The role of retinoic acid signaling at the early stage of zebrafish somitogenesis

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2012/02/10

Graduate School of Biological Sciences　Doctoral Thesis Abstract

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Abstract

During vertebrate development, the primary body axis elongates posteriorly and is periodically divided into embryonic structures called somites, which give rise to the vertebrae, skeletal muscles and dermis. It has been thought that the period of segmentation depends on the segmentation clock controlled by cyclic genes such as Notch effectors, while the position of segmentation is determined by the opposed gradients of fibroblast growth factor (FGF) and retinoic acid (RA). The anterior somites which create a part of the skull and the cervical vertebrae are formed early, and then the posterior somites which give rise to the thoracic, lumber and sacral vertebrae are progressively generated along with the axis elongation. A difference between the anterior and posterior somitogenesis has been observed in several species including amphioxus, mouse and zebrafish. In zebrafish, for instance, the anterior somites form every 20 min, and then the posterior somites from every 30 min. However, how the pace of somitogenesis is changed and whether the anterior-posterior difference is important for the later body plan remain unclear

We observed somitogenesis of the first 8 somites by time-lapse imaging, and found a clear difference between the first 4, which formed quickly within approximately 80 min, and the later 4 somites sequentially formed within 120 min. More posterior somites also formed in similar pattern with somites 5 – 8. These show a change of the anterior-posterior somitogenesis and a constant pace of segmentation throughout the posterior somites in zebrafish. Investigation of starts and ends point of somite formation didn't show obvious difference of segmentation period between somites 1 to 8 (28.9 ± 2.1 min). However, we found that overlapping rate of somitogenesis differs from somites 1 – 4 to 5 – 8. In the somites 1 to 4, segmentation between a somite and the next was timely overlapped for 13.6 ± 2.5 min, while in the somite 5 to 8, it became small by 20% (2.7 ± 2.5 min) relative to those of the anterior somites. Investigation on others strain indicating that the transition is not specific for a zebrafish strain. These results therefore suggest that the transition between the anterior and posterior somitogenesis is originated from the different overlapping rate of segmentation between a somite and next somite.

In zebrafish, somite segmentation is regulated by the combined action of Notch, FGF and RA activities. We inhibited the activity of these signals using antisense morpholino oligonucleotides. Although knockdown of a Notch ligand *deltad* or an FGF ligand *fgf8*, which is known to regulate Notch or FGF signaling in somitogenesis, respectively, did not resulted in a failure of the anterior-posterior transition, knockdown of *raldh2*, which is a major source of RA, did. *raldh2* morphants initiated somitogenesis similar to control-MO, but yielded the transient extension of segmentation period at somites 4 and 5. These results suggest that RA signaling ensures timely somite formation at the transition between the anterior and posterior somitogenesis.

Since the extension of segmentation period in *raldh2* morphants might decrease total number of somites, we counted somite number in zebrafish embryos at different stages. *raldh2* morphants lost a somite relative to control morphants throughout the posterior somite formation. These results suggest that RA signaling is essential for proper transition between the anterior and posterior somitogenesis, and that the failure of the transition then leads to loss of a somite.

Because a vertebra is created by the caudal part of somite and the rostral part of next somite, somite number is correlated with vertebra number. The first 2 somites do not contribute to the vertebral column, while somites 3 to 34 give rise to 32 vertebrae; which are consist of 2 cervical, 10 rib bearing, 2 rib and hemal arch bearing, 14 hermal arch bearing and 3 tail fin set vertebrae. Since *raldh2* morphants lost a somite by the extension of segmentation period at somites 4 and 5, we reasoned that *raldh2* morphants should result in loss of a vertebra at later stages. We investigated the number of vertebrae in *raldh2* morphants and found the loss of the second cervical vertebra, which is derived from both the caudal part of somite 4 and the rostral part of somite 5. These results therefore suggest that RA depletion fails to ensure timely segmentation of somites 4 and 5, eventually leading to loss of the second cervical vertebra. To confirm these, we tested whether administration of RA during early developmental stages restores these defects in *raldh2* morphants. RA administration in *raldh2* morphants for 4 – 10 but not 10 – 14 hour postfertilization (hpf), resulted in normal formation of somites 4 and 5, leading to recover loss of a somite and the second cervical vertebra. This is supported by the data showing that treatment of DEAB, an inhibitor of RA synthesis enzyme, for 4 – 10 but not 10 – 14 hpf, led to loss of a somites. These results suggest that RA supplied during blastula and gastrula stages controls segmentation period at the transition.

It is known that the future boundary of a somite is already determined at 120 – 150 min prior to formation of the last somite, suggesting that pre-patterns of somites 4 and 5 are already generated at late gastrulation. Because this time window is consistent with the temporal requirement of RA, we thought that RA is doing something during late gastrulation to modulate the segmentation period of somites 4 and 5. Since the segmentation clock regulates the segmentation period, we investigated whether RA modulates the somite segmentation clock during late gastrulation, by checking expression of a cyclic gene *her1,* which plays a major role in somite segmentation in zebrafish. *raldh2* morphants showed *her1* expression in a similar manner to control morphants. Although the number of *her1* stripes in control increased from two to three at a period between 9 to 10 hpf, the increase of *her1* stripes was delayed in *raldh2* morphants. These results suggest that the difference of the anterior-posterior somitogenesis is originated from increasing the cycle number of the clock within the paraxial mesoderm at late gastrulation, which is mediated by RA.

We investigated the mechanism how RA controls the cycle number of the clock. Since RA is implicated in the determination of somite size by antagonizing opposed FGF gradient. To test whether such a mechanism also contributes to RA-dependent regulation of the clock cycle, we investigated expression of *fgf8*, *mespb, papc* or *tbx16*, which are implicated in somite size determination. However, we could not detect obvious failures of their expression in *raldh2* morphants. This is also supported by the data showing no defect of the transition between the anterior and posterior somitogenesis in *fgf8* morphants. These results suggest that a mechanism in which RA regulates the cycle number of the clock is different from that of somite size determination mediated by opposed gradients of FGF and RA signaling.

Next, we investigated the expression of *ripply1,* which known to have role in

transcriptional termination of *her1*. We found the ectopic expression of ripply1 in *raldh2* morphant, which overlap with the supposed position of third stripe of *her1*. Showing the possibility that ectopic expression of *ripply1* inhibit the expression of *her1*, at the transition between the anterior and posterior somitogenesis. This is also supported by the data showing that, over expression of *ripply1* down regulate of *her1* expression on Zebrafish embryo. These results suggest that a mechanism in which RA regulates the cycle number of the clock is possibly trough controlling the *her1* repression by *ripply1*-co repressor association

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CHAPTER I
INTRODUCTION

During vertebrate development, the primary body axis elongates posteriorly and is periodically divided into embryonic structures called somites, which give rise to the vertebrae, skeletal muscles and dermis. The patterning of anterior-posterior in the vertebrate embryo could be divided into two major processes: an initiation phase, in which the embryo is forming the head and the body (primary body), and an elaboration phase, in which the body progressively forming the trunk and tail (secondary body). A difference between the anterior and posterior somitogenesis has been observed in several species including amphioxus, mouse and zebrafish, which leading to question; are there differences between anterior and posterior-somitogenesis in zebrafish? How the differences generate? And whether the anterior-posterior difference is important for the later body plan remain unclear?

In this study we employ the zebrafish as model animal, considering it advantages compare to other model organisms, such as transparency, high number of breed, short embryonic stage, and easy of genetic modification.

1.1. Zebrafish as a model animal

The use of zebrafish as a model organism was pioneered by Dr. George Streisinger, who produced the first zebrafish clone in the laboratory (Grunwald, 2002). Since then, the zebrafish popularly use as model organism for vertebrate development by developmental biologists. It is well known that zebrafish appears to combine several best features as a models organism.

1.1.1. Zebrafis origin

The zebrafish (*Daniorerio*, formerly also known as *Brachydaniorerio*) is a small tropical freshwater fish. The fish are well known as a pet fish, which is popular among the fish breeder. The zebrafish belongs to the family of the cyprinids (Cyprinidae) in the class of ray-finned fishes (Actinopterygii) and within the order of Cypriniformes. The fish origin and its natural habitats are rivers, small streams, stagnant or slow-moving pools near streams, and rice field. The fish is distributed in some part of South Asia, mainly northern India, Pakistan, Nepal, and Bhutan and also could be found on some river in Myanmar (Engeszer et al. 2007).

1.1.2. Evolutionary and History of zebrafish

Danio is a member of the order Cypriniformes (Table 1), a large group of freshwater fishes distributed throughout North America, Africa, and Eurasia. Cypriniformes is known as the most diverse group of freshwater fishes with estimates of diversity reaching close to 3,500 species (Nelson, 2006). Taxonomicaly, Cypriniforms are placed within the series Otophysi (a subgroup of the larger superorder Ostariophysi), a clade of freshwater fishes that also includes the tetras (order Characiformes), South American knifefishes (order Gymnotiformes), and catfishes (order Siluriformes).

Now days, scientists recognize that gene duplication is one of the sources of many new functions in living creatures. The importance of gene duplication as source of genetic material to biological evolution has been recognized since the 1930s. The presence of duplicate genes is sometimes beneficial because extra amounts of protein or RNA products are provided. This applies mainly to strongly expressed genes the

products of which are in high demand, such as rRNAs and histones (Fishman 2001).

Tabel 1. The taxonomy of zebrafish (Meyer, 1993)

The taxonomy of zebrafish	
Phylum	Chordata
Class	Actinopterygii
Order	Cypriniformes
Familiy	Cyprinidae
Genus	Danio
Species	*Danio rerio*

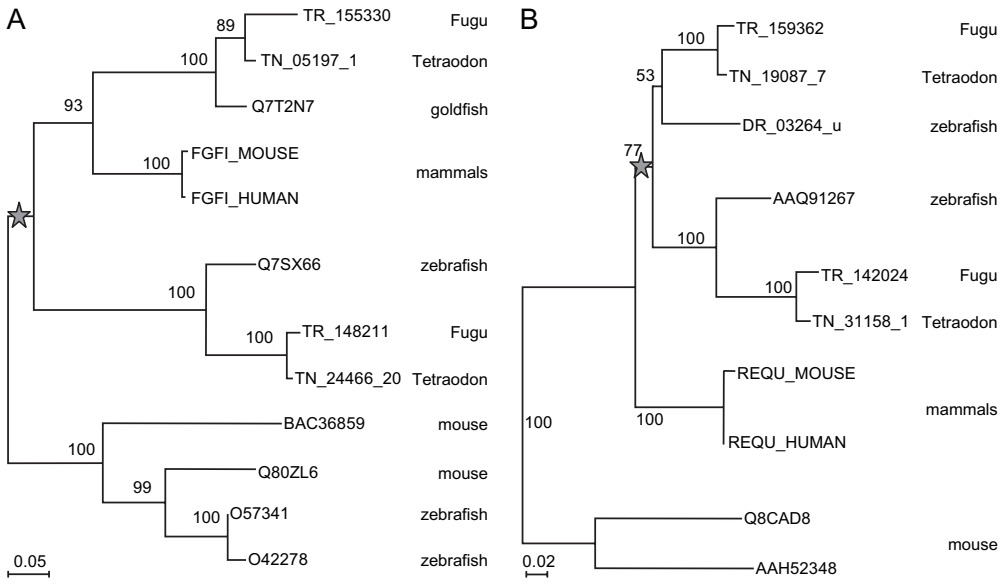

Figure 1. Examples of relative dating of gene duplications by phylogeny of zebrafish. (A) Fibroblast growth factor-18 precursor, duplicated before the tetrapod–actinopterygian split (ancestral vertebrate duplication), with secondary loss of 1 copy in mammals, leading to 2 copies in fishes but only 1 in human (B) Zinc-finger protein ubi-d4 (Requiem), duplicated anciently in fishes. (The stars represent the duplication that led to 2 Tetraodon paralogs in each case, modified from Brunet, (2006)

Many studies have reported that gene families in zebrafish tend to have expanded membership as compared with mammals (Force et al. 1999). It has been reported that

some gene families in zebrafish have more members than the corresponding families in mammals (Talbot, 2000). Engrailed gene for example, in tetrapod there is two engrailed gene family, named En1 and En2. However in Zebrafish, has been reported to have four *engrailed* genes, which are; *eng1, eng2*, and *eng3* (Ekker et al. 1992), and *eng1b* (Amores et al. 1998) another example is the identification of seven hox complexes in zebrafish, whereas mammals have only four known hox complexes (Ekker et al. 1992). Aldh gene family also believed as a result of gene duplication. Thus, sequence comparisons and mapping together suggest that most zebrafish duplicate gene pairs arose from duplication of chromosomes or chromosomal segments in the fish lineage after the split of teleost and mammalian ancestors (Woods et al. 2000, Woods et al. 2005). In some cases, however, zebrafish may have retained ancestral duplicates. In accord with previous mapping studies as with comparisons of individual genes between zebrafish and mammals, the presence of duplicates of some chromosomal segments in zebrafish implies that there is not a single zebrafish counterpart for every group of syntenic mammalian genes (Figure 1) (Postlethwait et al. 1998, Gates et al. 1999)

1.1.3. Life cycle and development of the zebrafish

The zebrafish development and it short period of embryonic stage are considered as an advantage compare to other vertebrate model organisms. In the laboratory set up, adult female zebra fish produced few hundred eggs, which can be obtained from a single spawning. The embryos need very short time to accomplished early stage of development. The fertilized egg only need approximately 48 hours or two days to develop become larva, which usually hatches on the third day of development. In this

stage fish already acquire the ability to seeking for food and also active avoidance behaviors. The fish only need 3 months to complete whole developmental stage, form single cell of egg to adult fish, which could start to produce new progeny. (Kimmel et al. 1995).

The newly fertilized egg is in the zygote period until the first cleavage occurs, about 40 minutes after fertilization. The onset of gastrulation occurs at 6hpf or at around 50% epiboly stage. At this time, Blastoderm remains uniform in thickness; a thickened marginal region termed the germ ring appears around the blastoderm rim. After epiboly, at the bud stage (10hpf) First somite furrow appears and neural tube develop, the rudiments of the primary organs become visible, the tail bud becomes more prominent and the embryo elongates. The first cells differentiate morphologically, and the first body movements appear. Organ and body compartments or structures such as the notochord, neural tube, heart, cerebellum, olfactory pit and dorsal aorta within 24 h of fertilisation of the egg. The embryo develops from fertilized egg to larva takes approximately two days. Stainier *et al,* (1993), reported that, the myocardial progenitors of zebrafish already identified at early blastula (512 cell satge), which is originally located in the ventrolateral marginal zone (Stainier, Lee & Fishman 1993). More over, it is reported that, in the late blastula, the myocardial progenitors are found within the three tiers of blastomeres closest to the embryonic margin (Warga, Nusslein-Volhard 1999).

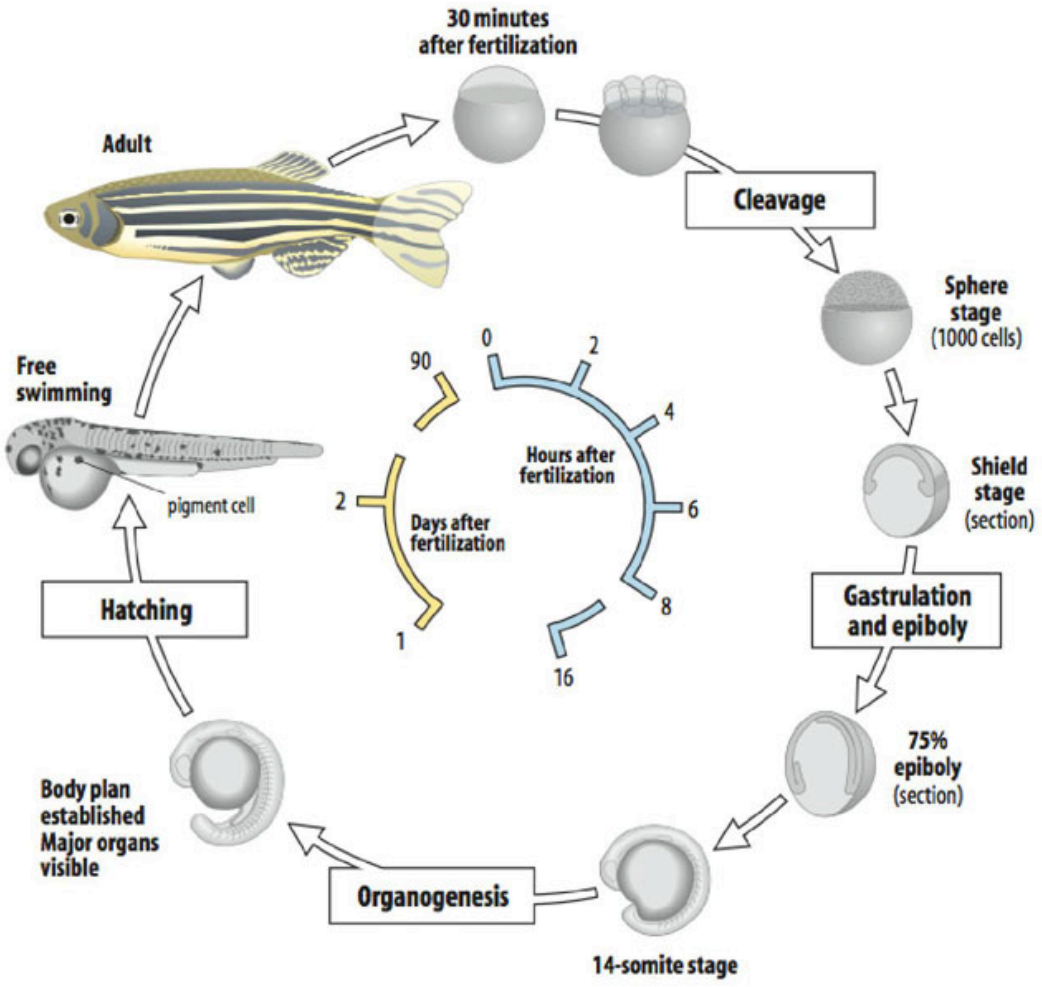

Figure 2. Life cycle of zebrafish showing a scheme of the development of zebrafish, from a single cell to adult stage, which occurs around 90 days. (adopted form Staveley, 2012)

The differentiation of embryonic cells to become internal organs such as the tail artery, intestine, stomach and liver, as well as the mouth, eyes and muscles are accomplished within around five days, and the organs have taken up their function (Ingham 1997, Kimmel et al. 1995, Vogel, 2000,). After 5 days of development, organs such as the tail artery, intestine, stomach and liver, as well as the mouth, eyes and

muscles are all clearly visible. Simply by observing the living embryo under a dissecting microscope it is possible to identify structures such as the notochord, neural tube, heart, cerebellum, olfactory pit and dorsal aorta within 24 h of fertilisation of the egg (Kimmel et al. 1995).

Table 2. Early developmental stage of zebrafish

Period	Time (hour)	Description
Zygote	0	The newly fertilized egg through the completion of the first zygotic cell cycle
Cleavage	3/4	Cell cycles 2 through 7 occur rapidly and synchronously
Blastula	2 1/4	Rapid, metasynchronous cell cycles (8,9) give way to lengthened, asynchronous ones at the midblastula transition; epiboly then begins
Gastrula	5 1/4	Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis; through the end of epiboly
Segmentation	10	Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears
Pharyngula	24	Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and tins begin development
Hatching	48	Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral tin; hatching occurs asynchronously
Early larva	72	Swim bladder inflates; food-seeking and active avoidance behaviors

Adopted from Kimmel, (1995)

Considering the short development period, the zebrafish provides the possibility of studying a complete developmental process in a short period of time (D'Costa, 2009). This rapid development allows the observation of developmental processes and the completion of experiments generally within a few hours to days. More over its

development could be observe easily in great detail (Kimmel et al. 1995, Ingham 1997, Vogel, 2000,).

1.1.4. History; saturation mutagenesis

The utilization of mutagenic screens and the isolation of zebrafish orthologues/analogues of human genes are providing great resources to reveal the normal and perturbed development of vertebrate (Dodd et al. 2000). Examining the mutants in zebrafish, provide clear clue to reveal the mystery of genetically inherited human diseases. It is a big hope that, in the coming years, that every genetic disease will have gene therapy as the treatment, as best solution and cure.

It is a fortunate that, recent days, Scientists have almost entirely mapped out the genetic structures of the zebra fish. In addition, the genetic screens, which have been conducted, also yielded many zebrafish mutant phenotypes. It is easy to induce new mutations in zebrafish and large-scale screens have been carried out to identify mutations causing defects in particular biological processes, such as the developing nervous system

There are several ways to induced mutation to the zebrafish, such by exposure the sperms of the fish to chemical mutagens; ethyl methanesulfonate (EMS) and N-ethyl-N-nitrosourea (ENU), which could induce mutations at different stages of spermatogenesis. Both EMS and ENU induced mutations at high rates in post-meiotic germ cells (Solnica-Krezel, 1994), Ionizing radiation, such as X-rays, also induce genetic alterations, and insertional mutagenesis with a retrovirus is mutagenic and allows for rapid cloning of the gene (Fishman 2001). Therefore, up to the present, many

useful zebrafish mutants who were produced by mutagenesis researches are available. Zebrafish Mutation Project (ZMP), reported it current achievement of a total number of 4469 gene with mutation in zebrafish. Genetic screen identifying over 4000 mutations were completed and published (Ingham 1997, Driever et al. 1996, Haffter et al. 1996). More over, a total of 6647 mutations from amongst 6194 mutagenised genomes, a frequency approaching 1.1 mutations per genome sampled have been reported (Driever et al. 1996, Haffter et al. 1996).

It has been reported that, among many of the mutant fish are exhibit various developmental and physiological disorders, which are resemble of human disease. These disorders include hemophilia, anemia, porphyria, and neuropathies of the peripheral nervous system and diseases of the central nervous system. The zebrafish haematopoiesis is appears to resemble haematopoiesis in higher vertebrates, in term of function of the gene and also its conserve expression. Brownlie (1998) reported that haem biosynthetic enzyme ALA52 is encoded by the sauternes (sau) (Brownlie et al. 1998). Recent studies have also led to the isolation of zebrafish mutants, designated jumbo and chihuahua, which may serve as models of obesity and osteogenesis imperfecta, respectively (Dodd et al. 2000).

1.1.5. Advantages of the Zebrafish as a Model Organism

The zebrafish has many properties that make it ideally suited as a model organism for experimental biologists:

a. The zebrafish are easy to be breed and maintained in the laboratory condition. Female and male are easily to be distinguished by naked eye. Size is small so

that easy to keep large populations of animals in a small space. Adults could be maintained in breeding condition on a year-round basis and individual females would give rise to hundreds of progeny. It is very good property and well suited for standard genetic analyses (Grunwald, Eisen 2002).

b. The zebrafish produced large numbers of offspring, when kept under optimal conditions, up to more than 200 eggs could be produce by a single female per week. Large numbers of offspring provide an easy way for experimental setup, greatly facilitates high-throughput approaches, statistical analyses of experimental data, and also facilitate the reliable identification of mutant phenotypes for a genetic screen (Kimmel et al. 1995, Ingham 1997, Vogel, 2000).

c. The zebrafish eggs are strong and large enough to be use in experimental manipulations, such as microinjections or transplantation of cells. However, the embryos are small enough to be maintained in a small container or space (Ingham 1997, de Jong, 2005).

d. The zebrafish embryos are developing outside of the mother fish (ex-utero); these properties provide an easy access for observation at all stages of development of the embryos. Moreover, unlike in birds and reptiles, the chorion of zebrafish eggs and the embryos themselves are completely transparent during early stage of development. This transparency of the zebrafish embryos and larvae, which due to their relatively small size and the absence of pigment throughout the first 36 h of development, allows the visualization (live imaging) of the developmental processes that occur on and inside of the developing animal

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e. The somitogenesis, external organ formation, and also the development of internal organs, such as nervous system, vascular system, as well as the digestive tract, can be visualized in real time measurement. These property facilitate numerous experimental approaches and also allowing an ideal observation of the developing embryo, without necessarily open the chorion and interfere the developmental process of the embryo it self.

f. The yolk of zebrafish embryo does not participate in the cleavage divisions but remains separated from the embryo throughout development, this property, together with their rapid embryonic development (within 72 hours zebrafish fry attain an adult-like stage), allow a simultaneous experiment to be conducted within relatively short period. Moreover, the development of the zebrafish is similar to the embryogenesis in higher-ordered vertebrates, including humans and other mammals. The zebrafish thus, seems to combines many of the advantages of model organisms for specific developmental phenotypes.

g. Embryonic development of the zebrafish is synchronous, where all of the embryos derived from one mating time, will have the same developmental stage. On the other hand, the organs in zebrafish larvae are composed of fewer cells. However, the organs still function in much the same way as those in larger animals making them easier to study.

h. Experimental treatment for both embryos and adult animals is relatively easy for zebrafish, chemicals for example, can be added directly to the medium in which embryo maintained. Moreover, the transparanccy of the embryo, supported for

the techniques, such as mRNA in situ hybridization and antibody staining to test for the expression patterns of genes and proteins, whole mount without any need for dissection during the processes of staining

i. Sperm samples can easily be obtained (even from live fish), stored, and later used for in vitro fertilizations.

j. Regeneration, Zebrafish have the ability to regenerate fins, skin, the heart. Zebrafish have also been found to regenerate photoreceptor cell and retinal neurons following injury (Ingham 1997, Raya et al. 2003). The small size of zebrafish embryos permits them to receive enough oxygen by passive diffusion to survive and continue to develop for many days even in the complete absence of blood circulation. This is particularly useful when examines genetically or experimentally manipulated animals with circulatory defect (Vogel, Weinstein 2000).

1.2. Somite; structure, formation and derivate

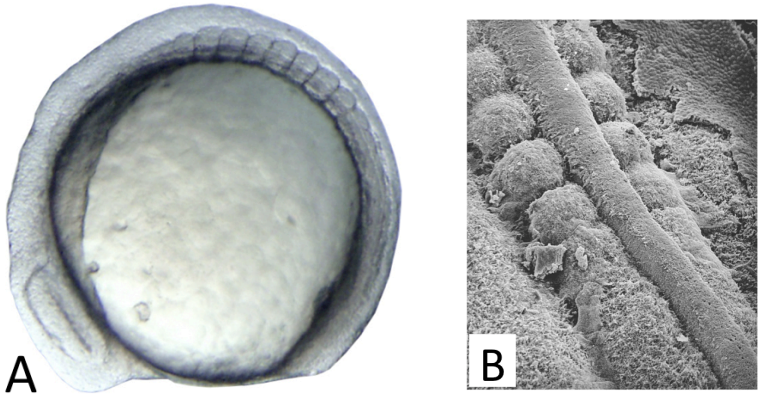
Somites are transient structures, which very important in organizing the segmental pattern of vertebrate embryos during the developmental stage. Progenitors of the somites arise from the ventral and lateral margin of the zebrafish blastula. Somites give rise to the vertebrae and ribs, axial skeleton, the dermis of the dorsal skin, the skeletal muscles of the back, and the skeletal muscles of the body wall and limbs. On the other hand, somites determine the migration paths of neural crest cells and spinal nerve axons. The important components of somitogenesis (somite formation) are time

arrangement (periodicity), segmentation (epithelialization), specification, and differentiation (Kimmel et al. 1995, Stickney, 2000, Morin-Kensicki, 2002).

