Many aspects of the world around us are based on patterns of repeated structures. From the physical repetition of a mountain range, or the trees that cover them, to the sequential construction of buildings, and the separate compartments of a train, segmentation of a common form enables the continued elaboration of that structure's successful function. How is repetition used during the development of living things? I am sure you see evidence of segmentation in the life all around you, such as the repeated growth of fern leaves, defined by the rules of apical dominance, or as clearly evident in the separated and evenly sized segments of this orange. Do such segments exist in animals?

Current research suggests that segmentation of the animal body plan evolved once from a common ancestor of arthropods, annelids, and vertebrates. Unlike the earthworm and scorpion whose segmentation is outwardly visible, segmentation in the giraffe is a little harder to imagine. However, if we were to examine its skeleton, then the separate units of bones from its most interior skull to the progressively more posterior cervical, thoracic, or lumbar vertebrae reveal an obvious segmental pattern. From an evolutionary perspective, segmentation of the body plan has provided smaller, repeated units of tissue for which new structural and functional diversification could emerge over the course of an evolutionary timescale.

The question and focus for this Dev Tutorial is how segments are formed in the vertebrate embryo, a process called somitogenesis. How can changes in the developmental mechanisms controlling somitogenesis give rise to some 300 vertebrae in the snake yet only around 30 in humans? Creation of segments in vertebrates occurs during embryonic development when the paraxial mesoderm located on either sides of the midline position neural tube and notochord divide up into repeated blocks of tissue. These blocks are called somites. Hence, the name for the process being somitogenesis.

Let's take a moment to define the tissue undergoing somite formation, that of the paraxial mesoderm. The paraxial mesoderm sits bilaterally adjacent to the notochord and neural tube, whereas the intermediate and lateral plate mesoderm tissues occupy

the positions lateral to the paraxial mesoderm. Unlike the kidneys and circulatory system that are derived from the intermediate and lateral plate mesoderm, the paraxial mesoderm gives rise to the somite, and cells of the somite will differentiate into cartilage and bone of the vertebrae, tendon and skeletal muscle, as well as contributions to the dorsal aorta.

Upon initial patterning, the somite can be divided into two specific regions. The more dorsolateral region is called the dermomyotome, and it generates the skeletal muscle and dermis of the back, body wall, and limbs. The opposing ventromedial region of the somite is the sclerotome, and, at least in amniotes, it constitutes a vast majority of the somite as compared to the dermomyotome. The sclerotome will contribute cells to a great diversity of different tissues. The sclerotome bordering the dermomyotome is termed the syndetome, and it will make tendons. In contrast, a large contribution from the central portion of the sclerotome is called the arthrotome, which creates several portions of the vertebral joints. In addition, the sclerotome found in the most ventromedial location will give rise to the vertebral body.

However, the distal portions of the ribs are derived from cells in the most lateral portion of the sclerotome. Yet, the most dorsomedial cells produce the spines and arch of the vertebrae. Lastly, the ventral sclerotome actually provides endothelial precursor cells to help build the dorsal aorta. Thus, the somite will generate many vitally important cell types, cell types that will differ in their final derived structures, depending on their sequential position along the anterior to posterior axis.

We will cover the development of the segment identity and somite differentiation in another Dev Tutorial. However, here, I want to focus on the basic mechanisms governing somite formation. How is a somite formed? What dictates the size of a somite, or how many somites a particular species may exhibit? Well, I think the best way to start to understand this process of somitogenesis is to simply watch it happen. Time-lapse microscopy has enabled researchers to capture the process by which the paraxial mesoderm becomes segmented. The first movie I will play is of a chick embryo,

and this movie was created by the Pourquie Laboratory. It shows this developing chick embryo from a dorsal view. As this movie loops several times, please, watch the progression of somite formation in this embryo very carefully. The second time-lapse movie was produced by Rolf Karlstrom and Don Kane, which is of a lateral view of the zebrafish embryo undergoing segmentation of its paraxial mesoderm. Again, please take a moment to truly admire the beautiful process that is somitogenesis. What do you see happening?

Now, as I show both of these movies to you again, I will give you 60 uninterrupted seconds to contemplate the following questions. Where are segments forming? Is there a directionality to their creation? Do they appear to be the same size upon formation, and is there any temporal pattern to their formation? Lastly and importantly, is anything else moving during segmentation? And five, four, three, two, one, your minute of thought is up. What did you come up with? Somites are forming where?

