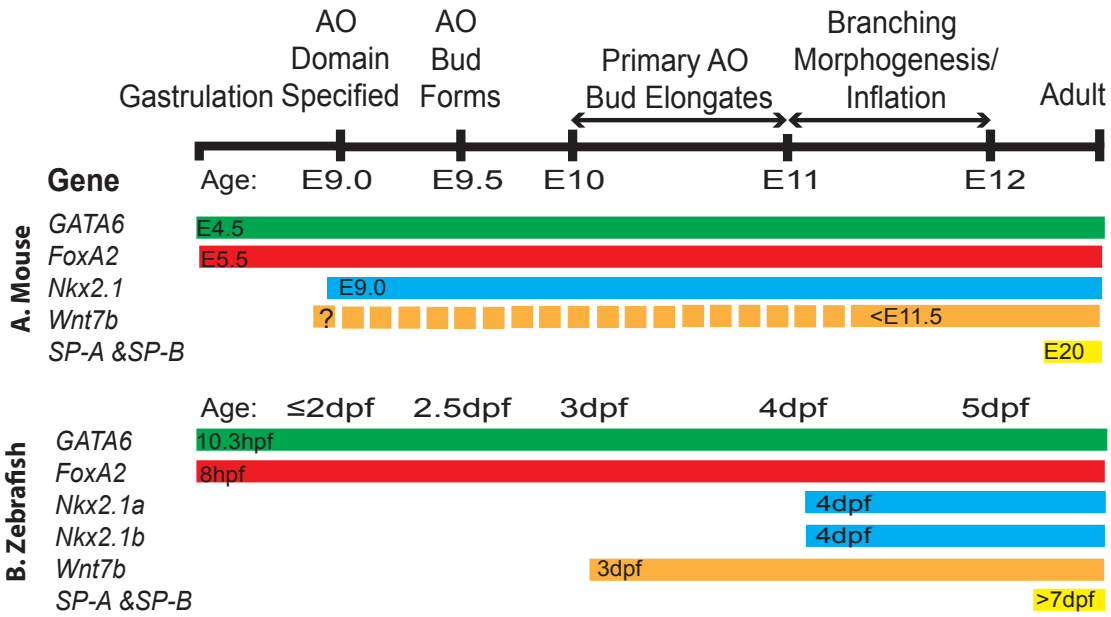
From these results we suggest that SP-A and SP-B expression is not necessary for swimbladder inflation in zebrafish, as this occurs around 4-5dpf (94) when neither is actively expressed in the swimbladder. It has previously been shown that the presence of a surfactant layer is necessary for inflation in other fish species (91, 92), but it is unclear if the critical component is the surfactant proteins proper or their complex surrounding phospholipid matrix (90). Though we examined the presence of SP-A and SP-B, we did not look for the presence of other components of this mixture. Considering the late onset of surfactant expression in zebrafish, it may be that other components of this surfactant mixture are present earlier in development.

Interestingly, the only swimbladder tissue known to actively secrete surfactant proteins is mature gas-gland tissue (93), the primary function of which is secreting oxygen to the swimbladder lumen for buoyancy regulation. For the fishes that lack a gas-gland, it has been suggested that surfactant proteins diffuse into the swimbladder from the adjoining gut tissue (92). It appears that zebrafish lack a gas-gland or that it is very poorly developed (107), so it may be that diffusion from the gut is the only source of surfactant proteins in the zebrafish swimbladder. Diffusion from the gut rather than expression in swimbladder tissue would explain the transient presence of SP-A and lack of SP-B in the swimbladder (Figure 6).

An interesting corollary of these results is that, despite the relatively delayed onset time of *Nkx2.1a* and *Nkx2.1b* in zebrafish (4dpf), SP-A and SP-B are still expressed considerably after (Figure 7). Due to the absence of SP-A and SP-B expression in mouse *Nkx2.1* null mutants (54), we expected that surfactant protein expression in zebrafish would be initiated after *Nkx2.1* (if at all). This further supports our hypothesis that the mouse *Nkx2.1* -/- lung phenotype is a phenocopy of the swimbladder condition, with a lack of branching and tracheal septation. Our results suggest that *Nkx2.1* in zebrafish does not in specify the AO domain in the primitive endoderm, but rather initiates downstream expression of SP-A and SP-B or serves no critical function in the swimbladder of this species.

The lack of SP expression during inflation of the swimbladder raises the question of what function, if any, surfactant proteins serve in the zebrafish swimbladder. In mouse, surfactants are critical for proper gas exchange, inhibition of lung wall adhesion upon expiration of gas and have a role in innate immunity (108, 109). In zebrafish, gas exchange for respiration does not occur through the swimbladder, and once inflated the swimbladder remains so; neither respiration nor collapse prevention seems likely. However, it is possible that surfactant proteins are in fact important in inflation, but that they are simply supplied by diffusion from the adjoining gut and not from expression within the swimbladder. This hypothesis is consistent with the apparent transient expression of SP-A in the zebrafish swimbladder at 3 and 7dpf (Figure 7). As surfactant proteins have been strongly implicated in innate immunity, this would be another logical functional prediction. It is also possible that surfactant

proteins assist with the exchange of gases with the bloodstream for buoyancy regulation in adults. Or it is even possible that SP expression in the AO is primitive (along with lungs) for Osteichthyes and it has been lost in fishes with non-respiratory



AOs

Figure 7: Timing of expression of candidate genes in mouse (A) and zebrafish (B). *FoxA2* and *GATA6* expression times are both expressed from early specification of the AO domain through adult in both mouse and zebrafish but divergent during very early development; they are expressed from early endoderm specification. *Nkx2.1* and *Wnt7b* expression differs during our period of interest. Expression of both *Nkx2.1a* and *Nkx2.1b* in zebrafish is initiated much later in morphological development than its mouse homolog. *Wnt7b* expression is also initiated later in zebrafish than mouse, though the time of onset of mouse *Wnt7b* expression has not been documented (indicated by dashed line) so the relative order of *Nkx2.1* and *Wnt7b* expression initiation may be the same in zebrafish and mouse (73, 75, 76, 97, 99, 100, 114, 115). Surfactant proteins A and B are not expressed in zebrafish swimbladder at least up to 7dpf and may only diffuse into the swimbladder from the gut (see text).

Implications for evolution

Functional studies and documentation of the onset of *Wnt7b* expression in mouse and *Nkx2.1* in both mouse and zebrafish will yield important insight into the extent to which gene expression and interactions are conserved between the AOs of these two taxa. Demonstration that network interactions are conserved between mouse and zebrafish would strengthen the hypothesis that similarities in AO gene expression patterns are due to common ancestry, that is, the networks themselves are homologous (7). However, differences in the network interactions do not necessarily weaken the hypothesis of common ancestry. The lineages leading to mouse and zebrafish have been evolving independently since at least the late Silurian (Janvier 1996), approximately 400 million years ago. Each has been on its own morphological and genetic trajectory, and considerable divergence is to be expected. Because our candidate genes are not co-expressed outside of the osteichthyan AO, and *Nkx2.1* is involved in so few developmental processes, it is most likely that its expression in both the mouse lung and zebrafish swimbladder is due to their common origin as AOs.

