# A genetic analysis of somitogenesis in the Medaka (*Oryzias latipes*)

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# Erklärung

Die vorliegende Dissertation mit dem Titel „A genetic analysis of somitogenesis in the Medaka (*Oryzias latipes*)“ wurde am Institute für Physiologische Chemie I der Bayrischen Julius-Maximilians-Universität Würzburg unter Anleitung von Herrn Prof. Dr. Manfred Schartl in der Zeit von März 2002 bis Juni 2005 angefertigt.

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Dissertation selbstständig angefertigt habe und dabei keine anderen als die von mir nur angegebenen Quellen und Hilfsmittel verwendet habe.

Würzburg, Juni 2005

# 1. Introduction

## *1.1 Somite formation in vertebrates*

Somites are epithelialized reiterated segments in the paraxial mesoderm that are present in developing cephalochordates and vertebrates. In vertebrate species, such as frog, chicken, mouse, zebrafish and medaka, somites develop as groups of cells, which bud off in a highly coordinated manner from the anterior end of the unsegmented presomitic mesoderm (PSM) (Fig. 1). The PSM is a growth zone at the posterior end of the embryo, which is derived from the primitive streak during gastrulation or tailbud tissue after gastrulation. The tailbud is a mass of undifferentiated cells which undergo complex stereotyped movements similar to gastrulation before contributing to the different final germ layers (Dubrulle and Pourquie, 2004). It was proposed that the tailbud functions as a blastema consisting of pluripotent cells (Pourquie, 2000a). Somites are transient structures that appear only for short periods during early development. Later, they differentiate into vital structures including the axial skeleton, the dermis of the back, and all striated muscle of the adult body, under the influence of different growth factors secreted from the surrounding tissues.

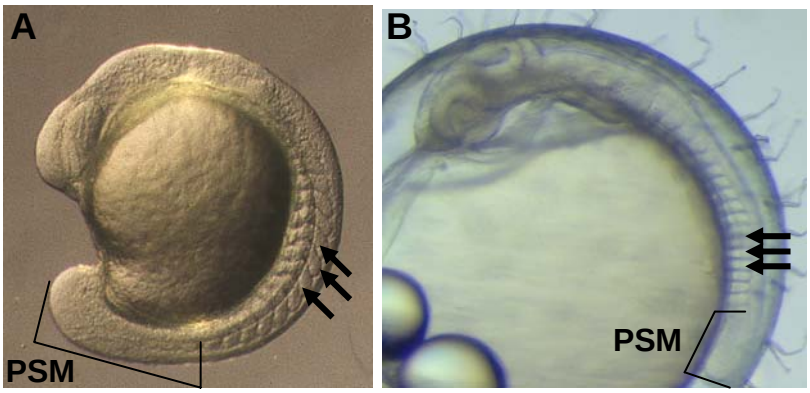


**Fig. 1.** Somite formation in zebrafish (A) and medaka (B): Lateral views of embryos showing the similarities in somite morphology between both species. Formed somites are located on both sides of notochord and neural tube along the anterior-posterior axis. The zebrafish embryo is at 16 hpf with 16 somites, the medaka embryo is at 24 hpf and exhibits 17 somites (picture A kindly provided by C. Winkler).

The segmental organization of somites along the anterior posterior axis of the embryo later becomes reflected in a segmental property on the spinal cord and spinal ganglia. In chicken, somitogenesis begins after the ingression of cells from the epiblast into the mesodermal layer,

through the anterior primitive streak (Garcia-Martinez and Schoenwolf, 1992; Psychoyos and Stern, 1996). The most anterior paraxial mesodermal cells form the head mesoderm and anterior somites, the remaining paraxial cells produce the posterior somites (Freund et al., 1996; Kuratani et al., 1999; Jouve et al., 2002). In zebrafish, the first somite appears after 10.5 hpf (hours post fertilization), when cells in the paraxial mesoderm undergo mesenchymal to epithelial transition. The next somites are produced in a similar fashion at regular and repeated intervals. Due to the coordinated periodicity of somite formation, which takes 30 min for one somite to form in zebrafish at 28°C (Kimmel et al., 1995), 60 min for medaka at 26°C (Iwamatsu, 2004), 90 min at 37°C for chicken (Pourquie, 2004) and 120 min for mouse, and the highly coordinated morphogenesis, somitogenesis and segmentation presents an attractive and fascinating system in developmental biology. Still, until recently, the mechanisms that regulate the periodicity of somite formation were largely unknown. One recent discovery was the finding of cyclic genes linked to the tightly regulated temporal periodicity of somite formation (Palmeirim et al., 1997). Since then, genetic and mutant analyses on the one hand, and transplantation experiments on the other revealed evidence as to how this temporal periodicity is established, how the positions of segment boundaries are determined and how anterior-posterior polarity within the somitomeres is generated. The periodicity underlying the process of somite formation is regulated by a molecular oscillator, the segmentation clock, which is present in the tailbud of all vertebrate embryos. This clock is antagonized through a wave front activity, which determines the position, where the next somite will emerge. Several studies manifested that the PSM region can be divided into two different domains based on their cellular states. Region I includes most of the PSM, including the tailbud, the posterior and intermediate PSM, where somite precursor cells are kept in an immature and undetermined state. Region II depicts the anterior PSM region, in which PSM cells receive the first determination signals and become specified with respect to their future segmental identity (Fig. 2; Saga and Takeda, 2001).

## *1.2 The different steps of somitogenesis*

The paraxial presomitic mesoderm periodically generates segmental borders and is thus divided into epithelial somites. Somite formation is a continuous process during early development that results from several sequential events. The formation of each pair of somites in all vertebrates can be simplified and divided into three timely separated steps (Pourquie, 2001; Fig. 2). First, precursor cells in the posterior PSM exit their undetermined state and

receive a basic prepatterning signal. As the tailbud growth zone extends posteriorly with time, these cells will become positioned in the anterior part of the PSM, where they are determined in their identity. By this time, cells of the future somite, the so-called somitomeres, have entered the next step in which they acquire distinct anterior-posterior (A-P) identity. The establishment of A-P polarity within somitomeres is a prerequisite for segmentation and the maintenance of segment borders. Finally, somitic cells become epithelialized through mesenchymal epithelial transition in which they change their adhesive properties and detach from the PSM to form the finished somite.

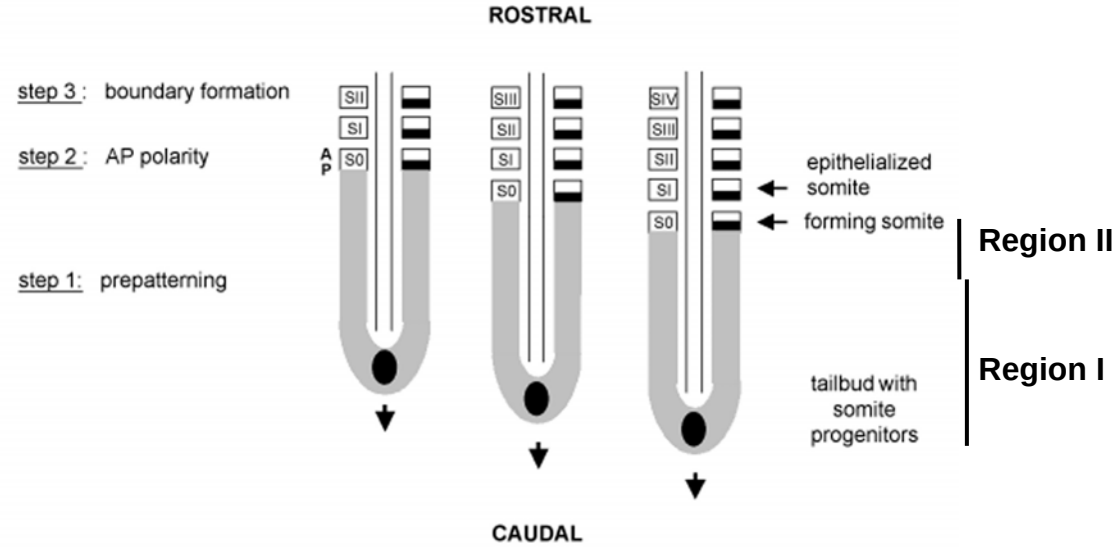


**Fig. 2.** Somitogenesis in vertebrates. A schematic dorsal view of the posterior embryo, illustrating the three timely separated steps that take place during the process of somite formation (prepatterning, establishment of A-P polarity and boundary formation). The PSM is given in a grey color (taken from Winkler and Elmasri, 2005).

Each of these steps is regulated through several molecular genes that are expressed at different developmental stages and positions and will be outlined in more detail below. Signaling factors that induce this differentiation include BMPs, Wnts and Noggin. The dorsal part of a somite differentiates into the dermomyotome, which give rise to all trunk and limb skeletal muscle and the dermis of the back. On the other hand, the ventral part of somites differentiates into the sclerotome, which gives rise to the axial skeleton.

## *1.3 The molecular factors regulating prepatterning of the presomitic mesoderm (PSM)*

### 1.3.1 The intrinsic clock in the PSM of vertebrates

Early reports showed that cells in the PSM remain at their position and show little movement during tailbud outgrowth (Stern et al., 1988). Prepatterning is the step during somitogenesis in which the somitic precursor cells in the posterior PSM are faced with the first signals in the tailbud. Theses signals induce the oscillating expression of several cyclic genes in the PSM region, which regulates the prepatterning of the PSM cells. The existence of a clock in the PSM of vertebrates that controls genes with cyclic expression was predicted several years ago by different theoretical models. Consistent with the widely accepted "Clock and Wave front" model (Cook and Zeeman, 1976), a clock establishes a cyclic wave in the PSM, which will be later interpreted by a determination front to build the spatial periodicity of somites. The determination front, which is called wave front, is located in the anterior PSM and travels gradually back into the tailbud at regular speed as somite formation proceeds and the tailbud grows posteriorly. Therefore, this interaction between the clock and the wave front will lead to the regularly timed boundary formation in the anterior PSM. The first molecular evidence for such a clock and the existence of genes with oscillating characteristics came from the chicken with the isolation of the *hairy* homologue, *c-hairy1* (Palmeirim et al., 1997). *C-hairy1* expression starts in the tailbud and travels anteriorly into the PSM region once per cycle of somite formation (Fig. 3). Subsequently, additional cyclic genes belonging to the family of *hairy*-(h) and *Enhancer of split*-[E (spl)] related genes were identified that possess an oscillating pattern of expression in the PSM. Examples for this are chicken *c-hairy2* (Palmeirim et al., 1997) and *c-hey2* (Leimeister et al., 2000), the mouse *hes1* (Jouve et al., 2000) and *hes7* (Bessho et al., 2001), and zebrafish *her1* (Holley et al., 2000) and *her7* (Oates and Ho, 2002). *Lunatic fringe* (*lfng*) is another member that shows oscillating expression in the PSM of mouse (McGrew et al., 1998) and chicken (Forsberg et al., 1998). In contrast, the zebrafish (Leve et al., 2001; Prince et al., 2001) *lfng* homologue does not exhibit an oscillating pattern.

### 1.3.2. The role of Notch/Delta signaling in PSM prepatterning

The Notch pathway is an intercellular signaling cascade consisting of the transmembrane receptor Notch and its transmembrane ligands, Delta and Serrate. When this signal is triggered, the extracellular domain of Notch is released by a Furin-protease and the remaining membrane bound Notch domain, the so-called Notch intracellular domain (NICD), interacts with suppressor of hairless/RPBJK. This complex then enters the nucleus to activate genes of the *hairy-enhancer of split* (*E* (*spl*)) family, such as *c-hairy1* in chicken, *Hes* in mouse and *Her* in zebrafish. The prepatterning of the PSM is established through the Notch/Delta pathway. This was confirmed by explants experiments in chicken (Palmeirim at al., 1998), Xenopus (Jen et al., 1997), and mouse (Kusumi et al., 1998) and in zebrafish mutant analyses (Holley et al., 2000). The uncoordinated "salt and pepper" expression of Notch/Delta downstream targets in Notch/Delta zebrafish mutants and further functional analyses suggested that the Notch/Delta pathway plays an important role in the synchronization of cyclic expression among neighboring cells (Jiang et al., 2000). Furthermore, the inactivation of the Notch target gene suppressor-of-Hairless (Su (H)) in different zebrafish somite mutants backgrounds, showed a strong defect in somite formation beyond the first 5-7 somites. Suppressor-of-Hairless (Su (H)) encodes a protein that interacts with Notch and mediates Notch/Delta signaling activation to several downstream factors of the Notch pathway. Therefore, this indicates that the Notch/Delta pathway is required also for the lack of expression initiation in addition to its function of synchronization between neighboring cells (Sieger et al., 2003). All zebrafish Notch/Delta mutant embryos lack posterior somites, and a knockdown of downstream Notch/Delta pathway targets like *her1* and *her7* resulted in a disruption of the posterior somites (Holley et al., 2000; Oates and Ho, 2002). These experiments and others manifested that the Notch/Delta pathway is essential for the formation of the posterior somites in zebrafish and for the maintenance and/or induction of cyclic gene expression in the PSM (Holley and Takeda; 2002). This was further supported by a y-ray induced zebrafish mutant, where a large deletion occurred which included the loci of *her1* and *her7* (Henry et al., 2002). Remarkably, in all these Notch/Delta zebrafish mutants only the posterior somites were disturbed, whereas the anterior somites were formed normally. This was explained by a genetic redundancy which might be responsible for the normal formation of the anterior somites in these zebrafish mutants (Oates and Ho, 2002), rather than excluding the function of Notch/Delta pathway in the formation of the anterior somites. This was supported by an experiment that combined a simultaneous reduction of *deltaC* and *her7*

