Limbs undeniably give us the greatest ability to do things. Our legs provide us with the locomotion to move. Whether for running, climbing or swimming through the water, our limbs help us to traverse sometimes great distances. The importance of our limbs is also self-evident in how they enable unique interaction between individuals such as in fighting off an enemy or supporting your loved one with a warm embrace. Most important is the utility of our hands and fingers. The form and function of our digits are so versatile that they help us to carry out the many dexterous tasks needed for our work and needed to catch and grow and prepare the foods that keep us healthy.

But what of this trout? Its fins certainly provide locomotion to the water. Are this trout's pectoral fins analogous to the human hands holding it? Developmental biologists have been long fascinated with limb development, in part due to the obvious homology that exists between the limbs of different vertebrate species. The curiosity lies in the hope that understanding the evolutionary history of the development of fish fins to the primate's opposable thumb might reveal how terrestrial tetrapods came to be.

This dev tutorial is designed to give you a basic understanding of the different elements involved in limb development, namely identifying the major structures of the embryonic limb; how these different structures contribute to the induction, growth and patterning of the forelimb and hindlimb; and introduce you to some of the molecular mechanisms governing this process. It is important to note that like pretty much all aspects of embryonic development, the story is much more complicated. And this reality is even more evident in the case of the limb where a long history of concerted research focus has provided a great many insights into the mechanisms of limb development. Therefore, I hope this introductory tutorial will make it easier for you to delve deeper into the complexities of limb development during your studies in and out of class.

Let's begin with a bit of anatomy. Literally put your own arms in "anatomical position" with your palms facing forward and thumbs up. First we need to identify the axes that define our limbs. Our limbs are appendages, off of the main body axis and as such possess a directionality away from the truck. We call this the proximal to distal axis. The

next two axes you're likely familiar with, the anterior to posterior and dorsal to ventral. In this position, your thumbs are pointed towards your head and are considered the anterior, while your pinky is posterior. Additionally, the backside of your arm and your knuckles are all dorsal; and conversely your palms ventral. Now let's go to an illustration to reveal the different elements that build the limb across these axes.

The skeletal elements of the forelimb nicely delineated three morphologically distinct regions that run along the proximal to distal axis such that the humerus represents the most proximal region known as the stylopod, which is followed more distally by the zeugopod, constituting the radius and ulnar bone; and lastly the autopod or hand with its carpals and phalanges, phalanges being the digits or fingers. Despite potentially dramatic differences in the skeletal elements of limbs from different species, the use of the stylopod, zeugopod and autopod can usually be defined no matter whether it's the chick's wing with only three digits or the horse's hoof, which is its only remaining digit. What developmental mechanisms do you think might be influencing the establishment of distinctly different skeletal elements along a specific axis? Do you recall a similar pattern anywhere else in the embryo and what processes governed its formation?

While I am sure many could come to mind, the determination of tissue identity along an axis is profoundly associated with hox genes. Just like the vertebral elements along anterior to posterior axis of the body, the hox family of transcription factors also control sulfates in the developing limb. Importantly, the rule of collinearity seems to continue to operate but along the proximal to distal axis. Collinearity, recall, is the predominant correlation of the order of hox gene expression along a given axis of the embryo, matching the three prime to five prime position of these genes along the chromosome. In both the forelimb and hindlimb, more three prime hox parallel groups are expressed more proximal; whereas more five prime position hox genes are sequentially expressed in more distal tissues of the limb. As you might predict, loss of these hox genes lead to severe limb deformities in mouse and humans. As in the trunk, collinear hox gene regulation does still operate along the anterior to posterior axis of the limb, but a significant difference exists that I will elaborate on later. Now you know the type of

elements that build a limb ultimately require proper hox gene expression. Thus, the next question is what developmental mechanisms function to set up this pattern of hox expression? To get at this larger question, I would like to describe limb development in two major events, the first being limb bud induction and outgrowth, and the second event focus on the mechanisms controlling cell fate specification in the limb. What does inducing a limb to form even mean?