Somite bud off as epithelial spheres from the cranial end of the unsegmented presomitic mesoderm (PSM) that lies on either side of the neural tube. The first somites appear in the anterior portion of the trunk, and new somites sequentially arise from the anterior to posterior of the body regular intervals. The segmentation period is specific and depends on the species; such as 30 minutes in the zebrafish, 90 minutes in chick and 120 minutes in mouse. Recently, it is revealed that this periodicity is controlled by oscillatory gene expression in the PSM, termed the somite segmentation clock (Kimmel et al. 1995, Stickney, 2000, Morin-Kensicki, 2002).

1.2.1. Morphology of the somite

Somite morphologically forms as a rectangular loosely packed block of mesenchymal cells, surrounded by epithelial layer (Stickney, 2000). As somite polarity is established, morphological segmentation commences. Somite morphogenesis involves a mesenchymal to epithelial transition (MET) of the boundary cells. The typical trunk somite averages around five cells in length and consists of epithelial anterior and posterior boundary cells separated by internal mesenchymal cells.



Figures 3. lateral and dorsal view of somite (A) zebrafish, and (B) chicken (Modified from; Gilbert, 2006)

The zebrafish (*Danio rerio*), first somites will start to appear at approximately 10 hour post fertilization (hpf). One pair of somites is formed every 20-30 minutes by formation of a new somitic furrow, which then completely divided by the formation of epithelial segment. In zebrafish, as in most other vertebrates, somites form as epithelial spheres from the presomitic mesoderm (PSM) in an anterior to posterior direction along the body axis (Stickney, 2000). Each single pairs of somites, are located symmetrically on either side of notochord. Roughly 30-34 somite pairs formed in a normal embryo, which consists of; 7 somites above the yolk cell, 10 above the yolk extension and 13-15 somites located posterior to the anus (van Eeden et al. 1996).

The AP polarity of somites is also necessary for the maintenance of segment boundaries, as when half somites are juxtaposed, boundaries only form when anterior and posterior halves are confronted (Stern, 1987). The establishment of anterior and posterior differences within segments is essential for the development of somites and the correct patterning of a number of other structures (Durbin et al. 2000). Recently,

signalling molecules such as Shh, BMP and Noggin have been identified and implicated in patterning and differentiation within the somites (Currie, 1998).

1.2.2. Derivative of somite; muscle, skin and bone

Somites, as a transient structure, give rise to the vertebrae, ribs, axial skeleton, skin and the skeletal muscles of the body wall and limbs. When the somite is newly formed and separated from the PSM, any of its cells could become any of somite-derived structures. However, as it's become mature, cells are differentiated further and region specified. Cell in various regions of somite become committed to form only certain cell types.

Shortly after somite formation, adaxial cells undergo a remarkable morphological change and migration; they begin as a sheet of about 20 cuboidal cells all adjacent to the notochord and end as a monolayer of muscle fibers on the surface of the somite (Devoto et al. 1996). The migration of adaxial cells can be divided into two phases: one in which they move dorsally and ventrally while remaining on the medial surface of the somite, and another in which they migrate radially toward the lateral surface of the somite. Adaxial cells elongate while still located medially, changing their shape from plump cubes into skinny rods. This elongation-driven shape change might be sufficient to displace the adaxial cells dorsally and ventrally. The second phase of adaxial cell migration is the movement of non-pioneer slow muscle fibers radially through the somite (Kimmel et al. 1995, Stickney, 2000, Morin-Kensicki, 2002).

The segmental plate mesoderm will be converted to somite, which is composed of simple epithelial spheres, and lack apparent polarity. As maturation proceeds, in the

dorso-ventral axis of each somite, morphological and functional differences start to establish along the line (Gomez, Pourquie 2009). The ventral part of the somite, epithelium develop to mesenchyme, which then converted to sclerotome. On the other hand the dorsal somite epithelium remains it state as epithelial cells, forming the dermomyotome. At the later stage dermomyotomes generates a second epithelial layer, which named myotome (Tam, Trainor 1994, Keynes, Stern 1988, Capdevila, Tabin & Johnson 1998). The dorso-ventral polarization of somite domain, resulted the distinct fates for later stage of development; the dermomyotome which is located in dorsoventral part, gives rise to the axial skeletal muscles and dermis, while the cell in the ventral medial part of the somite undergo mitosis, and lose their round epithelial characteristics, then become mesenchymal cells again. These portion is called the sclerotome, which differentiate become precursor cells (chondrocytes) of the ribs, and intervertebral discs. Moreover, the sclerotome, together with the notochord, gives rise to the vertebral column (Kimmel et al. 1995, Stickney, 2000, Morin-Kensicki, 2002)

It is reported by Morin-Kensicki (2002), that not all part of somite in zebrafish will contribute to the vertebrae bone. It is shown that, the alignment of the myotome derived from somite 5 with the second and third vertebrae, which provides evidence that the first two somites and perhaps part of the third may not contribute to the vertebral column. An average of 32 total vertebrae were reported, and categorized based on features characteristic of their AP position as follows: (1) cervical; (2) rib-bearing; (3) rib and hemal arch-bearing; (4) hemal arch-bearing and (5) tail fin set. Following the order of these categories, the axial formula most frequently observed was 2:10:2:14:4 (Table 3) (Morin-Kensicki, 2002).

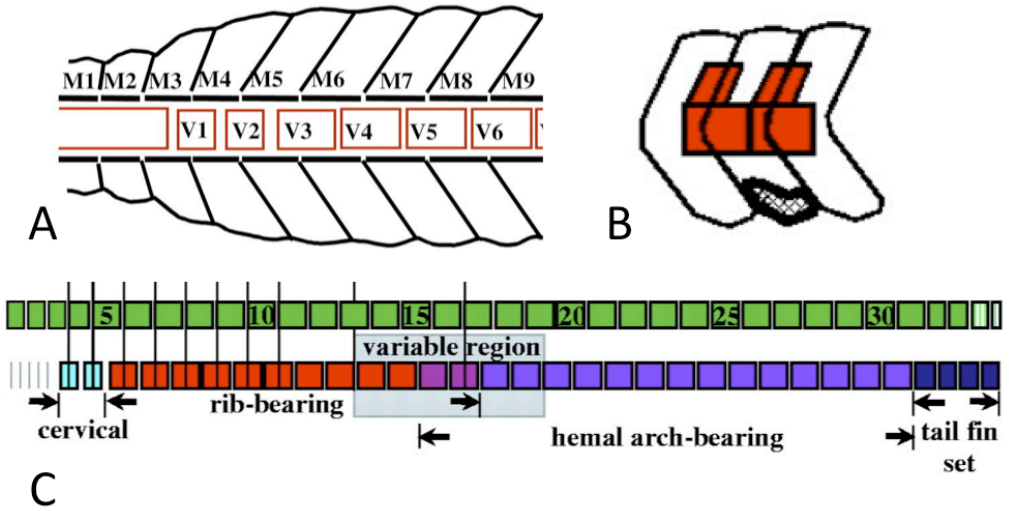


Figure 4. Somite to vertebrae segment relationship. (A,C) Somite number1 and 2 don't contribute to the vertebrae. (B) resegmentation model show; anterior part of a somite together with the posterior part of next somite, form a vertebrae segment (modified form; Morin-Kensicki,E.M. 2002).

somites contribute to the formation of vertebrae and according to the 'resegmentation' model, the vertebral segments are derived from the somites through sclerotome division. The sclerotome is divided into an anterior and posterior part, forming resegmented compartments from which the vertebrae are formed. Anterior sclerotome-half (A), posterior sclerotome-half (P), they have shown that cells derived from single somite halves contribute to vertebral formation in a non-lineage-restricted manner, thus contributing to the formation of more than one vertebra zebrafish (Morin-Kensicki, 2002, Bagnall, 1988, Bird, 2003a).

1.2.3. Formation of somite; cyclic segmentation

Somite formation (Somitogenesis) is highly robust process by which the vertebrate trunk is divided into a series of segments called somites (Gilbert, 1997). It is believed that the process of somitogenesis can be divided into three distinct stages, which may be regulated by different genetic mechanisms (Tam, 1994); (1) Specification as paraxial mesoderm: the mesoderm derived from the germ ring in fish or the tailbud, is arranged on both sides of the neural tube as the paraxial mesoderm; (Dequeant, 2008) (2) segmentation: the formation of epithelial block of cells in the paraxial mesoderm, which is called somites; (3) differentiation: the somites differentiate into the sclerotome and dermomyotome which subsequently segregates into the dermatome and myotome (Tam, 1994, Dequeant, 2008).

Somites are formed as the result of a complex interaction of processes that take place in the early vertebrate embryo, where two parallel bands of tissue known as the presomitic mesoderm (PSM), unsegmented paraxial mesoderm, that lie alongside the notochord, is sequentially organized into discrete blocks via a mechanism, which is tightly regulated both in space and time (Pourquie 2003, Giudicelli, 2004). However, The whole mechanisms of somite formation, until recently, still not yet clearly understood. Developmental biologists proposed model on how the somite is generated. Many models for somite formation have sought to explain somite formation such as*:* Cooke and Zeeman's original Clock and Wavefront model; Meinhardt's Reaction-Diffusion model; Stern's Cell Cycle model; *Clock and Induction models* (Baker, 2007a). Since, the clock and wave front model is ”widely accepted”, here we focus our discussion on the clock and wave front model.

The clock and wave front model is firstly proposed by Cooke & Zeeman (1975). The model proposed that; the periodic arrangement of somite blocks in amphibian embryos could be produced by the combined action of an intracellular oscillator, or clock, and the passage of a single 'kinematic wave' of somitogenic cell determination travelling along the A-P axis of the embryo, which occurs in presumptive somite cells (Pourquie 2003, Cooke 1975, Baker, Maini 2007b) there is some interaction between the wavefront of FGF8 (Dubrulle, McGrew & Pourquie 2001) or gradients of Wnt, FGF and retinoic acid (RA) (Dequeant, Pourquie 2008) and the segmentation clock in the PSM that acts to control cells into potential somites (Dubrulle, McGrew & Pourquie 2001). For a cell at a particular point, it is assumed that transformation to segment will only be achieved once FGF8 signalling has decreased below a certain threshold. The threshold level of FGF8 is the level expressed at the determination front (Dubrulle, 2001).

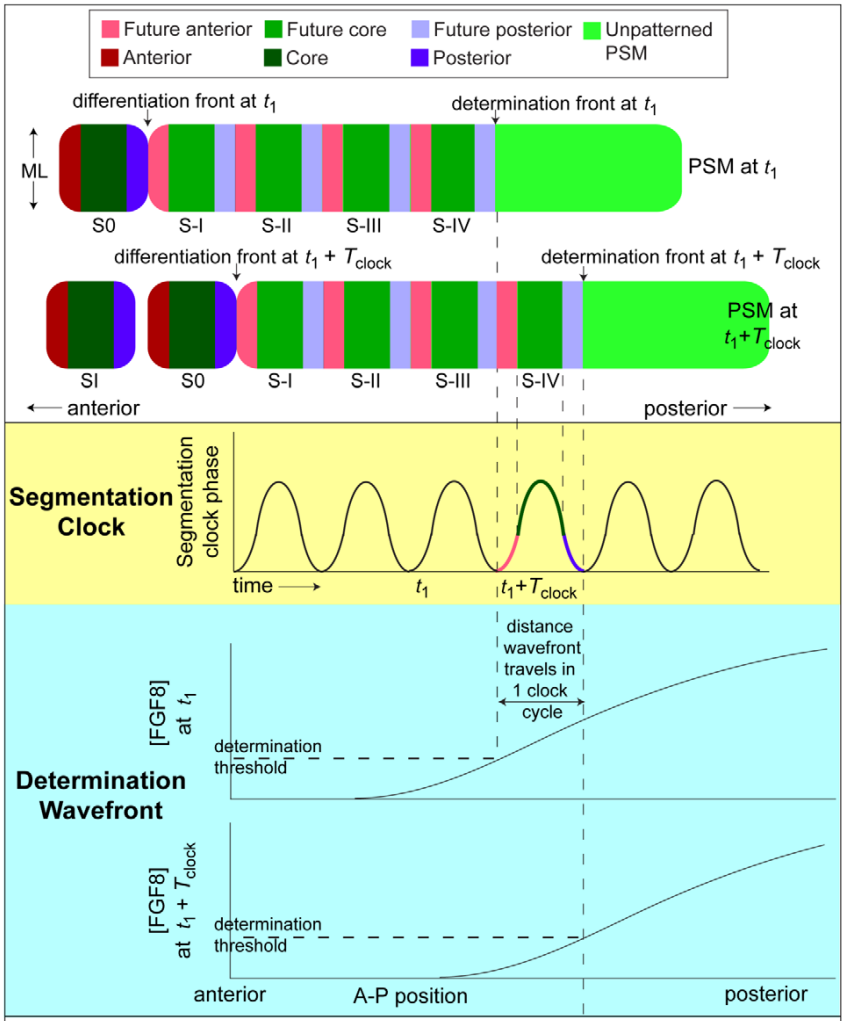


Figure 5. illustration of clock and wave front model. The temporally decreasing of FGF8 in the AP position results in a posterior-propagating determination front. Cell in the anterior of the front becomes competent to sense the state of its intracellular segmentation clock. The cell determine it state and somatic cell type based on the state of its segmentation clock. Cell differentiation follows four segmentation clock periods. The PSM grows continuously in the posterior direction through addition of cells from the tailbud, maintaining its length. Tclock is the period of the segmentation clock. (Modified from: Hester, 2011)

1.2.4. The segmentation clock

The rhythm of somite production is characteristic of the species as; 30 min for the zebrafish, 90 min in the chick embryo and 120 minutes in mouse. The molecular mechanism underlying the periodical formation of somites is coupled to an internal oscillator, and named the segmentation clock. The cyclic expression of genes in the PSM is a clear evidence of the existence of a molecular clock or oscillator that functions with a temporal periodicity (Palmeirim et al. 1997). Moreover, it is reported that the pre-patterning of the somites is a result of an oscillator mechanism, which work or occurs in the PSM (Meinhardt 1986, Dale, 2000). This molecular clock would translate a smooth maturational or positional gradient present in the PSM into a spatially periodic pattern, as a permissive signal for somitogenesis to occur at regular intervals in successive, uniformly sized blocks of cells (Stickney, Barresi & Devoto 2000).

Palmerim et al., (1997), identified the *c-hairy1* gene, as a first evidence for the oscillator mechanism in the chick embryo. The *c-hairy1* gene was reported to dynamically express in the PSM of embryos, in the direction from the posterior to the anterior PSM. The cells in the PSM region of the chick embryo undergo repeated on and off phases of *c-hairy1* transcription before the cells were transformed to become a somite. The recent report on the lunatic fringe of chick and mouse, and her1 in zebrafish provide the evident for the model (Palmeirim et al. 1997, Aulehla, 2006, Holley et al. 2002). The expression of each of these genes oscillates in cells in the PSM, cycling on and off, with a periodicity equal to the formation time of one somite (Stickney, 2000). Various hairy/Enhancer of split (Espl)-related basic helix–loop–helix (bHLH) genes such as Hes1, Hes5, Hes7, and Hey2 in the mouse; hairy-1, hairy-2, and Hey2 in the

chicken; her1 and her7 in the zebrafish (Jiang et al. 2000), which are down stream target of the Notch signaling are expressed in a dynamic pattern of stripes across the PSM in a posterior to anterior direction (Stickney, Barresi & Devoto 2000, Jiang et al. 2000). Moreover, it was reported that, most genes that exhibit a cyclic expression pattern in the PSM are involved in the Notch signaling pathway (Bessho et al. 2003, Bessho, 2003)

There is also reported that, Lunatic fringe, encoding a glycosyltransferase that modulates the Notch signaling in the chicken and mouse, and the Notch ligand deltaC in the zebrafish show an oscillatory expression pattern in the PSM. These cyclic genes, as well as other components of the Notch signaling pathway, determine the proper somite segmentation in mice (Bessho et al. 2001) and zebrafish (Jiang et al. 2000, Bessho et al. 2003, Bessho, 2003, Oates, Ho 2002, Lewis 2003)

The mechanisms for generation of the oscillation clock have been proposed: modulation of Notch signaling by Lunatic fringe, Her/Hes auto-repression, and negative feedback of Axin2 on the Wnt pathway (Cinquin 2007). Furthermore, the cyclic expression of Hes7, her1, and her7 requires their own activity, suggesting the existent of a negative feedback loop, which is a key role in and is a critical component of the oscillation machinery (Jiang et al. 2000, Bessho et al. 2003, Bessho, Kageyama 2003, Oates, Ho 2002, Lewis 2003).

Recently it was reported that Notch signaling pathway, plays important role in the specification of molecular prepattern in zebrafish. In fish, oscillations of deltaC were proposed to drive the periodic activation of Notch, leading to the cyclic expression of the genes coding for the Hairy and Enhancer of Split–Related (HER) b-HLH repressor genes her1 and her7 (Jiang et al. 2000, Oates, 2002, Lewis 2003). Moreover, several

report mention that; mutants fish of *deltaD* (*after eight*) and *Notch1* (*deadly seven*), as well as MO-knockdown of *deltaC* abolish the cyclic expression of *her1* and *her7* (Jiang et al. 2000, Oates, Ho 2002, Lewis 2003). In which, mutant or morphant only show an irregular expression in the anterior PSM and a weak, diffuse expression in the posterior part of the PSM and the tailbud. It is also reported that, *her1* and *her7* seem to cross regulate each other, and both are important factors fro the transcription of *deltaC* and *deltaD* (Gajewski et al. 2003).

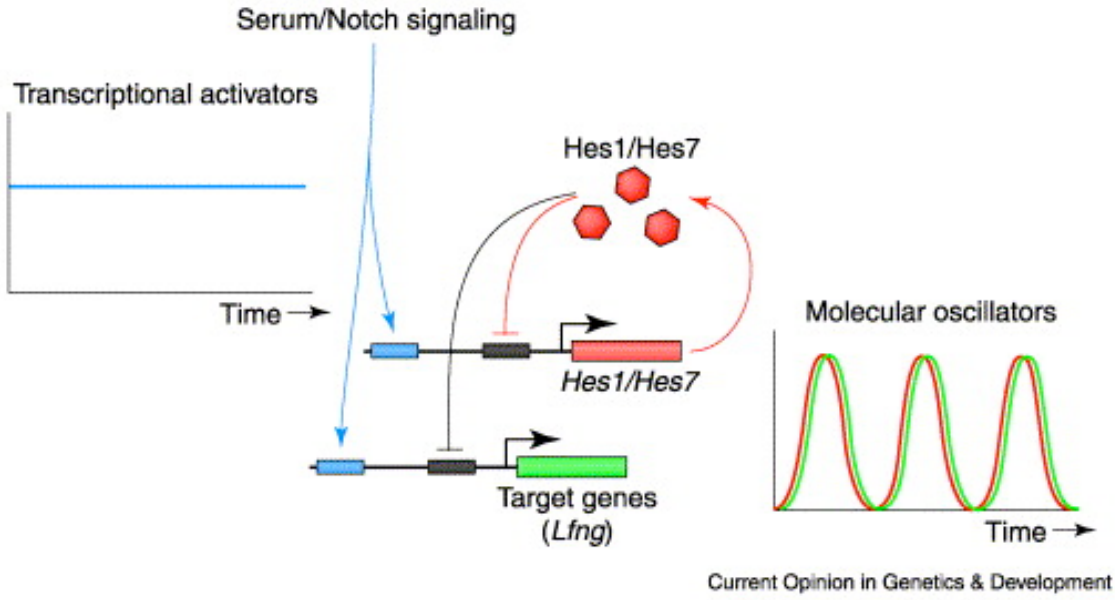


Figure 6. Model for Hes1/Hes7 oscillation. Transcriptional activators from serum stimulation or Notch signaling activate the *Hes1/Hes7* promoters constitutively, and then Hes1/Hes7 protein is produced after a short latent time. Hes1/Hes7 protein inhibits their own transcription by binding to their own promoter, resulting in reduction of the mRNA and protein as a result of their instability. After Hes1/Hes7 protein disappears, the transcription of Hes1/Hes7 is initiated again by the activators. Like *Hes1*/Hes7, the promoters of target genes, such as *Lfng*, are activated by the transcriptional activators and repressed by Hes1/Hes7 protein. Thus, *Lfng* expression oscillates in the same phase as *Hes7* expression. A direct current (activities of transcriptional activators) is transduced to an alternating current (the expression of molecular oscillators) by the negative feedback loop of Hes1/Hes7 (Bessho, Kageyama 2003)

1.2.5. The wave front

The wave front is represents the anterior to posterior progression of development during the segmentation period, as the link between axis elongation and morphological somite formation. The wavefront moves posterior during elongation of the embryonic axis but remains at a fixed-point relative to the position of the most recently formed somite, thus ensuring that somites are consistent in size.

It was reported that several factors including fibroblast growth factor (FGF), Wnt, and retinoic acid (RA) involve in positioning of the determination front. The factors are forming gradient in somite region and PSM, in controlling the paraxial mesoderm differentiation and in defining the somite-forming along the antero-posterior axis. Moreover, those factors also defining the state of PSM cells status; from a state permitting the oscillating gene expression, to a state driving the segmentation program (Dequeant, 2008).

The differentiation status of the un-segmented paraxial mesoderm (PSM) seems to be controlled by the existence of signaling gradients. Wnt and FGF are reported to work in antagonistic manner with RA. High levels of Wnt and FGF signaling maintain cells in an undifferentiated state, posterior PSM fate, whereas the exposure to high levels of RA signaling, which related to low level of fgf8 and wint3a, is responsible for initiating differentiation of the PSM cells. Moreover, It was reported that transition of the differentiation status of the cells occurs in the anterior one-third of the PSM (Dubrulle, 2001). This finding is supported by the report of Niederreither *et al*. (1997), that Raldh2, an RA-synthesizing enzyme, shows strongest expression in somites and the anterior part of PSM (Niederreither et al. 1997), in juxta position to the expression of

Cyp26A1, a cytochrome p450 enzyme involved in RA degradation. The Cyp26A1 is expressed in the posterior part of PSM (Sen et al. 2005, Fujii et al. 1997). Thus, it is suggest that the activity of RA, which has the ability to diffuse over long distances (Thaller, 1987), is spatially controlled by the expression of synthesizing and degrading enzymes such as Cyp26a1 (Niederreither, 2008). On the other hand, FGF signaling is highest at the posterior end of the PSM and show a gradual decrease toward the anterior, suggesting a role for FGF signaling in maintaining the characteristics of the posterior PSM cells and keeps the cells in undifferentiated state, (Dubrulle, 2001). only when the cells aren't under the influence of fgf in the anterior PSM, the oscillations cease and cells acquire a segmental identity (Baker, Schnell & Maini 2006a, Baker, Schnell & Maini 2006b).

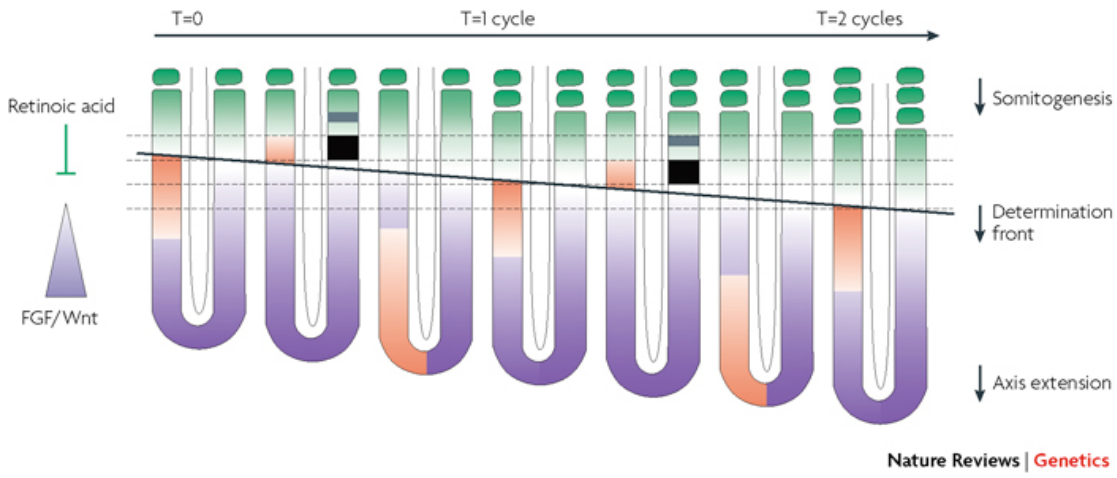


Figure 7, The system of opposing gradients of FGF–Wnt signalling (purple) and retinoic acid (green) signalling determine the front (black line) along the presomitic mesoderm (PSM). (modified from: Dequéant and Pourquié, 2008)

A mechanism must, therefore, be in place to maintain the undifferentiated state of cells in the posterior PSM and to control initiation of differentiation once cells reach

the anterior PSM. This mechanism is currently thought to be based on a combinatorial gradient system in the PSM that involves the activity of the fibroblast growth factor (FGF), the Wnt/ b-catenin, and the retinoic acid (RA) signaling pathways (Dubrulle, Pourquie 2004a). The FGF (Dubrulle, McGrew & Pourquie 2001, Dubrulle, Pourquie 2004b) and Wnt/ b-catenin signaling pathways(Aulehla, Pourquie 2006) form a posterior-anterior gradient, whereas the RA signaling pathway establishes an opposing gradient of activity (Diez del Corral et al. 2003, Moreno et al. 2008).