(Oates et al., 2005). In these embryos, a disruption of both anterior and posterior somites was observed. Thus, also anterior somite formation requires the parallel function of *her* and *delta* components, demonstrating that Notch/Delta signaling is involved in somite formation along the entire A-P axis in zebrafish (Oates et al., 2005). The involvement of Notch during anterior somite formation was additionally confirmed in a double mutant experiment, where mutants defective in *notch* and *integrinα5* showed somite boundary defects along the entire anterior-posterior axis. Furthermore, they showed that injection of fibronectin morpholinos into *integrinα5* mutants caused enhanced anterior somitic defects, though normal somite formation was still observed in the posterior somites. Therefore, this suggests that the Notch pathway in combination with Integrinα5 plays a role during boundary formation in the anterior as well as posterior somites (Julich et al., 2005). After showing the importance of Notch/Delta components in vertebrate somite formation, the next challenging question was how the generation and regulation of these cyclic expression patterns occur in the PSM. A three-phase model was postulated to explain the initiation and oscillating activity of the cyclic gene *her1* in the PSM (van Eeden et al., 1996; Holley et al., 2000, 2002). Generally, the signals in the posterior PSM, which initiate cyclic genes, are still not well understood. The postulated hypothesis predicted that inside each individual PSM cell, several regulation events take place within one oscillation cycle (Oates and Ho, 2002). According to this model, Delta ligands activate Notch receptors, which immediately results in the activation of the downstream target genes *her1* and *her7*. These *her* genes encode for transcriptional repressors. Following a short delay due to the translation of their proteins, they repress their own transcription and that of Delta ligands. After degradation of Her1 and Her7 repressor proteins, *delta* genes are turned on again which results in the reactivation of the Notch/Delta pathway (Oates and Ho, 2002). As Delta ligands non-autonomously activate receptors of adjacent cells, this oscillating activity is also transmitted to the neighboring cells. This is thought to result in a highly coordinated wave of transcription that travels through the PSM towards anterior. Previous work has already shown that the Notch/Delta interactions are also important for the synchronization of the oscillating transcription between neighboring cells (Jiang et al., 2000). This view of regulation of downstream *her* targets in the PSM was recently challenged through work with transgenic lines and *her1* and *her7* promoter analysis (Gajewski et al., 2003). In this report, the authors confirmed that the PSM exhibits an activation of Notch receptors via a Delta component which directly results in the activation of the targets *her1* and *her7*. Most importantly, they showed that, in contrast to previous suggestions (Oates and Ho, 2002), *her1* acts as an activator rather than a repressor, not only on its own but also on *her7*

transcription. On the other hand, *her7* acts as an interstripe repressor on *her1*, but as an activator on itself. Therefore, the exact function of both cyclic *her* genes in the PSM differs considerably (Gajewski et al., 2003). To separate these two functions definitely, it is important to analyze whether the segmentation clock is still running in the different zebrafish Notch/Delta mutants or whether the clock acts cell-autonomously.

## 1.3.3. The components of the Wave front activity

As previously mentioned, the postulated "Clock and Wave front" model (Cook and Zeeman, 1976) suggests the existence of a wave front activity in the anterior PSM to translate the temporal periodicity of oscillating gene activity driven by the intrinsic segmentation clock into a spatial periodicity. The aim of this wave front is to define the position in the PSM, where the determination signals are initiated to induce the building of the next somite boundary. Insights into the molecular mechanisms regulating such a front came from recent findings in mouse, chicken, Xenopus and zebrafish, in which dynamic antagonizing gradients of morphogenes were found to act in the PSM. A posterior/anterior gradient, established through FGF and Wnt gradients, and an anterior/posterior retinoic acid gradient (Dubrulle et al., 2001; Sawada et al., 2001; Aulehla et al., 2003; Diez del corral et al., 2003; Moreno and Kintner, 2004) (Fig. 3). Moreover in zebrafish, it was shown that a *t-box* gene, the *tbx24,* is a molecular component of the wave front activity found in the anterior PSM region (Nikaido et al., 2002). Experiments using the *fused somite fss/tbx24* mutants showed that this *t-box* gene is crucial for all events that occur before the final decision is made to form the epithelialized somite (Nikaido et al., 2002). Interestingly, no Tbx24 ortholog has been identified so far in higher vertebrates. Thus, it was suggested that this mechanism could be restricted to teleost fish.

### *1.3.3.1 A FGF gradient Positions the wave front in the anterior PSM*

Studies performed in chicken (Dubrulle et al., 2001) and zebrafish (Sawada et al., 2001) demonstrated that FGF activity mediated through *fgf8* in the tailbud is required to control the proper size of somites. Transplantation of beads soaked with FGF8 into the PSM of chicken embryos resulted in somites with small size. On the other hand, beads soaked with the FGF signaling inhibitor SU5402 increased the size of somites (Dubrulle et al., 2001). Also in zebrafish, somites change their size in a similar manner under the influence of SU5402

treatment and ectopic *fgf* activity in the PSM (Sawada et al., 2001). All together, this manifested that FGF signaling is responsible for determining the position of the presomitic maturation front and consequently the position, where the next somite epithelialization will take place. *Fgf8* is expressed in a graded fashion in the PSM of chicken (Dubrulle et al., 2001) (Fig. 3) and zebrafish (Sawada et al., 2001), with the highest levels in the posterior part of the tailbud. This demonstrates that the *fgf8* expression pattern is conserved during evolution among different vertebrates. It was shown that FGF signaling activity is mediated through a phosphorylated Mitogen Activated Protein Kinase (MAPK)/ERK1 and 2, a general downstream target of the FGF pathway, which is highest in the posterior PSM. The level of phosphorylated MAPK in the PSM correlates with the expression of *fgf8* in the tailbud. Intensive studies of gene expression in SU5402 treated embryos and mutants showed that FGF/MAPK signaling regulates the position of the maturation front in the PSM (Sawada et al., 2001). The FGF pathway seems to be functionally independent of Notch and Fss activity, because it was shown that in zebrafish Notch/Delta and *fss/tbx24* somite mutants *fgf8* expression and the level of phosphorylated MAPK remain constant (Sawada et al., 2001). Unfortunately, no FGF zebrafish mutant with somite defects has as yet been described so far. In the *acerebellar* (*ace*)/*fgf8* mutant in zebrafish, obvious defects in mid-hindbrain boundary formation were observed, whereas only a mild defect in the somites was reported (Reifers et al., 1998). In *fgf8* morpholino injected embryos, a weak disturbance in posterior development was observed (Araki and Brand, 2001; Draper et al., 2001). Intriguingly, the simultaneously knocking down of two important FGF ligands, *fgf8* and *fgf24,* in the tailbud by morpholino antisense oligonucleotide injection showed severe defects during posterior development and somite formation (Draper et al., 2003). This demonstrated that both FGF ligands act functionally redundant. When blocking only one *fgf* gene, the other closely related gene can compensate and take over the function. In contrast to this, in mice *fgf8* mutant embryos show a severe defect and do not form posterior mesoderm.

#### *1.3.3.2 Positioning the wave front in the anterior PSM through a retinoic acid gradient*

The first proof that RA is acting in determining the somitic front came from studies in vitamin A deficient (VAD) quail embryos, which do not synthesize RA. These embryos show significantly smaller somites than control embryos, as well as a longer PSM domain (Diez del corral et al., 2003). The expression of the RA synthesizing enzyme retinaldehyde dehydrogenase (Raldh2) was detected at high levels in the newly forming somites, and at a

lower level in the most anterior PSM region in chick and Xenopus (Haselbeck et al., 1999; Swindell et al., 1999). Inhibiting RA signaling in murine embryos affects PSM prepatterning and leads to somitic defects (Kessel and Gruss, 1991). Retinoic acid (RA) establishes an anterior/posterior gradient that antagonizes the FGF gradient in the PSM region in chicken and Xenopus (Diez del corral et al., 2003; Moreno and Kintner, 2004) (Fig. 2 and 3). It refines the position of the next forming somite boundary. It was shown that FGF8 represses Raldh2 and therefore controls the onset of RA synthesis, while RA down-regulates Fgf8 (Diez del corral et al., 2003; Moreno and Kintner, 2004). In Xenopus, an additional role for RA was demonstrated. It was shown that RA acts as a transcriptional activator of genes involved during A-P determination such as the *mesp* genes (Moreno and Kintner, 2004). However in mouse, RA activity was detected in all formed somites and in the anterior PSM, but without any gradient fashion (Vermot et al., 2005). In mouse, Raldh2 null mutants die early during development and display small somites (Niederreither et al., 1999). A further important function for RA during somite formation was observed in mouse, zebrafish and chicken. It was demonstrated that RA is able to tune the effect of the left-right asymmetry machinery into a symmetric elongation of the anterior-posterior axis on both sides of the vertebral column (Kawakami et al., 2005; Vermot and Pourquie, 2005). This was achieved by regulating the synchronization of the cyclic clock members along the PSM region (Kawakami et al., 2005; Vermot and Pourquie, 2005). Taken together, this all suggested that RA function during somite formation seems to be evolutionarily conserved among vertebrates (Vermot and Pourquie, 2005). In mouse and Xenopus, the RA metabolizing enzyme CYP26, belonging to the P450 cytochrom family, is highly produced in the tailbud region of the embryo (Sakai et al., 2001; Blentic et al., 2003; Niederreither et al., 2003). Using mice lacking CYP26, transcripts of *T* (*Brachyury*) and *wnt3a* were down-regulated in the tailbud, which may reflect the observed caudal defects. From this, the authors suggested that the positional degradation of RA by CYP26 in the caudal part of the embryos is needed for correct patterning in the tailbud (Sakai et al., 2001).

#### *1.3.3.3 Positioning the wave front in the anterior PSM through a Wnt gradient*

Wnt signaling comprises an additional pathway which was revealed to be essential for somite formation in mouse (Aulehla et al., 2003). It was shown that *wnt3a* shows a similar gradient of RNA expression in the tailbud like *fgf8*, also with the highest level in the posterior PSM. Upon this, it was suggested that like for *fgf8,* a specific threshold of *wnt3a* is needed to

determine the final maturation event in the anterior PSM and the progression into boundary formation (Aulehla et al., 2003). Detailed analyses of the Wnt pathway identified an antagonistic Wnt downstream target called *axin2*, which shows oscillating expression in the PSM (Aulehla et al., 2003). In *vestigial tail* (*vt*) mouse mutant embryos that lack *wnt3a*, *axin2* and *fgf8* expression are significantly down regulated. In contrast, *lunatic fringe* (*lfng*) expression is up regulated. This suggested that *wnt3a* might act upstream of both, the Notch/Delta and FGF pathways. Furthermore, it was suggested that the Wnt cascade regulates the oscillation of Notch/Delta pathway components (Galceran et al., 2004; Hofmann et al., 2004). Therefore, it appears that Wnt signaling links the Notch/Delta oscillating clock with the FGF gradient, and thus plays an important role in setting up the clock and the wave front activity (Aulehla and Hermann, 2004) (Fig. 4). Transplantation of cells overexpressing *wnt3a* into the posterior PSM region in chicken embryos caused the formation of smaller somites in the surrounding area of the cells (Aulehla et al., 2003). Unfortunately, no Wnt pathway mutant with defects in somite formation has to date been described in zebrafish. One interesting Wnt signaling mutant in zebrafish, in which tailbud formation is affected, is the *wnt5a* mutant *pipetail*. Despite defects in tailbud formation, it seems that somite formation occurs normally without any obvious changes (Rauch et al., 1997). On the other hand, the only *wnt5* gene so far described in medaka, is ubiquitously expressed throughout the embryo (Elmasri and Winkler, unpublished). Both, *axin1/masterblind* (Heisenberg et al., 2001) and its paralog *axin2* in zebrafish and in medaka (Elmasri and Winkler, unpublished) show no oscillating expression in either species. There are no reports from mutant studies in zebrafish showing any function of Wnt signaling during somite formation. Nonetheless, it cannot be excluded that the Wnt pathway is involved in the process of somitogenesis in teleost fish. Alternatively, it is possible that a Wnt role in somite formation might be restricted to higher vertebrates.

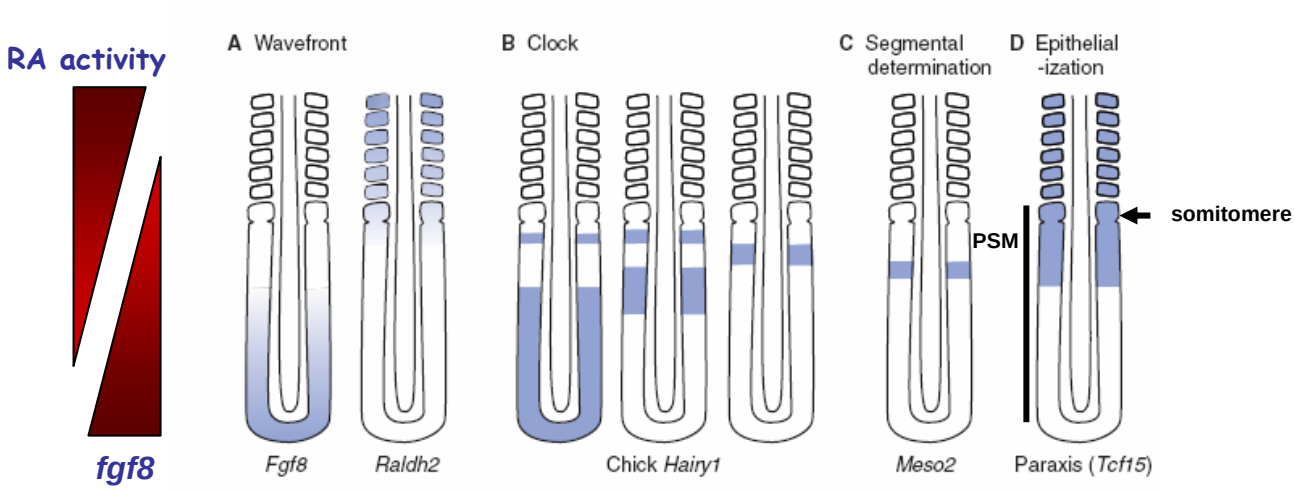


**Fig. 3.** The expression pattern of genes involved in somitogenesis of the chick embryo. A schematic dorsal view of the tailbud. A and B show the "Clock and Wave front" components in chicken. In A, the two antagonistic wave front factors are shown: fibroblast growth factor 8 (*fgf8)* (left) and Retinaldehyde dehydrogenase 2 (Raldh2) (right) form antagonistic gradients. In B, the clock is represented by one cyclic gene, chicken *Hairy1* that shows three different expression phases. In C, striped expression of bHLH transcription factor *Meso2/Mesp* gene is shown that is required for segmental determination and A-P establishment. In D, the expression pattern of *paraxis* is shown that is required during epithelialization of the somitomeres (taken from Dubrulle and Pourquie, 2004).