Well, the amazing thing about limbs is that they seemingly form from nothing. First bulges or buds raise off of the trunk in the presumptive locations of the forelimbs and hindlimbs. These limb buds continue to grow off of the truck, after which digits begin to become visible in the distal most portion of the elongated bud. From there the limb continues to grow in chondrogenesis, bone formation, muscle differentiation and nerve and blood vessel infiltration all commence. When needs to happen for this bud to emerge and grow? Think about it. What sorts of cell behaviors would lead to an enlargement of the focal area of the epithelium? If you contemplate in making more cells through proliferation, then you're right. If you thought of more cells moving into the presumptive limb field, then you are also right. Mesenchymal cells from the lateral plate mesoderm and from the hypaxial myotome actively migrate toward the limb field to create a heterogeneous mass of proliferative bone and muscle progenitor cells.

Now important for understanding how this bud forms is knowing that the bud itself has its own anatomy. All of the axes that we identified on your own adult arms are present even in the little limb bud. It has dorsal and ventral halves, as well as anterior and posterior halves. And relative to the flank of the trunk, it's proximal to distal axis extends as the limb bud grows. The limb bud can also be subdivided into three different parts of significance, the first being a structurally distinct ridge of epithelium located at the most distal extent of the limb bud where it perfectly bisects the dorsal and ventral halve of the bud. This structure is called the apical ectodermal ridge, or AER for short. The massive progenitor cells building underneath the AER is called the progress zone, and the last distinct region of the limb bud is restricted to a small portion of the posterior most bud, which was identified not because of a physical distinction but rather due to its unique

functional activity. When this region was ectopically transplanted to the anterior side of the chick forelimb bud, it resulted in the dramatic mirror image duplication of its digits. Yeah, that actually happened. And for this reason, it was aptly termed the zone of polarizing activity, or ZPA. I will address the nature of this activity later in the tutorial. These three parts: the AER, the progress zone and the ZPA all represent regions with the central roles in the development of the limb. You just saw what the ZPA was capable of. If you remove the AER from limb bud, it will cease to develop in a stage-dependent manner. If you replace the underlying mesenchyme of the forelimb bud with, say, hindlimb-derived mesenchyme, then a leg will form instead of an arm. These results suggest that the AER is necessary for limb outgrowth, and the progress zone is necessary for proper limb type specification.

I will review at this time that each of these limb bud domains are molecularly interacting with each in a positive manner, to drive the induction and sustained outgrowth of the limb bud. But where? How is the axial location of the limb bud growth initiated? To answer this question, we have to return to the trunk of the embryo. Have you learned about any powerful signaling systems that may already be operating along the, say, rostral or the caudal axis of the embryo? If you have researched somatogenesis even a little bit, then you would know that retinoic acid and fibroblast growth factor 8 have opposing gradients of expression along this axis and directly antagonize each other for the purpose of forming segments. Interestingly, FGF-8 is also expressed by cardiac mesoderm and the heart, which results in the graded restriction of retinoic acid activity. It is at this highest concentration of retinoic acid where the forelimb field is initiated.

Currently, there is less confidence in exactly how the hindlimb field is initially determined, but we do know quite a bit about the downstream molecular mediators of limb bud initiation for both the forelimb and hindlimb regions. Like any good developmental biologist, the first step would be to find which genes show early expression in the presumptive limb bud fields. The transcription factors Tbx5 and Tbx4 were found to exhibit these expression characteristics, with Tbx5 specifically expressing the forelimb field and Tbx4 in the hindlimb field. At this molecular level, it is not

surprising that there are some species' differences emerging. For instance, while Tbx4 is the main transcriptional inducer in the chick hindlimb, islet one appears to serve this inductive role for the mouse hindlimb. Whether forelimb or hindlimb, mouse or chick, they all lead to the expression of fibroblast growth factor 10 in the developing progress. This FGF-10 expression promotes proliferation and the maintenance of the different transcriptional regulation.