1.2.6. primary body and secondary body

The patterning of anterior-posterior in the vertebrate embryo could be divided into two major processes: an initiation phase, in which the embryo is forming the head and the body (primary body), and an elaboration phase, in which the body progressively forming the trunk and tail (secondary body)(Kimelman, Schier 2002). In zebrafish, the initiation phase occurs before the start of gastrulation, and the primary body axis grows posteriorly and is concomitantly segmented into somites, which arise sequentially. The anterior somites that give rise to the cervical vertebrae created early, while the more posterior somites that become the thoracic, lumbar, and sacral vertebrae form at progressively later times. During the axis elongation period, the embryo must parse the somite precursors appropriately so that there are enough cells remaining to make the most posterior somites at the end of somitogenesis (Szeto, Kimelman 2006).

Szeto and Kimelman (2006), reported that cells are specified to give rise to anterior trunk, posterior trunk, and tail somites. They find that this cell fate decision occurs surprisingly early in zebrafish development, prior to gastrulation, in response to

nodal, fgf, and bmp signaling. One consistent difference in anterior trunk somitogenesis observed in mice, zebrafish, and the cephalochordate amphioxus is the more rapid progression of the somite cycle relative to posterior somitogenesis. In the zebrafish, the anterior six somites form every 20 min, while the 24 posterior somites form every 30 min (Hanneman, 1989). In amphioxus, this temporal difference is even more extreme where, the anterior approximately eight somites form every hour but somite formation in the late phase is slower: on average, the larvae produce a new somite on either side of the body every 18 h (Schubert et al. 2001, Holley 2006).

In agreement with the findings of Szeto and Kimelman (2006), genetic and embryological experiments have uncovered several differences in the specification, formation, and differentiation of the anterior trunk, posterior trunk, and tail somites. Differences in the specification of the anterior paraxial mesoderm have been revealed by genetic experiments in mice and zebrafish. Sawada *et al*. (2001) reported that the precursors for the first three to four somites do not express *mesp-a*. On the other hand, the first stripe of *her1* expression, which corresponds to the fifth somite, is express at 70% epiboly satge. Therefore, it suggests that *mesp-a* does not function during the AP patterning of the first somites up to somite number 4. This supports the idea that anterior and posterior segments are patterned differently.

On the other hand, it is reported that, mice mutant for either of the transcription factors mesogenin or tbx6 form only the anterior trunk somites (Chapman et al. 2003, Yoon, 2000). Similarly, in zebrafish, Zoep; no tail double mutants lack all but the anterior trunk somites (Schier et al. 1997). Since no tail activates both spadetail and fgf8 expression, the loss of posterior mesoderm in Zoep; no tail embryos is a likely due to a

combined reduction of nodal, fgf, and t-box function (Draper, Stock & Kimmel 2003, Griffin, Kimelman 2003). As discussed above, analysis of t-box genes in zebrafish has uncovered differences in the genetic hierarchy that specifies trunk and tail MPCs, with spadetail and tbx6 involved in specifying anterior and posterior trunk, while no tail and tbx6 specify tail MPCs (Griffin, Kimelman 2003, Kimmel et al. 1989, Goering et al. 2003)

Numerous genetics studies reported that disorder of notch signaling in mice, and zebrafish, receptor tyrosine phosphatase in zebrafish, or mesp2 and wnt3a in mice leads to a segmentation defect in the posterior but not the anterior trunk somites. The defects could be located especially on the somites posterior to the fifth up to ninth somite in zebrafish (Holley 2006, Rida, Le Minh & Jiang 2004). Consequently, the *deltaD* mutant in zebrafish is called after eight, while the *notch1a* mutant is named deadly seven (Holley 2006). In contrast to perturbation of notch pathway function, zebrafish mutants for integrin5, called before eight, and fibronectin1 affect the formation of only the first approximately seven somites (Koshida et al. 2005, Julich et al. 2005).

1.2.7. Axial skeleton

The most prominent feature of vertebrate body plan is the axial skeleton (vertebrae), which number and structure are variable between the species; human beings have twenty-four cervical, thoracic, lumbar vertebrae as well as twelve pairs of ribs. On the other hand, zebrafish has 30-32 segments, while in snake could have more than 350 segments of vertebrae.

The vertebral column develops from sclerotome, a mesenchymal cell population derived from the ventral somite. Sclerotome cells that will contribute to the vertebral column in the zebrafish move to surround axial midline structures and differentiate as cartilage and bone (Bird, 2003b). Although all the somites look identical, they will form different structures at different positions along the anterior-posterior axis. The somites that form the cervical vertebrae of the neck and the lumbar vertebrae of the abdomen are not capable of forming ribs; ribs are generated only by the somites forming the thoracic vertebrae. Moreover, the specification of the thoracic vertebrae occurs very early in development. The somites are specified in this manner according to the Hox genes they express.

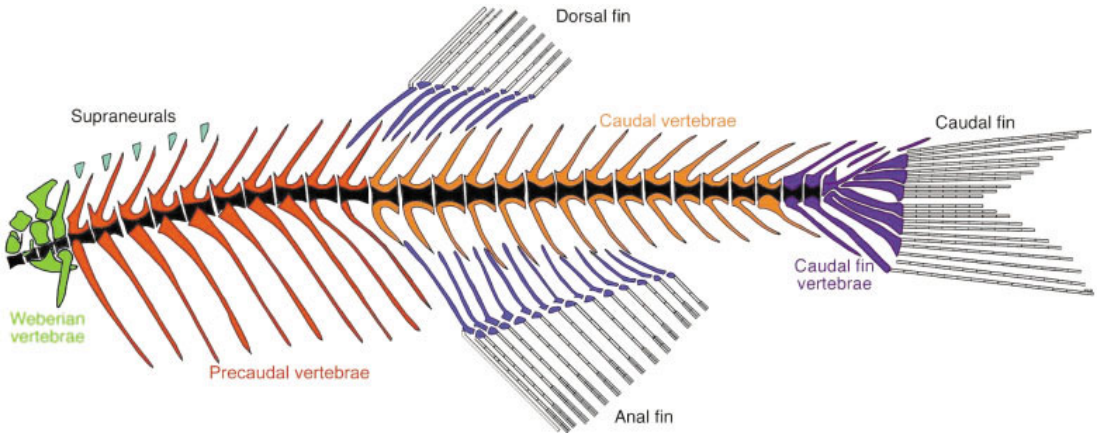


Figure 8. Generalized diagram of the zebrafish axial skeleton. Centra are black, the Weberian apparatus is green, supraneurals are light blue, precaudal vertebrae are red, caudal vertebrae are orange, the caudal fin skeleton is purple, and the dorsal and anal fin endoskeletons are blue (Bird, 2003b)

1.3. Retinoic acid

One important component of our dietary is vitamin A, a lack of vitamin A during development results in congenital malformations. In humans, the importance of this vitamin extends into adulthood, when it has important roles in regulating fertility, maintaining normal vision, and preventing neoplastic growth and neurodegenerative diseases. Retinoic acid (RA) is a lipid containing small molecule that is a bioactive derivative of vitamin A. It is a signaling molecule that regulates the expression of genes involved in the development of vertebrate embryos and also in the physiological processes in adults (Ross et al. 2000).

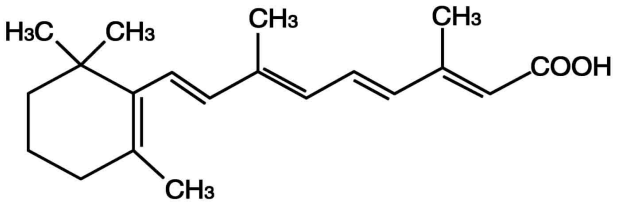


Figure 9. chemicals structure of retimnoic acid

Retinaldehyde dehydrogenase (Raldh) is the key enzyme of generation of RA. The endogenous RA is degraded become in active form by CYP26 enzymes. RA is known as a natural morphogen, and it has been known for over fifty years that either too much or too little RA during early development is teratogenic mainly due to anteroposterior patterning defects. In general, excess RA posteriorizes, while RA deficiency anteriorizes chordate embryos Conversely (Niederreither, 2008, Ross et al. 2000, Albalat, 2009a).

1.3.1. RA synthesis and metabolic pathways

Retinoic acid is an endogenous molecule in the embryonic and adult vertebrate, which is derived from vitamin A. It is lipophilic with molecular weight of 300 Da. In the adult RA is the most biologically active naturally occurring member of a family of molecules called retinoids. Retinoids are obtained from the diet in the form of retinyl esters. Cells in the embryo or adult that require RA obtain it from the blood, where it circulates as retinol bound to retinol binding protein. Retinol is the major source of retinoids, which in embryo stage, it obtained from the mother placenta, or from the egg yolk (for ovivarus species).

The synthesis of embryonic retinoic acid (RA) is widely known trough a canonical pathway. Endogenous RA is synthesized in two steps: the first is the reversible oxidation of retinol to retinal performed by alcohol dehydrogenases (ADHs or RDHs/SDRs) and the second is the oxidation of retinal to retinoic acid, a non reversible enzymatic reaction, which is carried out by retinaldehyde dehydrogenases (RALDHs). Retinol is taken up by binding of retinol to cellular retinol binding proteins (CRBPs) from yolk sac or placental transfer. Inside the cell, the retinol is enzymatically converted, to retinal by the retinol or alcohol dehydrogenases (RoDHs or ADHs), and then from retinal to Retinoic acid (RA), by the retinaldehyde dehydrogenases (RALDHs). There are several members of these enzyme classes, and the most important RALDHs for the embryo are RALDH1, RALDH2 and RALDH. After the synthesis, RA then bind to retinoic acid binding proteins (CRABPs) for further trans port. The excess

or un-used RA are further metabolized by two cytochrome P450 enzymes; CYP26A1 and CYP26B, to supposedly inactive products such as 4-oxo-RA, 4-OH-RA, 18-OH-RA and 5,8-epoxy-RA14–16, and is then excreted (figure).

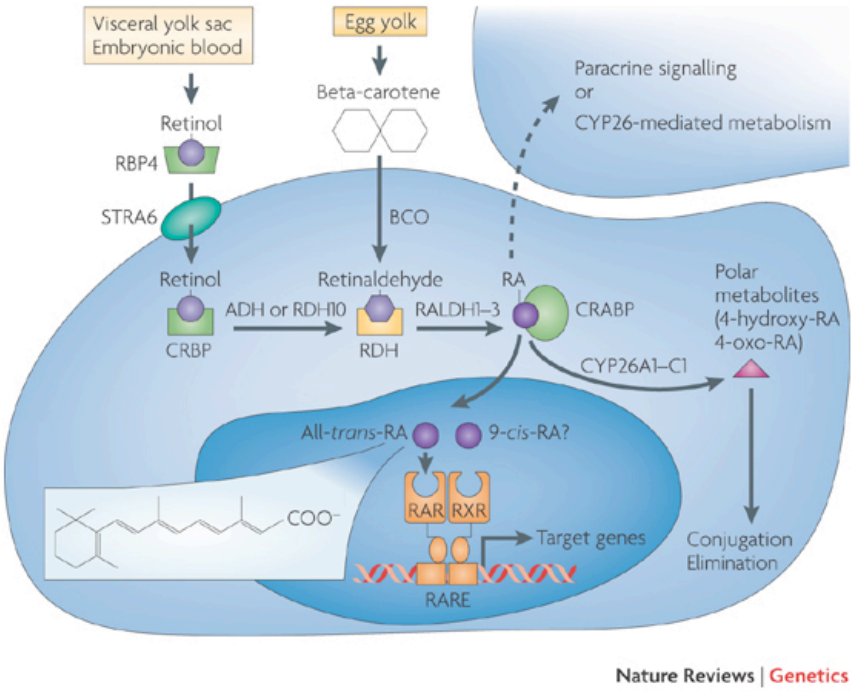


Figure 10. the retinoic synthesis pathway and its function on the gene transcription (modified from Niederreither,K. 2008)

1.3.2. History of RA in development

In early 50s, McCarthay and Cerecedo, (1951) conducted a study on rodents that were fed a vitamin A deficient (VAD) diet, and reported a complex neonatal syndrome (the VAD syndrome) in the rodents. The VAD diet affecting many organ systems, such as the reproductive function and vision. More over, several studies also reported that, the development of many chordate and vertebrate specific characters is controlled, directly or indirectly, by retinoic acid (RA), a vitamin A derived morphogen. In chordate

embryos, the availability of RA in optimum concentration is critically needed; too much or too little RA during early embryogenesis causes malformations, which are mainly due to a mispatterning of the embryo along the antero-posterior body axis. In humans, as in other vertebrates, the alteration of the RA signaling system causes congenital malformations, fertility problems and vision defects, and can lead to tumorigenesis and neurodegenerative (Niederreither, 2008, Albalat, 2009b). RA also controls heart morphogenesis and differentiation, and is involved in the development of several organs undergoing budding morphogenesis, the best studied being the lung, kidney and pancreas (Niederreither, 2008).

Several experimental approaches have been used to investigate functions of RA during early embryogenesis. These were performed in various species (such as zebrafish, *Xenopus laevis*, chicks, quail and mice) and strategies to inhibit RA signaling, such through dietary VAD, loss of function or pharmacological inhibition of synthesizing enzymes, inhibition of RARs, and through dominant-negative RARs. In the late 1980s, RA was shown to be a ligand for several nuclear receptors, thereby directly controlling the transcriptional activity of target genes (Niederreither, 2008, Albalat, 2009b). The roles of these proteins in regulating RA signaling in vertebrates also have been elucidated with gene knockouts (Campo et al. 2008, Marletaz et al. 2006).

Targeted disruption of the murine RA receptor (RAR) genes revealed mainly redundant roles; homozygous disruption of two RARs was necessary to induce abnormalities that recapitulate those of VAD syndrome (Niederreither, 2008). Gene-disruption studies also confirmed that RARs act *in vivo* as heterodimers with retinoid X

receptors (RXRs; nuclear receptors that bind the 9-*cis*-RA stereoisomer) (Nagpal et al. 1993).

RA was shown to regulate embryonic anterior–posterior (AP) patterning, in particular by controlling the expression of specific homeobox genes (Hox genes) (Durston et al. 1989, Simeone et al. 1995). RA has also been implicated as a putative morphogen controlling digit specification in tetrapod limbs, and in regeneration of urodele amphibian limbs (Maden 2002a). Moreover, RA also have been shown for many developing tissues or organ systems, including the facial region and forebrain (Maden 2002b), and pharynx (Mark, 2004).

1.3.3. RA-regulated growth and patterning

In vertebrates, RA seems to be involved in signaling or interpreting positional information at different times during development, for example during specification of the primary anterior-posterior axis. The roles of RA signaling in antero-posterior patterning of the body, central nervous system (CNS) and pharynx have received particular attention (Maden 2002b, Mark, 2004). In the CNS, when vertebrate embryos are treated with RA, anterior neural structures (like the forebrain) are lost and the hindbrain and spinal cord seem to expand to compensate (Campo et al. 2008, Marletaz et al. 2006, Maden 2002b). Retinoic acid (RA) signaling also plays an important role in somite segmentation, where its distribution shows a gradient in the PSM. Suggest that, RA signaling is supposed to be involved in the determination of the position of segmentation.

RA exerts its influence in growth and patterning of embryos by interacting with nuclear receptors or receptor-like proteins to modulate the transcription of genes. These proteins consisting of; transcription activation, **or** suppression regions, a DNA binding domain, a dimerization domain, and ligand binding domains. (Niederreither, 2008). The influence of gene expression by RA is mediated at the level of transcription through binding to dimeric transcription factors formed by RA receptors (RAR) and retinoid X receptors (RXR). The best-known mechanism of action of these receptors involves their binding to RA response elements (RARE) in the promoters of retinoid-responsive genes. RARE composed typically of two direct repeats of a core hexameric motif, PuG(G/T)TCA. The classical RARE is a 5-bp-spaced direct repeat (referred to as DR5). However, RAR/RXR heterodimers also bind to direct repeats separated by 1 bp (DR1) or 2 bp (DR2). RXRs also bind to DR1 as RXR/RXR homodimers (Bastien, 2004)

In the absence of ligand, retinoid receptors are found primarily in the nucleus. They bind as asymetric, oriented RAR/RXR heterodimers to specific DNA sequences or RA response elements (RAREs) (Bastien, 2004). unliganded and DNA-bound retinoid receptors repress transcription through the recruitment of the corepressors NCoR and SMRT (Glass, Rosenfeld 2000) and (Aranda, 2001). The corepressors recruit high molecular weight complexes endowed with histone deacetylase activity (HDACs), which increase the interaction of the N-terminal histone tails with the nucleosomal DNA. Upon recruitment of ligand, the ligand-induced conformational changes favor the interactions between RAR and RXR and therefore increase their DNA affinity (Rastinejad et al. 2000, Depoix et al. 2001). They also cause co-repressor release and create a new hydrophobic cleft formed between H3, H4 and H12 which constitutes a

surface where co-activators can bind. Then the retinoid receptors become able to recruit the transcription machinery (Bastien, 2004). Nevertheless, retinoid receptors also affect transcription through RARE-independent mechanisms, such as repression of transcription factor activator protein (Pfahl 1993), or by modulating the interaction of Sp1 and GC-rich DNA via ternary complex formation (Pfahl 1993, Husmann et al. 2000)

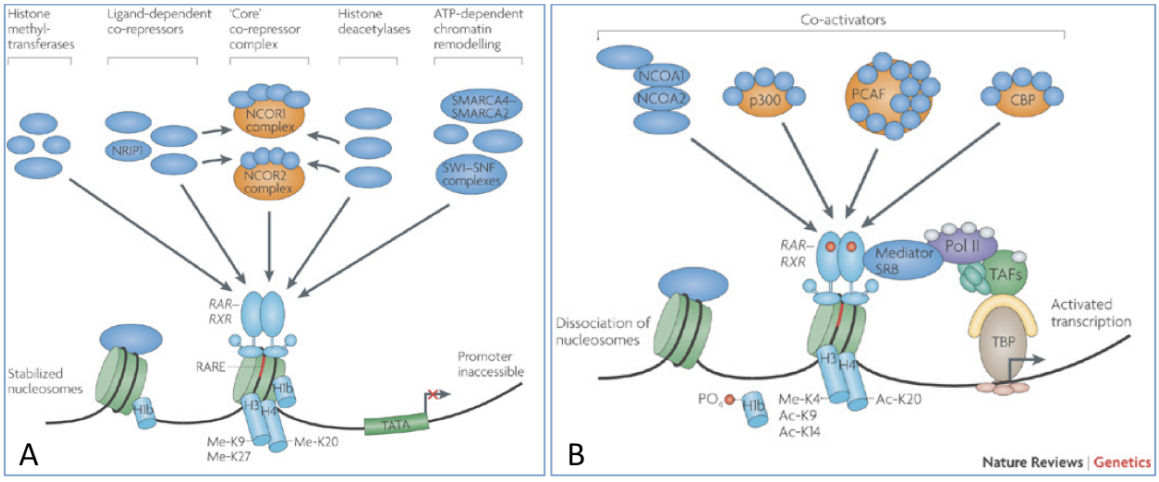


Figure 11 illustration of the mechanisms of RA induces the transcription of genes. (A) In the absence of ligand, RAR–RXR heterodimers bind to RA-response elements (RAREs) in the DNA sequences, and unliganded RARs recruit co-repressors, co-repressors, in turn, mediate their negative transcriptional effects by recruiting histone deacetylase and methyl-transferase complexes, which stabilize the structure, (B) The binding of RA leads to a conformational change of the RAR ligand-binding domain, releasing the co-repressors and recruiting co-activators. Whereas some co-activators interact with the basal transcriptional machinery, others induce chromatin remodelling, which activates transcription, modified from Niederreither,K. (2008)

1.3.4. Physiological activity of retinoic acid; limb development

The use of the retinoid ligand knockout models to study embryonic development has linked the physiological function of retinoic acid to developmental processes. An emerging theme is that retinoid signaling needs to be coordinated with other embryonic

signals, including FGFs and SHH, and that such interactions occur repeatedly during development. Unraveling interactions with other signaling pathways, during elongation of the rostrocaudal axis, development of secondary axial structures (the limbs), forebrain outgrowth, and in many organogenic processes. Imbalances in RA–FGF signaling might contribute to congenital human malformations such as spina bifida. (Xu et al. 2004)

The limb was, historically, the first developing system in which retinoic acid (RA) was postulated to act as a putative anterior–posterior (AP) morphogen. The presence of RA is necessary for the proper induction of Shh in posterior limb bud cells, which act as a signalling centre (the zone of polarizing activity, ZPA) to regulate AP patterning and digit specification. RA acts in concert with posteriorly restricted determinants to induce a functional ZPA that secretes SHH. Once the ZPA interactions with the apical ectodermal ridge (AER) have been established, RA synthesis remains confined to the proximal limb margin from which it probably acts on cell differentiation. The distal proliferating region must be devoid of RA signalling through the action of cytochrome P450 26 B1 (CYP26B1) (McGlinn, 2006, Tickle 2006). Antagonistic effects of RA and FGFs also take place during limb bud development, which has long been studied as a model system to characterize signals that regulate growth and patterning. RA is able to antagonize FGFs, including FGF4, FGF8 and FGF10, which are produced in the apical ectodermal ridge and in the distal mesenchyme and are necessary for sustained limb bud growth and maintenance of distal progenitor cells (McGlinn, 2006, Tickle 2006). Forelimb development is severely compromised in Raldh2–/– mouse mutants, correlating with an absence or poor induction of Shh in the rudimentary buds. Exogenous RA administration can significantly rescue forelimb growth, but is unable to

achieve proper Shh posterior restriction and hence leads to AP digit-patterning defects36. (Niederreither, 2008).

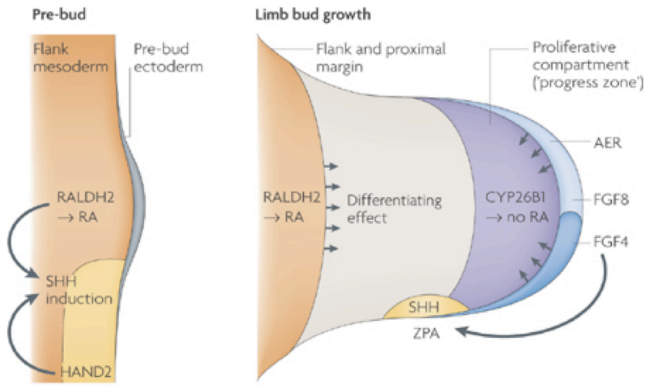


Figure 12 Scheme of limb bud initiation and formation under the influence of RA signaling. RA induce the activation of ZPA to be interact with the apical ectodermal ridge (AER), when it have been established, RA synthesis remains confined to the proximal limb margin from which it probably acts on cell differentiation. Meanwhile, the distal proliferating region must be devoid of RA signaling through the action of cytochrome P450 26 B1 (CYP26B1)

1.3.5. Roles in left-right axis formation

The establishment of the vertebrate body axis; ateroposterior (A/P), dorsoventral (D/V) and left–right (L/R) is central to the organization of the vertebrate body plan (Hamada et al. 2002). All vertebrates' body exhibits external bilateral symmetry, which is particularly obvious at during the somitogenesis, where somites were formed as two symmetrical columns of mesodermal segments on both sides of the embryonic axis (Vermot, 2005). However, in the adult asymmetrical organ positioning along the L/R axis are readily apparent, where internal organs such as the heart, stomach, and intestines all have a characteristic asymmetric structure and are asymmetrically positioned within the body cavity (Capdevila et al. 2000). The earliest morphological

manifestation of L–R asymmetry is the rightward looping of the developing heart. Asymmetric arrangement of internal organs is invariant within a given species and is conserved throughout evolution (Wasiak, 1999).

The process of L/R determination is categorized into four stages: (1) initial breaking of L/R symmetry, which leads to the establishment of specific patterns of gene expression in and around the embryonic organizer; (2) the relay or transfer of L/R-biased signals from the organizer to the lateral plate mesoderm (LPM); (3) the stabilization of broad domains of side-specific gene expression, and; (4) the transfer of L/R asymmetric information to the organ primordia, and the elaboration of specific programs of asymmetric morphogenesis. (Hamada et al. 2002, Vermot, 2005, Vermot et al. 2005)

Retinoic acid (RA) signaling also plays an important role in somite segmentation, and its distribution shows a gradient in the PSM. Thus, RA signaling is supposed to be involved in the determination of the position of segmentation. Because the somite segmentation shows asymmetry in the absence of RA, RA signaling is essential for the symmetry of somite segmentation. Recent report reveals that RA signaling is essential for the symmetry of somite segmentation, in mouse, chick and zebrafish (Hamada et al. 2002, Vermot, 2005, Capdevila et al. 2000, Kawakami et al. 2005).

The body plan of vertebrates has distinct left–right (LR) asymmetries in the disposition of internal organs. Cells and tissues are instructed as to their left or right identity at very early stages of embryo development. Taken together, our results demonstrate that the bilateral progression of somitogenesis in zebrafish embryos

depends on RA signalling and is tightly linked to the cascade of LR organ asymmetry information (Kawakami et al. 2005).

Retinoic acid is also required for normal specification of heart left-right asymmetry. In a large percentage of vitamin A-deficient quail embryos, exhibit the randomization of the heart, which appears on the wrong side (Twal, 1995, Twal, 997). Retinoids, although not directly involved in-assigning cardiac asymmetry genes to their asymmetry-specific sites, are absolutely essential for normal heart sidedness to occur administration of vitamin A to deficient embryos as late as stage 8 (neurulation) rescue the defect of vitamin A-deficient phenotype (Twal, 1995, Twal, 1997)

1.4. Summary of this study

During vertebrate development, the primary body axis elongates posteriorly and is periodically divided into embryonic structures called somites, which give rise to the vertebrae, skeletal muscles and dermis. It has been thought that the period of segmentation depends on the segmentation clock controlled by cyclic genes such as Notch effectors, while the position of segmentation is determined by the opposed gradients of fibroblast growth factor (FGF) and retinoic acid (RA). The anterior somites which create a part of the skull and the cervical vertebrae are formed early, and then the posterior somites which give rise to the thoracic, lumber and sacral vertebrae are progressively generated along with the axis elongation. A difference between the anterior and posterior somitogenesis has been observed in several species including amphioxus, mouse and zebrafish. In zebrafish, for instance, the anterior somites form every 20 min, and then the posterior somites from every 30 min. However, how the pace

of somitogenesis is changed and whether the anterior-posterior difference is important for the later body plan remain unclear.