Without further comparative data, the ancestral condition of the AO network cannot be inferred. Some might argue that the swimbladder must be primitive because it is found in a fish, and the swimbladder lacks the (presumably derived) tracheal septation and branching morphogenesis seen in modern mammalian lungs. However intuitive this might seem, it is equally likely that the mouse expression pattern is primitive, because ventral paired lungs are primitive for the Osteichthyes (bony vertebrates including both tetrapods and ray-finned fishes) and most tetrapods retain this ancestral

morphology. A third possibility, that both the lineage leading to mouse and the lineage leading to zebrafish have independently diverged from the ancestral state, is most likely. There is no reason to expect that even homologous structures will have a completely conserved patterning mechanism or that one or another extant species will have retained all elements of the ancestral network. Many studies of homologous structures in different taxa have borne this out, most famously, the case of insect segmentation. Insect segments are unquestionably homologous, but they are underwritten by both conserved and very divergent gene regulatory mechanisms (Patel et al. 1992; Dawes et al. 1994). Though we attempted to increase the possibility of identifying conserved elements by examining genes expressed during AO budding and not during the formation of teleost- or tetrapod-specific structures, there is no *a priori* reason to expect that this portion of the developmental pathway should be more conserved than any other.

There is no test that can rule out co-option and convergence completely, but further studies can provide evidence for or against our hypothesis that the AO and its underlying developmental regulatory network are similar due to common ancestry. First, the network interactions of these gene products must be determined in zebrafish. When reconstructing phylogenies, complex characters are traditionally believed to be more reliable than simple ones because identical complexes are thought less likely to originate twice. A gene regulatory network is a much more complex character than a collection of gene products, and the same network is not likely to have been assembled convergently multiple times (14). Co-option of the network as a whole from a

previous function is conceivable, but since this network has not been identified outside of the osteichthyan AO, it appears unlikely to have been co-opted as a unit from any other developmental process. However, surveys of gene expression in non-osteichthyan vertebrates are rare, and we would not know if the network was present in a non-osteichthyan fish and co-opted from a function subsequently lost in osteichthyans. This possibility can only be diminished by a broader phylogenetic survey of gene expression in basal osteichthyan and non-osteichthyan vertebrates. If these genes are not co-expressed anywhere in a non-osteichthyan, then the network can be considered a molecular synapomorphy or homology *sensu* (7, 8, 110) of the Osteichthyes (bony vertebrates).

If the same cassette of genes is conserved across (homologous for all) Osteichthyes and this cassette is only found in lungs and swimbladders, then the unique association of the network with osteichthyan AOs supports the idea that lungs and swimbladders are the same organ; that is, lungs and swimbladders have the same character identity(14). This is further evidence, in addition to classical morphology, that the swimbladder is a modified lung, that is, they are transformational homologues(7). We suggest that that the presence of mouse lung network genes in the zebrafish swimbladder makes this network a candidate ChIN for the osteichthyan AO.

Conclusions

A suite of genes, previously known to be co-expressed only in the tetrapod lung, is also co-expressed in the zebrafish swimbladder. For two genes, both involved in the

development of the primitive foregut, the timing and location of expression are conserved (*FoxA2* and *GATA6*). Three other genes (*Nkx2.1a*, *Nkx2.1b* and *Wnt7b*) are expressed at different times in development and in different sub-regions of the AO. However, the order of activation of these five genes may be the same and we have speculated on how the functions of these gene products may also be conserved.

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REFERENCES

1. Gould SJ, Vrba ES (1982) Exaptation-A Missing Term in the Science of Form. *Paleobiology* 8:4–15.

2. Sanetra M, Begemann G, Becker M, Meyer A (2005) Conservation and co-option in developmental programmes : the importance of homology relationships. 17:1–17.

3. Carroll S, Grenier J, Weatherbee S (2004) *From DNA to Diversity* (Wiley-Blackwell, New York)Second.

4. Kuratani S (2004) Evolution of the vertebrate jaw : comparative embryology and molecular developmental biology reveal the factors behind evolutionary novelty. *J Anat*:335–347.

5. Davis MC, Dahn RD, Shubin NH (2007) An autopodial-like pattern of Hox expression in the fins of a basal actinopterygian fish. *Nature* 447.

6. Prum RO (1999) Development and Evolutionary Origin of Feathers. *J Exp Zool* 306:291–306.

7. Mccune AR, Schimenti JC (2012) Using Genetic Networks and Homology to Understand the Evolution of Phenotypic Traits. *Curr Genomics* 13:74–84.

8. Patterson C (1982) in *Problems of Phylogenetic Reconstruction*, eds Joysey KA, Friday AE (London), pp 21–74.

9. Eldridge N (1979) in *Phylogenetic analysis and paleontology*, eds Cracraft J, Eldridge N (Columbia University Press, New York), pp 165–198.

10. Allin EF (1975) Evolution of the Mammalian Middle Ear. *J Morphol* 147:403–437.

11. Geoffroy Saint-Hilaire É (1830) *Principes de philosophie zoologique, discutée en mars 1830, au sein de l'Académie Royale des Sciences* (Pichon et Didier, Paris).

12. Appel TA (1987) *The Cuvier-Geoffroy Debate: French Biology in the Decades before Darwin* (Oxford University Press, Oxford).

13. Shubin NH, Alberch P (1986) in *Evolutionary Biology*, eds Hecht MK, Wallace B, Prance GI (Plenum Press, New York), pp 319–287.

14. Wagner G (2007) The Developmental Genetics of Homology. *Nat Rev Genet* 8:473–479.

15. Owen R (1843) *Lectures on the Comparative Anatomy and Physiology of the Invertebrate Animals, Delivered at the Royal College of Surgeons in 1843* (Longman, Brown, Green, and Longmans, London).

16. Owen R (1846) *Lectures on the comparative anatomy and physiology of the vertebrate animals* (Longman, Brown, Green, and Longmans, London).

17. Roth VL (1984) On homology. *Biol J Linn Soc* 22:13–29.

18. Vanvalen L (1982) Homology and Causes. *J Morphol* 312:305–312.

19. Wray GA, Abouheif E (1998) When is homology not homology? *Curr Opin Genet Dev* 8:675–680.

20. Wray GA (1999) Evolutionary dissociations between homologous genes and homologous structures. *Novartis Found Symp* 222:189–203; discussion 203–6.

21. Shubin, N. Tabin, C. Carroll S (2009) Deep homology and the origins of evolutionary novelty. *Nature* 457:818–823.

22. Rosen DE, Forey PL, Gardiner BG, Patterson. C (1981) Lungfishes, tetrapods, paleontology, and plesiomorphy. *Bull Am Museum Nat Hist* 167:163–275.

23. Lauder GV, Liem KF (1983) The evolution and interrelationships of the actinopterygian fishes. *Bull Museum Comp Zool* 150:95–197. Available at: http://biostor.org/reference/636.

24. Nelson JS (2006) *Fishes of the World* (Wiley). 4th Ed.

25. Budgett JS (1901) On some points in the anatomy of Polypterus. *Trans Zool Soc London* 15:323–338.

26. Rice AN, Bass AH (2009) Novel vocal repertoire and paired swimbladders of the three-spined toadfish, Batrachomoeus trispinosus: insights into the diversity of the Batrachoididae. *J Exp Biol* 212:1377–1391.

27. Greene HW (2000) *Snakes: The Evolution of Mystery in Nature* (University of California Press).

28. Graham JB (1997) *Air-Breathing Fishes: Evolution, Diversity and Adaptation* ed Graham J (Academic Press, San Diego, CA).

29. Thompson KS (1992) *Living fossil: the story of the coelacanth* (W.W. Norton & Company).

30. Veerappan N, Pandi V, Balasubramanian T (2009) Sound Production Behaviour in a Marine Croaker Fish, Kathala axillaris (Cuvier). *World J Fish Mar Sci* 1:206–211.

31. Webb JF, Smith WL (2000) The laterophysic connection in chaetodontid butterfly fish : morphological variation and speculations on sensory function. 1125–1129.

32. Grande T, Pinna M De (2004) in *Mesozoic Fishes 3 – Systematics, Paleoenvironments and Biodiversity*, eds Arratia G, Tintori A (Springer Verlag, München, Germany), pp 429–448.