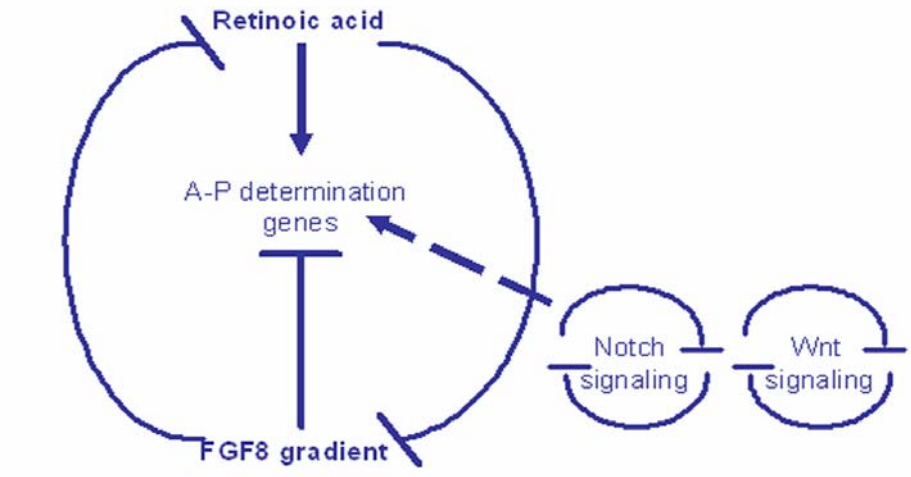


**Fig. 4.** A model showing the different molecular interactions that lead to somite formation in vertebrates. The presomitic mesoderm (PSM) prepatterning is regulated by Notch and Wnt signals, which represent the clock components. At the same time this clock is accompanied by two antagonistic gradients, retinoic acid (RA) and FGF8 that determine the maturation front in the PSM. Afterwards, RA induces and FGF8 inhibits the A-P polarity determination genes (from Dubrulle and Pourquie, 2004).

## *1.4 Setting up Anterior-posterior polarity in the anterior region of the presomitic mesoderm*

After determining a segmental prepattern for the presomitic cells in the posterior PSM, the next step of somite formation is the establishment of a proper anterior-posterior polarity before boundaries are formed and somite epithelialization occurs. During this step, cells in the somitomeres (the forming somites) acquire either an anterior or a posterior character based on the signaling that each part receives (Holley and Takeda, 2002; Dale et al., 2003). Despite the changes in their identities and adhesive properties, the cells remain in a mesenchymal form and stay attached to the anterior region of the PSM.

### 1.4.1. Notch/Delta signaling regulates A-P polarity of somitomeres

The expression of several Notch/Delta components, such as *deltaC* and *notch5,* shows anterior-posterior differences in the anterior PSM region. Experiments in *Xenopus* revealed that the acquisition of the posterior somite identity results from the periodic repression of genes belonging to the Notch pathway via *ESR5* (*enhancer of split-like* gene, acting downstream of the Notch signaling in the PSM) (Pourquie, 2000b). In *Xenopus* the transient repression of Notch pathway components is sufficient for determining the identity because once the initial subdivision is established, it is maintained by a positive feedback loop also mediated by *ESR5* (Pourquie, 2000b). Generally in Notch/Delta somite mutants in zebrafish (*aei*, *des*, and *bea*), anterior-posterior markers show a "salt and pepper" expression pattern. This suggests that A-P polarity is lost. Therefore, it appears that also in zebrafish the generation of A-P polarity is controlled by the Notch/Delta pathway (Sawada et al., 2000). Unfortunately, there is no Notch/Delta somite mutant available in zebrafish with a specific A-P polarity defect that might help to analyze anterior-posterior polarity independently of the molecular clock components.

### 1.4.2. The function of *mesp* in setting up anterior-posterior identity

The first genes to be expressed in a strict segmental manner are the transcription factors of the Mesp/Meso/Thylacine family (Sparrow et al., 1998; Buchberger et al., 2000; Sawada et al., 2000; Saga and Takeda, 2001) (Fig. 3). The bHLH transcriptional factor *mespa* in zebrafish is the homologue of mouse *Mesp-2*, expressed in the anterior regions of the somitomeres

(Durbin et al., 2000). The ectopic expression of *mespb* causes a loss in the posterior identity through the down-regulation of the posterior somite markers *myoD* and *notch5* (Sawada et al., 2000). Combined with mutants analysis, this indicated that *mespb* plays a crucial role during the establishment of anterior-posterior polarity within the somitomeres probably through interaction with FGFRs and the Notch/Delta pathway (Sawada et al., 2000). In the *fss/tbx24* zebrafish mutant, expression of both *mesp* genes is lost, indicating a possible role of the wave front in the induction of anterior-posterior markers (Durbin et al., 2000; Sawada et al., 2000).

## *1.5 Epithelialization: Formation of morphologically distinct somite boundaries*

The final step during somitogenesis is the formation of epithelialized somites. At this step, mesenchymal cells of the somitomeres located in the most anterior PSM region start a mesenchymal to epithelial transition. During this conversion, changes in cell shape, cell adhesion properties, and subcellular polarization of organelles and proteins are observed. This is followed by the formation of epithelialized somites, which are finally able to bud off from the PSM and create a distinguishable somite.

### 1.5.1. The Eph signaling and its role during boundary formation

The Notch/Delta pathway seems to be involved also during this final step of somitogenesis. It appears that boundary formation in the somitomeres is initiated by modulation of Notch activity (Sato et al., 2002). In addition to this, also the *Eph* pathway was confirmed to play an important role during boundary formation. The receptor *EphA4* is expressed in the anterior part of a somitomere, whereas transcripts of its transmembrane ligand *ephrinB2* are found in the posterior halves of a forming somite. The formation of the boundary seems to occur at the point where the receptor *EphA4* in the anterior interacts with its ligand *ephrinB2* in the posterior somitomere domain. Manipulation of Eph signaling results in a disturbance of boundary formation and produces irregular somites in zebrafish, despite normal A-P patterning (Durbin et al., 1998). Analyses in zebrafish *fss/tbx24* mutants showed that *EphA4* expression is lost in this mutant, while *ephrinB2* transcripts are spread over the entire somitomere region (Barrios et al., 2003). Intriguingly, transplantation of cells ectopically expressing *EphA4* into the PSM of *fss* mutants resulted in a rescue of boundary formation. This indicated that Eph signaling is sufficient to induce boundary formation in the anterior

PSM (Barrios et al., 2003). Although Eph components are similarly expressed in zebrafish, chicken and mouse no somitic defects were observed in mouse *EphA4* mutants (Helmbacher et al., 2000), nor in the *ephrinB2* mutants (Wang et al., 1998). This was explained by the existence of redundant activities among Ephrin family members in this species.

### 1.5.2. Fibronectin and its role during somite boundary formation

Possible interaction partners for the Eph receptor during epithelialization are members of the transmembrane receptor family of Integrins (Bokel et al., 2002). Fibronectin is another factor that might be involved in segmentation. Fibronectin is a major part of the extracellular matrix (ECM) and participates in several cellular processes including cell substrate adhesion, cell migration, organization of the cytoskeleton and cell proliferation (Henry et al., 2001; Crawford et al., 2003). Fibronectin signals to the cytoplasm via Integrin receptors found on the plasma membrane. During somitogenesis, Fibronectin protein, as well as other focal adhesion molecules like Paxillin and focal adhesion kinase (FAK) accumulates at somite boundaries. This indicates that the association of Fibronectin and Integrins may function in the formation of somite boundary formation (Henry et al., 2001; Crawford et al., 2003). *Integrinα5* (*itga5*), which encodes a sub-unit of the receptor binding to Fibronectin, is involved in epithelialization of somites in mice (Goh et al., 1997). In mouse null mutants for *integrinα5*, the paraxial mesoderm was segmented, but epithelialization of somites failed to form. Thus, this indicated a role for Intergrin signaling during somite boundary formation (Yang et al., 1999). Furthermore, mice deficient for *fibronectin* (*fn*) exhibit a similar but more severe phenotypical defect in somite boundary formation than that in *integrinα5* deficient mice (George et al., 1993; Yang et al., 1999).

### 1.5.3. The role of Cadherins during somite boundary formation

The Cadherin superfamily contains essential adhesion molecules influencing somite boundary formation in amniotes (Duband et al., 1987). N-cadherin, which is present in the anterior PSM and somitomeres, seems to be the major Cadherin related to somitogenesis. One major role of N-cadherin is to keep anterior and posterior parts of the somitomere together as one functional unit, the somite. This became evident in studies where somites lacking N-cadherin were cleaved into an anterior and a posterior part with epithelial morphology (Linask et al., 1998).

The role of N-cadherin during boundary formation in zebrafish is still unclear, although recently the mutant *parachute* (*pac*) was found to encode for the zebrafish N-cadherin homologue (Lele et al., 2002). The *pac/ncad* mutant shows defects in neural tube and eye development, but no somite phenotypic defects (Lele et al., 2002; Erdmann et al., 2003). This was explained by gene redundancy that might occur in zebrafish Cadherin family members. Another important member of this family that acts as a regulator of somite boundary formation in *Xenopus* is *protocadherin* (*papc;* Kim et al., 2000). *Papc* is detected in the anterior part of the somitomeres, and its inactivation leads to the formation of somites with abnormal boundaries (Kim et al., 2000). In the zebrafish *fss/tbx24* mutant, expression of *papc* in future somitic regions is lost, while *deltaC* still shows the normal cyclic pattern, thus indicating a defect in somite maturation (Jiang et al., 2000). *Papc* null mutant mice show no skeletal defects, and are viable and fertile (Yamamoto et al., 2000). Additional analyses in null mutant mice illustrated that the transcription of *papc* is dependent on *Mesp2*. Furthermore, the dynamic nature of *papc* transcription in mouse somitomeres requires the expression of *lfng*, which acts in cooperation with Notch signaling, and is essential during somite formation. Thus, *papc* is an important regulator of somite epithelialization (Rhee et al., 2003).

### 1.5.4. The involvement of *paraxis* in the boundary formation step

Paraxis is a bHLH transcription factor involved in somite boundary formation. *Paraxis* is expressed in the PSM and segmented somites and is highly conserved among different vertebrates like mouse (Burgess et al., 1996), chicken (Barnes et al., 1997) and zebrafish (Shanmugalingam et al., 1998). In mouse embryos carrying a null mutation in *paraxis,* expression of *Mesp2* and Notch pathway genes were not affected in the PSM. However, expression of genes that are normally expressed in the posterior part of the somitomerese was diffuse in the mutants (Johnson et al., 2001). Furthermore, in these *paraxis* null mice, the paraxial mesoderm exhibited characteristic furrows. This demonstrated boundary formation, but apparently cells at these boundaries failed to become fully epithelialized. This indicates that *paraxis* is involved in maintaining somite polarity, and later acts during epithelialization independent from Notch signaling (Johnson et al., 2001). Thus, it implies that segmentation and epithelialization are two separate processes under distinct genetic regulation (Burgess et al., 1996). Paraxis seems to regulate the activity of Rho GTPases, such as rac1 and Cdc42,

which were recently shown to mediate mesenchymal-epithelial transition during somite formation (Nakaya et al., 2004).

## *1.6 Fish as model systems to study somitogenesis*

Fish in general is an attractive model system to study the function of genes during vertebrate development and disease, due to several advantages which allow a combination of molecular and embryological experimental strategies. Zebrafish and medaka produce large number of eggs in which fertilization and development occurs externally. Furthermore, development is extremely fast and takes only few days from fertilization until a free swimming larvae hatches. Generally, in zebrafish this needs 2.5 days and in medaka 7 days. Additionally, embryos are highly transparent and large enough to allow micromanipulations like the injection of DNA, RNA or antisense morpholino oligos at early stages of development or to perform transplantation of cells or beads soaked with chemicals. This and the short generation time of approximately three months were the bases for an efficient mutagenesis screening procedure in zebrafish and medaka to identify recessive embryonic phenotypes which are mostly lethal. Several large scale screens were conducted in zebrafish, one in Boston (Driever et al., 1996) and two others in Tuebingen and in Eugene (Haffter et al., 1996; Knaut et al., 2003). Furthermore, several small-scale screens were additionally performed, e.g. recently in Japan (Koshida et al., 2005). Also in medaka, small and one large scale screens have to date been performed in Kyoto and elsewhere (Loosli et al., 2000; Furutani-Seiki et al., 2004). From these screens, thousands of mutants showing deficiencies in a broad spectrum of developmental processes were isolated. During the last few years, some of the zebrafish mutants were positionally cloned and the mutated genes identified. This was facilitated by recent genome sequencing projects, which were performed at the Sanger Center in the UK (http://www.sanger.ac.uk/Projects/D_rerio). In medaka on the other hand, the cloning of the mutants has recently been started, which is accompanied by a sequencing project performed at the National Institute of Genetics in Tokyo (http:dolphin.lab.nig.ac.jp/). Furthermore, high resolution genetic maps have been published in medaka to develop the appropriate resources for positional cloning of the many new isolated medaka mutants (Naruse et al., 2000; 2004).