In this study, we precisely observed somite segmentation using a time-lapse imaging in zebrafish and showed that the anterior-posterior transition of somitogenesis is caused by changing the overlapping rate of segmentation, not by the difference of segmentation period. We also showed that RA depletion by knockdown of a RA synthetic enzyme *raldh2* resulted in the extension of segmentation period in somites 4 and 5, which correspond to the transition, eventually leading to loss of a somite and the second cervical vertebra. Furthermore, RA administration in *raldh2* morphants resulted in normal segmentation period in somites 4 and 5, and restored loss of a somite and the cervical vertebra. Overall, our results uncover a novel mechanism mediated by RA that adjusts segmentation period to the anterior-posterior difference of somitogenesis, which is required to link the head and trunk properly in the zebrafish embryo.

CHAPTER II
MATERIALS AND METHODS

2.1. Test Organisms; Zebrafish and it maitenance

Wild-type zebrafish were maintained, reared and staged, under laboratory conditions according to standard protocol{{2 Kimmel,C.B. 1995;}}. The embryos for the experiments were obtained by natural mating, which was conducted by using 6 (six) set of matting box, each contain of 2 male and 2 female, respectively. The fishes were set at 07: ^{00}-09: ^{00} p.m. of one day prior to the mating, which were conducted at around 09:^{00} -10:^{00} a.m. Male and female fish were put together for about 15 minutes during the mating process, in order to obtain similar stage of embryo. The Embryos were collected, wash with medium and used for the experiment or kept in 28.5^{o}C.

2.2. Time-lapse Imaging

Time-lapse imaging was conducted to obtain life image of somite formation during early stage of zebrafish embryo. Picture series were recorded on a motorized stage Olympus FluoView FV1000 confocal microscope, which is driven by Fluoview software. Pictures were made using standard bright field optics by using multi-line Argon laser that was set on 488nm. UPLSAPO 5x10 objective lens were used and images were obtained with olympus digital camera. For every single take, the depth of picture was set up to 10 (ten) Z layer or stack, with 17.15μm spacing of each stack. Automated picture acquisition was set to take one picture for every two minutes

interval. Temperature of the stage was automatically set at 28.5 ^{o}C and real time temperature was regularly measured each time of experiment.

Embryos at shield stage were dechorionated in the medium and than mounted into a drop of 1% low melting agarose, which be positioned at the center of a glass-bottom dish. The embryos were carefully oriented and fixed in a latero-lateral position. When the desired position is fixed, the glass bottom disk than filed with fish medium. Two zebrafish embryos were used in each set of time lapse imaging. Two different position of embryo were set for different stage of embryo during the picture acquisition. Lateral view of time lapse imaging was conducted to record the formation of somite number 1 to 8, and dorsal view of time lapse was aimed to record somite formation at 12 to15 hpf. After the time lapse imaging, embryo were carefully recovered from the agarose, and maintain for the further examination or morphological determination.

The pictures were selected, appended and combined by Fluoview sofware. Picture file than where stacked from different Z-planes, time stamped and processed by Metamorph software to produce movies.

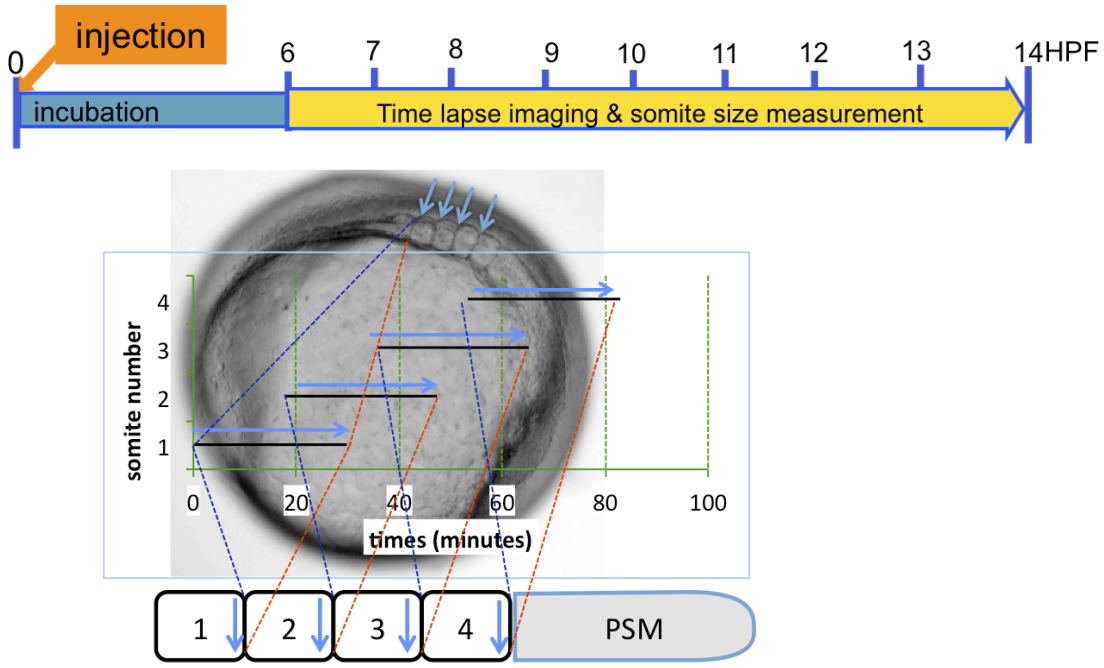
2.3. Whole-mount in situ hybridization

Embryos at desire stage were transferred to the flacon and fixed in 4% paraformaldehyde for overnight at 4^{o}C temperature. Embryos than were dechorionated, dehydrated and stored in 100% methanol at -20°C for next processes. Digoxigenin-labeled RNA probes were transcribed using a SP6/T7 DIG RNA

Labeling Kit (Roche) for: *her1, mespb, papc, ripply1, raldh2, fgf8, tbx16*. Whole-mount ISH was performed on fixed embryos as previously described.

2.4. Somitogenesis period and somite length measurement

Time-lapse movies were visually analyzed to determine the somite segmentation period. Starting point of boundary formation was decided when a furrow start to visible in the dorsal limit of the somitic mesoderm. These time point then noted as zero (0) minute. Somite was considered formed when it boundary was clearly visible, and separated the somite from dorsal to ventral side of embryos.



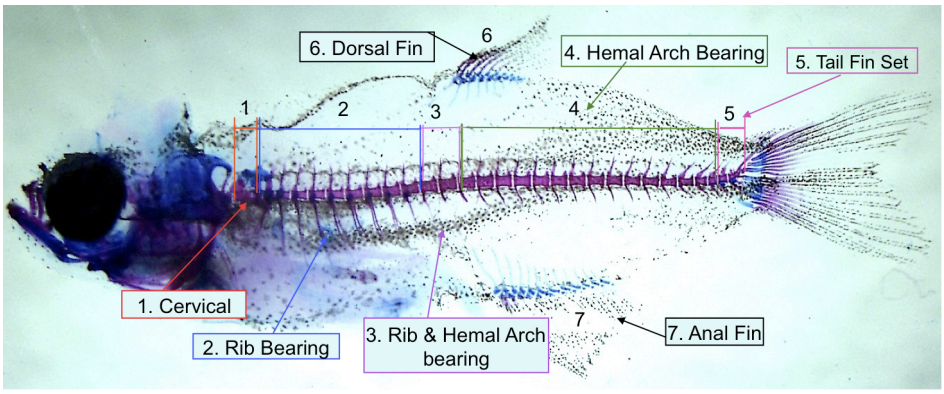
Figures 13. Illustration of segmentation period analysis on time lapse; the somite segment started to form as furrow in the dorsal part of unsegmented paraxial mesoderm (PSM), then continue to form segment dorso-ventral direction. The graph illustrate the progression of real time somite formation, where each somite is presented as a horizontal bar, each length of bar indicate the period of somite

formation in minutes. (overlapping rate = the conjoint of time when previous somite is formed and the next somite is started to form)

Somite length was measured on newly formed somite. The length of each somite, along the axis, was measured by drawing a straight line connecting the previously formed to newly formed somite boundary, in the middle part of the left somite.

2.5. Skeletal staining

Bone staining was conducted to visualize the vertebrae. Alcian Blue, 8GX (CI 74240), and Alizarin Red S (CI 58005), were used to stain non-mineralized cartilage matrix and mineralized cartilage and bone matrix, respectively. Zebrafish at the age of 1.5 months, were immobilized in ice water then fixed for 48 hours in ethanol 100%, remove scale and clean internal organ for adult fish. The fish than transferred to acethone to remove fat for 24 hours, and than stained with staining solution in 37^{o}C for 48hours. After the staining fish is washed using dH_2O and were placed in 0.05% KOH mg/ml until the vertebrae were clearly seen, than transferred to Moll solution containing 1%KOH until clear. The fish than transferred to 20% glycerin solution for picture making, than stored in 50% glycerin solution. Vertebrae of control morphants and raldh2morphants were characterized and the numbers were counted. Vertebrae where categorized following (Morin-Kensicki, 2002)



Figures 14. Bone staining for characterization and counting of vertebra. Vertebra were classified for anterior to posterior as: cervical, rib bearing, rib and hemal arch bearing, hemal arch bearing, and tail fin set

2.6. Microinjection of Morpholino Oligonucleotides (MOs).

Control and raldh2 MOs were obtained from Gene Tools as follows: universal control MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′; and raldh2mo-MO 1, 5' - GTTCAACTTCACTGGAGGTCATC - 3'. 0,2mM of control and raldh2 morpholino were injected into blastomere of one-cell-stage zebrafish embryos

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2.7. DEAB treatment

DEAB (diethylaminobenzaldehyde, Fluka) is a competitive reversible inhibitor of aldehyde dehydrogenases. A stock solution were prepared at the concentration of 10^{-2}M DEAB in dimethyl sulfoxide (DMSO). The stock was stored in the dark container and keep at temperature of -20°C. Embryos were treated in the dark in a final dilution of 10^{-5}M DEAB in fish medium. Controls were placed into equal concentration of DMSO. The chorion of experimental and control embryos

were manually tortured before treatments, to allow the penetration of DEAB, and the fish then placed into medium for the treatment.

DEAB was applied in the dark, in concentrations of 10^{-5} M and 2×10^{-5} M in embryo medium from a 10^{-2} M stock in DMSO. As controls, sibling embryos were treated with equivalent concentrations of DMSO

2.8. Retinoic acid (RA) rescue

All−trans−Retinoic acid (ATRA) from Sigma Aldrich is used for the reascue experiment. ATRA is a ligand for both the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Range finding test were conducted by exposing batches of wild type (WT) embryos to different concentration of RA, in order to determine the concentration suited for rescue experiment. The RA exposure procedure was done by putting embryos (n=30) in separate Petri dish contain fish medium. Embryos than were exposed from 4-12 and 4-15 hours post fertilization (hpf), respectively, at concentrations of 0, 0.05, 0.1, 1.0, 5.0, 15.0 and 50 nM of AT-RA. On the other hand, embryos treated with DMSO were used as controls. The dish than wrapped with aluminum foils to avoid the light and kept in 28.5^{o}C incubator. After exposure, the embryos phenotype was determined and somites were counted. At last, concentration of 1nM was chosen for the rescue experiment.

Rescue experiment was conducted following the work of range finding test, except for the concentration and the period of exposure. The control and *raldh2* morphant embryos were treated with 1nM of ATRA, for the period of 4-10hpf, 4-

14hpf, and 10-14hpf. The rescue were determined by the total number of somite of treated embryos which be compared to somite number of the control.

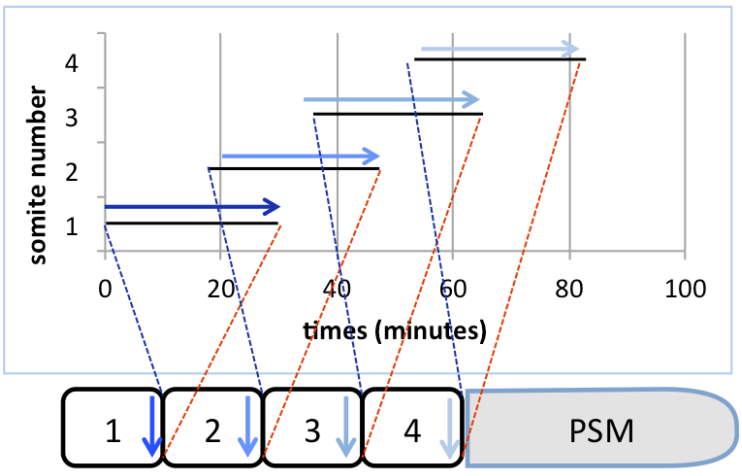
CHAPTER III
RESULTS

3.1. Anterior somitogenesis is different to posterior somitogenesis in zebrafish embryo

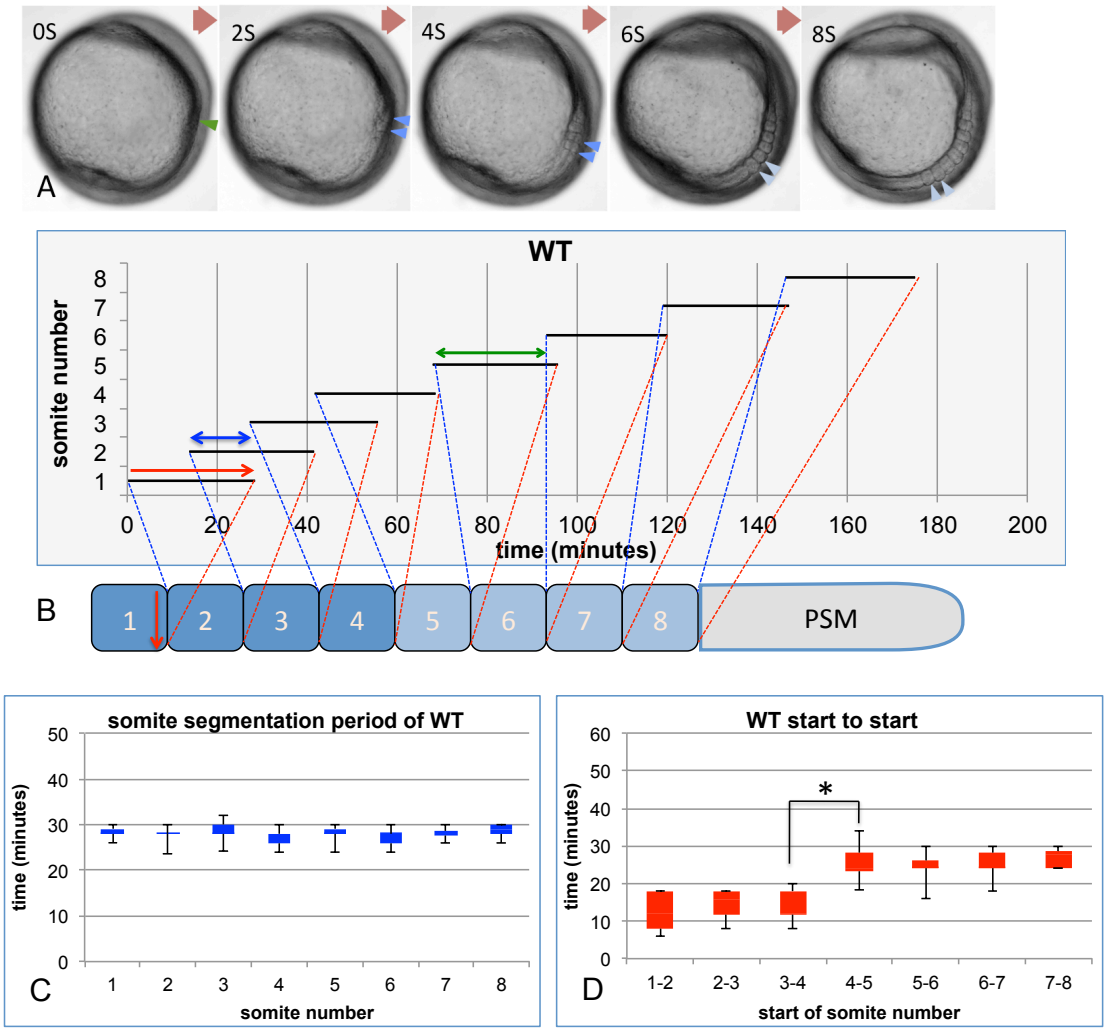
The patterning of anterior-posterior in the vertebrate embryo in general could be divided into two major processes: an initiation phase, when the embryo is forming the head and the body (primary body), and an elaboration phase, in which the body progressively forming the trunk and tail (secondary body) (Kimelman, 2011). The initiation phase, in zebrafish, occurs before the start of gastrulation, where the primary body axis grows posteriorly and is concomitantly segmented and arises sequentially into somites. The anterior somites that give rise to the cervical vertebrae created early, while the more posterior somites that become the thoracic, lumbar, and sacral vertebrae form at progressively later times. (Szeto, 2006; Kimelman, 2011). Anterior trunk somitogenesis observed in mice, zebrafish, and the cephalochordate amphioxus is more rapid progression of the somite cycle, relative to posterior somitogenesis . In the zebrafish, the anterior six somites form every 20 min, while the 24 posterior somites form every 30 min (Hanneman, 1989). In amphioxus, this temporal difference is even more extreme where, the anterior approximately eight somites form every hour but each subsequent somite cycle is 18 h (Hanneman, 1989, Kimmel et al. 1995, Irmler, Schmidt & Starck 2004).

To precisely measure segmentation period in each somite, we next investigated when somite formation starts and ends (Fig. 17). Although we did not recognize obvious difference of segmentation period between somites 1 to 8 (28.9 ± 2.1 min), we found that overlapping rate of somitogenesis differs from somites 1 – 4

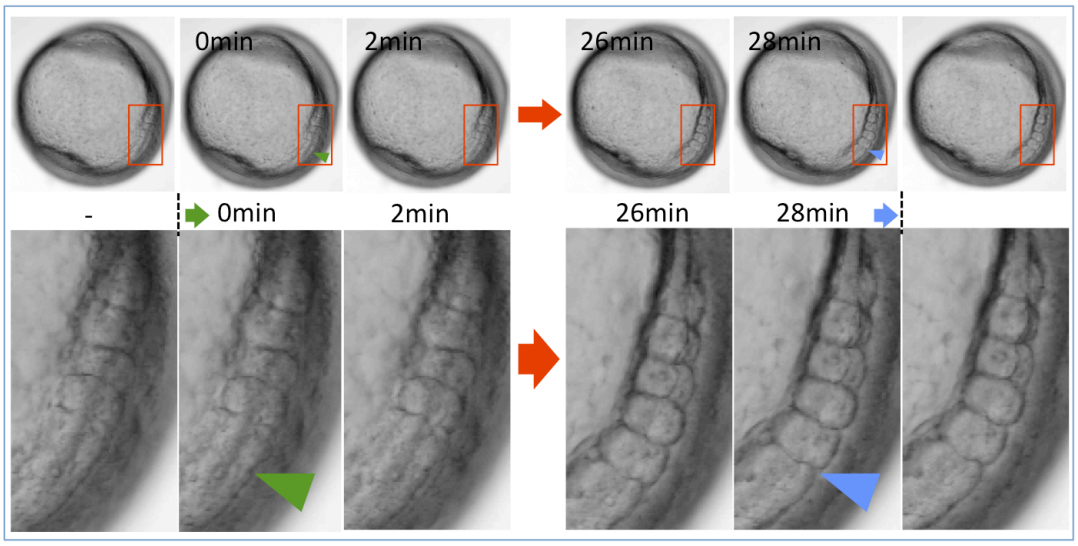
to 5 – 8. In the anterior somites 1 to 4, segmentation between a somite and the next somite was timely overlapped for 13.6 ± 2.5 min, while overlapping times during formation of the posterior 5 to 8 somites became small by 20% (2.7 ± 2.5 min) relative to those of the anterior somites (Fig. 16). The similar tendency of sudden change between the anterior and posterior somitogenesis was also seen in different zebrafish strains such as Riken-wako, India and TL, indicating that the transition is not specific for a zebrafish strain. These results therefore, suggest that the transition between the anterior and posterior somitogenesis is originated from the different overlapping rate of segmentation between a somite and next somite in zebrafish embryos.



Figures 15. Illustration of somites segmentation period on time-lapse analysis; the somite segment started to form as furrow in the dorsal part of unsegmented paraxial mesoderm (PSM), then continue to form the segment in dorso-ventral direction. The graph illustrate real time progression of somite formation, where each somite is presented as a horizontal bar, each length of bar indicate the period of somite formation in minutes.



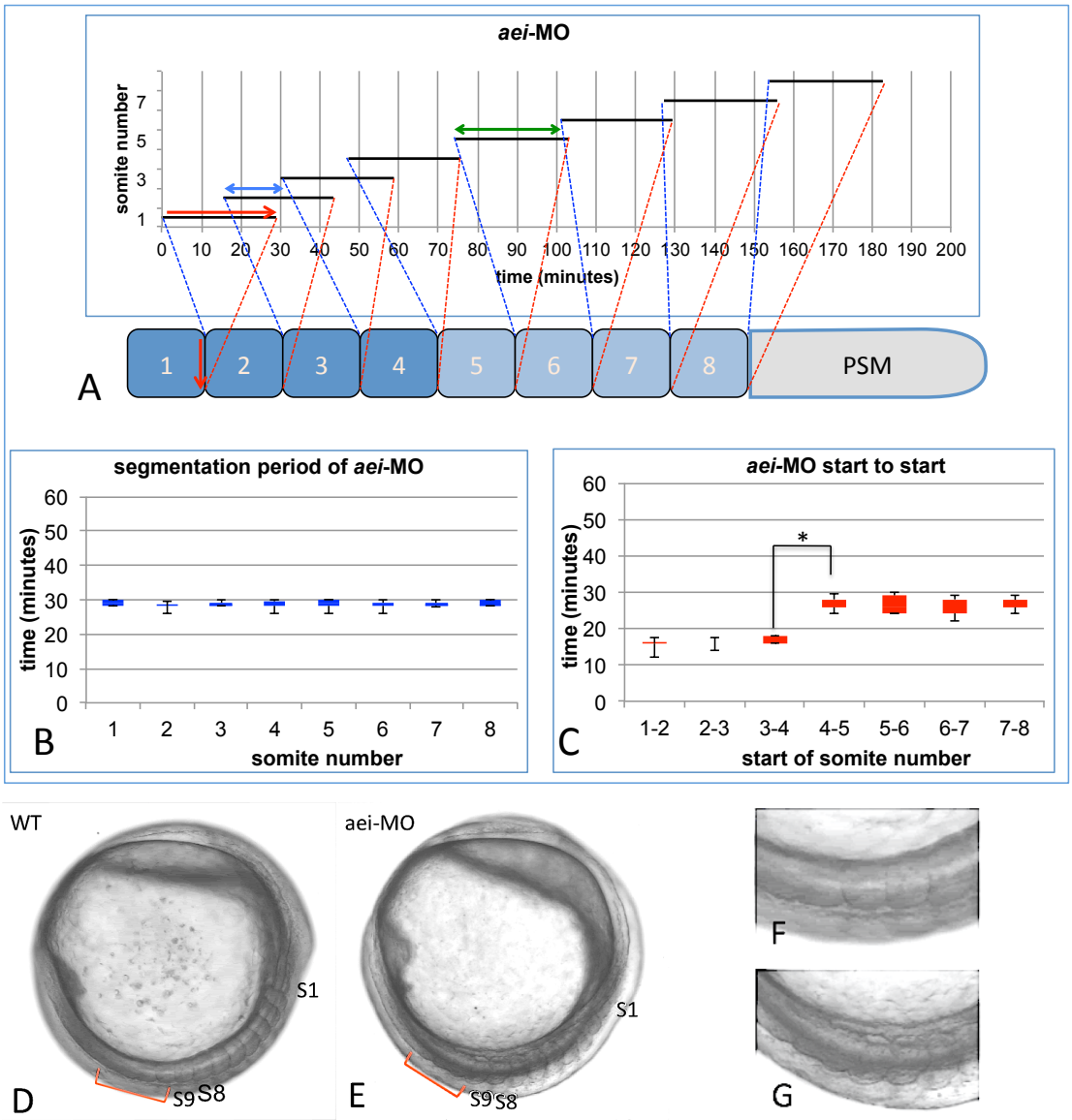
Figures 16. Lateral views time-lapse imaging at 1 to 8 somite stage of wild type (WT) zebrafish embryo, (A) the anterior to posterior sequential somite formation of somite number 1 to 8; arrowhead indicate number of somite formed on the embryo, (B) graph illustrate the progression of real time somite formation. The entire bar has similar length, but short of start-to-start interval shown by somite 1 to 4 (blue double head arrows) and long start to start interval on somite number 5-8. It is important to be noted that the transition of short and long interval is occurs between somite numbers 4 to 5, (C, D) Box-and-whisker plots representing comparison of start to start and segmentation period of somite 1 to 8. The plot shows short interval of each starting point of somite 1-4, but long interval between somite 5-8, which is significantly different (star) (C). Constant of segmentation period are shown by entire somite (D). (The box covers the interquartile range with the median indicated by the line within the box, small bars indicate higher and lower values, 0S=0 somite, 4S=4somite, 8S=8somite, overlapping rate = the conjoint of time when previous somite is formed and the next somite is started to form)