33. Schulz-Mirbach T, Heß M, Metscher BD, Ladich F (2013) A unique swim bladder-inner ear connection in a teleost fish revealed by a combined high-resolution microtomographic and three-dimensional histological study. *BMC Biol* 11:75–88.

34. Darwin C (1859) *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. (John Murray, Oxford). 1st Ed.

35. Romer AS (1970) *The vertebrate body* (Saunders, Philadelphia). 6th Ed.

36. Goodrich ES (1930) *Studies on the structure & development of vertebrates* (Macmillan and Co. Ltd, London).

37. Wassnetzov W (1932) Über die Morphologie der Schwimmblase. *Zool Jahrbücher Abteilung Anat und Ontog der Tiere* 56:1–36.

38. Denison RH (1941) The soft anatomy of Bothreolepis. *J Paleontol* 15:553–561.

39. Perry SF, Wilson RJ, Straus C, Harris MB, Remmers JE (2001) Which came first, the lung or the breath? *Comp Biochem Physiol A Mol Integr Physiol* 129:37–47.

40. Sullivan LC, Daniels CB, Phillips ID, Orgeig S, Whitsett JA (1998) Conservation of surfactant protein A: evidence for a single origin for vertebrate pulmonary surfactant. *J Mol Evol* 46:131–138.

41. Sagemehl M (1885) Beitrage zur vergleichenden anatomie der fische III. Das cranium der characiniden nebst allgemeinen bermerkungen uber die mit einen

Weber'schen apparat versehenen physostomen familien. *Morphol Jahrb* 10:1–119.

42. Wilder BG (1877) Gar-pikes old and young,. *Pop Sci Mon* 11:1–12.

43. Dean B (1895) *Fishes Living and Fossil* (Macmillan and Co. Ltd, New York).

44. Grigg GC (1965) Studies on the Queensland lungfish, Neoceratodus forsteri (Krefft). III Aerial respiration in relation to habits. *Aust J Zool* 13:413–421.

45. Rowntree WS (1903) On some points in the visceral anatomy of the Characinidae, with an enquiry into the relations of the ductus pneumaticus in Physostomi generally. *Trans Linn Soc London, Second Ser Zool* 9:8–83.

46. Winata CL et al. (2009) Development of zebrafish swimbladder: The requirement of Hedgehog signaling in specification and organization of the three tissue layers. *Dev Biol* 331:222–236.

47. Teoh P-H, Shu-Chien AC, Chan W-K (2010) Pbx1 is essential for growth of zebrafish swim bladder. *Dev Dyn* 239:865–874.

48. Yin A, Korzh S, Winata CL, Korzh V, Gong Z (2011) Wnt signaling is required for early development of zebrafish swimbladder. *PLoS One* 6:e18431.

49. Chuang PT, McMahon AP (2003) Branching morphogenesis of the lung: new molecular insights into an old problem. *Trends Cell Biol* 13:86–91.

50. Cardoso WV, Lü JN (2006) Regulation of early lung morphogenesis: questions, facts and controversies. *Development* 133:1611–1624.

51. Morrisey EE, Hogan BLM (2010) Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell* 18:8–23.

52. Lazzaro D, Price M, De Felice M, Di Lauro R (1991) The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* 113:1093–1104.

53. Cass AN, Servetnick MD, McCune AR (2013) Expression of a lung developmental cassette in the adult and developing zebrafish swimbladder. *Evol Dev* 15:119–132.

54. Minoo P, Su G, Hong D, Bringas P, Kimura S (1999) Defects in Tracheoesophageal and Lung Morphogenesis in Nkx2.1(–/–) Mouse Embryos. *Dev Biol* 209:60–71.

55. Domyan ET et al. (2011) Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Dev Cambridge Engl* 138:971–981.

56. De Robertis EM, Sasai Y (1996) A common groundplan for dorsoventral patterning in Bilateria. *Nature* 380:37–40.

57. Smith AB, Peterson KJ, Wray G, Littlewood DTJ (2004) in *Assembling The Tree of Life*, eds Cracraft J, Donoghue M (Oxford University Press, New York), pp 365–383.

58. Swalla B (2006) Building divergent body plans with similar genetic pathways. *Heredity (Edinb)* 97:235–243.

59. Morris VB (2007) Origins of radial symmetry identified in an echinoderm during adult development and the inferred axes of ancestral bilateral symmetry. *Proc R Soc London B* 274:1511–1516.

60. Dahn RD, Davis MC, Pappano WN, Shubin NH (2007) Sonic hedgehog function in chondrichthyan fins and the evolution of appendage patterning. *Nature* 445:311–314.

61. Gillis JA, Dahn RD, Shubin NH (2009) Shared developmental mechanisms pattern the vertebrate gill arch and paired fin skeletons. *Proc Natl Acad Sci* 106:5720–5724.

62. Kimmel CB, Miller CT, Keynes RJ (2001) Neural crest patterning and the evolution of the jaw. *J Anat* 199:105–120.

63. Cohn M (2002) Lamprey Hox genes and the origin of jaws. *Nature* 416:386–387.

64. Kuratani S (2004) Evolution of the vertebrate jaw: comparative embryology and molecular developmental biology reveal the factors behind evolutionary novelty. *J Anat* 205:335–347.

65. Hurley IA et al. (2007) A new time-scale for ray-finned fish evolution. *Proc R Soc London B* 274:489–498.

66. Stiassny MLJ, Wiley EO, Johnson GD, de Carvalho M. (2004) in *Assembling The Tree of Life*, eds Cracraft J, Donoghue MJ (Oxford University Press, New York), pp 410–429.

67. McCune AR, Carlson RL (2004) Twenty ways to lose your bladder: common natural mutants in zebrafish and widespread convergence of swim bladder loss among teleost fishes. *Evol Dev* 6:246–259.

68. McMahon B (1969) A functional analysis of the aquatic and aerial respiratory movements of an African lungfish, Protopterus aethiopicus, with reference to the evolution of the lung-ventilation mechanism in vertebrates. *J Exp Biol* 51:407–430.

69. Kerr J (1907) in *The work of John Samuel Budgett*, ed Kerr J (Cambridge University Press, Cambridge), pp 195–290.

70. Liem KF (1988) Form and function of lungs: the evolution of air breathing mechanisms. *Am Zool* 28:739–759.

71. Janvier P, Desbiens S, Willet JA (2007) New evidence for the controversial "lungs" of the late devonian antiarch Bothreolepis canadensis (Whiteaves, 1880) (Placodermi: Antiarcha). *J Vertebr Paleontol* 27:709–710.

72. Cardoso W (2001) Molecular regulation of lung development. *Annu Rev Physiol* 63:471–494.

73. Zeng X, Yutzey KE, Whitsett JA (1998) Thyroid transcription factor-1, hepatocyte nuclear factor-3β and surfactant protein A and B in the developing chick lung. *J Anat* 193:399–408.

74. Sakiyama J, Yamagishi A, Kuroiwa A (2003) Tbx4-Fgf10 system controls lung bud formation during chicken embryonic development. *Development*:1225–1234.

75. Field HA, Ober EA, Roeser T, Stainier DYR (2003) Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev Biol* 253:279–290.

76. Yee N.S., Lorent K, Pack M (2005) Exocrine pancreas development in zebrafish. *Dev Biol* 284:84–101.

77. Hollemann T, Pieler T (2000) Xnkx-2.1: A homeobox gene expressed during early forebrain, lung and thyroid development in Xenopus laevis. *Dev Genes Evol* 210:579–581.