### 1.6.1. Zebrafish as an established model to analyze somitogenesis in vertebrates

Based on their morphology, two groups of somitogenesis mutants were classified in zebrafish. The first group includes the *you*-type genes (so called because the somites of the homozygous mutants show a U shape) and the second group the fused somites (*fss)* type genes. The *you*-type mutants, *sonic-you* (*syu; shh*) (Schauerte et al., 1998), *you-too* (*yot; gli2*) (Karlstrom et al., 1999), *chameleon* (*con; dispatched homolog 1*) (Nakano et al., 2004) and *u-boot* (*ubo; prdm 1*) (Baxendale et al 2004), do not exhibit defects during early somite formation. Rather, the defects are seen later in the patterning of already formed somites such as during muscle differentiation. The molecular cloning of these mutants demonstrated that all you-type mutants represent deficiencies in the Hedgehog signaling pathway. The second group of zebrafish somite mutants, the *fss*-type (Table 1), includes eight complementation groups, *fused somites* (*fss*) (Nikaido et al., 2002), *beamter* (*bea*) (van Eeden et al., 1996), *deadly seven* (*des*) (Holley et al ., 2002), *after eight* (*aei*) (Holley et al., 2000), *spadetail* (*spt*) (Griffin et al., 1998), *mindbomb* (*mib*) (Itoh et al., 2003), *before eight* (*bfe*)/*kt293* (Julich et al., 2005; Koshida et al., 2005) and *kt259* (Koshida et al., 2005), and show defects in somite boundary formation. However, with exception of *fss, spt, kt293/bfe* and *kt259,* the rest of the mutants show characteristic similarities in their phenotypes. Cloning of the mutants belonging to this class showed that *kt293/bfe* and *kt259* are affected in *integrin-fibronectin* genes, and that *fss* and *spt* mutations affect T-box genes, whereas the remaining four mutants were defective in the Notch/Delta signaling pathway. The identification and characterization of the responsible genes for these Notch/Delta mutants significantly increased our understanding of somitogenesis in vertebrates. *After eight* (*aei)* was the first zebrafish somite mutant in which the affected gene was identified. The homozygous *aei* mutant embryos carry a premature stop codon in the *deltaD* gene (Holley et al., 2000). Phenotypically, they form the first 7-9 pair of somites in the anterior trunk, but after that the process of boundary formation ceases. Neuronal hyperplasia was the only other process beside somitogenesis that was reported to be affected in the *aei* mutant (Jiang et al., 1996). Interestingly, homozygous *aei* mutants are viable and develop into fertile adults. In the zebrafish Notch/Delta mutants, the expression of different downstream genes of the Notch/Delta cascade like *her1, her7*, *deltaC, her11* and *hey1* is disturbed.

**Table 1: The zebrafish *fss*-type somitogenesis mutants**

| Mutant | Gene | Somite phenotype | Additional phenotypes | Reference |
|---|---|---|---|---|
| ***after eight* (*aei*)** | ***deltaD*** | **Forms only first 7-9 somite pairs** | **None** | **Holley et al., 2000** |
| ***deadly seven* (*des*)** | ***notch1a*** | **Forms only first 7-9 somite pairs** | **None** | **Holley et al., 2002** |
| ***Beamter*** | **n.d** | **Forms only first 2-4 somite pairs** | **None** | **van Eeden et al., 1996** |
| ***mind bomb* (*white tail*)** | ***RING ubiquitin ligase*** | **Irregular formed somite boundaries in posterior segments** | **Increased neural progenitor numbers** | **Itoh et al., 2003** |
| ***fused somites* (*fss*)** | ***tbx24*** | **No somite boundaries** | **None** | **Nikaido et al., 2002** |
| ***spadetail* (*spt*)** | ***tbx16*** | **No trunk somites** | **Normal notochord, otherwise defective trunk mesoderm** | **Griffin et al., 1998** |
| ***kt259*** | ***fibronectin*** | **The anterior 2-10 somites were fused. Posterior somites formed normally. Less severe somite boundary defects than in *kt293*/*bfe* mutants.** | | **(Koshida et al., 2005)** |
| ***kt293/ before eight (bfe)*** | ***itga5*** | **The anterior 2-10 somite boundaries are fused. Posterior somites form normally.** | | **(Koshida et al., 2005; Julich et al., 2005)** |

For example, in *aei/deltaD* embryos, expression of cyclic genes including *her1, her7, deltaC* (Holley et al., 2000, 2002; Oates and Ho, 2002), and non cyclic targets like *her11* (Sieger et al., 2004) and *hey1* (Winkler et al., 2003) has vanished or is greatly reduced in the posterior PSM. On the other hand, a disorganized "salt and pepper" expression pattern was observed in the anterior PSM. In *deadly seven* (*des)* embryos, the affected gene encodes for Notch1 protein (Holley et al., 2002). Examining the somitic phenotype, as well as gene expression in the PSM has failed to show any obvious difference between *des* embryos and *aei/deltaD*. Moreover, expression of Notch/Delta downstream target genes including *her1, her7,* and *deltaC* is abolished in the posterior PSM. At the same time, the typical "salt and pepper" pattern was detected in the anterior PSM region of *des* embryos (Holley et al., 2002). In all

*fss*-type zebrafish mutants, not only the expression of the oscillating genes *her1, her7* and *deltaC* is disrupted (Holley et al., 2000, 2002; Oates and Ho, 2002), but also other Notch/Delta targets in the PSM are affected. An example is *her11* (Sieger et al., 2004) and *hey1* (Winkler et al., 2003). This is consistent with the idea that Notch/Delta pathway genes can be an output or a central component of the oscillator. However, functional analyses in zebrafish supported that Notch/Delta activity itself establishes the oscillator. On the one hand, overexpressing the receptor *notch1a,* results in an up-regulation of *her1* transcription (Takke and Campos-Ortega, 1999). On the other hand, overexpression of *her1* down-regulates transcription of *deltaC* and *deltaD*. Thus, this demonstrates a negative feedback loop in the Notch/Delta pathway and could provide the molecular basis for an oscillatory circuit. To confirm this, antisense morpholino oligos were injected into zebrafish embryos. Knocking down *her1* and *her7* resulted in a direct up-regulation of *her1* and *her7* transcription (Holley et al., 2002; Oates and Ho, 2002). From these results and mutant analysis it was concluded that the intrinsic clock during somite formation is established by cyclic Notch/Delta activity. Analyzing the somitogenesis mutant *fused somite* (*fss*) provided the evidence for a zebrafish mutation, which affect a wave front component (Nikaido et al., 2002) (Table 1). *Fss* mutant embryos are phenotypically different from all other described *fss*-type zebrafish somite mutants that were found to belong to the Notch/Delta signaling pathway or Integrin-Fibronectin system. The *fss* mutant is characterized by a complete lack of somite formation along the entire anterior-posterior axis. Recently, cloning of this mutant showed that the *fss* gene encodes for *tbx24,* which is exclusively expressed in the PSM region. Comparing the amino acid sequences of the conserved T-domains revealed that this new T-box protein shows no homology to any known T-box protein in higher vertebrates (Nikaido et al., 2002). Analyzing the behavior of cyclic clock components in the *fss/tbx24* mutant showed that Fss and the Notch pathway function independently. In *fss/tbx24* mutants, the anterior most PSM stripe of *her1* is always missing, whereas the two posterior stripes of *her1* appear normal in the PSM. In contrast, in *aei/deltaD* mutant embryos the posterior stripes of *her1* are disturbed or vanished, while the anterior stripe is detectable in the PSM. This demonstrates that *fss* does not affect oscillation and is required to induce and maintain *her1* in the anterior PSM (Sawada et al., 2000). Furthermore, somite formation problems in the *fss* mutants are not restricted to stabilization defects of oscillating genes in the anterior PSM region. In this mutant, also the induction of key genes like *mespb* and *papc* that are required later for setting up anterior-posterior polarity is affected (Sawada et al., 2000). Therefore, the *fss/tbx24* gene is essential for all events that occur before the final decision is made to form the epithelialized somite

(Nikaido et al., 2002). In applying the different zebrafish ENU screens, two further *fss*-type mutants affecting somite formation were identified with point mutations in *integrinα5* (*itga5*) and *fibronectin* (*fn*) (Julich et al., 2005; Koshida et al., 2005) (Table 1). Both mutants *kt293/before eight (bfe)/integrinα5* and *kt259/fibronectin*, show defective boundary formation in anterior somites (Julich et al., 2005; Koshida et al., 2005). In the *bfe* mutants the anterior two to ten somites are fused, whereas the posterior somites form normally (Julich et al., 2005). A similar phenotype was detected in *kt259* mutants, however, with the difference that the defects are less severe because boundaries are usually formed in these embryos (Koshida et al., 2005). Careful analysis of both mutants indicated that the accumulation of Fibronectin protein is an Integrinα5 dependent process. This accumulation is required for the maintenance of somite boundaries and for the epithelialization of cells at the connecting boundaries (Koshida et al., 2005). Further functional work demonstrated an interaction between the Integrin-Fibronectin system and Ephrinb2. This suggested that the Integrin-Fibronectin and the Eph-Ephrin system might function in a redundant manner during boundary formation in the posterior somites. Therefore, the process of morphological segmentation can be divided into two events, somite boundary establishment, which might occur without the help of *integrinα5* and *fibronectin,* and boundary maintenance which requires both *integrinα5* and *fibronectin* as well as *ephrinb2* (Koshida et al., 2005). At the same time, double mutant analysis between *before eight/integrinα5* and Notch/Delta mutants showed that somite formation is impaired along the entire anterior-posterior axis. Therefore this suggests that Notch/Delta and Integrinα5 function in parallel to regulate somite boundary morphogenesis, mesenchymal-epithelial transition and assembly of the Fibronectin matrix during posterior somite formation (Julich et al., 2005).

### 1.6.2. Medaka as a new complementary model to study somitogenesis in vertebrates

Similar to zebrafish, medaka is an oviparous fish that is able to produce large numbers of eggs on a daily basis during the whole year. The embryos have a chorion which is harder than that of zebrafish, but is still transparent enough to follow the fast development of the embryos. Depending on the incubation temperatures, medaka embryos need between 7-12 days to hatch. The embryo is large enough to perform manipulations like cell transplantations and microinjections. In the last few years, several embryological and genetic methods have been established, including RNA overexpression and gene knockdown using morpholino antisense

oligo nucleotides for functional analysis. Furthermore, different transgenic approaches were established including endonuclease (Thermes et al., 2002) and transposon-mediated transgenesis (Grabher et al., 2003). In addition, the availability of embryonic stem (ES) cells allows the analysis of cell differentiation processes *in vitro* (Hong et al., 1998; Bejar et al., 2003). The size of the medaka genome (1.0Mbp) is roughly a quarter of that of humans and almost half of that of zebrafish (1.8Mbp). Several sequencing projects are currently going on in Japan. Until recently, partially assembled whole genome shotgun reads representing approximately 10 fold genome coverage are accessible through the website of the National Bio-Resource Project (http://shigen.lab.nig.ac.jp/medaka/genome/indexen.html). Until now, many essential developmental processes are intensively studied on a molecular basis in medaka, like eye development (Del Bene et al., 2004), sex determination (Nanda et al., 2002; Schartl, 2004), and somitogenesis (Elmasri et al., 2004; 2004a; own publication number 9). Morphologically, somitogenesis occurs in a similar fashion among different teleost fish. Differences between different species for example include the total number of somites (30 pairs in zebrafish and 35 in medaka). The time required for the formation of one somite is 30 minutes at 28°C in zebrafish (Kimmel et al., 1995) and 60 minutes at 26°C in medaka (Iwamatsu, 2004). Out of different mutagenesis screens performed in the last few years in zebrafish, seven complementation groups were identified with specific somitogenesis defects. These include mutants in the Notch/Delta pathway and in the Integrin-Fibronectin system. Other studies in mouse, chicken and zebrafish have shown that additional components are required for somitogenesis. Most importantly, these are mediated by the FGF, Wnt and RA signaling pathways. Unfortunately, however, no zebrafish mutants with defects in these pathways showing specific somite defects are known to date. This was explained by functional redundancies among related genes that have resulted from a whole genome duplication, which occurred in a teleost fish ancestor approximately 350 Mio years ago (Volff, 2005). In fish, many developmental regulatory essential genes exist as duplicates that originated in the teleost lineage (Amores et al., 1998; Meyer and Schartl, 1999; McClintock et al., 2001; Wittbrodt et al., 2002; Winkler et al., 2003a). The duplicated genes were reported to show partially overlapping functions, as for the Wnt and Hedgehog pathways in zebrafish (Lewis and Eisen, 2001; Lekven et al., 2003). Intriguingly, the number of duplicated genes in the genome varies among different fish species (Amores et al., 2004; Naruse et al., 2004). Therefore, important differences were obtained when gene families were compared between zebrafish, Fugu and medaka (Amores et al., 1998, Winkler et al., 2003a; Amores et al., 2004; Naruse et al., 2004). Based on these deviations between teleost fish, a large scale mutagenesis

screen in the medaka fish (*Oryzias latipes*) was established in Kyoto, Japan to isolate mutants acting in various processes during development (Own publications number 7). We attempted to isolate mutants in the medaka fish in order to understand the molecular interactions and regulations of different signals involved during somitogenesis, and possibly to identify novel components implicated in this process. Thus, this screen aimed to increase the level of saturation regarding the number of mutants that affect specific processes during development, like somitogenesis. In medaka, we expected to find mutants that show phenotypic similarities to zebrafish somite mutants, however, we expected to find novel phenotypes that were not described in zebrafish before. In this screen, a broad spectrum of somitic phenotypes was obtained with 15 different isolated medaka somite mutants that we are currently analyzing. Their phenotypes range from a complete or partial loss of somite boundaries, to fused somites, or somites with irregular sizes and shapes. Thus, some of these mutants share characteristics with described zebrafish somite mutants, while several others represent phenotypes that have not yet been reported in zebrafish so far. With this screen (see medaka special issue of Mechanism of Development 2004. Vol. 121 and references therein) previous work was supported which established the Japanese teleost medaka (*Oryzias latipes*) as a complementary model system to zebrafish for developmental studies (Wittbrodt et al., 2002).