They are first forming in the anterior of the embryo. And progressively, segmentation occurs in the posterior direction. And amazingly, I hope you agree that at the point of formation, each somite does appear quite similar in size, and created in about the same amount of time. But what about the last question? Was anything else moving? Well, if you studied it carefully, you might have noticed that the entire tail of both the chick and fish grew longer as new somites formed. Although, due to the near entirety of somitogenesis captured in the movie of the chick, you can see that eventually, the tail stops growing and segmentation catches up, which terminates both somite formation and trunk elongation. Isn't it just inspiring what you can learn by simply watching the embryo develop?

It reminds me of an inspirational quote by one of the greatest chicken embryologists, Viktor Hamburger, who said, "Our real teacher has been and still is the embryo, "who is, incidentally, "the only teacher who is always right." All right, back to the embryo. And let's make sure you understand some of the anatomical differences of the paraxial mesoderm that exists along the anterior to posterior axis over the course of

somitogenesis. Here again, is our scanning electron micrograph image of a chick embryo's somites. Four to five balled up somites are beautifully seen on each side of the neural tube. Interestingly, positioned more posterior is the segmental plate, which constitutes the paraxial mesoderm that has not yet become subdivided into somites. This region can also be referred to as the presomitic mesoderm.

Recall that we concluded somite formation occurs in an anterior to posterior progression, which also suggests that more anterior somites are temporally older, or more mature, than the more posteriorly positioned somites. To be able to discuss a somite relative to its age, the developmental biology community has adopted a nomenclature for naming each bilateral somite pair. The most recently formed somite is designated S1, and each successively more anterior somite is S2, S3, S4, S5, and so on. Most importantly, is the naming of the cells that make up the segmental plate into something called somitomeres, or those regions destined to generate a future somite.

Although no clear boundaries are visible in the presomitic mesoderm, based on the distance from the last formed somite, meaning from the posterior border of S1, we can delineate the next to form somite, or somitomere, which would be S0. Just posterior to that somitomere would be S minus one, then S minus two, and so on. Now that we know some anatomy, let's return to the process of somitogenesis and ask, what is actually happening when a somite boundary forms in the segmental plate? In other words, how do those specific cells at the presumptive fissure region change to create the new posterior border of somite one and a new anterior border of somitomere zero?

In this movie of zebrafish somitogenesis by the Holley Lab, you can see cell membranes labeled in green and the nuclei red. Now, take careful note of how it starts as two rows of seemingly intermixed mesenchymal cells, within which successive and evenly spaced boundaries magically form. What sort of cell behavior do you think is happening at the locations of boundary formation? Well, let's consider what a boundary is. Like these two separate peaks, a boundary could be the physical separation between two significantly rigid structures. The early formed somite is in fact a rigid block of

epithelial cells, as seen here, pseudo-colored blue in this scanning electron micrograph of a chick cross section. And a true separation does exist between the newly opposing somite borders, a space that is filled by important extracellular matrix components, such as fibronectin. So have you figured out what the key cell behavior is?

The paraxial mesoderm starts off as a population of mesenchymal cells, and then these cells become an epithelial cell of the somite. Thus, somite boundary formation is physically created by a mesenchymal to epithelial transition. The cells that happen to find themselves at the presumptive boundary location will change from loose mesenchyme to tightly held epithelial border cells. Examination of gene expression patterns has revealed the Eph and ephrin signaling system is present at the right time and place to influence epithelialization. Here, in this in situ hybridization for EphA4 expression, you see that EphA4 specifically gets upregulated within the anterior half of newly formed somites. The mesenchymal to epithelial transition is triggered by the reciprocal signaling interactions between EphA4 receptors in the anterior half of somites with their ephrin ligands that are restricted to the posterior half of somites. Now, importantly, this leads to an ephrin-mediated fibronectin-rich extracellular matrix, which is critical for proper somite formation. Therefore, Eph-ephrin signaling plays a major role in regulating the structural properties essential for epithelialization of the segmental plate into somite boundaries.

I covered only a brief overview of epithelialization so that we might have further time to investigate the principles governing more of the where and when of somitogenesis. More specifically, how do subsets of cells in the segmental plate get triggered to undergo these precisely timed mesenchymal to epithelial transitions, which are the localized cell shape changes that create the correct number of appropriately sized and bilaterally symmetrical somites?

Let’s break this question down a bit into several different parameters. Let's see. Number one, how does the anterior to posterior axis elongate? Additionally, what dictates where a boundary will form? And third, what signals the alarm for when a boundary will form?

Let's start with the first question. How does the anterior to posterior axis elongate? This is an important question because axis elongation essentially creates the segmental plate, which serves as the substrate for somite formation. Moreover, it would be a safe assumption that the rate of axis elongation could potentially influence the size and or number of the somites in a given organism. Here's an illustration of the developing trunk of the zebrafish embryo, two pairs of bilateral somites in the presomitic mesodermal shell. So what are you thinking? How might this tail grow? In a moment, consider pausing this Dev Tutorial to contemplate the following potential ideas. Could it be A, cell proliferation, B, cell migration, C, cell size expansion? If you feel it could be any of these, then how and where are they employed?