78. Small EM, Vokes SA, Garriock RJ, Li DL, Krieg P. (2000) Developmental expression of the Xenopus Nkx2-1 and Nkx2-4 genes. *Mech Dev* 96:259–262.

79. Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH (2004) Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* 132:35–47.

80. Goss A.M. et al. (2009) Wnt2/2b and b-Catenin Signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev Cell* 17:290–298.

81. Meyer A, Schartl M (1999) Gene and genome duplications in verterbrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* 11:699–704.

82. Hoegg S, Brinkmann H, Taylor JS, Meyer A (2004) Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J Mol Evol* 59:190–203.

83. Rohr KB, Barth KA, Varga ZM, Wilson SW (2001) The nodal pathway acts upstream of hedgehog signaling to specify ventral telencephalic identity. *Neuron* 29:341–351.

84. Weidenfeld J, Shu W, Zhang L, Millar SE, Morrisey EE (2002) The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. *J Biol Chem* 277:21061– 21070.

85. Oguchi H, Kimura S (1998) Multiple transcripts encoded by the thyroid-specific enhancer-binding protein (T/EBP)/thyroid-specific transcription factor-1 (TTF-1) gene: evidence of autoregulation. *Endocrinology* 139:1999–2006.

86. Boggaram V (2009) Thyroid transcription factor-1 (TTF-1/Nkx2.1/T1F1) gene regulation in the lung. *Clin Sci* 116:27–35.

87. Boggaram V (2003) Regulation of lung surfactant protein gene expression. *Front Biosci* 8:751–764.

88. Bohinski RJ, Di Lauro R, Whitsett JA (1994) . The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol Cell Biol* 14:5671–5681.

89. Bruno M, Bohinski R, Huelsman K, Whitsett J, Korfhagen T (1995) Lung cell specific expression of the murine surfactant protein A (SP-A) gene is mediated by interaction between the SP-A promoter and thyroid transcription factor-1. *J Biol Chem* 270:6531–6536.

90. Daniels CB et al. (2004) The origin and evolution of the surfactant system in fish: insights into the evolution of lungs and swim bladders. *Physiol Biochem*

Zool PBZ 77:732–749. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15547792.

91. Zwerger P., Nimeth K, Würtz J, Salvenmoser W, Pelster B (2002) Development of the swimbladder in the European eel (Anguilla anguilla). *Cell Tissue Res* 307:155–164.

92. Marty, G.D., Hinton DE, Summerfelt RC (1995) Histopathology of swimbladder noninflation in walleye (Stizostedion vitreum) larvae: role of development and inflammation. *Aquaculture* 138:35–48.

93. Prem, C., Salvenmoser W, Wurtz J, Pelster B (2000) Swim bladder gas gland cells produce surfactant: in vivo and in culture. *Am J Physiol Regul Integr Comp Physiol* 279:R2336–R2343.

94. Nüsslein-Volhard C, Dahm R (2002) *Zebrafish: a practical approach* (Oxford University Press, New York).

95. Wilkinson DG (1992) in *In In Situ Hybridization: A Practical Approach*, ed Wilkinson DG (IRL Press, Oxford), pp 75–83.

96. Wallace KN, Pack M (2003) Unique and conserved aspects of gut development in zebrafish. *Dev Biol* 255:12–29. Available at: http://cat.inist.fr/?aModele=afficheN&cpsidt=14649401 [Accessed April 9, 2013].

97. Cheng PY et al. (2008) Zebrafish cdx1b regulates expression of downstream factors of Nodal signaling during early endoderm formation. *Development* 135:941–952.

98. Carl M, Bianco IH, Bajoghli B, Aghaallaei N, Czerny T (2007) Wnt/Axin1/β-Catenin Signaling Regulates Asymmetric Nodal Activation, Elaboration, and Concordance of CNS Asymmetries. *Neuron* 55:393–405.

99. Cai KQ, Capo-Chichi CD, Rula ME, Yang DH, Xu XX (2008) Dynamic GATA6 expression in primitive endoderm formation and maturation in early mouse embryogenesis. *Dev Dyn* 237:2820–2829.

100. Morrisey EE et al. (1998) GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev* 12:3579–3590.

101. Cohen E.D. et al. (2009) Wnt signaling regulates smooth muscle precursor development in the mouse lung via a tenascin C/PDGFR pathway. *J Clin Invest* 9:2538–2549.

102. Rajagopal J. et al. (2008) Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. *Development* 135:1625–1634.

103. Shu W, Jiang YQ, Lu MM, Morrisey EE (2002) Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* 129:4831–4842.

104. DeFelice M et al. (2003) TTF-1 phosphorylation is required for peripheral lung morphogenesis, prenatal survival and tissue-specific gene expression. *J Biol Chem* 278:35574–35583.

105. Rubio S et al. (1995) Pulmonary Surfactant Protein A (SP-A) is expressed by epithelial cells of the small and large intestine. *J Biol Chem* 270:12162–12169.

106. Sofi MH, Bhatnagar A, Sapra S, Mahmood A, Majumdar S (2010) Influence of intestinal sufactant like particles on differential activation of secondary signaling molecules during Salmonella typhimurium infection. Journal. *J Food Saf* 30:455–469.

107. Finney JL, Robertson GN, McGee CA, Smith FM, Croll RP (2006) Structure and autonomic innervation of the swim bladder in the zebrafish (Danio rerio). *J Comp Neurol* 495:587–606.

108. Mendelson C (2000) Role of transcription factors in fetal lung development and surfactant protein gene expression. *Annu Rev Physiol* 62:875–915.

109. Wright JR (2004) Host defense functions of pulmonary surfactant. *Biol Neonate* 85:75–83.

110. Abouheif E (1997) Developmental genetics and homology: a hierarchical approach. *TRENDS Ecol Evol* 12:405–408.

111. Grande L (2010) An Empirical Synthetic Pattern Study of Gars (Lepisosteiformes) and Closely Related Species, Based Mostly on Skeletal Anatomy. The Resurrections of Holostei. *Am Soc Ichthyol Herpetol Spec Publ* 6:1–871; i–x.

112. Pough FH, Janis CM, Heiser JB (2008) *Vertebrate Life* (Benjamin Cummings). 8th Ed.

113. Ikeda K, Shaw-White JR, Wert S.E., Whitsett J (1996) Hepatocyte nuclear factor 3 activates transcription of thyroid transcription factor 1 in respiratory epithelial cells. *Mol Cell Biol* 16:3626–3636.

114. Perea-Gomez A., Shawlot W, Sasaki H, Behringer RR, Ang S (1999) HNF3β and Lim1 interact in the visceral endoderm to regulate primitive streak formation and anterior-posterior polarity in the mouse embryo. *Development* 126:4499–4511.

115. Zhou L, Lim L, Costa RH, Whitsett JA (1996) Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *J Histochem Cytochem* 44:1183–1193.

CHAPTER 3

THE EVOLUTION OF A DORSAL SWIMBLADDER FROM VENTRAL LUNGS INVOLVED DORSO-VENTRAL INVERSION OF REGULATORY GENE EXPRESSION[2]

Abstract

A central problem of evolutionary biology is to decipher the genetic mechanisms that create novel adaptive features characterizing major groups of organisms. New features are always modifications of existing features and thus it is important to identify the antecedents of novelties as well as expose the genetic underpinnings of their transformation. One poorly understood transformation is how the developing lungs of bony vertebrates were modified to develop as a swimbladder. While lungs and swimbladders have been considered homologous by most comparative morphologists since the mid 1800s, a major challenge to that view has been that lungs evaginate ventrally from the developing gut tube, while swimbladders evaginate dorsally. We asked whether the expression pattern of two lung genes, the dorsal-ventral patterning transcription factors *Sox2* and *Nkx2.1*, is conserved in the developing zebrafish swimbladder. We show that the dorsal-ventral expression pattern of these two mutually regulating genes is inverted in zebrafish swimbladder relative to mouse lung. Our data support the hypothesis that swimbladders are modified lungs, and that modification is linked to the inverted expression of *Sox2* and *Nkx2.1*. Our study addresses a fundamental question in vertebrate evolution, and begins to illuminate the mechanism underlying this critical innovation of nearly half

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the vertebrates. This is the second known case of dorsal-ventral inversion of gene expression associated with a major evolutionary novelty, the other being the inversion from a ventral to dorsal neurectoderm in the chordate ancestor and the underlying inversion of body-axis patterning genes.