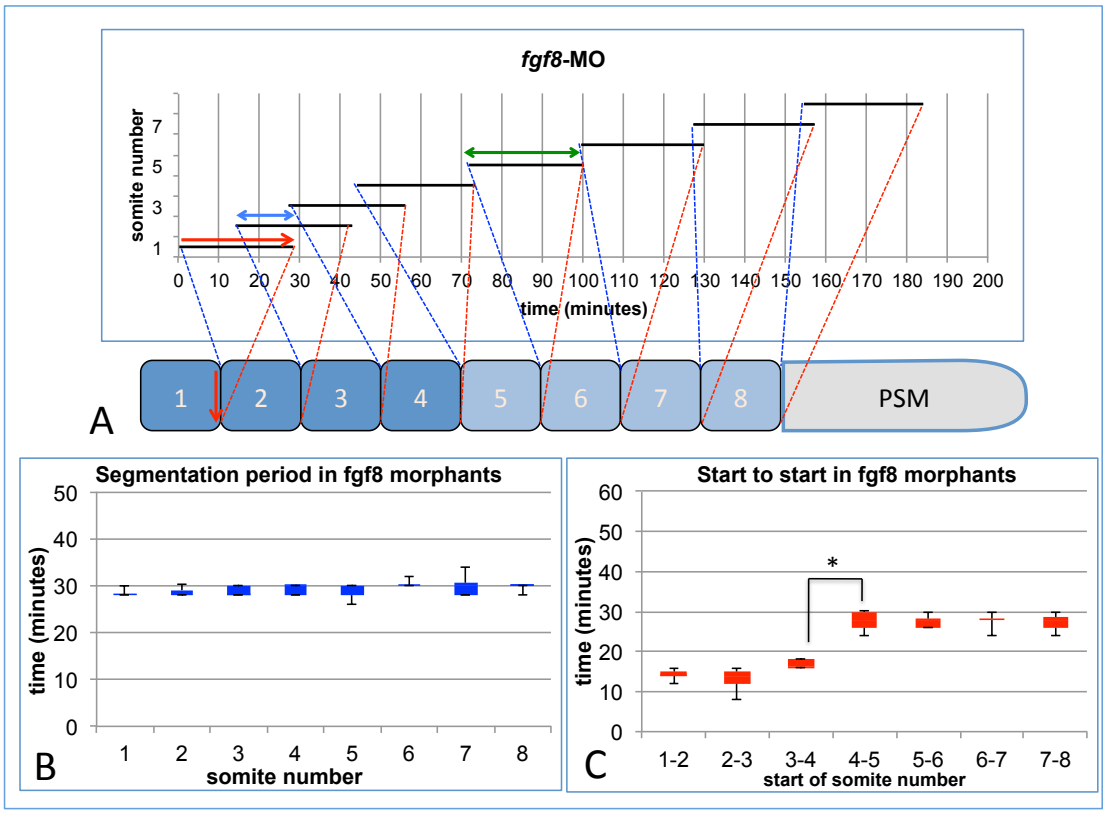
Figures 17. Determination of start to end point of one somite formation. The start of segmentation of a somite was decided when a furrow appear in the dorsal part of unsegmented PSM. This is considered as starting point of new somite formation (green arrowhead) and set as zero minutes. The completion of somite formation decided when a clear segment or somite boundary was formed and completely separate somite from PSM (blue arrowhead denoted the complete somite segmentation)

3.2. Notch effectors and FGF8 do not control anterior-posterior somite transition

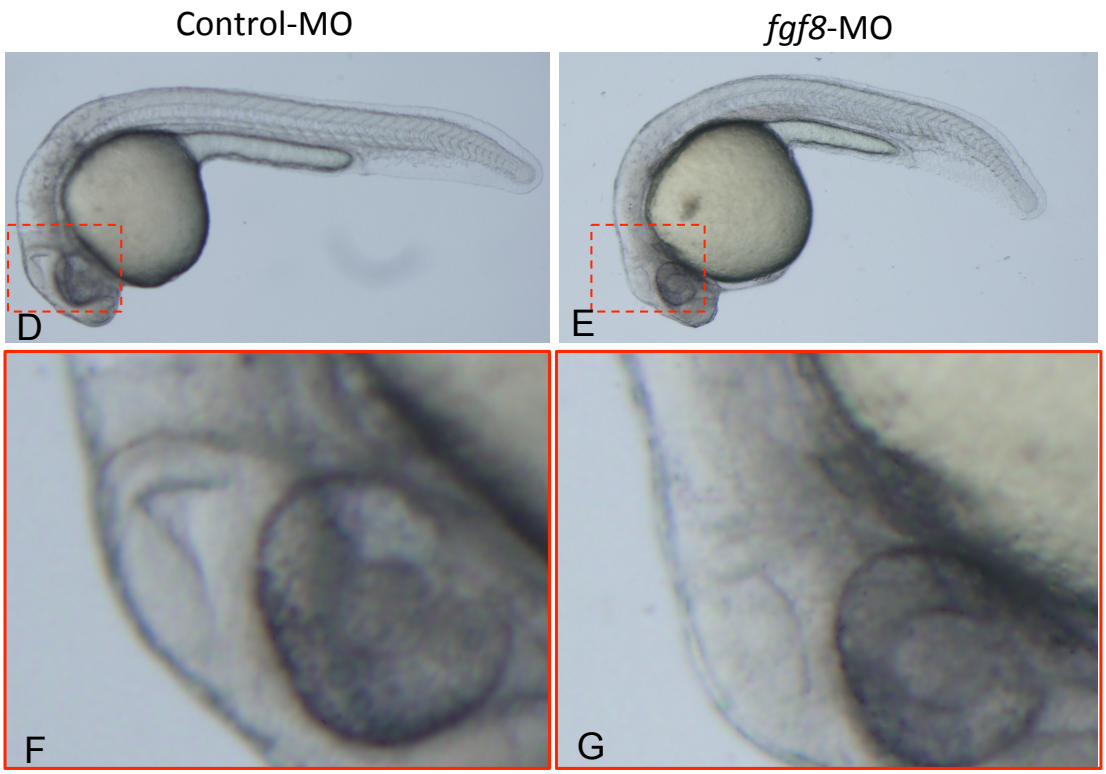
In zebrafish, somite segmentation is regulated by the combined action of Notch, FGF and RA activities. To test their involvement in the anterior-posterior transition of somitogenesis, we inhibited the activity of these signals using antisense morpholino oligonucleotides. The result shows that knockdown of a Notch ligand *deltad* or an FGF ligand *fgf8*, which is known to regulate Notch or FGF signaling in the context of somitogenesis, respectively, did not resulted any defects or failure of the anterior-posterior transition of somit formation (Fig. 18, 19).



Figures 18. Lateral views time-lapse imaging at 1 to 8 somite stage of *aei* morphants embryo, (A, B, C) anterior to posterior sequential somite formation with short of start-to-start interval shown by somite 1 to 4 (anterior somite), followed by long start to start interval on somite number 5-8 (posterior somite). The graph also indicates that the transition of anterior to posterior somite occurs between somite numbers 4-5. (B,C) Box-and-whisker plots of the somite formation show similar feature as seen in WT zebra fish embryo, suggesting no discrepancy of somite formation and anterior posterior transition on somite 1 to 8, in *aei* morphant. (D,F) Recovered embryo after time lapse imaging shows later stage phenotype of WT, which has regular somite boundary on somite no 8, 9, 10, in contrast to (E,G) irregular of somite boundary in *aei* morphant on somite number 9-10 and later somite. These both phenotypes provide an evident of morpholino oligo efficacy in knock down experiments.



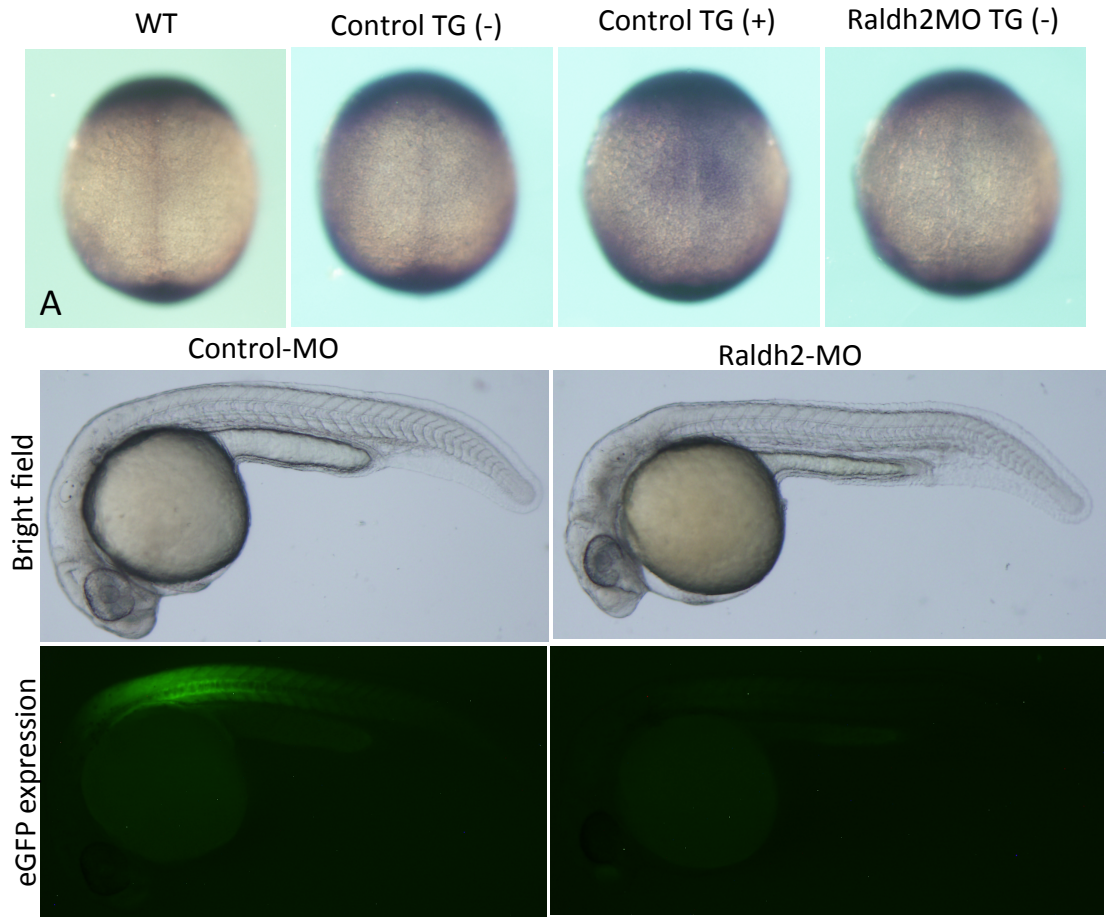
Figures 19. Lateral views time-lapse imaging at 1 to 8 somite stage of *fgf8* morphant embryo (A, B, C) anterior to posterior sequential somite formation with high overlapping rate of anterior somite, followed by low overlapping between posterior somite (5-8). The graph also indicates the transition of anterior to posterior somite, which occurs between somite numbers 4-5. (B,C) Box-and-whisker plots of the somite formation, show similar feature as seen in WT zebra fish embryo suggested no discrepancy of somite formation and anterior posterior transition on somite 1 to 8 in *fgf8* morphants. (D,F) WT phenotype of recovered embryo after time lapse imaging, shows the clear "cerebellum and the mid-hindbrain fold", in contrast to (E,G) *fgf8* morphant which is lack of the fold. The both phenotypes provide an evident of morpholino oligo eficacy in knock down experiments



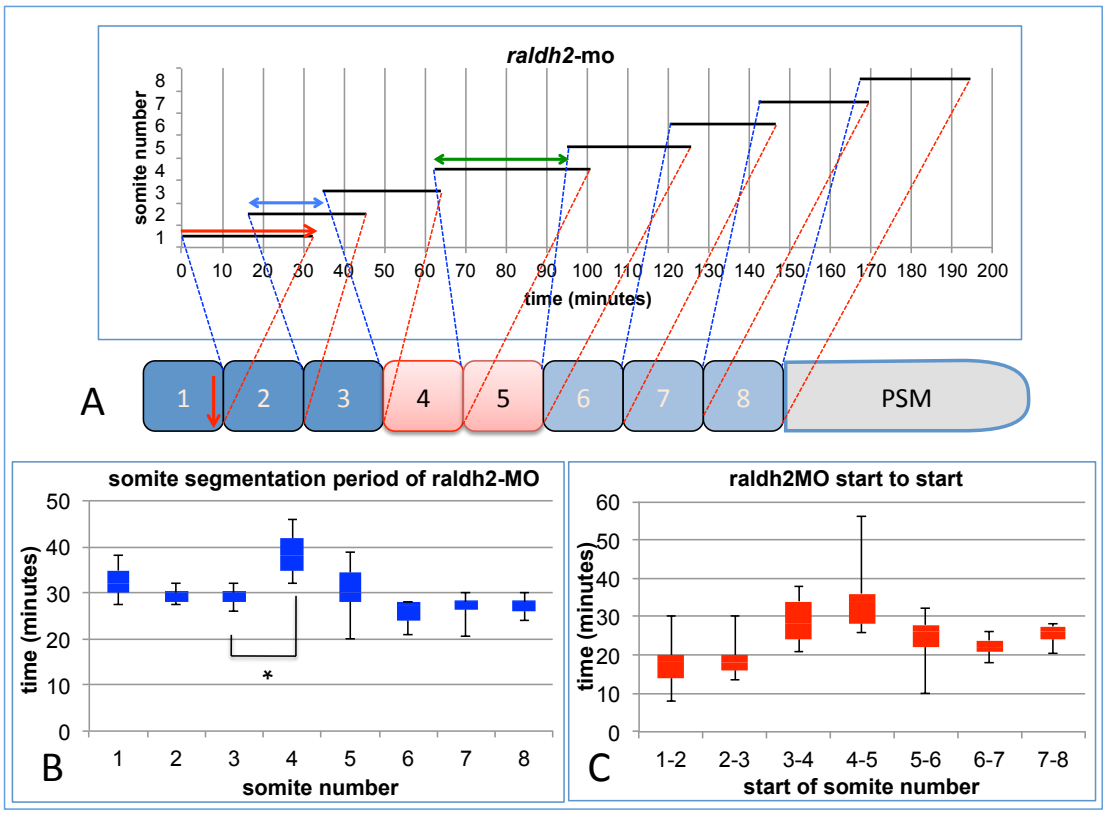
Figures 20. Lateral views time-lapse imaging at 1 to 8 somite stage of *fgf8* morphant embryo (A, B, C) anterior to posterior sequential somite formation with high overlapping rate of anterior somite, followed by low overlapping between posterior somite (5-8). The graph also indicates the transition of anterior to posterior somite, which occurs between somite numbers 4-5. (B,C) Box-and-whisker plots of the somite formation, show similar feature as seen in WT zebra fish embryo suggested no discrepancy of somite formation and anterior posterior transition on somite 1 to 8 in *fgf8* morphants. (D,F) WT phenotype of recovered embryo after time lapse imaging, shows the clear "cerebellum and the mid-hindbrain fold", in contrast to (E,G) *fgf8* morphant which is lack of the fold. The both phenotypes provide an evident of morpholino oligo eficacy in knock down experiments

3.3. RA signaling is essential for proper transition between the anterior and posterior somitogenesis

We knockdown of *raldh2* which is a major source of RA in the context of somitogenesis by following the same strategy as in *eai*-MO and *fgf8-MO*. Surprisingly knock down of *raldh2* resulted a defect on the anterior to posterior somitogenesis transition (Fig. 22).



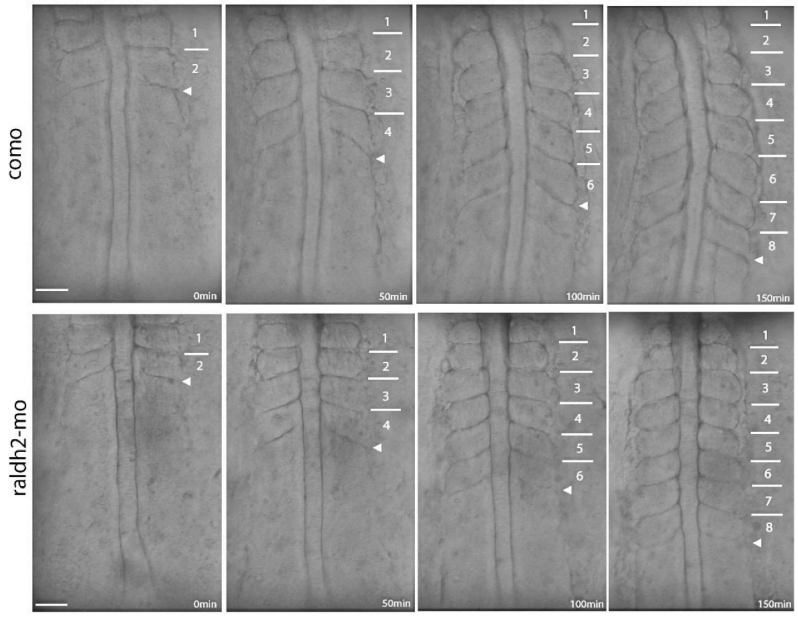
Figures 21. ***raldh2*-morpholino efficacy;** the somite segment started to form as furrow in the dorsal part of unsegmented paraxial mesoderm (PSM), then continue to form the segment in dorso-ventral direction. The graph illustrate real time progression of somite formation, where each somite is presented as a horizontal bar, each length of bar indicate the period of somite formation in minutes.



Figures 22. Lateral views time-lapse imaging at 1 to 8 somite stage of *raldh2* morphant embryo (A,B,C) anterior to posterior sequential somite formation of somite 1-8. Graph shows regular formation and high overlapping rate of somite number 1-3, and low overlapping rate in somite 5-8. Note; low overlapping rate between somite 3-4 (double head blue arrow), and extension of segmentation period of somite 4 and 5. (B,C) Box-and-whisker plots show the of the somite formation, show 8 somite formation, where in somite number 1-3 segmentation period occurs normally, but followed by high interval of start to start between somite number 3-4 and 4-5. The graph also shows longer somite segmentation period of somite 4 and 5, which is significantly different (star) compare to same somite in WT ones. On the posterior part (somite 6-8) low overlapping rate (double head green arrow) and normal segmentation period could be seen. These suggest that a discrepancy occurs on the transition of anterior to posterior somite in *raldh2* morphant.

raldh2 morphants initiated somitogenesis similar to that of embryos injected with control-MO. However, as somite formation proceeded, *raldh2* morphants yielded the transient extension of segmentation period at somites 4 and 5 (10 ± 3min;

about 38% longer than that of control). However, when we measure the somite formation in l2 to 15 hpf (Fig. 23), we couldn't find any different of somite number formed in control and *raldh2* morphant. During 150 minutes 6 somites were formed in both morphants. These results suggest that RA signaling ensures timely somite formation at the transition between the anterior and posterior somitogenesis.

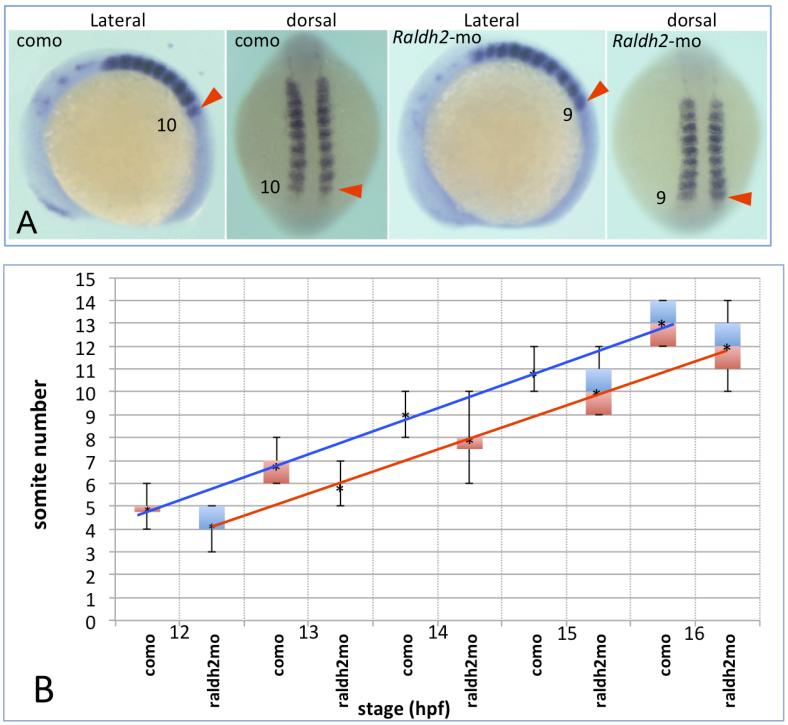


Figures 23. Dorsal view time-lapse imaging represents the somite formation of control and *raldh2* morphants zebrafish at 12-15hpf**,** show that within 150 minutes 6 somites were formed in both control and *raldh2* morphant. Suggested no differences on somite formation in the posterior part of embryo in control and *raldh2* morphants

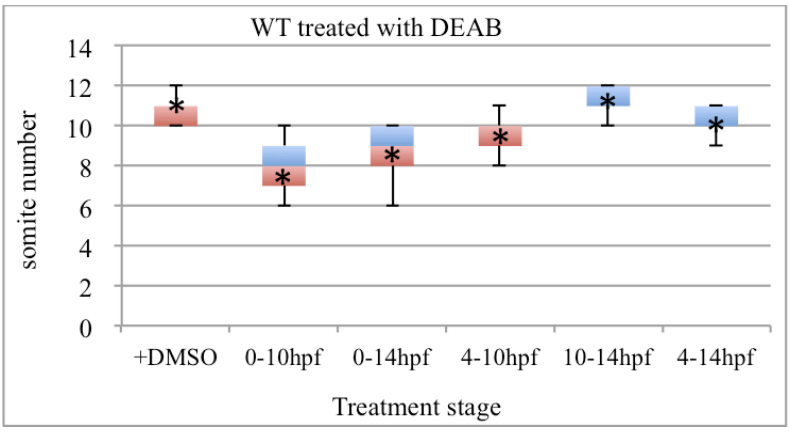
3.4. The transient extension of segmentation period at somites 4 and 5 in RA depleted embryos leads to loss of a somite.

Since the extension of segmentation period in *raldh2* morphants might decrease total number of somites, we counted somite number in zebrafish embryos at different embryos stages. Consistent with the data observed by time-lapse imaging,

raldh2 morphants lost a somite relative to control morphants throughout the posterior somite formation (Fig. 24).



Figures 24. *in situ* hybridization for *Uncx 4.1* was conducted to determine total somite number of control and *raldh2* morphant, in several times point of embryo development. (A) Dorsal and lateral view of control and *raldh2* morphant, show a total of 10 somite in control, and 9 somite were formed in *raldh2*-morphant (red arrowheads) (B) graph shows one somite is missing in *raldh2*-morphants compare to control, at 12hpf, which also could be observed on 13, 14, 15, and 16 hpf of measurement time point (The box covers the interquartile range with the median indicated by the line within the box, small bars indicate higher and lower values, stars indicate the average of somite number).



Figures 25. Box-and-whisker plots of somite number of DEAB embryo. Embryos were treated with 1mM of DEAB, for the period of 0-10, 0-14, 4-10, 10-14 1nd 4-14hpf respectively, and DMSO as control. The somite number were counted and plotted as in graph. DMSO and DEAB treated embryo for 10-14hpf show similar number of somite (11 somites), while treatment of 0-10 and 0-14 caused severe effect on embryo. DEAB treated embryo for 4-10 and 4-14, have 10 somiet in total, which is 1 somite less in average compare to control **(**DEAB=inhibitor of RA synthesis enzyme**)**

Next, in order to confirm that the lost of somite is specifically caused by *raldh2* knock down. We treated the embryo with DEAB, an inhibitor of RA synthesis enzyme for several different time windows (Fig. 25). The result shows that treatment of DEAB for 4 – 10 but not 10 – 14 hpf, led to loss of a somites in zebrafish embryo.

These results suggest that RA signaling is essential for proper transition between the anterior and posterior somitogenesis, and that the failure of the transition then leads to loss of a somite.

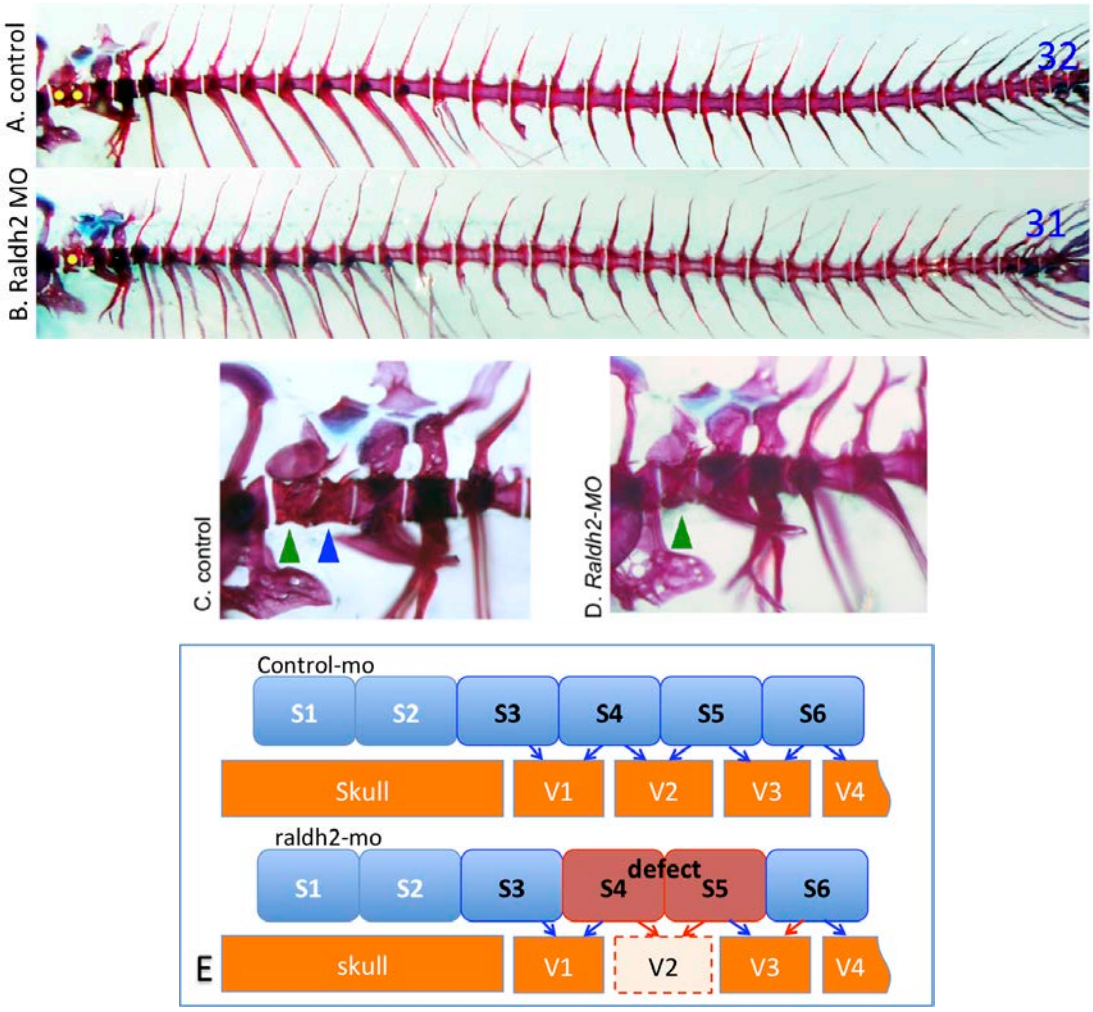
3.5. RA depletion cause a defect on somites 4 and 5, eventually leading to loss of the second cervical vertebra

Because a vertebra is created by the caudal part of somite and the rostral part of next somite, somite number is correlated with vertebra number. The first two somites do not contribute to the vertebral column, while somites 3 to 34 give rise to 32 vertebrae; in an anterior to posterior sequence, 2 cervical, 10 rib bearing, 1 rib and hemal arch bearing, 14 hermal arch bearing and 4 tail fin set vertebrae are formed. Since *raldh2* morphants lost a somite by the extension of segmentation period at somites 4 and 5, we reasoned that *raldh2* morphants should result in loss of a vertebra at later stages.