# 2. Results and discussions

## *2.1 Characterization of somitogenesis related genes in medaka*

The medaka represents an established model for developmental and genetic studies for many years, however, until recently somitogenesis has not been studied in this species. Therefore, we started to isolate the medaka orthologs of zebrafish genes expressed during somite formation in order to obtain marker genes for characterizing the medaka somitogenesis mutants. We isolated several genes that are implicated in the different steps of somite formation. First, genes were identified, which act as clock elements during PSM prepatterning and belong to the *hairy-enhancer of split (E (spl))* family. We focused on those members of this family that exhibit oscillating patterns of expression including *her1*, *her5* and *her7*. These *her* targets are the first genes isolated so far with a cyclic expression in medaka. All described cyclic *her* genes in zebrafish showed more than one stripe of transcripts that bud off from the posterior expression domain in the PSM (Holley et al., 2000; Oates and Ho, 2002; Gajewski et al., 2003). In contrast to this, all medaka oscillating *her* genes shared the characteristic of having a single stripe of RNA, which sweeps anteriorly from the posterior region (Elmasri et al., 2004; own publication number 9). Therefore, these results showed the existence of a clock in the PSM region of medaka embryos during somitogenesis similar to all other vertebrates. It suggests that also in medaka, these oscillating genes are core components of the oscillating clockwork acting in the posterior PSM (Elmasri et al., 2004; own publication number 9). Next, we isolated *mesogenin,* which is ubiquitously expressed in the tailbud and seems to be involved in the early process of tailbud formation in medaka (Elmasri et al., 2004). The behavior of *mesogenin* in medaka is similar to its ortholog in zebrafish (Yoo et al., 2003).We then identified *mesp,* an important component involved in segmental determination and specification of A-P polarity in higher vertebrates and zebrafish. Medaka *mesp* is expressed in the anterior PSM in one or two stripes depending on the phase of somite formation. Thus, it shows an identical expression as its ortholog in zebrafish, *mespb* (Sawada et al., 2000) and *Mesp2* in mouse (Saga et al., 1997). This suggests that the function of this crucial somitogenesis gene is conserved during evolution of teleost fish to higher vertebrates (Elmasri et al., 2004). Next, we isolated genes expressed either in the anterior or posterior regions of the formed somites. These genes represent markers for correct anterior-posterior compartmentalization of formed somites and boundary formation. *Lunatic fringe (lfng)* encodes a glycosyltransferase implicated in Notch/Delta signaling and shows oscillating

expression in higher vertebrates (Forsberg et al., 1998; McGrew et al., 1998). Unlike in mouse and chicken, but similar to zebrafish (Leve et al., 2001; Prince et al., 2001), medaka *lfng* shows a non-dynamic expression in the anterior half of all formed somites (Elmasri et al., 2004). This indicates that the role of *lfng* during somite formation has changed during evolution, from a non oscillating expression in teleost fish, into an oscillating pattern in higher vertebrates. In chicken, *lfng* establishes a feedback loop and thus functions as a component of the molecular oscillator (Serth et al., 2003). The second analyzed marker for formed somites is the myogenic gene *myf5*. Medaka *myf5* RNA is expressed in the most anterior PSM region, in the adaxial mesoderm and in the posterior half of each formed somite similar to the situation in zebrafish (Coutelle et al., 2001; Elmasri et al., 2004a). This suggests a conserved mechanism of regulation during somite differentiation into muscles in teleost fish. Finally, we analyzed *hey1* as a member of a gene family with highly divergent expression and function in vertebrates (Leimeister et al., 2000b; own publication number 1; own publication number 2). *Hey* genes encode a subfamily of *Hairy/Enhancer-of-split* bHLH transcription factors that are involved in somitogenesis, blood vessel and heart development. They are downstream targets of Notch signaling and show broad expression in mice (Leimeister et al., 1999, 2000a) and restricted expression in zebrafish (own publication number 2). In mouse, all three *hey* genes *hey1*, *hey2*, and *heyL* are expressed in the PSM, whereas *hey1* is the only *hey* gene expressed in the PSM and somites in zebrafish. Interestingly, in mice and chicken *hey2* shows oscillating expression (Leimeister et al., 2000b) and in zebrafish *hey1* is dynamic in the anterior PSM (own publication number 2). In medaka, *hey1* shows a broader expression than in zebrafish (Elmasri et al., 2004). During somitogenesis, *hey1* expression is found in the PSM, all formed somites, mesencephalon, segments of the hindbrain, dorsal aorta, and the heart. Similar to the pattern of *hey1* expression in zebrafish, *hey1* in medaka shows a dynamic mode of expression in the anterior PSM region (Elmasri et al., 2004). Overall, despite some species-specific differences in the behavior of single clock components (e.g. *lfng*) or later genes expressed in somites (e.g. *hey1*), it seems that the molecular mechanisms controlling segmentation are more or less conserved from teleost fish to higher vertebrates.

## *2.2 Isolation and characterization of medaka mutants*

### 2.2.1 The Kyoto large scale screen

In this screen, approximately 1300 F2 families were raised and 1137 F2 families were used for producing F3 progeny for further screening. From more than 6700 crossing of F2 fish, 6088 crosses were successful and yielded 24,887 clutches (a set of eggs produced per mating) that were used for screening. A total number of 260,000 F3 embryos were screened (Own publications number 7). Mutations were generated by treatment of male medaka founder fish with Ethyl-nitroso-urea (ENU) to introduce mutations into the germline (Shima and Shimada, 1988). The same method was used in the zebrafish mutagenesis screens (Mullins et al., 1994; Solnica-Krezel et al., 1994) and in mice (Russell and Montgomery, 1982). ENU is known to introduce point mutations very efficiently and randomly in the genome of spermatogonia (Russell and Montgomery, 1982). Although 2031 isolated mutations were embryonic lethal, 312 mutations with organogenesis defects could be identified. Out of this medaka large screen two main goals were attempted. The first one was to reach a high level of saturation in the number of mutated genes in the genome of teleost fish. The second aim was to isolate novel mutant phenotypes that have not been reported earlier in zebrafish. In zebrafish, the identified mutants have dramatically enriched our knowledge about molecular functions and regulation of different processes during development. The screen in medaka resulted in the identification of a large number of recessive, embryonic lethal mutants often with novel phenotypes. Based on their morphological defects and *in-situ* analyses several groups of mutants affecting different development processes including somitogenesis, retina and forebrain development were isolated (Elmasri et al., 2004a; own publications number 7, 5, 6). 60 isolated recessive mutants affected retina development (own publication number 6). These mutants were classified into five groups. The first group includes 11 mutants, in which the neural plate and optic vesicle growth are affected. The second group contains 15 mutants, in which the optic vesicle growth ceases. The third group comprises 18 mutants that are affected in optic cup development. The fourth group consists of 13 mutants with defects in retinal differentiation. The last group contains three mutants with defects in retinal pigmentation (own publication number 6). Furthermore, from this screen, 21 mutants with defects in forebrain formation were isolated (own publication number 5). Based on their phenotypes, these mutants were classified into two groups. Group I contains 11 mutants showing a reduction in the size of the telencephalon. Group II shows defects in the morphology of the telencephalon without

affecting its size. In zebrafish, several mutants affecting the development of the telencephalon were previously isolated and characterized, including *knollnase* (*kas*), *masterblind* (*mlb*) and *silberblick* (*slb*) (Heisenberg et al., 1996). In contrast to the situation in medaka, in all of these mutants the defects in the telencephalon are accompanied with defects in midline structures, resulting in cyclopia or curly tails (Brand et al., 1996). As midline structures are normal in medaka, the medaka forebrain mutants seem to be distinct from those isolated in zebrafish (own publication number 5). Moreover, 15 mutants with somitic phenotypes were isolated and nine of them were characterized in detail in the Winkler laboratory (Elmasri et al., 2004a). Depending on their morphological phenotypes and the expression of somitic marker genes, these mutants were separated into two classes. Class I includes the somite mutants with severe defects in tailbud formation and PSM prepatterning. This was evident by the randomized and impaired oscillation of *her7* expression and disturbed or absent *mesp* transcription. Consequently, these mutants fail to complete somite development (Table 2). Class II consists of the somite mutants with regular PSM prepatterning and normal oscillation of the cyclic *her7* gene. Nevertheless, they fail to form regular morphological somite boundaries and show variable deficiencies in expression of the A-P polarity gene *mesp*, the myogenic gene *myf5* in posterior somite regions and the anterior somitic marker *lfng*. This suggests that these mutants show defects during later phases of somite formation (Table 3) (Elmasri et al., 2004a). Until now, five of these somite mutants were mapped to specific positions on linkage groups (Table 4).

**Table 4: Chromosomal mapping of five mutant loci in medaka somitogenesis mutants**

| Mutant | Linkage group (LG) | Closest marker and distance |
|---|---|---|
| ***orgelpfeifen*** | **LG 20** | **Ola2311a (segment14), 5.3cM** |
| ***schnelles ende*** | **LG 9** | **AU168825 (segment31), 6.4cM** |
| ***doppelkorn*** | **LG 19** | **Olb2211h (segment10), 1.3cM** |
| ***planlos*** | **LG 14** | **AU169080 (segment20), 13.5cM** |
| ***zahnluecke*** | **LG 22** | **Ola2302d (segment 21), 14.1cM** |

### 2.2.2 Class I: Medaka somite mutants with defects in tailbud formation and PSM prepatterning

In class I medaka mutants, PSM prepatterning is severely affected. *Her7* and *mesp* expression is strongly disturbed or down-regulated in the PSM and the dynamic expression pattern of *her7* is altered. In the *bremser* (*bms*) mutant, we were unable to detect any obvious somite boundaries. In this mutant, the expression of *her7* was reduced and its oscillations were disrupted (Table 2a). On the other hand, *mesp* transcripts were observed in the anterior PSM region, but *mesp* expression was not restricted to one or two somitomeres as in wildtype sibling embryos and, instead, was broadened. On the other hand, *myf5* RNA was almost completely down-regulated. Therefore, these results indicate that in this mutant somite differentiation seems to be completely abolished. Phenotypically similar to *bms*, also in *planlos* (*pll*) mutants no somite boundaries are visible throughout the trunk. Transcripts of *her7* were present in the posterior PSM, however, the dynamic pattern of *her7* was abolished (Table 2). Moreover, *mesp* and *myf5* expression was completely down-regulated. This suggests that PSM cells in this mutant lack the capacity to regulate oscillation and induction of A-P polarity genes in the anterior PSM region. This was supported by the analyses of two newly isolated oscillating *her* genes, *her1* and *her5* (own publication number 9). For both genes, we noticed a complete down-regulation of expression in the posterior PSM and only remaining traces of RNA in the most anterior PSM region. At the same time, also oscillation of both dynamic *her* genes was abolished (own publication number 9). The differences in the expression of these three cyclic genes in the *pll* mutant suggest differences in the regulation between *her1* and *her5* on the one hand and *her7* on the other in these *pll* mutants. In the *schnelles ende* (*sne*) mutant, the first two to three anterior somites are visible, after that somite boundary formation ceases. On the molecular level, we observed weak expression of *her7* in the posterior PSM (Table 2). Furthermore, *myf5* transcripts are limited to the formed somites, and thus detected only in the first two to three somites. However, *mesp* expression was completely lost. *Mesogenin* is an early gene which is ubiquitously distributed in the tailbud of zebrafish (Yoo et al., 2003) and medaka (Elmasri et al., 2004a). Only remnants of *mesogenin* RNA are detected in the most anterior PSM domain of *sne* and *pll* mutants. Therefore, it seems that the defects observed in *sne* and *pll* are caused by deficiencies that occur during tailbud formation even before the process of somitogenesis is started. In the *samidare* (*sam*) mutant embryos, the first four to six somites are formed normally, but the process of boundary formation in the posterior somites is impaired. In *sam* embryos, the expression of *her7* shows an unequal distribution of RNA among neighboring cells throughout the PSM. This “salt and

pepper" pattern of expression was described before for *her1* and *her7* in Notch/Delta zebrafish somite mutants (Holley et al., 2002; Oates and Ho, 2002). The transcripts of *mesp* were not disturbed in the somitomeres. *Myf5* expression in the first formed somites was normal however, in the posterior somites *myf5* RNA was down-regulated. An additional defect detected in *sam* mutant embryos was the failure to form a correct mid-hindbrain boundary. A specific marker to demonstrate MHB defects is *engrailed 2* (*eng2*), which is exclusively expressed in the MHB region in medaka during somitogenesis (Ristoratore et al., 1999). In *sam* embryos, *eng2* transcripts were reduced. This correlates with the situation in the zebrafish MHB mutants *acerebellar* (*ace*/*fgf8*) (Reifers et al., 1998) and *no-isthmus* (*noi*/*pax2b*) (Scholpp and Brand, 1998), in which *eng2* is also down-regulated. Some of the phenotypic defects observed in medaka class I mutants show similarities to zebrafish somite mutants.