Introduction

A fundamental goal of evolutionary biology is to understand how novel traits originate. To fully address this question, biologists must identify both the morphological antecedent of the novel structure and the developmental genetic differences that produce a new phenotype. An important morphological innovation that defines most of the 24,000 species of ray-finned fishes (1) is the swimbladder. Swimbladders are surprisingly diverse in structure and function, having been repeatedly co-opted and reshaped for an impressive array of roles in respiration, hearing, sound production, and buoyancy, making them critical to the ecology, physiology and daily life of the majority fishes (2-5).

Swimbladders have many developmental, morphological and functional similarities to tetrapod lungs. These similarities led early comparative anatomists to propose that the respiratory swimbladder was a transitional form between gill-breathing fishes and air-breathing tetrapods (6, 7). Intuitive as this progression may seem, most modern comparative anatomists accept that swimbladders are actually derived from lungs, not the reverse (2, 8-10) (Figure 1). While swimbladders and lungs have convergently evolved a number of anatomical and functional characteristics (i.e. bilateral pairing (3) and gas-exchange (2)), the dorsal shift of the

pneumatic duct connecting the swimbladder to the gut has occurred only once (Figure 1). Swimbladders always bud from the dorsal endoderm while lungs always bud from the ventral endoderm. This difference has led some to question whether swimbladders and lungs are the same organ, or rather independent derivations of the posterior pharynx (11-13).

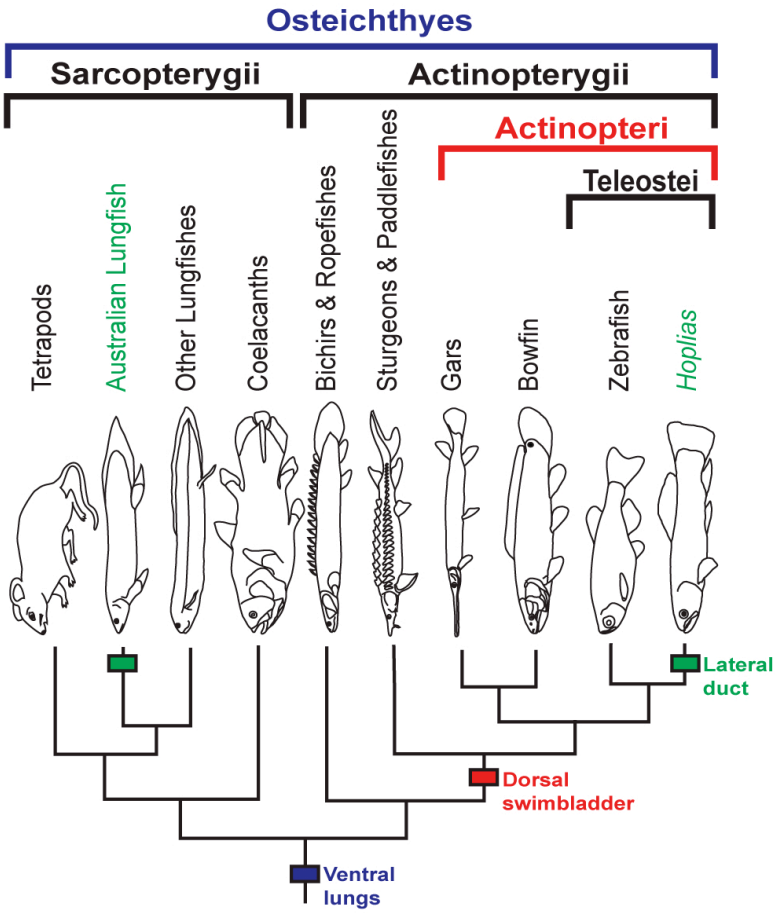

Figure 1: Phylogeny of living groups of Osteichthyes (bony vertebrates) showing distribution of lungs and swimbladders and the position of their gut connection. Phylogeny after Stiassny et al. (36) and Grande (37). Ventral lungs have long been interpreted as a character defining the Osteichthyes (3, 38); the origin of ventral budding lungs is marked by the blue bar. Nested within the Osteichthyes, the origin of the dorsal swimbladder phenotype is marked by a red bar. The Actinopteri, the sub-group of ray-finned fishes that have swimbladders, are bracketed in red. Historically, it has been hypothesized that the transformation from ventral to dorsal occurred through a series of laterally budding intermediates, such as seen in Neoceratodus (20), the Australian lungfish and Hoplias (20), a South American characiform fish, indicated in green. However, the phylogenetic position of these two taxa makes it clear that they are not intermediates, but rather have secondary modifications of either the lung or swimbladder condition. Figure modified from (16).

Recent molecular genetic data from tetrapod and fish model organisms (mouse and zebrafish) have shown that the development of lungs and swimbladders are regulated by shared signaling cascades (14, 15), and a core set of developmental regulatory genes that are co-expressed in no other organ (16), supporting the hypothesis that swimbladders are the same organ. However, to date there have been no insights into the mechanism that might transform a ventral lung into a dorsal swimbladder. Here we present gene expression data suggesting that an inversion of dorso-ventral patterning gene expression underlies the structural inversion of ventral lungs to a dorsal swimbladder.

Genetics of foregut patterning

The vertebrate foregut is patterned along both anterior-posterior and dorsal-ventral axes (17, 18). During early organogenesis in tetrapods, as exemplified by the mouse, the lung evaginates ventrally from the anterior primitive gut tube, whereas the dorsal portion takes on an esophageal fate. Throughout the anterior foregut, the SRY-related HMG box transcription factor *Sox2* is expressed. Within this *Sox2* expression domain, additional regionalizing transcription factors mark foregut organ domains. The first molecular marker of the lung bud location is *Nkx2.1* (also known as TTF-1 or T/EBP), a homeodomain transcription factor expressed only in the lung, thyroid and regions of the forebrain (19). *Nkx2.1* expression is restricted to the ventral foregut, and *Sox2* is expressed most highly in the dorsal foregut (20, 21). The upstream mechanisms responsible for establishing this pattern are not fully understood, but are known to include regulation by signaling molecules secreted from the surrounding mesenchyme (17). Specifically, BMP4 is expressed in the region surrounding the ventral foregut, and represses *Sox2* expression in this area, where noggin represses

BMP4 dorsally and allows *Sox2* expression to persist (17, 21). Mouse conditional knockdowns of BMP type 1 receptor genes lead to an expanded *Sox2* domain, a lack of *Nkx2.1* expression, tracheal agenesis and a ventral re-specification of the presumptive lung bud tissue to a dorsal esophageal fate (20). Once expressed, *Sox2* and *Nkx2.1* mutually antagonize each other and maintain regional polarity (22). *Sox2* is expressed throughout the pharynx to the mid-stomach where its expression is antagonized by *Cdx2* (18) and surrounding mesenchyme (23).