All right, I hope you did take a moment to think about these ideas and that potentially the correct answer is in fact all of them. Elongating the axis will need new cells to be made in the tail bud, cells that will migrate into the segmental plate. And quite amazingly, enlargement of notochord cells will also help drive this process. A population of neuromesodermal progenitor cells resides in the tail bud in the region called the dorsal medial zone. In amniotes, this zone is similar to the caudal lateral epiblast. As their name would imply, these neuromesodermal progenitors will give rise to both cells of the growing neural tube and cells of the growing paraxial mesoderm. And as we postulated, these progenitor cells will in fact migrate into these two different tissues, the neural plate and the segmental plate. But before reaching these destinations, they will undergo proliferation, predominantly within a domain called the maturation zone. So that accounts for the cell migration and proliferation that we hypothesized would be, in part, powering axis elongation.

However, I also mentioned contributions from the process of cell enlargement. The chordamesoderm develops into the notochord, which starts out as something akin to like a stack of coins. However, these coins, or rather cells, inflate vacuoles inside them, which, like a plant cell, leads to the expansion or enlargement of that cell. As all of these chordamesoderm cells are exhibiting this vacuole filling in an anterior to posterior direction, it results in the progressive elongation of the notochord. Concomitantly, both

the notochord and developing presomitic mesoderm are secreting extracellular matrix components that link the two mesodermal tissues together. Therefore, as the notochord elongates, the presomitic mesoderm actually hitches a ride and gets pulled along with the notochord.

So in summary, the combination of proliferating and migrating neural mesodermal progenitors in the tail bud, and the expansion of chordamesodermal cells powers axis elongation. In this way, the embryo is generating the segmental plate. But what determines where and when an epithelial boundary will be created in this plate? Some elegant rearrangement experiments done with chick segmental plate tissue revealed that the cells anterior to somitomere minus four are quite committed to their destined fate in those specific regions of specific somites. However, cells more posterior to somitomere minus four are undetermined, meaning their fates can be changed. Due to these and other experiments, the region of somitomere minus four has been deemed the determination zone, or the point at which there is literally no going back. Now, the use of the word front should suggest to you that this location represents the edge of something grander, such as the edge or front of a wave. Although, at this point, we are simply describing a location of cell maturation.

The true question is, what is regulating cell determination along the posterior to anterior axis of the segmental plate? I like to compare this phenomenon to that of the sun's effect on plant growth. Now, provided our other conditions are constant, if a plot of land had constant cloud cover on one side while the other a perfect amount of sunlight, then the growth rates for these plants on the two sides would differ. Right? Now, let's say this cloud cover on the shaded side started to move off the land over time, such that it slowly provided the perfect amount of sunlight across the land. Then you would see a similar path of gradual growth and maturation of those plants over time.

Is the more posterior presomitic mesoderm covered by a similar cloud that prevents somitic maturation? Indeed, it is. Gene expression analysis has visually demonstrated the presence of a graded morphogen of fibroblast growth factor-8, which is expressed

and secreted by the caudal progenitor cells, creating a gradient that extends well into the segmental plate. In support of FGF-8 functioning as a negative regulator of cell determination, this expression of FGF-8 in cells anterior to somitomere minus four does prevent those cells from differentiating. Therefore, as the tail bud grows posteriorly, and the natural mechanisms of RNA and protein decay limit the life of FGF-8, the shallow edge of this gradient also shifts posteriorly. The threshold of FGF-8 levels around somitomere minus four drops low enough to permit boundary specification.

But what is positively promoting somite formation? An opposing gradient of retinoic acid from the anterior paraxial mesoderm functions to directly antagonize FGF-8 and induce somitic maturation. But when? I mean, as the tail bud grows, at some point every cell in the segmental plate experiences the right balance of FGF-8 and retinoic acid to induce epithelialization. But not every cell directly contributes to the formation of a boundary. What tells a group of competent cellsthat they are the lucky ones, that, in fact, it is time to develop into somite border? Presomitic cells need a repetitive alarm clock. But how do cells keep track of time? Keep track of time in a way that enables them to make behavioral changes at precise and regular intervals, like taking a walk every morning. We have the convenience of looking at a clock to know it is noon and time for that meeting with friends for lunch. But what do cells have to look at to tell when it is time to do something? Time to change from a mesenchymal morphology to the epithelial one.