**Table 2: Class I mutations affecting tailbud formation and PSM prepatterning**

| Mutant | Somitic Phenotypes | Head Phenotypes |
|---|---|---|
| ***bremser (bms)*** | **no somites formed, only partial boundaries** | **arrested eye and forebrain morphogenesis** |
| ***planlos (pll)*** | **partial formation of only 1-2 anterior somites** | **arrested eye and forebrain morphogenesis** |
| ***schnelles ende (sne)*** | **only the first two somite pairs form, tailbud reduced** | **normal** |
| ***samidare (sam)*** | **no segmentation of posterior trunk, anterior six pairs of somites form normally** | **arrested eye and forebrain morphogenesis** |
| ***doppelkorn (dpk)*** | **individually fused somites, irregular somite size** | **arrested eye and forebrain morphogenesis** |

For example *planlos* (*pll*), *schnelles ende* (*sne)* and *samidare (sam*) all members of the class I medaka somite mutants, develop the first few anterior somites normally but the formation of the posterior somites ceases in these mutants (Elmasri et al., 2004a). This is a feature also observed in the zebrafish Notch/Delta mutants *after eight* (*aei*) (Holley et al., 2000), *beamter* (*bea*) (van Eeden et al., 1996) and *deadly seven* (*des*) (Holley et al., 2002). In contrast, the *bremser* (*bms*) mutants failed to establish any somite boundaries. In addition, also *dpk* has a unique phenotype in this class and forms partially fused somites and somites with irregular sizes (Elmasri et al., 2004a). Surprisingly and despite some phenotype similarities among the medaka mutants, *her7,* a clock component implicated in PSM prepatterning showed clear

difference between these mutants from a nearly complete loss of *her7* transcription (e.g. in *bms*) to an almost unchanged expression (e.g. in *dpk*) (Elmasri et al., 2004a). However, one common feature found in all class I mutants was the disruption of the oscillating character of cyclic *her7* expression. This suggests that clock components or clock regulating elements appear to be affected in this class of mutant. Alternatively, early processes during tailbud formation could also be affected (Elmasri et al., 2004a). The molecular identification of the genes deficient in these mutants will help to understand the functional interactions of different signals involved in the process of PSM prepatterning and tailbud formation. The appearance of so far unrecorded phenotypes makes it likely that novel components acting during this period of somitogenesis will be isolated.

#### *2.2.2.1 Dpk is a medaka mutant with deficiencies in the stabilization of the wave front*

*Doppelkorn* (*dpk*) is member of the class I medaka mutants and shows a unique and novel phenotype that was not reported until now in zebrafish (Table 2). Phenotypically, in *dpk* mutants single somites are fused or enlarged on an irregular basis along the entire A-P axis. Based on our preliminary analysis using *her7* as a marker, we obtained first evidence that the medaka *dpk* mutant is probably affected in PSM prepatterning or tailbud formation. We noticed that the PSM clock component *her7* was not properly down-regulated in the most anterior part of the PSM as it is in wildtype. Instead, *her7* expression extends beyond that of *mesp* and is detected in the segmented paraxial mesoderm. In contrast, in wildtype sibling embryos the A-P polarity marker *mesp* is always expressed anteriorly to *her7* (Fig. 5). However, in zebrafish *fss/tbx24* mutants, the stabilization of oscillating genes in the anterior PSM fails and therfore, the expression of the most anterior *her1* stripe lack in the *fss* mutants (Nikaido et al., 2002). Therefore, the excess in the amount of *her7* RNA is an indication for a stabilization defect rather than a complete lack of stabilization in zebrafish *fss/tbx24* mutants (Nikaido et al., 2002). Thus, the stabilization of cyclic genes in the anterior PSM seems to be affected in *dpk* mutants. This proposes that in the *dpk* mutant, the wave front activity is de-regulated and gives a first hint that in *dpk* a componenet upstream of the wave front might carry the mutation (Elmasri et al., 2004a).

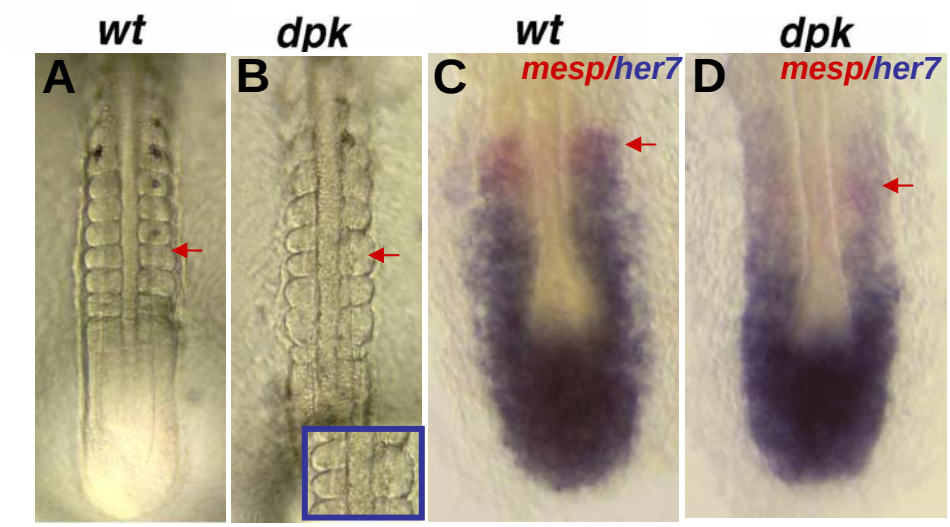


**Fig. 5.** Somite formation in medaka wildtype (wt) and *doppelkorn* (*dpk*) somite mutant embryos. A-B, images of live embryo that show the regular somite size in a wt embryo (A) and fused somites in *dpk* (B). Higher magnifiction of one fused somite shown in the insert in B. The irregular sized somite is markerd with an arrow. C-D, *in-situ* analysis of expression of the A-P marker *mesp* in red and the cyclic PSM prepatterning gene *her7* in blue. *Her7* transcripts are down-regulated at the level of *mesp* in wt (C), while *her7* RNA passes the *mesp* domain and sweeps into the formed somites of *dpk* mutants (D). Dorsals view of medaka embryos at the 12 somite stage at 23 hpf (from Elmasri et al., 2004a).

In order to investigate this in detail we analyzed this stabilization defect in *dpk* mutant also for other *her* members expressed in the PSM that belong to the clock (Elmasri in preparation; Elmasri et al., 2004a). For this we used the two newly isolated oscillating *her* genes *her1* and *her5* (own publication number 9). At one phase during the oscillation cycle, when the RNA was reaching the most anterior PSM region. We noticed a broad and diffuse domain for both genes in the *dpk* mutants. In this phase, the level of disturbance differs among the different *her* members. In case of *her1,* a slight broadening of its expression was observed in *dpk*. Furthermore, we observed a mild delay in the wave of RNA sweeping toward anterior on one side of the somitic columns. Thus, the bilateral symmetry of this cyclic gene was affected. Similar to the situation for *her1,* an obviously broader domain of *her5* transcripts was noticed in the anterior PSM of *dpk* embryos. In this case, also a delay in the wave traveling rostrally was observed on one side of the embryo. Interestingly, the disturbances observed for *her1* and *her5* are different from those previously discussed for *her7*. Nevertheless, a stabilization defect seems to be present for all three analyzed *her* genes in *dpk* mutants. However, the mode of oscillation remains normal for all three analyzed *her* genes in the *dpk* mutants (Elmasri in preparation; Elmasri et al., 2004a). In zebrafish, the stabilization of *her1* and *her7* in the most anterior PSM region is essential for the establishment of the following somite boundaries (Holley et al., 2002; Oates and Ho, 2002). This stabilization process seems to be

regulated through several signals belonging to the wave front activity in zebrafish. This wave front is positioned in the anterior PSM and acts as an antagonist to the oscillating clock genes traveling towards anterior (Nikaido et al., 2002). The observed continuous and normal oscillation of cyclic *her* genes in medaka *dpk* is totally different to the reported "salt and pepper" pattern of *her1* and *her7* genes in Notch/Delta deficient zebrafish mutants (Holley et al., 2002; Oates and Ho, 2002). Therefore, it excludes the possibility that *dpk* is a Notch/Delta deficient medaka mutant. Altogether, this suggests that *dpk* is a likely candidate for a mutant with deficient wave front, which is necessary to down-regulate oscillating genes in the anterior PSM. Alternatively, it could also regulate segmental determination factors to establish the position of the newly forming somitomeres. However, the *dpk* mutant must be different from *fss/tbx24* (the only wave front mutant isolated in zebrafish), because the A-P marker *mesp* was not repressed in *dpk,* in contrast to the ortholog *mespb* in zebrafish *fss* mutants (Nikaido et al., 2002). In *dpk*, *mesp* rather shows a diffuse and broadened pattern of expression. A further alternative is that *dpk* could affect a component of a negative feedback loop in the clock that is responsible for the down-regulation of *her7* in the anterior PSM region (Elmasri et al., 2004a). In zebrafish it was shown that *her7* acts as an interstripe repressor on *her1*, but as an activator on itself in the generated oscillatory circuits (Holley et al., 2002; Oates and Ho, 2002; Gajewski et al., 2003). Finally, the mutated gene could also belong to the Retinoic acid (RA) signaling pathway or to a component that interacts with RA signaling. This is suggested because the delay in the bilateral symmetry of all used cyclic genes in the PSM of *dpk* embryos is similar to the situation in RA deficient embryos (Kawakami et al., 2005; Vermot and Pourquie, 2005). Work in mouse, zebrafish and chicken showed that RA signaling promotes the synchronization of the molecular clock between the left and right PSM in the tailbud. Therefore, they confirmed the importance of RA in modulating the bilateral symmetric elongation during somitogenesis (Kawakami et al., 2005; Vermot and Pourquie, 2005).

### 2.2.3 Class II: Medaka somite mutants with defects in A-P polarity and somite boundary formation

In medaka mutants belonging to class II PSM prepatterning is unaffected (Table 3). Thus, *her7* and *mesp* transcripts are normally expressed in the PSM and the dynamic expression pattern of *her7* is not disturbed. However, class II mutants exhibit deficiencies in genes like *myf5* and *lfng* that are expressed later in the anterior respectively posterior areas of

epithelialized somites. In the *kurzer* (*krz*) mutant embryos, irregular somite boundaries and variable somite sizes were observed. Furthermore, also development of the head region was delayed and a slight shortening in the A-P length of the body axis was detected. *Myf5* transcripts were not restricted to the posterior halves of epithelialized somites. Instead *myf5* RNA was detected in a discontinuous and non-segmental manner throughout the formed somites. In addition, *lfng* expression was reduced in the anterior halves of formed somites. This suggests that a posteriorization in the identity of the somitomeres could have occurred in *krz* embryos. We also analyzed transcription of the newly isolated *her1* gene in *krz* mutants. Surprisingly and differently to the unaffected behavior and dynamics of *her7* expression in these mutants, *her1* RNA distribution was disturbed and the transcripts were often compressed into the anterior PSM. However, the dynamic behavior of *her1* was unaffected in *krz* embryos. Thus, regulation of *her1* was slightly affected in *krz,* whereas regulation of *her7* expression and dynamic behavior was completely intact. This proposes differences in the regulation of *her1* and *her7* cyclic genes in *krz* mutants (own publication number 9).