In zebrafish (*Danio rerio*) pattern formation in the foregut and specification of several major endodermal derivatives, liver, pancreas and thyroid has been studied (24) and expression of genes marking the organ domains is largely conserved with mouse. For example, *Sox2* expression in zebrafish is posteriorly restricted by expression of another Cdx transcription factor, *Cdx1b* (25). However, as in tetrapods, the upstream signaling molecules initiating these processes remain poorly understood, as are the genetic mechanisms specifying the location and identity of the swimbladder.

Given the role of *Nkx2.1* and *Sox2* interaction in D-V patterning of the mouse foregut, and the dorsal expression pattern of zebrafish *Nkx2.1b,* inverted relative to mouse *Nkx2.1* (16), we hypothesized that expression of *Sox2* in the zebrafish foregut might also be inverted at the point of swimbladder budding, demonstrating conservation of the site of evagination relative to the expression of these two important D-V patterning genes.

Results

Two copies of *Nkx2.1* exist in zebrafish, *Nkx2.1a* and *Nkx2.1b*. We examined

the expression pattern of both in a previous study (16). We showed that endodermal expression of *Nkx2.1b* posterior to the gill arches is restricted to the developing swimbladder bud starting at 4dpf (Figure 2) and that *Nkx2.1a* is expressed at the point of swimbladder evagination, but its distribution differed from *Nkx2.1b*. Whereas *Nkx2.1b* expression was restricted to the swimbladder bud, *Nkx2.1a* was expressed dorsally in the developing swimbladder, as well as in the ventral and lateral regions of the gut tube around the point of evagination (Figure 2). Endodermal expression of *Nkx2.1a* also does not begin until 4dpf (16).

Here, we examined the expression of *Sox2* in zebrafish because in mouse, *Sox2* antagonizes *Nkx2.1*. *Sox2* was detected in the pharyngeal endoderm from the posterior buccal cavity extending posteriorly to the point of swimbladder evagination. Expression throughout the dorsal and ventral endoderm was evident at 2dpf when we began sampling. As development progressed, *Sox2* expression became restricted ventrally approaching the position of the swimbladder bud; this restriction was most noticeable between 2.5 and 3dpf (Figure 2). *Sox2* expression is reciprocal to that of *Nkx2.1b*, grading from greatest expression directly opposite *Nkx2.1b* expression to least expression overlapping with *Nkx2.1b* and swimbladder bud (Figure 2).

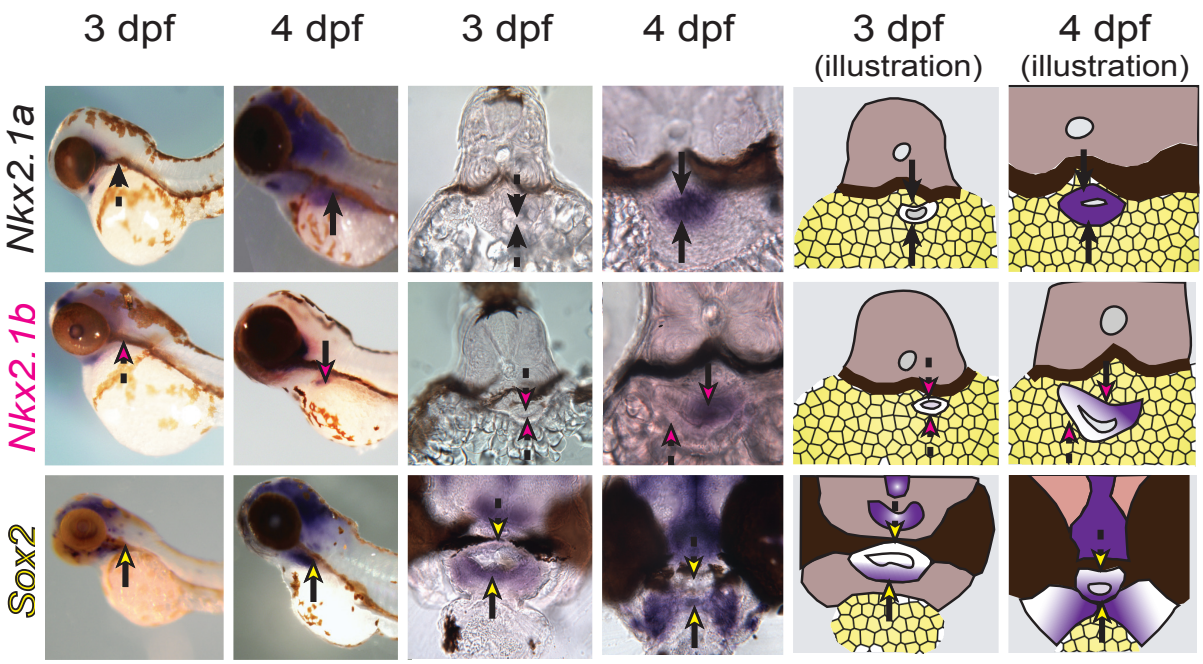

Figure 2: Whole mount *in situ* hybridization of zebrafish embryos show dorsal expression of *Nkx2.1b* and ventral expression of *Sox2*. Panels at left show samples in whole-mount lateral view. Panels at right show the same specimens in 100um transverse sections from the posterior pharynx of the larva. Solid arrows indicate expression; dashed arrows indicate lack of expression. *Nkx2.1a* patterns are indicated in black, *Nkx2.1b* patterns are indicated in pink and *Sox2* patterns are indicated in yellow (color coding continued in Figure 3). At 4dpf, *Nkx2.1a* is expressed in the dorsally developing swimbladder as well as the ventral and lateral regions of the gut tube around the point of evagination. At this same time, *Nkx2.1b* expression is localized dorsally to the point of evagination. Expression data for *Nkx2.1a* and *Nkx2.1b* reprinted from (16).

Discussion

Our results show conservation of the spatial relationship between the site of evagination, the expression pattern of an *Nkx2.1*, and its antagonist *Sox2* (Figure 3). In tetrapods, *Nkx2.1* is the earliest known marker of the lung primordium, and its expression is restricted to the ventral portion of the gut tube where the bud will form. In zebrafish, *Nkx2.1b* is expressed at the dorsal site of swimbladder evagination, though its expression is not initiated until after budding (16). The tetrapod lung and *Nkx2.1* expression domain are positioned ventrally on the gut tube. In zebrafish, both

the expression domain of *Nkx2.1b* and the swimbladder bud are inverted relative to the A-P axis of the fish, and appear in the dorsal portion of the gut tube. In mouse, *Sox2* is an important regulator of the lung *Nkx2.1* expression domain (20, 22), and is expressed in a reciprocal fashion with highest levels directly opposite of the lung bud (20). We have shown that zebrafish *Sox2* also shows this pattern, with an inversion that coincides with the inversion of the evagination site, making the *Sox2* expression