To test this, we investigated the number and characteristics of vertebrae in *raldh2* morphants by staining the bones and cartilages. We then compare the morphology of the bone in both control and *raldh2* morphant, which show no different on both morphant.

Next, we compare the number of each segment of vertebra of control and *raldh2* morphant. Although bones and cartilages within the vertebral column of *raldh2* morphants morphology were indistinguishable from those of control morphants, this manipulation resulted in loss of the second cervical vertebra (Fig. 26 and table 3). Table 3 showed the average number of vertebrae in WT, control morphant and *raldh2* morphant. WT and control morphants has an average of 32.1±0.9, with the individual range from 31 to 34 of total vertebrae number in the group. On the other hand, *raldh2* morphant has 31.2±1.1 vertebrae in average; ranged from 29 to 33 of total vertebrae number in the group. Interestingly, only in the

cervical bone control and *raldh2* morphant show a signifant different in average number. Control has 2.0±0.0 cervical bones in average, while *raldh2* morphant has 1.3±0.5 cervical bones. The other bone type; rib bearing, rib and hemal arch bearing, hemal arch bearing and tail fin set vertebrae show no significant different in average number between control and raldh2 morphant. Because the second cervical vertebra is derived from both the caudal part of somite 4 and the rostral part of somite 5, loss of the second cervical vertebra in *raldh2* morphants is consistent with the earlier defect in the anterior-posterior transition at somites 4 to 5. These results therefore suggest that RA depletion fails to ensure timely segmentation of somites 4 and 5, eventually leading to loss of the second cervical vertebra.



Figures 26. Skeletal staining with alizarin red and alcian blue of 1.5 month of Zebrafish (A-B). Side by side comparison of control and *raldh2* morphant, shows similar morphological pattern of control and *raldh2* morphant vertebrae. the counting reveal a less number of vertebrae in *raldh2* morphant; a total of 32 vertebrae were observed in control compare to 31 on *raldh2* morphant. (C) control has two cervical vertebrae compare to (D) one cervical in *raldh2* morphant (E) Diagrammatic representation of the somite and vertebrae relationship (modified from Morin-Kensicki, 2002). The diagram illustrate that somite number 1 and 2, didn't contribute to the vertebrae formation, while, posterior part of somite 3 and anterior part of somite 4 together form 1st cervical vertebrae. These patterns were followed by 2nd cervical and next vertebrae formation in the same manner. On the other hand, defect on somite 4-5 on raldh2 morphant, resulted the lost of second cervical vertebrae in adult stage

Table 3. Vertebrae characteristic and number of WT, control-MO and *raldh2* morphant

WT

Vertebrae	Averages±s.d.	Minimum	Maximum	Mode	n
Total	32.5±1.0	31	34	33	21
cervical	2.0±0.0	2	2	2	21
Rib Bearing	10.2±0.4	10	11	10	21
Rib and hemal arch bearing	1.9±0.4	1	2	2	21
hemal arch bearing	15.4±0.9	14	17	16	21
Tail fin set	3.0±0.2	3	4	3	21

CoMo

Vertebrae	Averages±s.d.	Minimum	Maximum	Mode	n
Total	32.1±0.9	31	34	32	18
cervical	2.0±0.0	2	2	2	18
Rib Bearing	10.2±0.4	10	11	10	18
Rib and hemal arch bearing	1.9±0.3	1	2	2	18
hemal arch bearing	15.1±0.8	14	16	15	18
Tail fin set	3.0±0.0	3	3	3	18

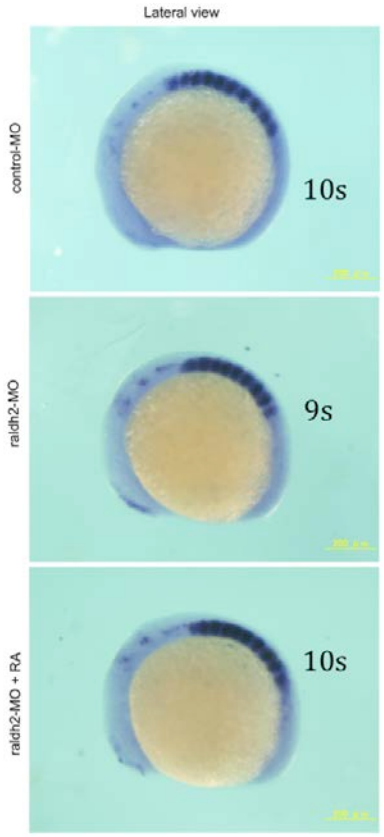
raldh2-mo

Vertebrae	Averages±s.d.	Minimum	Maximum	Mode	n
Total	31.2±1.1	29	33	31	17
cervical	1.3±0.5	1	2	1	17
Rib Bearing	9.9±0.3	9	10	10	17
Rib and hemal arch bearing	1.9±0.3	1	2	2	17
hemal arch bearing	15.2±0.9	14	17	15	17
Tail fin set	3.0±0.0	3	3	3	17

3.6. Retinoic acid application partially restores the defect of somite and and vertebra number

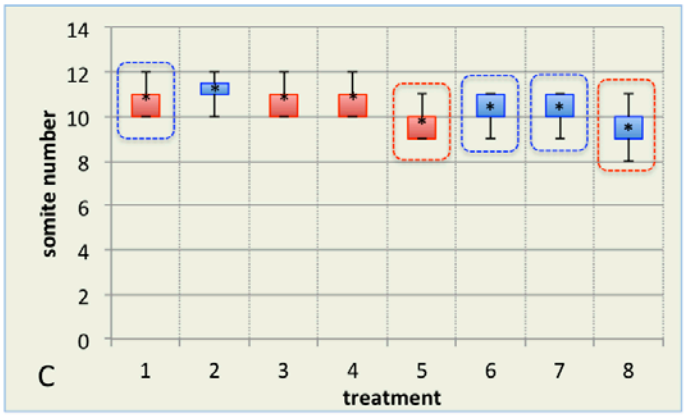
To confirm our model proposed above, we tested whether administration of RA during early developmental stages restores these defects in *raldh2* morphants. We were applying 1nM of All Trans Retinoic acid (ATRA), directly to the medium of intended embryo, at different time point. RA administration in *raldh2* morphants for

4 – 10 hours post fertilization (hpf) resulted in normal formation of somites 4 and 5, leading to recover loss of a somite and the second cervical vertebra. Since RA administration in *raldh2* morphants for 10 – 14 hpf did not rescue any defects in the transition, somite number and vertebra number (Fig. 27, 28 and table 4).

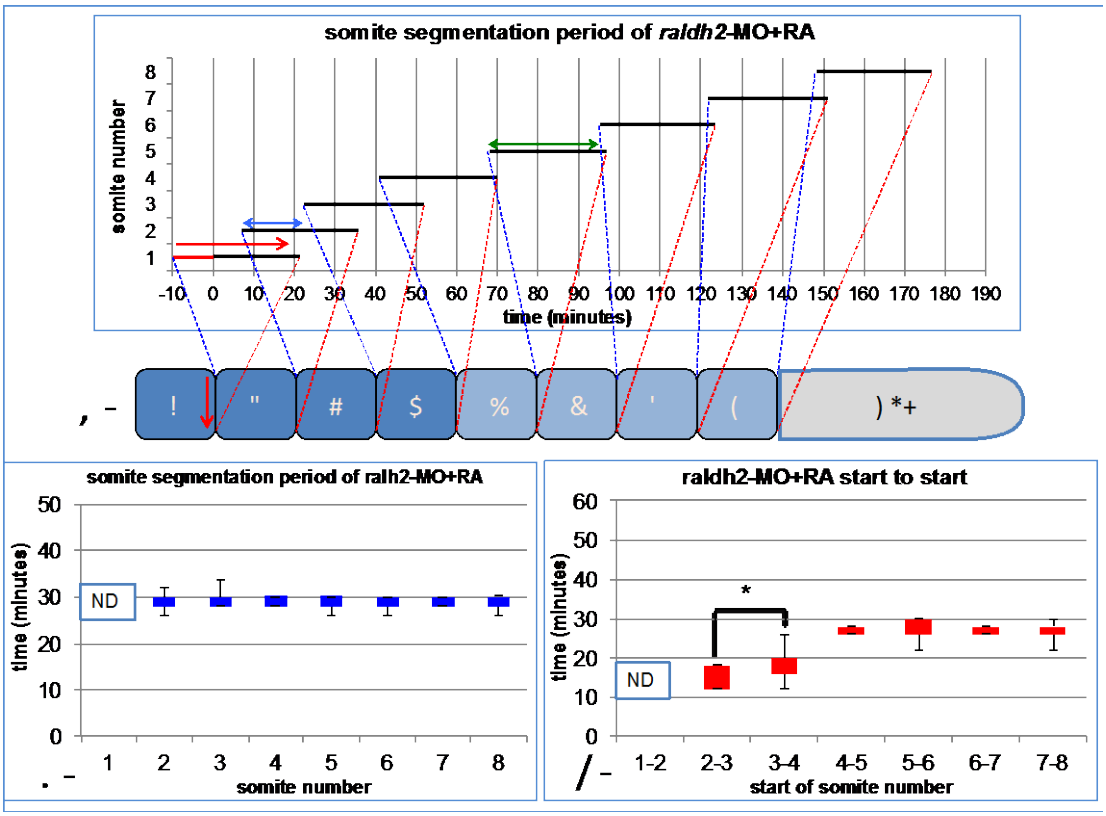


No. of somite / treatment	8	9	10	11	12	N	Average
Control-MO + DMSO 4-14hpf (1)			14	21	2	37	10.7±0.6
Control-MO +1nMRA 4-14hpf (2)			6	14	7	27	11.0±0.7
Control-MO +1nMRA 4-10hpf (3)			15	20	4	39	10.7±0.6
Control-MO +1nMRA 10-14hpf (4)			14	23	2	39	10.7±0.6
ralh2-MO + DMSO 4-14hpf (5)		14	29	5		48	9.8±0.6
ralh2-MO + 1nM RA 4-14hpf (6)		5	23	11		39	10.2±0.6
ralh2-MO + 1nM RA 4-10hpf (7)		6	22	11		39	10.1±0.6
ralh2-MO + 1nM RA 10-14hpf (8)	8	19	15	2		44	9.3±0.8

B



Figures 27. RA rescue experiment for *raldh2* morphants, (A) Whole-mount *in situ* hybridization for *Uncx 4.1*, for measuring the somite number of control, *Raldh2* and *Raldh2* morphants treated with RA. The application of single dose ATRA (1nM) and DMSO (as control), were conducted in several time windows; 4-14, 4-10, and 10-14 hpf respectively. At the end of the treatment, embryo where fixed and processed for *in situ* hybridization, and continued for the somite counting.



Figures 28. Lateral views time-lapse imaging at 1 to 8 somite stage of *raldh2* morphant embryo treated with ATRA (A,B,C) anterior to posterior sequential somite formation with high overlapping rate at anterior somite, followed by low overlapping rate at posterior somite. The graph also indicates that the transition of anterior to posterior somite occurs between somite numbers 4-5, with no extension of segmentation period on somite 4-5. (B,C) Box-and-whisker plots of the somite formation show similar feature as seen in WT zebra fish embryo, suggesting no discrepancy of somite formation on somite number 1 to 8, in RA rescued morphant. We started the time lapse after 10hpf of embryo stage, in order to allow the RA to work up to 10hpf. Therefore, as a consequence, we weren't able to fully monitor the whole period of 1st somite, which have been started to form during the time-lapse period. (ND = not determined)

Table 4 Vertebrae characteristic and number of *raldh2* morphant rescued by RA

raldh2-mo+RA

Vertebrae	Averages±s.d.	Minimum	Maximum	Mode	n
Total	31.8±0.6	31.0	33.0	32.0	31
cervical	1.5±0.5	1.0	2.0	2.0	31
Rib Bearing	10.1±0.2	10.0	11.0	10.0	31
Rib and hemal arch bearing	2.0±0.0	2.0	2.0	2.0	31
hemal arch bearing	15.2±0.6	14.0	16.0	15.0	31
Tail fin set	3.0±0.0	3.0	3.0	3.0	31

The result suggests that ATRA application could partially restore the defect of proper transition between the anterior and posterior somitogenesis on *raldh2*-morphant. Therefore, RA supply for blastula and gastrula stages is quite important for normal transition of the anterior-posterior somitogenesis.

3.7. How RA controls the cycle number of the clock; Mechanisms of action

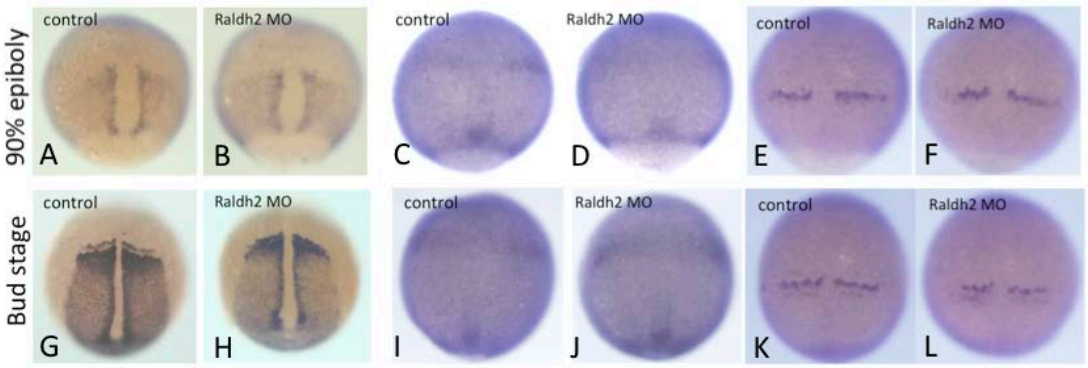
We investigated the mechanism how RA controls the cycle number of the clock. Since RA is implicated in the determination of somite size by antagonizing opposed FGF gradient. To test whether such a mechanism also contributes to RA-dependent regulation of the clock cycle, we investigated expression of *fgf8*, *mespb*, and *papc*, which are implicated in somite size determination.

We conducted *in situ* hybridization for; *papc*, which encoding for a cell adhesion molecule expressed in gastrulating mesoderm, *fgf8* and *mespb*, encoding Mesp-b which involved in establishing the anterior fate within the presumptive somites. The *in situ* hybridization were conducted for the 90% epiboly and Bud stage of control and *raldh2* morphant. Control and *raldh2* morphant show similar expression of *papc, fgf8, and mespb*. *papc* expression in 90% epiboly is broader along

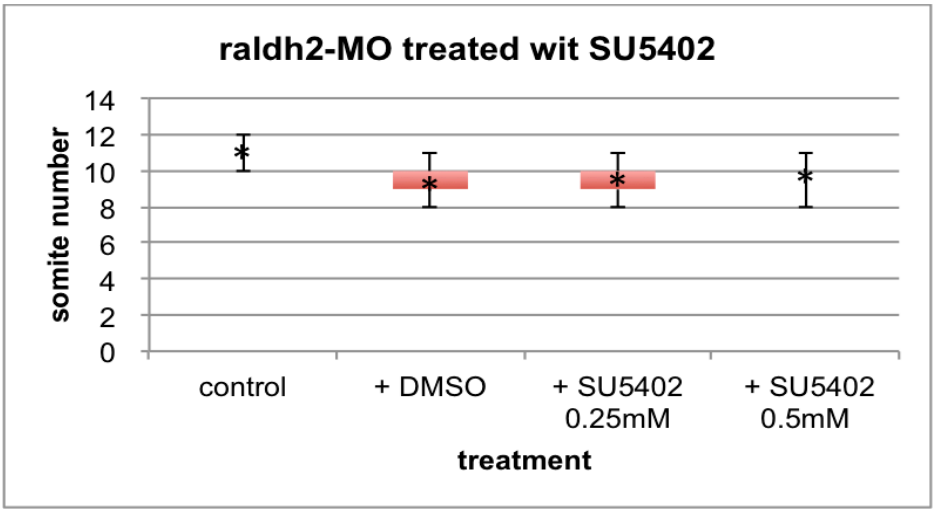
the animal-vegetal, with no expression in the dorsal midline and getting broader in the entirely anterior to posterior PSM in bud stage. On other hand, *fgf8* is expressed in mid line and the posterior PSM of both stage, mean while, *mespb* is expressed in one or one to two bilaterally symmetric stripes in 90% epiboly and bud stage. *In situ* result suggest that depletion of retinoic acid don't caused obvious failures of their expression in *raldh2* morphants (Fig. 29).

Next, to confirm further the possible involvement of fgf8 in the RA controlling clock mechanism by conducting the following experiment. Assuming that depletion of retinoic acid in *raldh2* morphant resulted in anteriorly shifted of RA-FGF8 antagonist gradient, which probably leading to transition defect. Therefore, we try to re-balance the gradient by mildly inhibit the FGFR. We treat the *raldh2* morphant with mild concentration of SU540 (0.25 and 0.5mM), an inhibitor of FGFR, then count the somite formed on embryos.

The data of *in situ* hybridization and fgfR inhibition are also supported by the time lapse data showing no defect of the transition between the anterior and posterior somitogenesis in *fgf8* morphants (Fig. 28). And therefore confirmed no involvement of t he fgf8 on the mechanism on controlling of the clock during the anterior-posterior transition.



Figures 29. *In situ* hybridization for *papc*, *fgf8* and *mespb* in *raldh2* morphants. (A,B,C,D,E,F) represent 90% epiboly stage embryo (G,H,I,J,K,L) represent bud stage of embryo. (A,B) *papc* broadly express along the animal-vegetal axis with no expression in the dorsal midline, (G,H) the anterior and mediolateral borders become sharp, and expanded to the entire PSM. (C,D,I,J) fgf8 express on the mid line and posterior PSM (E,F) one pair of symmetrical strip at the dorsal embryo and become two stripe at bud stage (K,L)



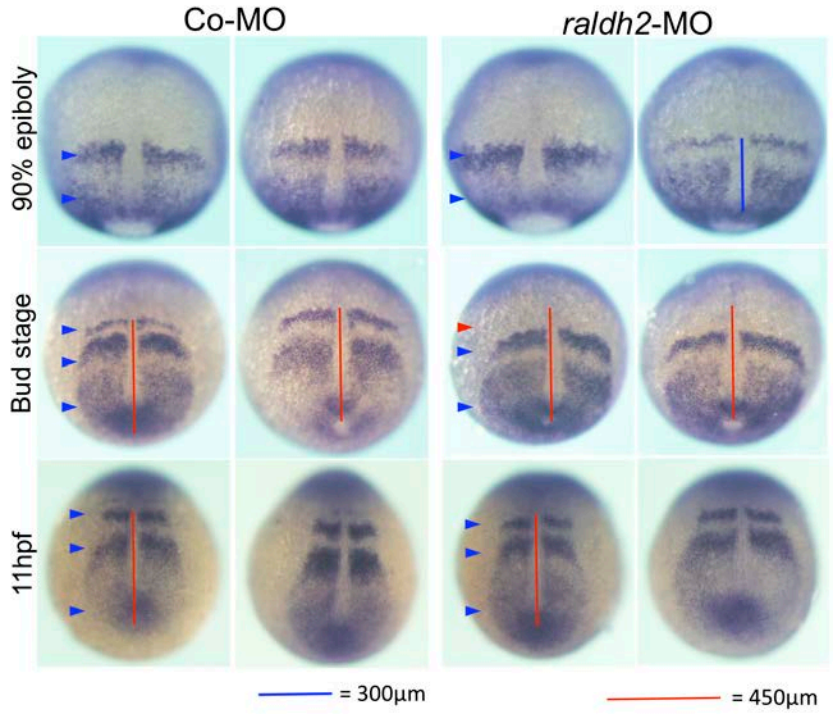
Figures 30. Box-and-whisker plots of SU5402 treated *raldh2* morphant. Morphant where treated with 0.25, 5.0mM of SU5402, and DMSO as control. The somite number were counted and plotted as in graph. DMSO and SU5402 treated mophant don't show any differences in somite number, and 1 somite less in average compare to WT control. These suggest no effect of fgf8 inhibition on somite number reduction in raldh2 morphant, showing no relathionsip on the defect of somite number with fgf8 concentrtaion. (SU5402 = an inhibitor of fgfR)

It is known that the future boundary of a somite is already determined at 120 – 150 min prior to formation of the last somite, suggesting that pre-patterns of

somites 4 and 5 are already generated at late gastrulation. Because this time window is consistent with the temporal requirement of RA, we thought that RA is doing something during late gastrulation to modulate the segmentation period of somites 4 and 5. Since the segmentation clock regulates the segmentation period, we investigated whether RA modulates the somite segmentation clock during late gastrulation, by checking expression of a cyclic gene *her1*, which plays a major role in somite segmentation in zebrafish (Fig. 31).

We conducted *in situ* hybridization for *her1* on 90% epiboly and Bud stage of control and *raldh2* morphant. *raldh2* morphants showed a wave-like propagation of *her1* expression in a similar manner to control morphants on 90% epiboly stage. Although the number of *her1* stripes in control morphants increased from two to three at a period between 9 to 10 hpf, the increase of *her1* stripes was delayed in *raldh2* morphants: some of *radlh2* morphants at 10 hpf remained two stripes of *her1*, and then almost all of them had three stripes by 11 hpf.

These results suggest that the difference of the anterior-posterior somitogenesis is originated from increasing the cycle number of the clock within the paraxial mesoderm at late gastrulation, which is mediated by RA.



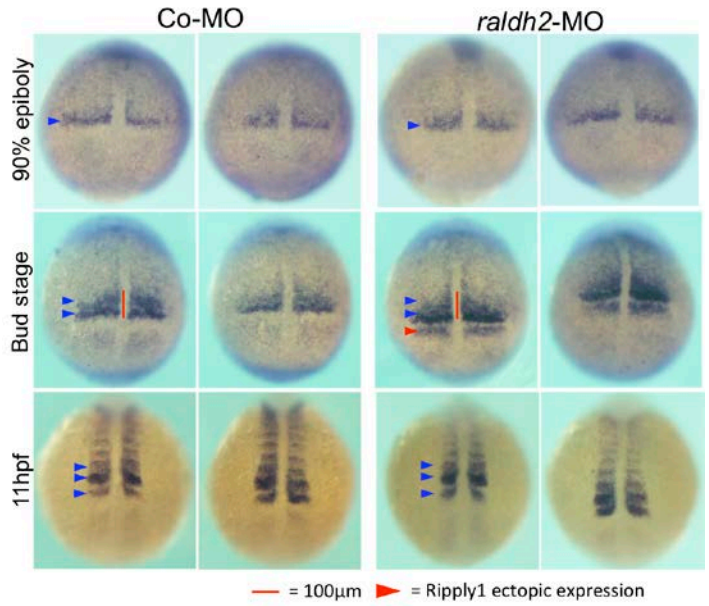
Figures 31 Whole mount *in situ* hybridization of *her1* on control and *raldh2* morphant. (A) Dorsal view of embryos at 90% epiboly stage, show the expression of *her1*, which is detected as two pair of stripe at the PSM. Both control and *raldh2* morphant shows no different of *her1* expression pattern. (B) Dorsal view of Bud stage embryos shows the expression of *her1* as three pair of stripe from the posterior to anterior of PSM in the control, in contrast to, only two pair in *raldh2* morphant embryo PSM.

3.8. Depletion of retinoic acid alter the expression of *ripply1* on *raldh2* morphant

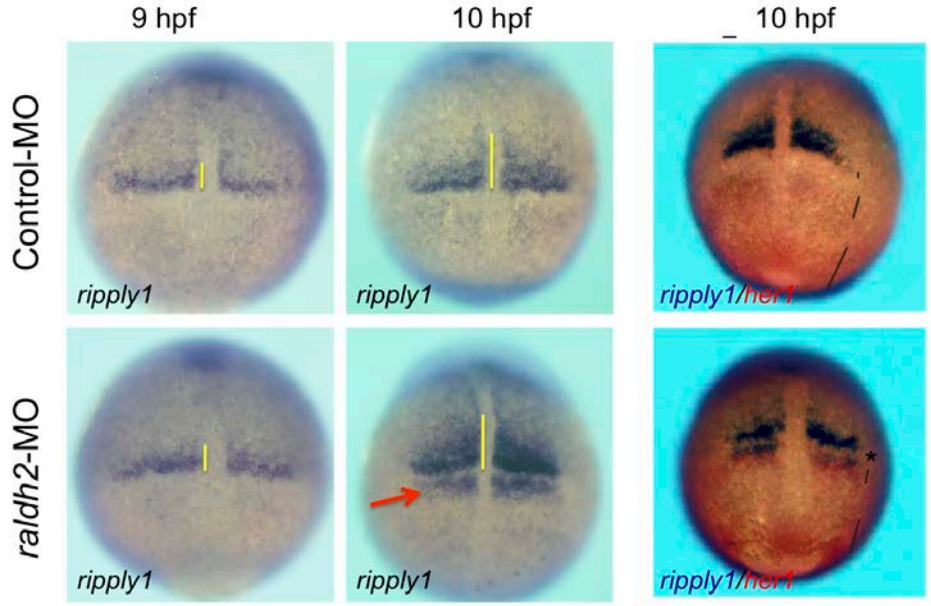
It has been reported by Kawamura *et al*, (2005) that knockdown of a transcriptional repressor *ripply1* leads to expansion of *her1* expression in the site of mature somites. To investigate whether *ripply1* is involved in *her1* expression during gastrulation, we next investigated the expression of *ripply1* in *raldh2* morphants.

We conducted the *in situ* hybridization of *ripply1* on 90% epiboly, bud stage and 11 somite stage. Control morphant and *raldh2* morphant shows similar expression pattern of *ripply1* at 90% epiboly, as one pair of stripe on the dorsal

compartment of embryo. At bud stage two stripe were observed at the anterior end of unsegmented PSM, surprisingly, *raldh2* morphant shows the posterior expansion of ripply1. While at 11 somite stage, control and raldh2 morphant show similar expression of *ripply1*, which is shown as 9-11 stripe, polarize from anterior PSM to anterior somite. To precisely determine the position of *ripply1* and *her1* expression pattern, two color *in situ* hybridization of her1 (fastred/red) ripply1 (DIG/purple) was conducted. The result, confirmed that the ectopic expression of *ripply1* is located at adjacent to the second stripe of *her1* in *raldh2* morphants, which lead to the possible relation of *her1 ripply1* expression (Fig 32, 33).