**Table 3: Class II mutations affecting A-P-polarity and epithelialization**

| Mutant | Somitic Phenotypes | Head Phenotypes |
|---|---|---|
| ***kurzer (krz)*** | **variable somite shapes and sizes** | **arrested eye and forebrain morphogenesis** |
| ***orgelpfeifen (opf)*** | **mild morphological phenotype with slightly irregular somite shapes and variable mediolateral extension** | **arrested eye and forebrain morphogenesis** |
| ***fussel (fsl)*** | **individually fused somites, variable somite sizes** | **necrotic** |
| ***zahnluecke (zlk)*** | **irregular sizes and shapes of anterior somites, posterior somites partially missing** | **arrested eye and forebrain morphogenesis** |

On the ther hand in the *orgelpfeifen* (*opf*) mutants, we detected a mild reduction in the size of somites and slightly irregular orientation of somite boundaries in the formed somites. Intriguingly, expression of *myf5* was strongly down-regulated in the *opf* mutants, while *lfng* transcripts were expanded along the anterior-posterior axis. This indicates that in this mutant an anteriorization in the identity of the somitomeres might have occurred. In *fussel* (*fsl*) mutants, we noticed that some somites are enlarged and that individual somites are fused. In this mutant, expression of *myf5* was expanded into the anterior domain of the epithelialized

somites. At the same time, a strong reduction of *lfng* transcription was observed. In contrast to the *opf* mutant, it seems that a posteriorization in the identity of somitomeres has probably occurred in this mutant. However, in the *zahnluecke* (*zlk*) mutants, the first few somites show irregular boundaries and in more posterior regions, somite boundary formation is impaired. Transcription of *myf5* is slightly down-regulated in the posterior somites. We also examined expression of *her1* gene in the *zlk* mutant and found that transcription was unaffected in *zlk* embryos. Also oscillation was normal and similar to *her7* as previously described (own publication number 9). In *zlk*, these results suggest a defect in later steps during somitogenesis, probably in the phase of somite boundary formation, independent from PSM prepatterning and establishment of A-P polarity. The A-P polarity found in the somites is already determined in the cells located in the most anterior PSM. This was supported by transplantation experiments performed in chicken (Aoyama and Asamoto, 1988). In mouse, molecular analyses showed that a complex interplay of different feedback loops in the anterior PSM causes the restriction of transcripts found later in the anterior, respectively posterior part of the formed somite (Takahashi et al., 2003). In these loops, the most important players are the Delta ligands, *DII1* and *DII3* which show non-overlapping expression and non redundant function in the anterior PSM (Takahashi et al., 2003), and *Mesp*, which modulates Notch signaling pathway (Takahashi et al., 2005). Further genes required in this complex network in the anterior PSM are not yet identified. In zebrafish, despite the fact that several known zebrafish somite mutants (*aei*, *des*, *wit*, *bea*, and *fss*) have defects in the formation of somite boundaries (van Eeden et al., 1996). These mutants show alterations in the cyclic behavior of oscillating genes. Moreover, in these mutants also expression of A-P polarity genes is de-regulated. These A-P defects were argued to be secondary defects, because all mutated genes play a role in PSM prepatterning or in establishing the wave front (Holley et al., 2000, 2002; Nikaido et al., 2002). Thus, separating PSM prepatterning from the A-P polarity step using zebrafish seems to be impossible. Therefore, the described zebrafish somite mutants so far are not suitable to study regulation of A-P polarity and the signals required during this step of somite formation. In contrast, members of the class II medaka somite mutants possess normal *her7* oscillating expression in the PSM, whereas defects are observed in the specification of anterior-posterior identity. This is evident by disturbed *mesp* expression in the anterior PSM or later defects in *lfng* and *myf5* expression (Elmasri et al., 2004a). Therefore, class II medaka somite mutants affect targets involved in the establishment of A-P polarity, boundary formation, epithelialization or later in the differentiation of somites. Alternatively, these mutants could disturb genes that translate oscillation output information into the A-P identity

of cells in the anterior PSM. This class of medaka mutants seems to open new perspectives to analyze A-P polarity regulation, determination and boundary formation in the presence of a normal functioning clock in the PSM. Intriguingly, in these medaka class II mutants we were successful in finding mutants with either anterior or others with posterior defects (Elmasri et al., 2004a). It is important to notice that the mutants showing different deficiencies in A-P polarity were unable to be distinguished on a morphological basis. However, these mutants were distinguished by molecular analyses using specifc anterior (e.g. *lfng*) or posterior (e.g. *myf5*) markers. Therefore, medaka class II somite mutant seems to be a novel group of mutants with specific A-P polarity deficiencies in the somitomeres. Such a group of mutants has not been isolated so far in zebrafish, mice or chicken. Therefore, the identification of the encoding genes for these new mutants with A-P defects will help to understand the molecular interactions of different signals involved in establishment of A-P polarity (e.g. the example the *mesp* genes) that occur in the somitomeres in the anterior PSM region. Furthermore, identifying these mutated genes could result in the isolation of novel components involved in A-P specification of the forming somites. It could even elucidate the interaction of these networks with early steps during somite formation, such as PSM prepatterning or later during boundary formation after the specification of PSM cell identities.

### 2.2.4 Phenotypic differences and similarities between medaka and other vertebrate somite mutants

On the morphological level, there are several similarities, but also important differences between distinct zebrafish and medaka somite mutants. For example, all *fss*-type zebrafish mutants, except *mind bomb* (*mib)* (Itoh et al., 2003), a *her1*/*her7* deficient mutant (Henry et al., 2002) and *before eight (bfe)* (Julich et al., 2005), are homozygous viable. They restore their early embryonic defects and develop into mature and fertile adults. In contrast in medaka, all isolated somite mutants are embryonic lethal. Homozygous mutant embryos die during late stages of somite formation, before reaching the hatching stage. One exception is *schnelles ende* (*sne)*, which fails to develop posterior somites and survives for four days after hatching. The reason for this early lethality in medaka somite mutants seems not to be a direct consequence of the somite defects that are characterized by fusion of somites or irregularly formed somites. Rather, the cause for the early death probably comes from other defects seen in these mutants, especially during head development. This hypothesis was supported by an apoptosis test. This showed that for one of the severely affected somite mutants *planlos* (*pll*),

an obvious increase in the number of apoptotic cells was present in the head region, whereas no significant change was detected in the PSM and trunk region (Elmasri et al., 2004a). Furthermore, another morphological difference between zebrafish and medaka somite mutants is that, in addition to the somite defects, no other obvious deformations were observed in zebrafish mutants. In contrast, all of the described somite medaka mutant exhibit necrosis and retarded development of structures in the head or arrest in eye and forebrain morphogenesis, except *schnelles ende* (*sne)*, which seems to develop a normal head. Early lethality and additional defects in the head and other tissues beside somites were also reported in mouse somite mutants. In mouse, the knockout mutants for Notch/Delta signaling members including *Delta1*, *Lfng* and *Hes7* die shortly before or after birth and show severe defects in somites and other organs during early development, including in the nervous system (Hrabe de Angelis et al., 1997; Evrard et al., 1998; Bessho et al 2001). Furthermore, some medaka mutants with individual fused somites and irregular somite boundaries are almost similar to mouse segmentation mutants, in which somite fusion was reported, like in knockout mice with defects for *Hes7* (Bessho et al., 2001). This suggests that on the morphological level, somite formation in medaka seems to be evolutionarily much more closer to higher vertebrates, like mouse than to other teleost fish, e.g. zebrafish. Most importantly, some medaka mutants showed novel phenotypes that have never been observed before in either mouse or zebrafish somite mutants. On the other hand, several class I mutants like *schnelles ende* (*sne*), *planlos* (*pll*) and *samidare* (*sam*) show high similarities to previously described zebrafish somitogenesis mutants. They only form anterior somitic boundaries, a general characteristic for the zebrafish Notch/Delta mutants *after eight* (*aei*) (Holley et al., 2000), *beamter* (*bea*) (van Eeden et al., 1996) and *deadly seven* (*des*) (Holley et al., 2002). However, further analyses on *sam* mutant embryos showed novel differences. Morphologically, medaka *sam* mutants are similar to the zebrafish *after eight* (*aei*) mutant carrying a mutation in the *deltaD* gene (Holley et al., 2000). In both cases, the first 7-9 somites are formed normally, but after that somite formation is impaired. On the molecular level, *her7* shows a randomized "salt and pepper" expression pattern in *sam* mutant, which is a general characteristic of the Notch/Delta signaling mutants in zebrafish (Holley et al., 2000, 2002; Oates and Ho, 2002). However, different to the situation in *aei, sam* mutant embryos have an additional defect in the formation of the mid-hindbrain boundary (MHB) region. A similar defect in the MHB region was found in the zebrafish FGF8 mutant *acerebellar* (*ace*) (Reifers et al., 1998). Intriguingly, in *ace* zebrafish mutants only very mild defects were observed in the somites. This was explained by the presence of functional redundancy among different FGF ligands acting in the

tailbud of zebrafish. This hypothesis was supported through the simultaneous knockdown of both *fgf8* and *fgf24* in zebrafish, which resulted in severe somitic defects (Draper et al., 2003). Thus, it is interesting to suggest that the *sam* mutant, based on the parallel defects in somites and MHB, is potentially deficient in the FGF signaling pathway. The FGF pathway was shown to be involved in MHB formation (Carl and Wittbrodt, 1999) and in the regulation of somite formation (Elmasri in preparation) in medaka embryos. As expression of *her7,* a downstream target of the Notch/Delta pathway is affected in *sam* mutants, we speculate that the deficient factor must be located in or upstream of the Notch/Delta circuit. Therefore, the ongoing work to identify the affected gene in *sam* will possibly yield an interaction between the Notch/Delta pathway and FGF signaling in the PSM of medaka.

### 2.2.5 Molecular differences and similarities between somitogenesis marker genes in medaka and other vertebrates

On the molecular level, many similarities can be observed in the expression of somite marker genes between medaka and zebrafish. One example is *lunatic fringe* (*lfng*), which is characterized as an oscillating gene in the PSM of mice and chicken (Forsberg et al., 1998; McGrew et al., 1998). This is in contrast to the situation in zebrafish and medaka, where the cyclic expression behavior of *lunatic fringe* (*lfng*) in the PSM region is lost. Instead, it is expressed in the anterior PSM, the anterior somites half, as well as in the neural tube (Leve et al., 2001; Prince et al., 2001; Elmasri et al., 2004). Furthermore, another example is the *hairy/Enhancer-of-split*- related gene *c-hairy2* in chicken (Palmeirim et al., 1997) and *c-hey2* in mice, which show cyclic PSM expression (Leimeister et al., 2000b). In contrast, it is the paralog *hey1* that has a dynamic mode of expression in the PSM in zebrafish (own publication number 2), as well as in medaka (Elmasri et al., 2004). However, there are also differences between the two fish species. *Her1*, a *hairy-related* gene, which is considered a central component of the segmentation clock, shows cyclic transcription in the PSM during somite formation in zebrafish (Holley et al., 2000). Its transcripts are found in the posterior part of the PSM and subsequently a wave of transcription sweeps towards the anterior. By the end of this cycle, two stripes have detached and remain in the anterior PSM region (Holley et al., 2000). The situation of two separated stripes in zebrafish differs significantly to the situation in medaka. For medaka *her1*, one U-shape domain remains in the posterior PSM and one stripe travels to the most anterior PSM region. Furthermore, additional expression of the medaka *her1* ortholog was found in the mid-hindbrain boundary (MHB) region (own

publication number 9). On the other hand, the *hairy-related* gene *her5* reveals even more obvious differences between zebrafish and medaka. This gene is exclusively expressed in the MHB region in zebrafish and plays a role as a prepatterning factor in the spatial definition of proneural domains in the neural plate (Mueller et al, 1996; Geling et al., 2004). Intriguingly, the *her5* ortholog in medaka is also expressed in the MHB region similar to zebrafish, but additional expression was detected in the tailbud. *Her5* expression in the PSM region is oscillating like for medaka *her1* (own publication number 9) and *her7* (Elmasri et al., 2004). Therefore, *her5* in medaka seems to have evolved a new function in the segmentation clock, which appears different to the *her5* ortholog in zebrafish (own publication number 9). *Her7*, shows oscillating expression in the tailbud region in zebrafish and has a central function during somite formation (Oates and Ho, 2002; Gajewski et al., 2003). During one somite formation cycle, expression is first detected in the posterior PSM, and then gradually travels anteriorly. Similar to *her1*, two discrete stripes are found in the anterior PSM at the end of this process. At this phase, transcription of *her7* is completely down-regulated in the most caudal PSM region as the expression extends rostrally (Oates and Ho, 2002). In contrast to this in medaka, the posterior expression domain of *her7* remains stable and only one stripe of *her7* transcripts buds off from this posterior domain and sweeps anteriorly (Elmasri et al., 2004). Nevertheless, interesting similarities in expression patterns were found when the different cyclic medaka *her* genes were compared with the corresponding oscillating genes in higher vertebrates, like the *hairy* clock genes in chicken and mouse. Thus, both medaka oscillating *her* genes and higher vertebrates *hairy* clock genes share the common feature of having a single stripe of transcripts that detaches from the posterior expression domain and sweeps anteriorly (Jouve et al., 2000; Bessho et al., 2001a; own publication number 9). This proposes that the mechanism regulating the clock seems to be more in common in medaka and higher vertebrates than to zebrafish. Thus this suggests that the regulation of cyclic genes in zebrafish has changed during evolution differently than in other vertebrates. Alternatively, the somitogenesis clock in zebrafish is the ancestral form of regulation in vertebrates, with the patterns seen in medaka and higher vertebrates being more derived (own publication number 9). The differences observed between *her* genes in medaka and zebrafish, especially the lack of the intermediate stripe in medaka, indicate differences in the regulation elements that control the dynamic expression of cyclic genes. Alternatively, also more general deviations might have occurred in the core mechanism controlling the clockwork in teleost fish. From an evolutionary point of view, the additional intermediate stripe in zebrafish could reflect an ancestral character in teleost fish that was lost specifically in medaka and pufferfish, but not

in zebrafish (own publication number 9). As previously mentioned, a gradient of FGF signaling regulates the maturation of cells in the PSM region of teleosts fish and higher vertebrates (Dubrulle et al., 2001; Sawada et al., 2001; Elmasri in preparation). The lack of the intermediate stripe could be explained by a steeper gradient of FGF activity in the PSM region of medaka, while in zebrafish a shallower gradient causes the presence of two maturation zones. On the other hand, the additional expression of medaka *her1* and *her5* in the medaka embryos can be explained by a decrease in the number of *her* genes found in medaka in comparison to other teleost fish. *Her* genes seem to be an example for a gene family that shows complex scenarios of gene duplication. Teleost fish have several duplicated copies of genes, where higher vertebrates have only one unique ortholog. This is due to a whole genome duplication that occurred shortly after the separation of teleosts from the tetrapod lineage. In the pufferfish *Takifugu*, 21 *her* genes were found in the genome (Gajewski and Voolstra, 2002). On the other hand in zebrafish, at least 23 *her* family members were identified until now (Sieger et al., 2003). In medaka, 10 *her* genes were recently isolated and described (own publication number 9). It seems that the copy number of genes in the analyzed *Hes* homologue groups in medaka and pufferfish is smaller than in zebrafish (own publication number 9). This suggests that for *her* genes a strong conservation appears between medaka and pufferfish during evolution, but obviously this conservation is less strong in the zebrafish (own publication number 9). The decrease in the number of *her* genes in medaka suggests that single members in medaka have take over more than one function to perform the same roles acquired by additional *her* genes in zebrafish. To support this hypothesis, we analyzed the expression pattern of the three cyclic *her* genes in medaka, *her1*, *her5* and *her7,* that all belong to the mouse *Hes7* homologue group (Sieger et al., 2004). There are four general expression domains for *her* genes in teleost fish: the mid-hindbrain boundary domain, the somites, the anterior PSM and the posterior PSM. One scenario to explain the deviation in the expression patterns among this subclass is that *hes7* in mouse is considered to reflect the ancestor situation (Sieger et al., 2004). *Hes7* expression in mouse was found to be restricted to the tailbud with an oscillating pattern (Bessho et al., 2001). Based on this scenario, all other expression domains were added secondarily and then later lost differentially for *her1*, *her5,* and *her7* in medaka and zebrafish. For *her1,* only the MHB expression domain was additionally lost in zebrafish in comparision to medaka (Holley et al., 2000; own publication number 9). On the other hand for *her5*, two expression domains, the anterior and posterior PSM region were further lost in zebrafish than in medaka (Müller et al., 1996; own publication number 9). Finally for *her7* in zebrafish as well as in medaka, the same

two domains, the expression in the MHB region and in the somites disappeared (Oates and Ho, 2002; Elmasri et al., 2004). This demonstrates that the regions of expression are differentially lost in zebrafish *her1*, in which the MHB domain is absent, and *her5,* in which the expression in the anterior and posterior PSM region were lost. Therefore, these results suggest an additional function in medaka for *her1* in the MHB region and for *her5* in the PSM region.