As it turns out, there is a molecular clock, in the form of the periodic turning on and off of gene expression. There are many genes found to cycle on and off in the presomitic mesoderm, such as Lunatic Fringe, Hairy, Hes, or Delta and Notch. I think this is best seen. The Pourquie Lab generated a transgenic mouse that expresses a destabilized form of the Venus fluorescent reporter under the control of the Lunatic Fringe regulatory elements. All that means is whatever cell in the mouse embryo actively expresses the Lunatic Fringe gene, then, they will also express the green fluorescent protein in the same exact pattern. If a cell normally turns on Lunatic Fringe, it will also fluoresce green.If a cell stops expressing Lunatic Fringe, then it will no longer be fluorescent. As you can see in this movie, cells in the most posterior region first express this reporter.

However, it appears to move up the segmental plate as if it were a passing wave. When this wave of expression reaches the most anterior extent of the segmental plate at somitomere zero, then a new somite border is created, after which, a new wave begins again. These gene expression patterns continue to cycle in regular, repeated waves, each time, ending with a new boundary being built until there is no more segmental plate to segment.

I find somitogenesis to truly be one of the most beautiful processes in developmental biology. Let's review how somite formation is paired with the oscillating expression patterns of specific genes in the presomitic mesoderm. Here is a schematic showing in purple the gene expression pattern of Hairy, which is a gene that encodes a transcription factor. Hairy expression is maintained in the posterior half of somites. However, a large band of expression can be first seen throughout the posterior presomitic mesoderm. This band of Hairy expression appears to move anteriorly, up into the segmental plate, and encompasses a smaller region of cells. And it continues to move like a cresting wave front until it ends in a domain restricted to the posterior half of somitomere zero, at which point a new boundary is created to form a new somite, somite number one. And the tail bud continues to extend. Let's see another wave of Hairy expression create the next somite.

So the next logical question is how are these oscillations in gene expression achieved? But kind of like a crowd of spectators doing the wave, even more complicated is how this cyclical pattern of expression is passed from a posteriorly positioned cell to a more anteriorly positioned cell, and then passed on again and again, such that it literally looks like a wave. Let's think this through. To regulate a gene on and off, its transcription must be controlled. And the primary culprits for this role are, of course, transcription factors. Okay, there's that. However, to relay a signal from one cell to another, then this would require the function of, I don't know, transmembrane receptors. Can you think of a protein that may actually be capable of doing both? Now, if you were listening carefully, I even mentioned that it was itself an oscillating gene, the Notch gene. Notch and its receptor binding partner provide the mechanism for cell-to-cell communication. Upon

signaling with Delta, Notch will get cleaved and then function intracellularly as a transcription factor to upregulate a variety of genes, such as Lunatic Fringe and Hes transcription factors. Yet, the most important aspect of this signaling event is that these and other transcriptional targets will also function to negatively feedback on Notch to turn it off. It is this cell-to-cell Notchâ Delta activation followed by a robust negative feedback loop that together establishes the molecular clock of somitogenesis. Loss of function mutations in Notch, Delta, or their downstream transcriptional targets result in major segmentation defects that cause severe malformations in vertebral development.

In summary, I have described several key elements to somitogenesis, such as an antagonistic system of FGF-8 and retinoic acid to delineate where a somite should form, a Notch-Delta-mediated molecular clock to signal when a somite should form, and an ephrin-Eph mechanism for how epithelialization physically happens. To keep things short and relatively basic, I have left quite a bit out. For instance, these systems are all interconnected. FGF-8 works in concert with another morphogen called Wnts, that together maintain a proliferative pool of caudal progenitors that support axis elongation.

Moreover, Delta signaling will operate through progressively more five prime Hox genes to attenuate FGF-8 and Wnts function, which eventually slows tail bud elongation to a stop. Hopefully this tutorial has provided you with some of the basic knowledge of somitogenesis to begin investigating on your own this most remarkable event to our own evolutionary history. Try playing with these different mechanisms to postulate how a snake's body can be divided some 300 times while our own, only a mere of 33 or so. Play with the rate of axis growth, the rate of the clock, and see what you can come up with.

My own laboratory has, at times, witnessed amazing alterations to the formation of somites. For instance, in the background of this Dev Tutorial is an image of zebrafish muscle fibers that show the boundaries between somites. However, something has occurred to interrupt proper boundary formation. Look carefully. A perfectly normal boundary formed in the dorsal half of one of the somites, whereas in the ventral portion,

muscle fibers span the full length of two somites, suggesting no boundary formed in that region. This malformation was the result of exposure to fossil fuel pollutants. But I have no idea which component caused this, nor what the developmental mechanism was that went awry.What do you think? Was is it a problem with the determination front, perhaps the molecular clock, or maybe even defects in the extracellular matrix that followed up axis elongation? I hope you can help me find the answers to these questions and many more as you uncover the mysteries of the development of segmentation. Thanks for listening and happy developing.