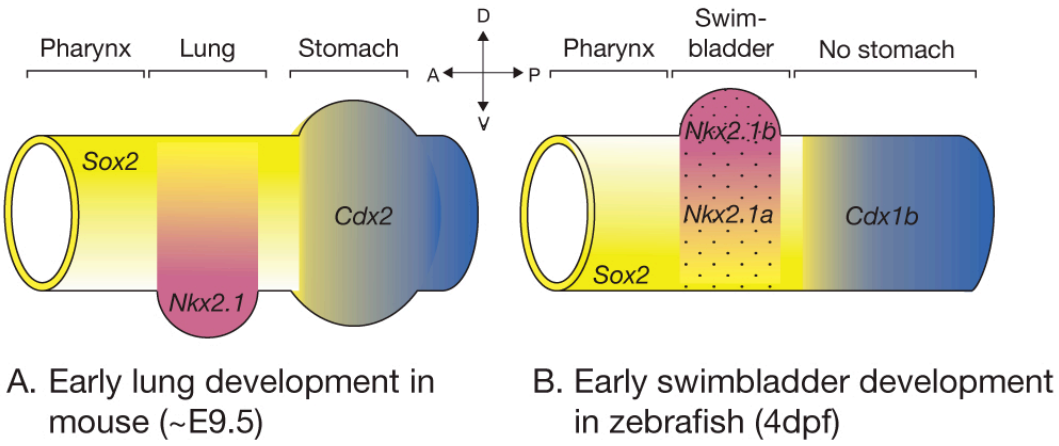

Figure 3: Schematic of known transcription factor expression domains surrounding the tetrapod lung and zebrafish swimbladder bud. In mouse, the lung bud is marked by ventrally restricted expression of Nkx2.1 (pink), which is bordered dorsally and mutually antagonized by Sox2 (yellow). In zebrafish, the swimbladder bud is inverted relative to its tetrapod counterpart, and it buds from the dorsal pharynx. As in mouse lung, the swimbladder bud is marked by expression of Nkx2.1. Zebrafish Nkx2.1a (black stippling) is expressed dorsally in the swimbladder bud as well as laterally and ventrally at the point of evagination, but both Nkx2.1b (pink) expression and the swimbladder bud are inverted to a dorsal position. Further, expression of Sox2 (yellow) is inverted relative to the mouse homolog, and its expression is concentrated ventrally instead of dorsally as in mouse. In both taxa the posterior boundary of Sox2 expression (blue) is defined by a Cdx gene (39).

highest ventrally instead of dorsally as in tetrapods (Figure 3).

Previous studies of *Sox2* expression in zebrafish endoderm have reported contradictory results. Some studies observe *Sox2* expression in the developing

esophagus and swimbladder, while others have shown expression restricted to the gut. Most recently, Yin et al. (15) proposed that *Sox2* is the earliest known marker of swimbladder development, starting at 24 hours post fertilization (hpf), though there is no morphological evidence of a swimbladder at this time. They defined a *Sox2* expression domain posterior to and separate from expression in the branchial arches that becomes continuous by 36hpf. Previously, Thisse et al. (26) and Rausch et al. (27) described *Sox2* expression through 60hpf and 96hpf respectively, and though they examined the whole larva, neither observed expression in the swimbladder. Muncan (28) reported expression in the swimbladder at 96hpf as being continuous with the expression in the pharynx, but did not address relative intensity or other stages of development. It is unclear if the variability in these results stems from technical or biological causes.

Technically, it is possible that variable results were obtained due to differences in wash stringency, probe concentration or stain development time, which would lead all studies to document the high levels of expression in the ventral endoderm, but only some to document the relatively lower levels of expression dorsally and in the swimbladder. A second technical source of variability could result from probe length and specificity. Primer and probe sequences were not supplied by all studies, so the length and region of the gene detected are unknown. Though there are no splice variants of *Sox2*, the zebrafish genome has 20 SRY-related HMG box transcription factors, and probes that bound only this conserved region could cross-hybridize with other *Sox* transcripts. Finally, the *Danio Sox2* gene is embedded in a region of non-coding DNA that is transcribed in the same direction and termed *Sox2ot* (overlapping

transcript) (29), and which could have been detected along with true *Sox2* transcripts.

Biologically, it is possible that all results are accurate representations of *Sox2* expression, but that temporal and spatial expression of this gene varies. In the mouse lung, *Sox2* expression is known to be cyclic and transient (30), and expression may vary temporally in zebrafish too.

Importantly, and regardless of the cause of reported variation in *Sox2* expression, all data are consistent with an inversion of regulatory gene expression in zebrafish relative to mouse. It is likely that the disputed regions, the dorsal pharynx and swimbladder bud, express *Sox2* at a significantly lower level than regions that are consistently observed in all studies. There is agreement that *Sox2* is expressed in the ventral portion of the pharynx up to the point of evagination, which supports our observation that *Sox2* is most highly expressed here. Similarly, in mouse, *Sox2* is partially antagonized by the expression of *Nkx2.1* and *Cdx2*, and low levels are expressed intermittently in the developing lung and stomach (20, 22). For this reason, some expression within the swimbladder, or a posteriorly overlapping expression domain with *Cdx1b* is consistent with inversion.

Though *Nkx2.1* has been implicated in the ventral restriction of *Sox2* expression in mouse, in zebrafish *Nkx2.1b* expression in the endoderm is not initiated until 4dpf, after the swimbladder has budded (36-48hpf (14)), and *Sox2* ventralization has occurred (Figure 2). For this reason it is clear that another factor is controlling the initial ventral concentration of *Sox2* expression. The upstream initiators of endodermal D-V patterning are not fully understood in any taxon, making it is difficult to propose mechanisms generating ventral restriction independent of *Nkx2.1*

antagonism. Because expression of *Nkx2.1a* and *Nkx2.1b* is not initiated until after the swimbladder buds, and because the mouse *Nkx2.1* null still forms a properly located lung, we propose that it is not the presence of *Nkx2.1* expression that specifies the location of evagination, but rather a relatively reduced level of *Sox2* expression, or the upstream mechanisms for which *Sox2* expression is a proximate marker.

The presence of an air-filled organ, whether lungs or swimbladder, characterizes the bony vertebrates (Osteichthyes), and most evolutionary morphologists accept that the swimbladder is derived from ancestral lungs. The most critical sticking point for those who have not accepted this hypothesis is that lungs evaginate ventrally from the gut and swimbladders evaginate dorsally, and that there is no ontogenetic or phylogenetic evidence of migration of the air-filled organ from a ventral to dorsal position; it is either dorsal or ventral, never lateral in any transitional taxon. The several cases of slightly lateral swimbladder evagination occur in relatively derived taxa (Figure 1). Due to the conserved and inverted pattern of expression of two critical D-V patterning genes in the pharynx of the zebrafish swimbladder relative to their expression in the developing tetrapod pharynx, we propose that an inversion of the ancestral genetic patterning mechanism is responsible for the transition of the ancestrally ventral lung to a dorsal swimbladder in the subgroup of the ray-finned fishes, the Actinopteri (Figure 1). Further functional studies demonstrating the interactions of these gene products and a better understanding of the upstream patterning mechanisms in both fishes and tetrapods will help to clarify the mechanism of this transition.

Only one other case of dorso-ventral inversion of morphology and patterning

gene expression is known: the transformation from a ventrally located neurectoderm in non-chordate bilaterians to a dorsal position in chordates (31, 32). The inversion reported here, that underlies the lung-to-swimbladder transformation differs from the prior case in two ways. First, only a single structure is inverted, rather than the whole body plan. Second, inversion of these patterning genes (*Sox2* and *Nkx2.1b*) occurs much later in development. These fundamental differences show that inversion can be a mechanistically simple, but powerful, generator of novelty dramatic across a spectrum of tissue types and developmental stages.

Methods

Zebrafish culture

Zebrafish were kept in Aquatic Ecosystems re-circulating racks, fed twice daily on brine shrimp and bred according to protocols outlined by Nüsslein-Volhard and Dahm (2002). After fertilization, eggs were collected and reared in mesh tubes in the recirculating rack system. Beginning at 24 hours, embryos were collected in 6 hour intervals, fixed in 4% Paraformaldehyde in 1X PBS overnight, then transferred to 100% MeOH and stored at -20°C until hybridization.