Previously I mentioned that this mesenchymal proliferation is influenced by interactions of the apical ectodermal ridge. Signaling from the AER to the progress zone is mediated by a different member of the fibroblast growth factor family but one you have met before, FGF-8. FGF-8 is only expressed in the AER of the limb bud and it alone can compensate for the removal of the AER and promote normal limb development. Further dramatic evidence of the inductive powers of FGF-8 and limb outgrowth is demonstrated by the administration of ectopic FGF-8 protein to the body wall between the limb fields. This results in the induction of an ectoptic limb with chimeric identity such that the more anterior half of this ectopic limb expresses Tbx5 and develops into a wing, while the posterior half expresses Tbx4 and adopts leg identity.

To summarize, limb bud initiation and outgrowth, the forelimb field is determined by the highest concentration of axial retinoic acid, which induces Tbx5 expression and forelimb specification. Tbx4 or islet one are required for hindlimb field determination, however FGF-10 ultimately drives proliferation of the progress zone for both limb fields. FGF-8 in the AER is critical for the sustained outgrowth of the limb bud through the establishment of a positive feedback loop of signaling between FGF-10 and FGF-8 and WNT3A. Now we have made a limb bud and placed it in the correct position along the body. We still need to determine how the correct cell identities are established along the different axes of the limb.

How is that sequential pattern of hox gene expression orchestrated along these axes to build the correct elements? One important principle about developmental biology is that it's kind of like a broken record but broken in the right way. What I mean is that the

embryo steals mechanisms that worked before and repeats, to some extent, in an organ-specific way. In the case of the limb, the embryo has co-opted the mechanisms of known morphogenetic signaling systems. The antagonistic relationship of retinoic acid and FGF-8 that we refered to earlier in the trunk is replicated along the proximal to distal axis, while the limb utilizes a posterior source of sonic hedgehog for anterior to posterior fates, and then when some BMP morphogens or the differences between dorsal and ventral development.

As I alluded to earlier, this can quickly get complex largely due to the challenge of deciphering how each of these signaling systems is integrated together for the coordinated development of the limb. While you keep that reality in mind, for simplicity I would like to still explore each of these mechanisms in more detail, but as separate parts. As the limb bud grows, cell fates along the proximal to distal axes are predominantly influenced by a proximal source of retinoic acid in the trunk and the opposing distal most expression of FGF-8 from the apical ectodermal ridge. The model posits that these opposing antagonistic signals establish two critical thresholds of specification resulting in differential hox gene expression.

Two independent groups showed an ectopic limb grafting paradigm that the administering of retinoic acid was capable of promoting more proximal cell identities, while adding FGF fostered only more distal hox gene expression. How are anterior to posterior fates determined? Well, do you recall that amazing ZPA transplant experiment that caused the mirror image digit duplication? That has everything to do with understanding anterior-posterior cell specification. This ZPA results presented a perfect opportunity to apply our developmental bio experimental design of find it, move it and use it. Sonic hedgehog was discovered to be solely expressed in the ZPA domain. Have you been acquainted with the function of the sonic hedgehog protein before this tutorial? Can you recall another part of the embryo where sonic hedgehog influenced cell fate determination? Sonic hedgehog is one of the most well-known morphogens for its role in the developing spinal cord, where its spatial and temporal graded secretion leads to differential gene expression along the ventral to dorsal axis of the neural tube.

This expression of sonic hedgehog on the anterior side of the limb bud produces the very same mirror image duplication, suggesting sonic hedgehog is sufficient for the activity of the ZPA. Amazingly, natural mutations in a sonic hedgehog enhancer were found in mouse, cats and humans that all cause the similar misexpression of sonic hedgehog on the anterior side of the limb bud. Consequently, this individual has showed digit duplications.

These results support the notion that sonic hedgehog is also functioning as a morphogen in the limb bud. Increasing the concentration of sonic hedgehog on the anterior side ends up inducing posterior digits with a gradiation to the middle of the autopod, thus creating a mirror image duplication. Does sonic hedgehog influence hox gene expression along the anterior-posterior axis? Remember I mentioned hox collinearity does apply to this axis, but there was an important difference. That difference is due to the effects of sonic hedgehog signaling on a different hox's regulatory enhancer element. The sonic hedgehog responsive global control region enhancers are found on the five prime side of the HoxD cluster of genes, which cause an inversion in HoxD expression patterns. It literally flips the hox gene expression along the anterior to posterior axis in the late limb bud. The interaction of sonic hedgehog signaling with the HoxD cluster was demonstrated with careful combinatorial genetic experiments by showing how five prime hox genes functionally interact with the downstream sonic hedgehog transcription factor GLI3 to influence digit number in a dose-dependent manner.