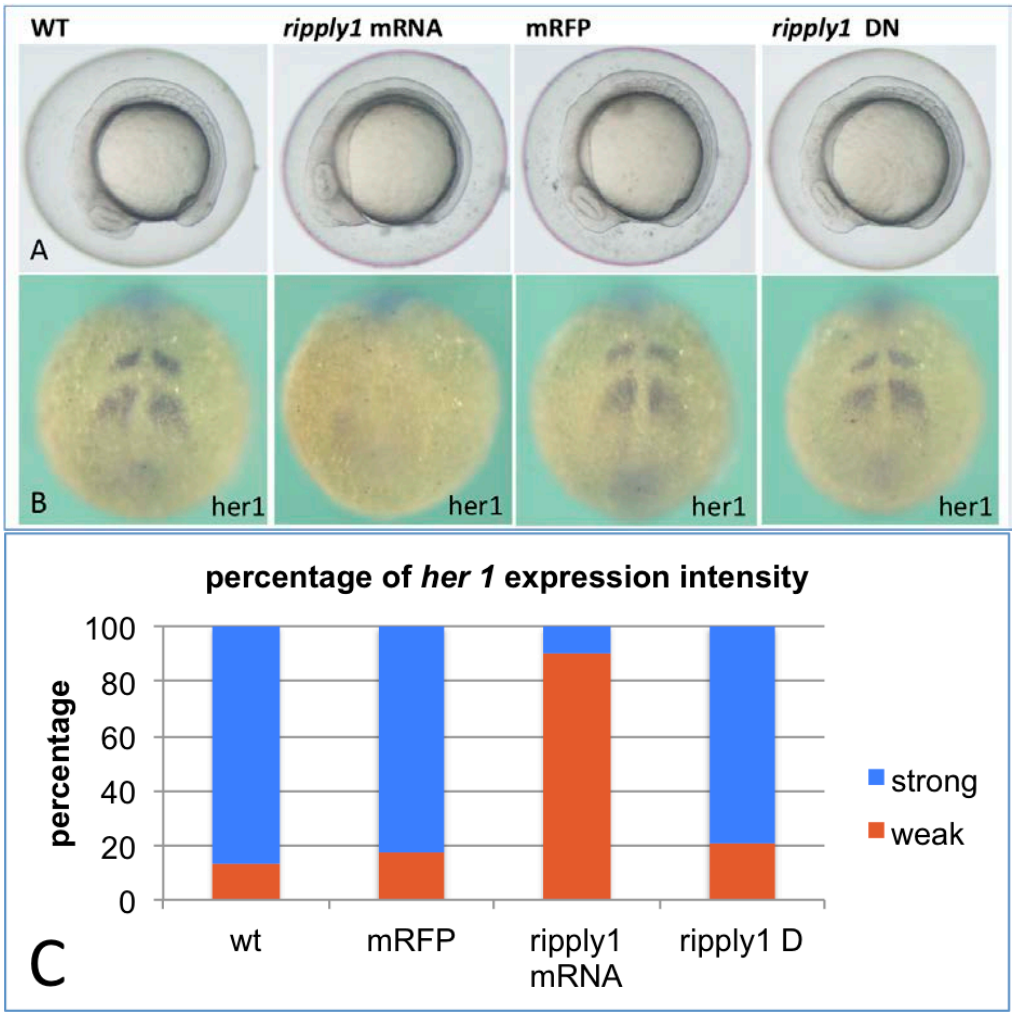
Figures 32. Whole mount *in situ* hybridization for *ripply1* on control and *raldh2* morphant, Dorsal view of embryos at 90% epiboly stage, show the expression of *ripply1* is detected as one pair of stripe along the notochord. Both control and *raldh2* morphant shows similar of *ripply1* expression pattern. At bud stage embryos show the expression of *ripply1* as two pair of stripe along the notochord. Ectopic expression of *ripply1* could be observed on the posterior part of second strip in *raldh2* morphant embryo, The 11 somite stage as 9-11 stripe, polarize from anterior PSM to anterior somite.



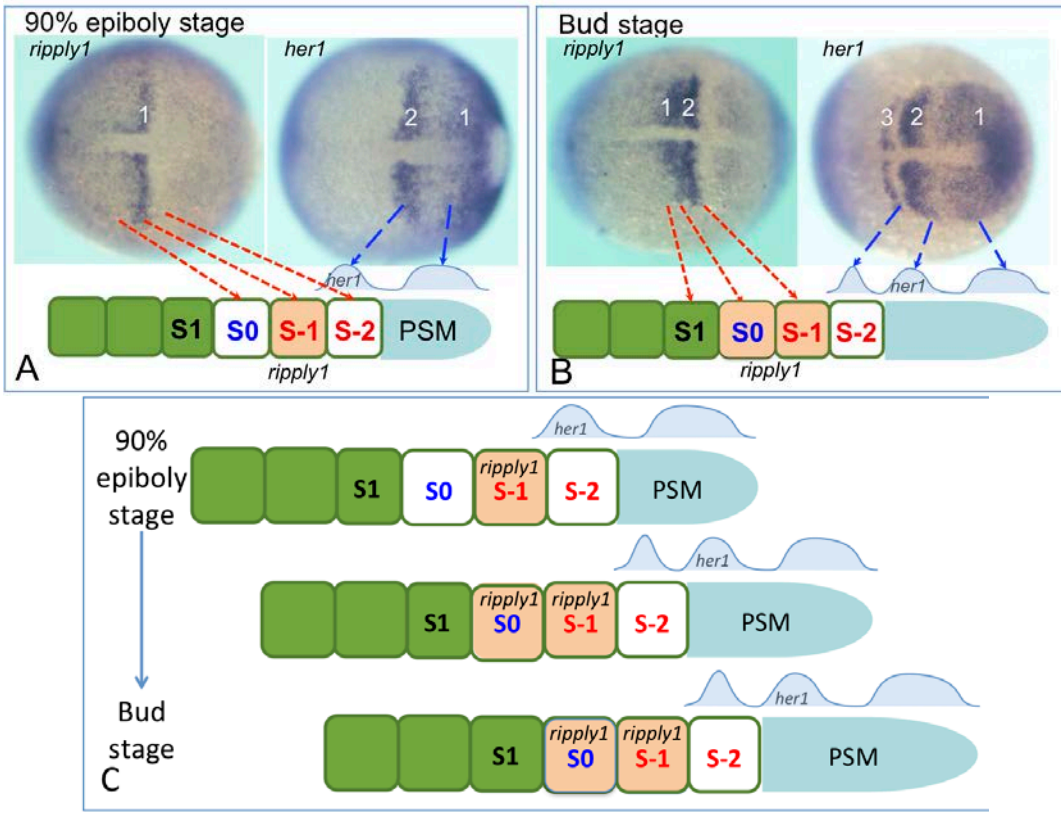
Figures 33 *In situ* and two-color *insitu* hybridization for *ripply1 and her1*. 9-10 hpf, control and raldh2 morphant shows ectopic expression of *ripply1* (red arrow) in the adjacent of 2nd strip of *her1* (star).

3.9. *ripply1* over expression down regulate *her1* expression

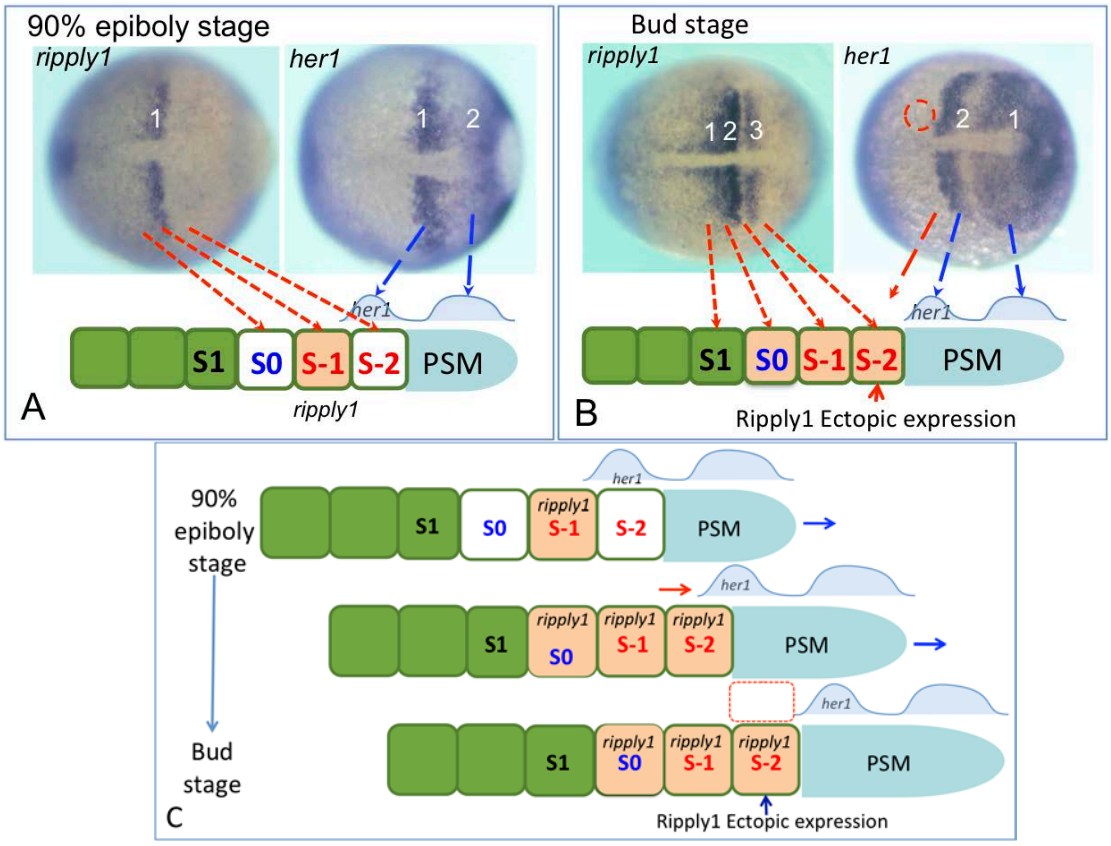
Next we overexpress *ripply1*, by injection of mRNA to the zebrafish WT embryo. A full set of WT uninjected, mRNA ripply1, ripply1ΔWRPW, and mRFP, were used in the experiment. To test for the specific effect of *ripply1* over expression, we use ripply1ΔWRPW, a *ripply1* lacking the WRPW motif, as one of the control. Over expression of *ripply1* resulted in down regulation of *her1* expression, showing the adequacy of *ripply1* to repress *her1* expression.



Figures 34 *ripply1* overexpressed embryos. Embryos were injected with *ripply1* mRNA, mRFP, and ripply1ΔWRPW as control. (A) The figure shows that overexpression of *ripply1* resulted in failure of the somite segmentation (indicated by arrowheads), while injection of mRFP and ripply1ΔWRPW; fail to disturb the segmentation of the somite. (B) *In situ* hybridization for *her1* shows down regulation of *her1* expression on *ripply1* injected embryo, compare to control and WT.



Figures 35 illustration of *ripply1* and *her1* expression at the transition of 90% epiboly to bud stage in control morphant embryo (A) 90% epiboly stage 1 pair of *ripply1* stripe express on S-1, while 2 stripe of her1 are express at the S-2 and posterior of PSM. (B) Bud stage, 2 stripe of *ripply1* express on S0 and S-1, while her1 express on S-2, anterior part of PSM and 3rd stripe following the expansion of PSM, express at the posterior of PSM. During the transition of 90% epiboly and bud stage, both ripply1 and her1 express on different domain, without overlapping each other

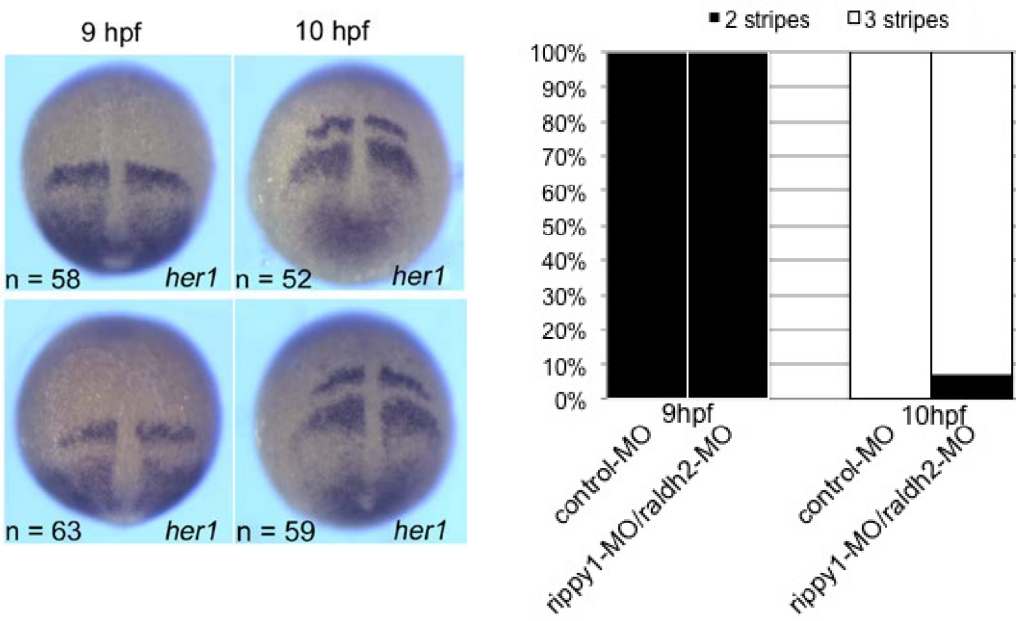


Figures 36 illustration of *ripply1* and *her1* expression at the transition of 90% epiboly to bud stage in *raldh2* morphant embryo (A) 90% epiboly stage 1 pair of *ripply1* stripe express on S-1, while 2 stripe of her1 are express at the S-2 and posterior of PSM. (B) Bud stage, there are 2 stripe of *ripply1* express on S0 and S-1, and 1 extra stripe in the S-2 (ectopic expression), while her1 express on 2 stripe on anterior part of PSM and posterior PSM. During the transition of 90% epiboly and bud stage, there is ectopic expression of *ripply1* at adjacent to the second stripe of *her1*, suggested the ripply1 mediated suppression of cyclic her1 expression.

We Illustrated the possible mechanisms on how RA controlling the cycle number of her1, which are presented in figure 22 and 23. These results suggest that, at the transition stage of 9 to10 hpf, Retinoic acid regulates the clock cycle number, by transient inhibition of *ripply1* expression.

3.10. *ripply1*-MO/*raldh2*-MO double knockdown, restore the *her1* stripe number

Next we did double knockdown for *ripply1 and raldh2*, by co-injection of both morfolino to the zebrafish WT embryo.



Figures 37 Epistasis experiment for ripply1-raldh2. We co-injected *raldh2*-MO and *ripply1*-MO into zebrafish embryos and found that knockdown of *ripply1* could rescue the number of *her1* stripe at 10 hpf

we co-injected *raldh2*-MO and *ripply1*-MO and the result shows a significant rescue phenotype for the number of *her1* stripe ($P < 0.05$, Figure 37) on the *ripply1*-MO/*raldh2*-MO. Therefore, the results suggest a possibility that the failure of AP transition is caused by RA/Ripply1-mediated spatial and temporal inhibition of *her1* expression

CHAPTER IV

DISCUSSION

The patterning of anterior-posterior in the vertebrate embryo could be divided into two major processes: an initiation phase, in which the primary body of embryo is formed and the elaboration phase, in which the body progressively forming the trunk and tail (secondary body). It is become very interesting when recent finding reveal the different of mechanism, which control the primary and secondary body. (Tam 1981; Hanneman and Westerfield 1989; Schubert et al. 2001), reported the evident of these different mechanisms, somitogenesis between the anterior and posterior somitogenesis has been observed in several species including amphioxus, mouse and zebrafish anterior somitogenesis occurs the more rapid progression of the somite cycle relative to posterior somitogenesis.

Here we seek for the information behind the different of anterior and posterior somite formation. We observed somitogenesis of the first 8 somites by time-lapse imaging, and interestingly found a clear difference between the first 4 and the later 4 somites; the first 4 somites formed quickly within approximately 80 min, while the rest of 4 somites are periodically/sequentially formed within 120 min. This observation result is agreed with previous findings showing a change of the anterior-posterior somitogenesis in zebrafish as those of the anterior and posterior somites in zebrafish are formed every 20 and 30 min, respectively. We also found that more posterior somites were also formed within a constant pace. These observations agreed with

previous findings showing a constant pace of segmentation throughout the posterior somites (Schroter, 2010, Baker, R.E. 2007)

We reveal an interesting fact when we investigated the starts and ends of every individual somite. Although we couldn't recognize obvious difference of segmentation period between somites 1 to 8, we found that overlapping rate of somitogenesis differs from somites 1 – 4, which show high overlapping rate compare to small overlapping rate on somite 5 – 8. The result therefore, suggest that the transition between the anterior and posterior somitogenesis is mainly originated from the different overlapping rate of segmentation between a somite and next somite in zebrafish embryos. We also confirmen that the sudden change between the anterior and posterior somitogenesis is not specific on one fish strain also seen our observation on the different zebrafish strain such as Riken-wako, India and TL, provide the evident of the similarity among the fish strain.

Our result is in line with previous report, which uncovered several differences in the specification, formation, and differentiation of the anterior trunk, posterior trunk, and tail somites, in mice and zebrafish (Szeto and Kimelman, 2006). However, up to recently, no report mentioning of the overlapping rate differences on the anterior and posterior somite formation. Moreover, there is remaining question on how the transition of primary and secondary body occurs in embryo and what mechanisms control the transition? Therefore we need to uncover the detail information about this new finding.

Next we seek for the possible mechanisms, which involved in the anterior-posterior transition. In zebrafish, somite segmentation is regulated by the combined action of Notch, FGF and RA activities. We seek the possible mechanisms by knock down those factors employing anti sense morpholino oligo for the experiment. Knock down of Notch ligand *deltad* or an FGF ligand *fgf8*, which is known to regulate Notch or FGF signaling in the context of somitogenesis, respectively, show no failure of the anterior-posterior transition, suggesting that those factors doesn't contribute during the anterior somite formation. This result agree with the report on mutants fish of *deltaD* (*after eight*) and *Notch1* (*deadly seven*), which normally form initial 7-8 somite, but show defect on later stage (Oates,A.C. 2002, Jiang,Y.J. 2000, Lewis,J. 2003).

We then knockdown of *raldh2*, which is a major source of RA in the context of somitogenesis, and resulted a discrepancy on anterior-posterior transition. Moreover, our experiment also reveal a specific defects on the *raldh2* morphants, which are the transient extension of segmentation period at somites 4 and 5, and small overlapping rate of somite 3 to 4. These results suggest that RA signaling ensures timely somite formation at the transition between the anterior and posterior somitogenesis.

One of characteristic of the somite is the constant clock, which related to constant somite number and it size. Extension of segmentation period, resulted in a change of somite consistency or loss of somite number. Resende, (2010), reported that the ablation of notochord, which lead to the depletion of Retinoic acid causes a delay in somite formation, accompanied by an increase in the period of molecular clock oscillations, and failed to form the expected number of somites (Resende,T.P. 2010).

So, we counted somite number in zebrafish embryos at different stages, and found *raldh2* morphants lost of a somite relative to control morphants throughout the posterior somite formation. This is consistent with the data observed by time-lapse imaging. These results suggest that, RA signaling is essential for proper transition between the anterior and posterior somitogenesis, and that the failure of the transition then leads to loss of a somite.

Since a vertebra is created by the caudal part of somite and the rostral part of next somite, suggesting that somite number is correlated with vertebra number (Morin-Kensicki, E.M. 2002). When we identify the number and characteristics vertebrae, we could not find obvious morphological differences of control and *raldh2* morphants. But, interestingly, the numbers of vertebrae in *raldh2* morphants reduce by 1 vertebrae compare to control morphant. The manipulation resulted in specific loss of the second cervical vertebra. Since the second cervical vertebra is derived from both the caudal part of somite 4 and the rostral part of somite 5, loss of the second cervical vertebra in *raldh2* morphants is consistent with the earlier defect in the anterior-posterior transition at somites 4 to 5. These results provide a good evident for the RA role on the transition of anterior to posterior segmentation. Therefore suggest that RA depletion fails to ensure timely segmentation of somites 4 and 5, eventually leading to loss of the second cervical vertebra.

The specific time windows of RA activity on the early stage embryo development was reported by Grandel (2011), which found that, an early gastrula stage RA signal triggers the process that leads to determination of tbx5-expressing limb

precursors (Grandel,H. 2011). So we tested whether administration of RA during early developmental stages restores these defects in *raldh2* morphants. Our rescue experiment, therefore, shows that RA administration only on a specific time windows (4 – 10 hpf) in *raldh2* morphants, resulted in normal formation of somites 4 and 5, leading to recover loss of a somite and the second cervical vertebra. Similar rescue experiment by application of RA, on the incubation medium of chick embryo was conducted by Resende (2010), which show, that the external RA supply for 9hours, is able to rescue timely somite formation in the absence of Shh (Resende,T.P. 2010). Moving further, we conducted the inhibitory experiment of RA synthesis by application of DEAB, an inhibitor of RA synthesis enzyme, which showing consistent result, that only the treatment period for 4-10 led to loss of a somites. These results suggest that endogenous RA supplied during blastula and gastrula stages controls segmentation period at the transition.

It is known that the future boundary of a somite is already determined at 120 – 150 min prior to formation of the last somite, suggesting that pre-patterns of somites 4 and 5 are already generated at late gastrulation. Because this time window is consistent with the temporal requirement of RA, we thought that RA is doing something during late gastrulation to modulate the segmentation period of somites 4 and 5. Since the segmentation clock regulates the segmentation period, we investigated whether RA modulates the somite segmentation clock during late gastrulation, by checking expression of a cyclic gene *her1,* which plays a major role in somite segmentation in zebrafish. Our result shows that depletion of RA didn't change the expression pattern

of a wave-like propagation of *her1*. However, in term of the stripe number, we note that the number of *her1* stripes in control morphants increased from two to three at a period between 9 to 10 hpf, in contrast to some of *raldh2* morphants embryos which still had two stripes of *her1,* suggesting a delay of *her1* cyclic expression in *raldh2* morphants. The observation of *her1* expression on 11hpf, show all of embryos in control and *raldh2* morphants had three stripes. These results suggest that the difference of the anterior-posterior somitogenesis is originated from increasing the cycle number of the clock within the paraxial mesoderm at late gastrulation, which is mediated by RA.

We investigated the mechanism how RA controls the cycle number of the clock. Since RA is implicated in the determination of somite size by antagonizing opposed FGF gradient. To test whether such a mechanism also contributes to RA-dependent regulation of the clock cycle, we investigated expression of *fgf8*, *mespb, papc*, which are implicated in somite size determination. However, we could not detect obvious failures of their expression in *raldh2* morphants. This is also supported by the data showing no defect of the transition between the anterior and posterior somitogenesis in *fgf8* morphants. These results suggest that a mechanism in which RA regulates the cycle number of the clock is different from that of somite size determination mediated by opposed gradients of FGF and RA signaling.

When we carefully compared *her1* expression between control and *raldh2* morphants at 10 phf, we found that the upper stripes of *her1* became thinner than those of control morphants, suggesting that RA suppresses *her1* expression at the anterior

part of the paraxial mesoderm at late gastrulation. Interestingly, it has been reported that knockdown of a transcriptional repressor *ripply1*, which encodes a nuclear protein associated with the transcriptional repressor Groucho, leads to expansion of *her1* expression in the site of mature somites. Moreover, it is reported that *ripply1* is required for the proper transcriptional termination of genes involved in somite segmentation, such as *mespb* and *her1,* at the transition from the PSM to somite (Kawamura,A. 2005). We investigated the expression of *ripply1* in *raldh2* morphants. We found the ectopic expression of *ripply1* at adjacent to the second stripe of *her1* in *raldh2* morphants. It is reported by Kawamura (2005) that, injection of a large amount (100 pg) of synthesized ripply1 mRNA into embryos at the 1- to 4- cell stage caused a severe reduction in the trunk length, while a lower dose (25 pg) of exogenous ripply1 mRNA resulted in a distinctive segmental disruption of somite boundaries in addition to a slight defect in elongation of the trunk (Kawamura,A. 2005).

Reffering to the finding, we conducted overexpression of *ripply1* in zebrafish emryo, resulted in down regulation of *her1* expression, showing the adequacy of *ripply1* to repress *her1* expression. Therefore, these results suggest that, at the transition stage of 9 to10 hpf, Retinoic acid regulates the clock cycle number, by transient inhibition of *ripply1* expression

Overall, our results uncover a novel mechanism mediated by RA that adjusts segmentation period to the anterior-posterior difference of somitogenesis, which is required to link the head and trunk properly in the zebrafish embryo.

REFERENCES

Albalat, R. & Canestro, C. 2009a, "Identification of Aldh1a, Cyp26 and RAR orthologs in protostomes pushes back the retinoic acid genetic machinery in evolutionary time to the bilaterian ancestor", *Chemico-biological interactions,* vol. 178, no. 1-3, pp. 188-196.

Albalat, R. & Canestro, C. 2009b, "Identification of Aldh1a, Cyp26 and RAR orthologs in protostomes pushes back the retinoic acid genetic machinery in evolutionary time to the bilaterian ancestor", *Chemico-biological interactions,* vol. 178, no. 1-3, pp. 188-196.

Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker, M. & Postlethwait, J.H. 1998, "Zebrafish hox clusters and vertebrate genome evolution", *Science (New York, N.Y.),* vol. 282, no. 5394, pp. 1711-1714.

Aranda, A. & Pascual, A. 2001, "Nuclear hormone receptors and gene expression", *Physiological Reviews,* vol. 81, no. 3, pp. 1269-1304.

Aulehla, A. & Pourquie, O. 2006, "On periodicity and directionality of somitogenesis", *Anatomy and Embryology,* vol. 211 Suppl 1, pp. 3-8.

Bagnall, K.M., Higgins, S.J. & Sanders, E.J. 1988, "The contribution made by a single somite to the vertebral column: experimental evidence in support of resegmentation using the chick-quail chimaera model", *Development (Cambridge, England),* vol. 103, no. 1, pp. 69-85.

Baker, R.E. & Maini, P.K. 2007a, "Travelling gradients in interacting morphogen systems", *Mathematical biosciences,* vol. 209, no. 1, pp. 30-50.