Whole-mount *in situ* hybridization

RNA probes for *in situ* hybridization were synthesized using the Ambion Maxiscript in vitro transcription kit and Roche digoxygenin-labelled UTP nucleotide mix via supplier protocols. *In situ* hybridization was conducted on the InSituProVS fluid-handling robot (Intavis) using a protocol modified from Wilkinson (1992). Embryo digestion was conducted at concentration of 10mg/ml Protenase K for 15

minutes at 37°C. A 20-minute incubation in RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma-Aldrich) was added for additional permiablization. Specimens were pre-hybridized in hybridization buffer with 100ug/ml yeast tRNA (Sigma-Aldrich) for 3 hours at 68°C. Probe solution contained ~10ug probe in 500ul hybridization buffer with 100ug/ml tRNA and an additional 500ug/ml salmon sperm DNA per robot well. Specimens were then treated with RNase cocktail (Invitrogen) and blocked in 5% Blocking Solution (Roche) in 1x maleic acid buffer (MAB). Anti-digoxigenin-AP Fab fragments (Roche) were diluted 1:5000 in 5% Roche Blocking Solution, and washes were conducted in .2X SSC and stained at room temperature in BM Purple (Roche) alkaline phosphatase, until developed. Multiple rounds of *in situ* hybridization with replicates of each probe and stage combination were run to ensure consistent results.

To determine the dorsal-ventral pattern of gene expression, whole-mounts were photographed, and well-stained representatives of each stage and probe were embedded in 4% low-melting point agar for sectioning. 100μm sections were cut on a Micro-Cut H1200 vibrating microtome, slide-mounted in glycerol and immediately photographed.

Whole mounts were imaged using an Olympus XZX16 dissecting microscope with an Olympus DP25 camera. Images were acquired in 8-bit RGB and managed in cellSens Entry software. Sections were photographed on a Leitz Diaplan compound scope at 25x magnification with an Optronix Magnafire SP camera with 10-bit RGB acquisition.

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REFERENCES

1. Nelson JS (2006) *Fishes of the world*, (Wiley-Blackwell, Hoboken, NJ), pp 624.

2. Graham JB (1997) *Air-Breathing Fishes: Evolution, Diversity, and Adaptation,* (Academic Press, New York), pp 299.

3. Rice AN & Bass AH (2009) Novel vocal repertoire and paired swimbladders of the three-spined toadfish, batrachomoeus trispinosus: Insights into the diversity of the batrachoididae. *Journal of Experimental Biology* 212(9): 1377-1391.

4. McCune AR & Carlson RL (2004) Twenty ways to lose your bladder: Common natural mutants in zebrafish and widespread convergence of swim bladder loss among teleost fishes. *Evolution and Development* 6(4): 246-259.

5. Helfman G, Collette B, Facey D & Bowen BW (2009) *The Diversity of Fishes: Biology, Evolution and Ecology,* (Wiley-Blackwell, Hoboken, NJ), pp 720.

6. Owen R (1846) *Lectures on the comparative anatomy and physiology of the vertebrate animals,* (Longman, Brown, Green, and Longmans, London), pp 308.

7. Darwin C (1859) *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. (John Murray, Oxford), pp 502.

8. Dean B (1895) *Fishes, living and fossil,* (Macmillan, New York), pp 300.

9. Goodrich ES (1930) *Studies on the structure \& development of vertebrates,* (Macmillan and Co. Ltd, London), pp 837.

10. Romer AS (1970) *The vertebrate body,* (Saunders, Philadelphia), pp 679.

11. Sullivan LC, Daniels CB, Phillips ID, Orgeig S & Whitsett JA (1998) Conservation of surfactant protein A: Evidence for a single origin for vertebrate pulmonary surfactant. *Journal of Molecular Evolution* 46(2): 131-138.

12. Perry SF, Wilson RJ, Straus C, Harris MB & Remmers JE (2001) Which came first, the lung or the breath?. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* 129(1): 37-47.

13. Daniels CB*, et al* (2004) The origin and evolution of the surfactant system in fish: Insights into the evolution of lungs and swim bladders. *Physiological and Biochemical Zoology* 77(5): 732-749.

14. Winata CL*, et al* (2009) Development of zebrafish swimbladder: The requirement of hedgehog signaling in specification and organization of the three tissue layers. *Developmental Biology* 331(2): 222-236.

15. Yin A, Korzh S, Winata CL, Korzh V & Gong Z (2011) Wnt signaling is required for early development of zebrafish swimbladder. *PloS One* 6(3): e18431.

16. Cass AN, Servetnick MD & McCune AR (2013) Expression of a lung developmental cassette in the adult and developing zebrafish swimbladder. *Evolution and Development* 15(2): 119-132.

17. Morrisey EE & Hogan BLM (2010) Preparing for the first breath: Genetic and cellular mechanisms in lung development. *Developmental Cell* 18(1): 8-23.

18. Zorn AM & Wells JM (2009) Vertebrate endoderm development and organ formation. *Annual Review of Cell and Developmental Biology* 25(1): 221-251.

19. Kimura S*, et al* (1996) The T/ebp null mouse: Thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes & Development* 10(1): 60-69.

20. Domyan ET*, et al* (2011) Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Development* 138(5): 971-981.

21. Que J, Choi M, Ziel JW, Klingensmith J & Hogan BLM (2006) Morphogenesis of the trachea and esophagus: Current players and new roles for noggin and bmps. *Development* 74(7): 422-437.

22. Que J, Luo X, Schwartz RJ & Hogan BLM (2009) Multiple roles for Sox2 in the developing and adult mouse trachea. *Development* 136(11): 1899-1907.

23. Ishii Y, Rex M, Scotting PJ & Yasugi S (1998) Region-specific expression of chicken Sox2 in the developing gut and lung epithelium: Regulation by epithelial-mesenchymal interactions. *Developmental Dynamics an Official Publication of the American Association of Anatomists* 213(4): 464-475.

24. Ober Ea, Field Ha & Stainier DYR (2003) From endoderm formation to liver and pancreas development in zebrafish. *Mechanisms of Development* 120(1): 5-18.

25. Flores MVC*, et al* (2008) Intestinal differentiation in zebrafish requires Cdx1b, a functional equivalent of mammalian Cdx2. *Gastroenterology* 135(5): 1665-1675.

26. Thisse B*, et al* (2001) Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402)

27. Rauch GJ*, et al* (2003) Submission and curation of gene expression data

28. Muncan V*, et al* (2007) T-cell factor 4 (Tcf7l2) maintains proliferative compartments in zebrafish intestine. *EMBO Reports* 8(10): 966-973.

29. Amaral PP*, et al* (2009) Complex architecture and regulated expression of the Sox2ot locus during vertebrate development. *RNA* 15(11): 2013-2027.

30. Gontan C, *et al* (2008) Sox2 is important for two crucial processes in lung development: Branching morphogenesis and epithelial cell differentiation. *Developmental Biology* 317(1): 296-309.

31. De Robertis E.M. & Sasai Y (1996) A common groundplan for dorsoventral patterning in bilateria. *Nature* 380: 37-40.

32. Nübler-Jung K & Arendt D (1994) Is ventral in insects dorsal in vertebrates?. *Roux's Archives of Developmental Biology* 203: 357-366.

33. Stiassny MLJ, Wiley EO, Johnson GD & de Carvalho MR (2004) in *Assembling The Tree of Life,* eds Cracraft J & Donoghue MJ (Oxford University Press, New York), pp 410-429.

34. Grande L (2010) An empirical synthetic pattern study of gars (lepisosteiformes) and closely related species, based mostly on skeletal anatomy. the resurrections of holostei. *American Society of Ichthyologists and Herpetologists Special Publication* 6: 1-871; --.

35. Rosen DE, Forey PL, Gardiner BG & Patterson. C (1981) Lungfishes, tetrapods, paleontology, and plesiomorphy. *Bulletin of the American Museum of Natural History* 167: 163-275.

36. Wallace KN & Pack M Unique and conserved aspects of gut development in zebrafish255(1): 12-29.