The last axis of specification to attend with is along the dorsal to ventral axis. There is a clear difference between the two sides of the axis with knuckles and footpads developing on the dorsal and ventral sides of the mouse paw, respectively. It was discovered that loss of the transcription factor LMX1B resulted in a transformation of the mouse's knuckles into duplicated foot pad on the presumptive dorsal side. This was an amazing transformation of cell fates due to this one transcription factor. Predictably, LMX1B is normally only expressed on the dorsal side of the limb bud. LMX1B is induced by the dorsally restricted WNT7A in the apical ectodermal ridge; whereas mesenchymal

bone morphogenic protein signaling appears to be promoting ventral fates in the limb bud. As I warned you earlier, all of these different signaling systems about every axis are intimately interacting with temporal dynamics to coordinate limb bud initiation, growth, differentiation and ultimately its termination.

I really have not spent much time discussing the many ways in which BMP signaling influences limb development. Like its celebrated role in triggering the programmed cell death of inter digit cells to remove the embryonic webbing between fingers, a property of BMP signaling in the autopod that is inhibited by Gremlin in the duck foot to maintain its webbing for paddling in the water. Although it is an important feature of BMP signaling, I decided to wait until the end of this tutorial to bring up BMPs so that you remember they are responsible for stopping limb bud outgrowth, BMP's function to inhibit FGF-8 in the AER, which like in the duck's foot, is kept in check by a mesenchymally expressed Gremlin. Gremlin expression is upregulated by sonic hedgehog, and the early positive feedback loops between FGF-8, WNT3 and FGF-10 and sonic hedgehog, all lead to the continued propagation of the limb. However, as the expression of FGF-8 climbs to a higher concentration, it hits a threshold for Gremlin downregulation. Therefore, as the limb bud grows, Gremlin expression decreases, as well as physically becomes gradually distant from the AER, which reduces Gremlin's repression of BMP, freeing BMP to inhibit FGF-8 and putting the brakes on limb bud outgrowth. It is perhaps easy to imagine the morphological connections between the development of the chick and duck autopods. Perhaps even their connection with the human hand.

Yet more obtuse is seeing the evolutionary history that led to the emergence of tetrapod limbs. Have you noticed the images of the developing fish fin in the background? We began this dev tutorial highlighting the functional advantages limbs provide to humans and fish alike. Now there are obvious differences between fish fins with its rays and the human limb lacking rays but possessing these digity things. Nevertheless, is it possible that today's terrestrial vertebrates are derived from an ancient aquatic creature that up and walked out of the water? Here's where I would cue Tiktaalik, to walk on the stage if

it were living. Neil Shubin's group discovered the fossils of this ancient flat headed lobe finned fish which revealed fins with skeletal elements homologous in both form and function to that of a jointed shoulder, radius and ulna and wrist, all of which would be necessary for limb-like postures to support movement about a surface. Phylogenetically, Tiktaalik sits right at the transition between fish and primitive tetrapods. This was one of the greatest discoveries to fuel our understanding of tetrapod evolution.

Can developmental biology confirm what Tiktaalik appears to be suggesting? Importantly, fish fin bud development shows the presence of all the same major signaling systems we discussed earlier: the FGF, sonic hedgehog and BMPs, all similarly positioned in time and space as is the same proximal to distal hox gene correspondence. In fact, several labs have now shown that conserved enhancer regions for proximal or distal hox genes in fish are capable of functionally driving similar expression patterns in the mouse limb bud. So the answer is yes, tetrapod limbs are in fact derived from fish fins. I hope you have enjoyed this dev tutorial on limb development and encourage you to use those limbs of yours. Raise a hand and ask a question about development.