Baker, R.E. & Maini, P.K. 2007b, "Travelling gradients in interacting morphogen systems", *Mathematical biosciences,* vol. 209, no. 1, pp. 30-50.

Baker, R.E., Schnell, S. & Maini, P.K. 2006a, "A clock and wavefront mechanism for somite formation", *Developmental biology,* vol. 293, no. 1, pp. 116-126.

Baker, R.E., Schnell, S. & Maini, P.K. 2006b, "A mathematical investigation of a Clock and Wavefront model for somitogenesis", *Journal of mathematical biology,* vol. 52, no. 4, pp. 458-482.

Bastien, J. & Rochette-Egly, C. 2004, "Nuclear retinoid receptors and the transcription of retinoid-target genes", *Gene,* vol. 328, pp. 1-16.

Bessho, Y., Hirata, H., Masamizu, Y. & Kageyama, R. 2003, "Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock", *Genes & development,* vol. 17, no. 12, pp. 1451-1456.

Bessho, Y. & Kageyama, R. 2003, "Oscillations, clocks and segmentation", *Current opinion in genetics & development,* vol. 13, no. 4, pp. 379-384.

Bessho, Y., Miyoshi, G., Sakata, R. & Kageyama, R. 2001, "Hes7: a bHLH-type repressor gene regulated by Notch and expressed in the presomitic mesoderm", *Genes to cells : devoted to molecular & cellular mechanisms,* vol. 6, no. 2, pp. 175-185.

Bird, N.C. & Mabee, P.M. 2003a, "Developmental morphology of the axial skeleton of the zebrafish, Danio rerio (Ostariophysi: Cyprinidae)", *Developmental dynamics : an official publication of the American Association of Anatomists,* vol. 228, no. 3, pp. 337-357.

Bird, N.C. & Mabee, P.M. 2003b, "Developmental morphology of the axial skeleton of the zebrafish, Danio rerio (Ostariophysi: Cyprinidae)", *Developmental dynamics : an official publication of the American Association of Anatomists,* vol. 228, no. 3, pp. 337-357.

Brownlie, A., Donovan, A., Pratt, S.J., Paw, B.H., Oates, A.C., Brugnara, C., Witkowska, H.E., Sassa, S. & Zon, L.I. 1998, "Positional cloning of the zebrafish sauternes gene: a model for congenital sideroblastic anaemia", *Nature genetics,* vol. 20, no. 3, pp. 244-250.

Brunet, F.G., Roest Crollius, H., Paris, M., Aury, J.M., Gibert, P., Jaillon, O., Laudet, V. & Robinson-Rechavi, M. 2006, "Gene loss and evolutionary rates following whole-genome duplication in teleost fishes", *Molecular biology and evolution,* vol. 23, no. 9, pp. 1808-1816.

Campo-Paysaa, F., Marletaz, F., Laudet, V. & Schubert, M. 2008, "Retinoic acid signaling in development: tissue-specific functions and evolutionary origins", *Genesis (New York, N.Y.: 2000),* vol. 46, no. 11, pp. 640-656.

Capdevila, J., Tabin, C. & Johnson, R.L. 1998, "Control of dorsoventral somite patterning by Wnt-1 and beta-catenin", *Developmental biology,* vol. 193, no. 2, pp. 182-194.

Capdevila, J., Vogan, K.J., Tabin, C.J. & Izpisua Belmonte, J.C. 2000, "Mechanisms of left-right determination in vertebrates", *Cell,* vol. 101, no. 1, pp. 9-21.

Chapman, D.L., Cooper-Morgan, A., Harrelson, Z. & Papaioannou, V.E. 2003, "Critical role for Tbx6 in mesoderm specification in the mouse embryo", *Mechanisms of development,* vol. 120, no. 7, pp. 837-847.

Cinquin, O. 2007, "Understanding the somitogenesis clock: what's missing?", *Mechanisms of development,* vol. 124, no. 7-8, pp. 501-517.

Cooke, J. 1975, "Control of somite number during morphogenesis of a vertebrate, Xenopus laevis", *Nature,* vol. 254, no. 5497, pp. 196-199.

Currie, P.D. & Ingham, P.W. 1998, "The generation and interpretation of positional information within the vertebrate myotome", *Mechanisms of development,* vol. 73, no. 1, pp. 3-21.

Dale, K.J. & Pourquie, O. 2000, "A clock-work somite", *BioEssays : news and reviews in molecular, cellular and developmental biology,* vol. 22, no. 1, pp. 72-83.

D'Costa, A. & Shepherd, I.T. 2009, "Zebrafish development and genetics: introducing undergraduates to developmental biology and genetics in a large introductory laboratory class", *Zebrafish,* vol. 6, no. 2, pp. 169-177.

de Jong, J.L. & Zon, L.I. 2005, "Use of the zebrafish system to study primitive and definitive hematopoiesis", *Annual Review of Genetics,* vol. 39, pp. 481-501.

Depoix, C., Delmotte, M.H., Formstecher, P. & Lefebvre, P. 2001, "Control of retinoic acid receptor heterodimerization by ligand-induced structural transitions. A novel mechanism of action for retinoid antagonists", *The Journal of biological chemistry,* vol. 276, no. 12, pp. 9452-9459.

Dequeant, M.L. & Pourquie, O. 2008, "Segmental patterning of the vertebrate embryonic axis", *Nature reviews.Genetics,* vol. 9, no. 5, pp. 370-382.

Devoto, S.H., Melancon, E., Eisen, J.S. & Westerfield, M. 1996, "Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation", *Development (Cambridge, England),* vol. 122, no. 11, pp. 3371-3380.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M. & Storey, K. 2003, "Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension", *Neuron,* vol. 40, no. 1, pp. 65-79.

Dodd, A., Curtis, P.M., Williams, L.C. & Love, D.R. 2000, "Zebrafish: bridging the gap between development and disease", *Human molecular genetics,* vol. 9, no. 16, pp. 2443-2449.

Draper, B.W., Stock, D.W. & Kimmel, C.B. 2003, "Zebrafish fgf24 functions with fgf8 to promote posterior mesodermal development", *Development (Cambridge, England),* vol. 130, no. 19, pp. 4639-4654.

Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J. & Boggs, C. 1996, "A genetic screen for mutations affecting embryogenesis in zebrafish", *Development (Cambridge, England),* vol. 123, pp. 37-46.

Dubrulle, J., McGrew, M.J. & Pourquie, O. 2001, "FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation", *Cell,* vol. 106, no. 2, pp. 219-232.

Dubrulle, J. & Pourquie, O. 2004a, "Coupling segmentation to axis formation", *Development (Cambridge, England),* vol. 131, no. 23, pp. 5783-5793.

Dubrulle, J. & Pourquie, O. 2004b, "fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo", *Nature,* vol. 427, no. 6973, pp. 419-422.

Durbin, L., Sordino, P., Barrios, A., Gering, M., Thisse, C., Thisse, B., Brennan, C., Green, A., Wilson, S. & Holder, N. 2000, "Anteroposterior patterning is required within segments for somite boundary formation in developing zebrafish", *Development (Cambridge, England),* vol. 127, no. 8, pp. 1703-1713.

Durston, A.J., Timmermans, J.P., Hage, W.J., Hendriks, H.F., de Vries, N.J., Heideveld, M. & Nieuwkoop, P.D. 1989, "Retinoic acid causes an anteroposterior transformation in the developing central nervous system", *Nature,* vol. 340, no. 6229, pp. 140-144.

Ekker, M., Wegner, J., Akimenko, M.A. & Westerfield, M. 1992, "Coordinate embryonic expression of three zebrafish engrailed genes", *Development (Cambridge, England),* vol. 116, no. 4, pp. 1001-1010.

Engeszer, R.E., Patterson, L.B., Rao, A.A. & Parichy, D.M. 2007, "Zebrafish in the wild: a review of natural history and new notes from the field", *Zebrafish,* vol. 4, no. 1, pp. 21-40.

Fishman, M.C. 2001, "Genomics. Zebrafish--the canonical vertebrate", *Science (New York, N.Y.),* vol. 294, no. 5545, pp. 1290-1291.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L. & Postlethwait, J. 1999, "Preservation of duplicate genes by complementary, degenerative mutations", *Genetics,* vol. 151, no. 4, pp. 1531-1545.

Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S. & Hamada, H. 1997, "Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos", *The EMBO journal,* vol. 16, no. 14, pp. 4163-4173.

Gajewski, M., Sieger, D., Alt, B., Leve, C., Hans, S., Wolff, C., Rohr, K.B. & Tautz, D. 2003, "Anterior and posterior waves of cyclic her1 gene expression are differentially regulated in the presomitic mesoderm of zebrafish", *Development (Cambridge, England),* vol. 130, no. 18, pp. 4269-4278.

Gates, M.A., Kim, L., Egan, E.S., Cardozo, T., Sirotkin, H.I., Dougan, S.T., Lashkari, D., Abagyan, R., Schier, A.F. & Talbot, W.S. 1999, "A genetic linkage map for zebrafish: comparative analysis and localization of genes and expressed sequences", *Genome research,* vol. 9, no. 4, pp. 334-347.

Giudicelli, F. & Lewis, J. 2004, "The vertebrate segmentation clock", *Current opinion in genetics & development,* vol. 14, no. 4, pp. 407-414.

Glass, C.K. & Rosenfeld, M.G. 2000, "The coregulator exchange in transcriptional functions of nuclear receptors", *Genes & development,* vol. 14, no. 2, pp. 121-141.

Goering, L.M., Hoshijima, K., Hug, B., Bisgrove, B., Kispert, A. & Grunwald, D.J. 2003, "An interacting network of T-box genes directs gene expression and fate in the zebrafish mesoderm", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 16, pp. 9410-9415.

Gomez, C. & Pourquie, O. 2009, "Developmental control of segment numbers in vertebrates", *Journal of experimental zoology.Part B, Molecular and developmental evolution,* vol. 312, no. 6, pp. 533-544.

Griffin, K.J. & Kimelman, D. 2003, "Interplay between FGF, one-eyed pinhead, and T-box transcription factors during zebrafish posterior development", *Developmental biology,* vol. 264, no. 2, pp. 456-466.

Grunwald, D.J. & Eisen, J.S. 2002, "Headwaters of the zebrafish -- emergence of a new model vertebrate", *Nature reviews.Genetics,* vol. 3, no. 9, pp. 717-724.

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., Kelsh, R.N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C. & Nusslein-Volhard, C. 1996, "The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio", *Development (Cambridge, England),* vol. 123, pp. 1-36.

Hamada, H., Meno, C., Watanabe, D. & Saijoh, Y. 2002, "Establishment of vertebrate left-right asymmetry", *Nature reviews.Genetics,* vol. 3, no. 2, pp. 103-113.

Hanneman, E. & Westerfield, M. 1989, "Early expression of acetylcholinesterase activity in functionally distinct neurons of the zebrafish", *The Journal of comparative neurology,* vol. 284, no. 3, pp. 350-361.

Hanneman, E. & Westerfield, M. 1989, "Early expression of acetylcholinesterase activity in functionally distinct neurons of the zebrafish", *The Journal of comparative neurology,* vol. 284, no. 3, pp. 350-361.

Holley, S.A. 2006, "Anterior-posterior differences in vertebrate segments: specification of trunk and tail somites in the zebrafish blastula", *Genes & development,* vol. 20, no. 14, pp. 1831-1837.

Holley, S.A., Julich, D., Rauch, G.J., Geisler, R. & Nusslein-Volhard, C. 2002, "Her1 and the Notch Pathway Function within the Oscillator Mechanism that Regulates Zebrafish Somitogenesis", *Development (Cambridge, England),* vol. 129, no. 5, pp. 1175-1183.

Husmann, M., Dragneva, Y., Romahn, E. & Jehnichen, P. 2000, "Nuclear receptors modulate the interaction of Sp1 and GC-rich DNA via ternary complex formation", *The Biochemical journal,* vol. 352 Pt 3, pp. 763-772.

Ingham, P.W. 1997, "Zebrafish genetics and its implications for understanding vertebrate development", *Human molecular genetics,* vol. 6, no. 10, pp. 1755-1760.

Jiang, Y.J., Aerne, B.L., Smithers, L., Haddon, C., Ish-Horowicz, D. & Lewis, J. 2000, "Notch signalling and the synchronization of the somite segmentation clock", *Nature,* vol. 408, no. 6811, pp. 475-479.

Irmler, I., Schmidt, K. & Starck, J.M. 2004, "Developmental variability during early embryonic development of zebra fish, Danio rerio", *Journal of experimental zoology.Part B, Molecular and developmental evolution,* vol. 302, no. 5, pp. 446-457.

Julich, D., Hwee Lim, C., Round, J., Nicolaije, C., Schroeder, J., Davies, A., Geisler, R., Lewis, J., Jiang, Y.J., Holley, S.A. & Tubingen 2000 Screen Consortium 2005, "beamter/deltaC and the role of Notch ligands in the zebrafish somite segmentation, hindbrain neurogenesis and hypochord differentiation", *Developmental biology,* vol. 286, no. 2, pp. 391-404.

Kawakami, Y., Raya, A., Raya, R.M., Rodriguez-Esteban, C. & Izpisua Belmonte, J.C. 2005, "Retinoic acid signalling links left-right asymmetric patterning and bilaterally symmetric somitogenesis in the zebrafish embryo", *Nature,* vol. 435, no. 7039, pp. 165-171.

Keynes, R.J. & Stern, C.D. 1988, "Mechanisms of vertebrate segmentation", *Development (Cambridge, England),* vol. 103, no. 3, pp. 413-429.

Kimelman, D. & Schier, A.F. 2002, "Mesoderm induction and patterning", *Results and problems in cell differentiation,* vol. 40, pp. 15-27.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. & Schilling, T.F. 1995, "Stages of embryonic development of the zebrafish", *Developmental dynamics : an official publication of the American Association of Anatomists,* vol. 203, no. 3, pp. 253-310.

Kimmel, C.B., Kane, D.A., Walker, C., Warga, R.M. & Rothman, M.B. 1989, "A mutation that changes cell movement and cell fate in the zebrafish embryo", *Nature,* vol. 337, no. 6205, pp. 358-362.

Koshida, S., Kishimoto, Y., Ustumi, H., Shimizu, T., Furutani-Seiki, M., Kondoh, H. & Takada, S. 2005, "Integrinalpha5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos", *Developmental cell,* vol. 8, no. 4, pp. 587-598.

Lewis, J. 2003, "Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator", *Current biology : CB,* vol. 13, no. 16, pp. 1398-1408.

Maden, M. 2002a, "Retinoic acid and limb regeneration--a personal view", *The International journal of developmental biology,* vol. 46, no. 7, pp. 883-886.

Maden, M. 2002b, "Retinoid signalling in the development of the central nervous system", *Nature reviews.Neuroscience,* vol. 3, no. 11, pp. 843-853.

Mark, M., Ghyselinck, N.B. & Chambon, P. 2004, "Retinoic acid signalling in the development of branchial arches", *Current opinion in genetics & development,* vol. 14, no. 5, pp. 591-598.

Marletaz, F., Holland, L.Z., Laudet, V. & Schubert, M. 2006, "Retinoic acid signaling and the evolution of chordates", *International journal of biological sciences,* vol. 2, no. 2, pp. 38-47.

McGlinn, E. & Tabin, C.J. 2006, "Mechanistic insight into how Shh patterns the vertebrate limb", *Current opinion in genetics & development,* vol. 16, no. 4, pp. 426-432.

Meinhardt, H. 1986, "Hierarchical inductions of cell states: a model for segmentation in Drosophila", *Journal of cell science.Supplement,* vol. 4, pp. 357-381.

Meyer, A., Biermann, C.H. & Orti, G. 1993, "The phylogenetic position of the zebrafish (Danio rerio), a model system in developmental biology: an invitation to the comparative method", *Proceedings.Biological sciences / The Royal Society,* vol. 252, no. 1335, pp. 231-236.

Moreno, T.A., Jappelli, R., Izpisua Belmonte, J.C. & Kintner, C. 2008, "Retinoic acid regulation of the Mesp-Ripply feedback loop during vertebrate segmental patterning", *Developmental biology,* vol. 315, no. 2, pp. 317-330.

Morin-Kensicki, E.M., Melancon, E. & Eisen, J.S. 2002, "Segmental relationship between somites and vertebral column in zebrafish", *Development (Cambridge, England),* vol. 129, no. 16, pp. 3851-3860.

Nagpal, S., Friant, S., Nakshatri, H. & Chambon, P. 1993, "RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization in vivo", *The EMBO journal,* vol. 12, no. 6, pp. 2349-2360.

Niederreither, K. & Dolle, P. 2008, "Retinoic acid in development: towards an integrated view", *Nature reviews.Genetics,* vol. 9, no. 7, pp. 541-553.

Niederreither, K., McCaffery, P., Drager, U.C., Chambon, P. & Dolle, P. 1997, "Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development", *Mechanisms of development,* vol. 62, no. 1, pp. 67-78.

Oates, A.C. & Ho, R.K. 2002, "Hairy/E(spl)-related (Her) genes are central components of the segmentation oscillator and display redundancy with the Delta/Notch signaling pathway in the formation of anterior segmental boundaries in the zebrafish", *Development (Cambridge, England),* vol. 129, no. 12, pp. 2929-2946.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. & Pourquie, O. 1997, "Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis", *Cell,* vol. 91, no. 5, pp. 639-648.

Pfahl, M. 1993, "Nuclear receptor/AP-1 interaction", *Endocrine reviews,* vol. 14, no. 5, pp. 651-658.

Postlethwait, J.H., Yan, Y.L., Gates, M.A., Horne, S., Amores, A., Brownlie, A., Donovan, A., Egan, E.S., Force, A., Gong, Z., Goutel, C., Fritz, A., Kelsh, R., Knapik, E., Liao, E., Paw, B., Ransom, D., Singer, A., Thomson, M., Abduljabbar, T.S., Yelick, P., Beier, D., Joly, J.S., Larhammar, D., Rosa, F., Westerfield, M., Zon, L.I., Johnson, S.L. & Talbot, W.S. 1998, "Vertebrate genome evolution and the zebrafish gene map", *Nature genetics,* vol. 18, no. 4, pp. 345-349.

Pourquie, O. 2003, "The segmentation clock: converting embryonic time into spatial pattern", *Science (New York, N.Y.),* vol. 301, no. 5631, pp. 328-330.

Rastinejad, F., Wagner, T., Zhao, Q. & Khorasanizadeh, S. 2000, "Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1", *The EMBO journal,* vol. 19, no. 5, pp. 1045-1054.

Raya, A., Koth, C.M., Buscher, D., Kawakami, Y., Itoh, T., Raya, R.M., Sternik, G., Tsai, H.J., Rodriguez-Esteban, C. & Izpisua-Belmonte, J.C. 2003, "Activation of Notch signaling pathway precedes heart regeneration in zebrafish", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100 Suppl 1, pp. 11889-11895.

Rida, P.C., Le Minh, N. & Jiang, Y.J. 2004, "A Notch feeling of somite segmentation and beyond", *Developmental biology,* vol. 265, no. 1, pp. 2-22.

Ross, S.A., McCaffery, P.J., Drager, U.C. & De Luca, L.M. 2000, "Retinoids in embryonal development", *Physiological Reviews,* vol. 80, no. 3, pp. 1021-1054.

Sawada, A., Fritz, A., Jiang, Y.J., Yamamoto, A., Yamasu, K., Kuroiwa, A., Saga, Y. & Takeda, H. 2000, "Zebrafish Mesp family genes, mesp-a and mesp-b are segmentally expressed in the presomitic mesoderm, and Mesp-b confers the anterior identity to the developing somites", *Development (Cambridge, England),* vol. 127, no. 8, pp. 1691-1702.

Schier, A.F., Neuhauss, S.C., Helde, K.A., Talbot, W.S. & Driever, W. 1997, "The one-eyed pinhead gene functions in mesoderm and endoderm formation in

zebrafish and interacts with no tail", *Development (Cambridge, England),* vol. 124, no. 2, pp. 327-342.

Schubert, M., Holland, L.Z., Stokes, M.D. & Holland, N.D. 2001, "Three amphioxus Wnt genes (AmphiWnt3, AmphiWnt5, and AmphiWnt6) associated with the tail bud: the evolution of somitogenesis in chordates", *Developmental biology,* vol. 240, no. 1, pp. 262-273.

Sen, J., Harpavat, S., Peters, M.A. & Cepko, C.L. 2005, "Retinoic acid regulates the expression of dorsoventral topographic guidance molecules in the chick retina", *Development (Cambridge, England),* vol. 132, no. 23, pp. 5147-5159.

Simeone, A., Avantaggiato, V., Moroni, M.C., Mavilio, F., Arra, C., Cotelli, F., Nigro, V. & Acampora, D. 1995, "Retinoic acid induces stage-specific antero-posterior transformation of rostral central nervous system", *Mechanisms of development,* vol. 51, no. 1, pp. 83-98.

Solnica-Krezel, L., Schier, A.F. & Driever, W. 1994, "Efficient recovery of ENU-induced mutations from the zebrafish germline", *Genetics,* vol. 136, no. 4, pp. 1401-1420.

Stainier, D.Y., Lee, R.K. & Fishman, M.C. 1993, "Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation", *Development (Cambridge, England),* vol. 119, no. 1, pp. 31-40.

Stickney, H.L., Barresi, M.J. & Devoto, S.H. 2000, "Somite development in zebrafish", *Developmental dynamics : an official publication of the American Association of Anatomists,* vol. 219, no. 3, pp. 287-303.

Szeto, D.P. & Kimelman, D. 2006, "The regulation of mesodermal progenitor cell commitment to somitogenesis subdivides the zebrafish body musculature into distinct domains", *Genes & development,* vol. 20, no. 14, pp. 1923-1932.

Talbot, W.S. & Hopkins, N. 2000, "Zebrafish mutations and functional analysis of the vertebrate genome", *Genes & development,* vol. 14, no. 7, pp. 755-762.

Tam, P.P. & Trainor, P.A. 1994, "Specification and segmentation of the paraxial mesoderm", *Anatomy and Embryology,* vol. 189, no. 4, pp. 275-305.

Thaller, C. & Eichele, G. 1987, "Identification and spatial distribution of retinoids in the developing chick limb bud", *Nature,* vol. 327, no. 6123, pp. 625-628.

Tickle, C. 2006, "Making digit patterns in the vertebrate limb", *Nature reviews.Molecular cell biology,* vol. 7, no. 1, pp. 45-53.

Twal, W., Roze, L. & Zile, M.H. 1995, "Anti-retinoic acid monoclonal antibody localizes all-trans-retinoic acid in target cells and blocks normal development in early quail embryo", *Developmental biology,* vol. 168, no. 2, pp. 225-234.

Twal, W.O. & Zile, M.H. 1997, "Retinoic acid reverses ethanol-induced cardiovascular abnormalities in quail embryos", *Alcoholism, Clinical and Experimental Research,* vol. 21, no. 6, pp. 1137-1143.

van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Warga, R.M., Allende, M.L., Weinberg, E.S. & Nusslein-Volhard, C. 1996, "Mutations affecting somite formation and patterning in the zebrafish, Danio rerio", *Development (Cambridge, England),* vol. 123, pp. 153-164.

Vermot, J., Gallego Llamas, J., Fraulob, V., Niederreither, K., Chambon, P. & Dolle, P. 2005, "Retinoic acid controls the bilateral symmetry of somite formation in the mouse embryo", *Science (New York, N.Y.),* vol. 308, no. 5721, pp. 563-566.

Vermot, J. & Pourquie, O. 2005, "Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos", *Nature,* vol. 435, no. 7039, pp. 215-220.

Vogel, A.M. & Weinstein, B.M. 2000, "Studying vascular development in the zebrafish", *Trends in cardiovascular medicine,* vol. 10, no. 8, pp. 352-360.

Warga, R.M. & Nusslein-Volhard, C. 1999, "Origin and development of the zebrafish endoderm", *Development (Cambridge, England),* vol. 126, no. 4, pp. 827-838.

Wasiak, S. & Lohnes, D. 1999, "Retinoic acid affects left-right patterning", *Developmental biology,* vol. 215, no. 2, pp. 332-342.

Woods, I.G., Kelly, P.D., Chu, F., Ngo-Hazelett, P., Yan, Y.L., Huang, H., Postlethwait, J.H. & Talbot, W.S. 2000, "A comparative map of the zebrafish genome", *Genome research,* vol. 10, no. 12, pp. 1903-1914.

Woods, I.G., Wilson, C., Friedlander, B., Chang, P., Reyes, D.K., Nix, R., Kelly, P.D., Chu, F., Postlethwait, J.H. & Talbot, W.S. 2005, "The zebrafish gene map defines ancestral vertebrate chromosomes", *Genome research,* vol. 15, no. 9, pp. 1307-1314.

Xu, Q., Lucio-Cazana, J., Kitamura, M., Ruan, X., Fine, L.G. & Norman, J.T. 2004, "Retinoids in nephrology: promises and pitfalls", *Kidney international,* vol. 66, no. 6, pp. 2119-2131.

Yoon, J.K. & Wold, B. 2000, "The bHLH regulator pMesogenin1 is required for maturation and segmentation of paraxial mesoderm", *Genes & development,* vol. 14, no. 24, pp. 3204-3214.

ACKNOWLEDGEMENT

Bismillahirahmanirrahim , The name of Allah most beneficent most merciful.

I would like to express my profound and sincere appreciation to the following for their much-needed assistance during the performance of this study, and without whom, this study would not have been possible:

Special thank are addressed to my sensei Prof. Yasumasa Bessho; my adviser for his great guidance, inspiration, and discussion during my PhD course.

Special thank are addressed: Prof. Kinichi Nakashima, Dr. Naoyuki Inagaki, Dr. Kousuke KATAOKA, member of advisory committee

Special thank are addressed to Dr. Takaaki Matsui his supervision, guidance and encouragement in the whole study,

Special thank are addressed to Prof. Masashi Kawaichi for his supervision, guidance and encouragement in the whole study,

sincere appreciation to NAIST GCOE program for the great support

Special respect and grateful to Mrs. Sachiko Iida for her unvaluable help, support and encourage during this PhD course.

Thank very much for all my friends in Bessho laboratory Matta-san, Akiyama-San and all member, Parents pak ***Warsid***, ibu ***Suratinah***, pak Mardi ibu Mardi, Retno Wahyu untari, Jumiyo, Fitri, Huda, Tri Wahyu Riyadi, Lina, Eni Sugiharti Fajarsari, Marsono, and Raffi, Momon, and also dik Nanik,
Etik Widayati and ***Daffa'***, ***Accel*** wife and son, whom the author wishes to dedicate a big part of his thesis, for their unfailing support, sacrifices