# 3. The role of FGF signaling during medaka somitogenesis

## *3.1 Expression of FGF receptors and ligands*

In the medaka screen, a broad spectrum of several novel somitic phenotypes was identified, like irregular sizes and fused or enlarged somites. We therefore estimated that some of these new phenotypes are caused by mutations in new signaling components, e.g. in the Wnt or FGF pathways. Corresponding defects were not described in any zebrafish somite mutant so far. In this study, we concentrated on the FGF cascade as previous work in chicken and zebrafish have shown the importance of this pathway in the establishment of the determination front in the PSM during somite formation (Dubrulle et al., 2001; Sawada et al., 2001). To search for differences in FGF signaling that might have occurred in different vertebrates during evolution, we investigated the expression of different components of this pathway in medaka starting with the FGF receptors. Expression of the receptor *fgfr1* was detected in the PSM and in the anterior part of formed somites in mice and zebrafish (Yamaguchi et al., 1992; Sawada et al., 2000). In addition, *fgfr1* RNA was also found in the adaxial mesoderm, otic vesicles, branchial arches and specifically in the MHB region. Knockdown experiments in zebrafish showed a similar phenotype for *fgfr1* as reported for the ligand *acerebellar* (*ace*)/*fgf8* (Reifers et al., 1998; Scholpp et al., 2004). In contrast, in *fgfr1* knockout mice somite formation was impaired (Yamaguchi et al., 1994). Therefore, *fgfr1* shows differences between mouse and zebrafish similar to that described for *fgf8* (Scholpp et al., 2004). On the other hand in medaka, we confirmed by *in-situ* expression pattern analyses that *fgfr1* transcripts are only detected in the tailbud region, somites and notochord, but not in the MHB (Carl and Wittbrodt, 1999; Elmasri in preparation). We showed that *fgfr1* transcripts in the tailbud are not equally distributed. A high level of RNA can be detected in the anterior PSM and in the last somites, while a weak expression of *fgfr1* was observed in the

intermediate and posterior PSM region (Elmasri in preparation). The difference in the expression patterns of *fgfr1* between medaka and mouse in comparison to zebrafish is suggestive of differences in the functions of *Fgfr1* among these vertebrates. Thus, in medaka the role of *fgfr1* seems to be restricted to tailbud and somite formation. In contrast to this, in zebrafish and mouse *fgfr1* was shown to be involved in MHB boundary formation (Trokovic et al., 2003; Scholpp et al., 2004). In line thes findings, we also investigated the expression of two other FGF receptors, *fgfr3* and *fgfr4*. For both receptors, transcripts were observed in the head region in the posterior forebrain and anterior midbrain. Moreover, we confirmed previous results and observed additional expression of *fgfr3* and *fgfr4* in the anterior somites. *Fgfr3* was detected in the first 3-4 anterior somites. In these anterior somites, *fgfr3* showed an equal distribution of its RNA. In contrast, for *fgfr4* we detected a stripe of transcripts adjacent to the neural tube along the first 3-4 somites (Carl and Wittbrodt, 1999; Elmasri in preparation). These results showed that *fgfr1* is the only receptor expressed in the PSM and therefore important during somite formation. Next, we concentrated our interests on the isolation of new FGF pathway components in medaka. By data bank searches, we identified another FGF ligand in medaka, *fgf3*. In zebrafish *limbabsent* (*lia)*/*fgf3* mutants, it was shown that *fgf3* primarily promotes the transcriptional activation of genes that regulate early specification of adenohypophyseal progenitor cells, but these mutants show no somitic defects (Herzog et al., 2004). Furthermore, an additional function for *fgf3* in combination with *fgf8* was postulated as signaling center required for the initiation of retina differentiation in chicken and zebrafish (Martinez-Morales et al. 2005). In contrast to zebrafish, mutational analyses of *fgf3* in mice showed defects in morphogenesis and differentiation of the inner ear and importantly also in somites (Mansour et al., 1993). Our work showed that *fgf3* transcripts in medaka are detected in the tailbud region, the adaxial mesoderm of the latest formed somites, in heart, eyes, rhombomere five and strongly in the MHB region. Based on this, we suggest that *fgf3* plays a role during the establishment of the MHB and is involved in tailbud formation and somitogenesis in medaka embryos (Elmasri in preparation). On the other hand, *fgf8* is another FGF ligand that we have analyzed in medaka. We have shown a similar expression pattern for *fgf8* in medaka tailbud as previously detected in chicken and zebrafish (Dubrulle et al., 2001; Sawada et al., 2001). *Fgf8* transcripts were observed in the MHB region (Ristoratore et al., 1999; Elmasri in preparation), as well as in a gradient manner in the tailbud, with the highest level in the posterior PSM. This indicates that the *fgf8* expression pattern is conserved throughout evolution in vertebrates. Moreover, this suggests a conserved role of the FGF pathway during somitogenesis mediated through *fgf8* (Elmasri in preparation).

## *3.2 SU5402 inhibiter experiments show the importance of FGF signaling for controlling somite size*

Similar to the situation in chicken and zebrafish, treating medaka embryos with the FGF signaling inhibitor SU5402 causes an increase in the size of somites (Elmasri in preparation). This demonstrates a similar function for the FGF pathway during somitogenesis in medaka as in other vertebrates (Dubrulle et al., 2001; Sawada et al., 2001). Moreover, the enlarged somites after SU5402 treatment were similar to the phenotype obtained in the *doppelkorn* (*dpk*) somite mutant, as described above (Elmasri et al., 2004a). In zebrafish, it was shown that the activity of the doubly phosphorylated ERK (dpERK), a general downstream target of activated receptor tyrosine kinase (RTK), including FGF receptors, was highly upregulated in the tailbud at the same positions where *fgf8* transcripts are found (Sawada et al., 2001). Similar as in zebrafish, phosphorylated MAPK in medaka was also localized in the tailbud with the highest level in the posterior PSM (Fig. 6). The similarities of *fgf8* transcript position and its gradient pattern on the one hand, and phosphorylated MAPK location on the other, is another indication for the conservation of the FGF pathway function in teleost fish. Despite these similarities between medaka and zebrafish, also species-specific deviations can be observed. One difference between both fish species was the obviously lower level of phosphorylated MAPK in medaka. Another difference was noticed with respect to *fgf8* transcripts. In zebrafish, broad *fgf8* expression was demonstrated to correlate with phosphorylated MAPK strong level in the PSM. In contrast, in medaka, *fgf8* expression was observed only in a small subregion of the elevated MAPK domain in the PSM (Elmasri in preparation).

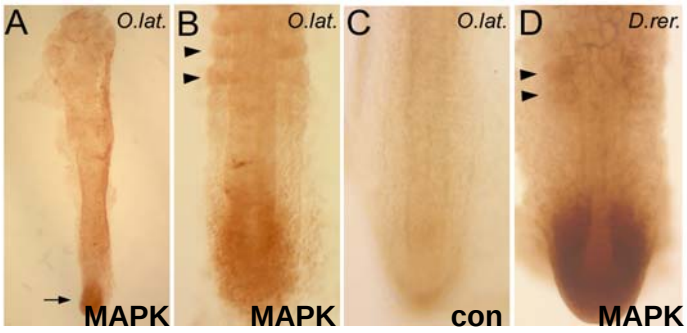


**Fig. 6.** Localization of MAPK/ERK1/2 in medaka (A-C) and zebrafish (D), using immunostaining. Dorsal views of medaka embryo (A) showing elevated levels of phosphorylated MAPK in the tailbud. B is a magnification of A. C, medaka control embryo after immunostaining in the absence of the first antibody. D, zebrafish embryo with strongly elevated levels of phosphorylated MAPK in the tailbud. The medaka embryos are at 23 hpf and exhibit 12 somites, and the zebrafish embryo is at 14 hpf with 14 somites (picture from Elmasri, in preparation).

## 3.3 Functional analyses for FGF8 using morpholino and overexpression approaches

To analyze the role of *fgf8* in medaka embryos, we blocked FGF8 protein production using a morpholino antisense approach. Interestingly, the *fgf8* knockdown experiment revealed severe defects during the formation of tailbud and somites after the first 3-4 somites (Fig. 7). The obtained phenotypes in the FGF8 morpholino injected embryos resembled the phenotypes of the *schnelles ende* (*sne*), as described above (Elmasri et al., 2004a). This suggests a function of *fgf8* during somitogenesis and tailbud formation in medaka. A different situation was observed in the *acerebellar* (ace)/*fgf8* zebrafish mutant, in which only weak defects during somite formation were reported (Reifers et al., 1998). The simultaneous knockdown of both *fgf8* and *fgf24* on the other hand showed severe somitic and tailbud defects in zebrafish (Draper et al., 2003). This confirmed the presence of functional redundancy between *fgf8* and *fgf24* in the zebrafish tailbud. Thus, this explains the lack of FGF pathway somite mutants in the zebrafish mutagenesis screens as other closely related genes can compensate any FGF deficient defects. The *fgf8* gene knockdown results in medaka indicate that *fgf8* apparently does not have a closely related paralog in medaka that might take over its function during somitogenesis. From this, we conclude that different gene duplicates of FGF signaling components are present in medaka when compared to zebrafish. Therefore, it seems highly possible to isolate an *fgf8* mutant with somite defects from the medaka mutagenesis screen.

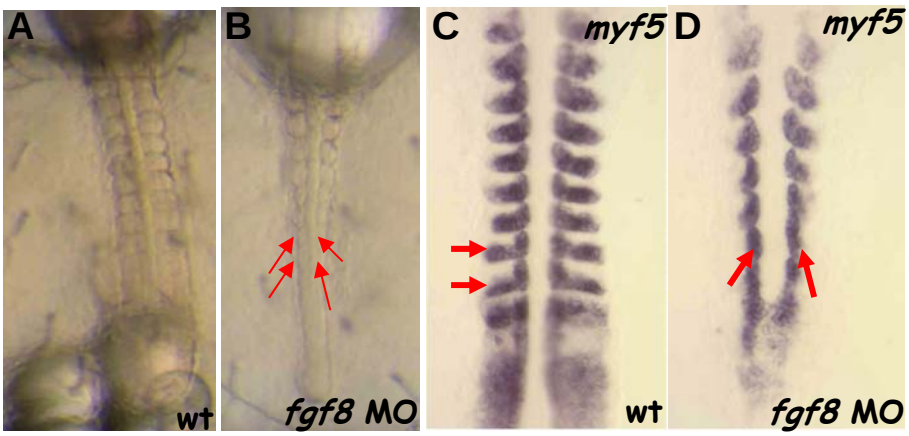


**Fig. 7.** Severe somite formation and tailbud defects in medaka embryos after injection of *fgf8* Morpholinos (MO). Dorsal views of medaka embryos with 10 somites at 22 hpf. A-B, live pictures of medaka wildtype (wt) embryo that shows normal tailbud and somite formation (A) and *fgf8* MO injected embryos that show severe defects during formation of tailbud and somites after the first 3-4 somites (B). C-D, *in-situ* anaylsis. C, wt embryo that shows *myf5* myogenic marker expression in the adaxial mesoderm and posterior halves of formed somites, D, MO injected embryo with highly reducted *myf5* expression in the posterior somites (picture from Elmasri, in preparation).

## *3.4 Medaka candidates for FGF signaling pathway mutants*

### 3.4.1 *Doppelkorn* (*Dpk)* as a potential candidate for an FGF pathway mutant

FGF is the main signal known until now in zebrafish and medaka that establishes the determination front and thus the size of the formed somites (Sawada et al., 2001; Elmasri in preparation). Phenotypically, as mentioned before, the *dpk* mutant shows individually fused somites or somites with increased size. This feature of the *dpk* mutants gave us the first hint to assume that in *dpk* embryos FGF signaling is affected. Based on this, we examined the expression of different FGF signaling components in *dpk* embryos. We started with the receptor *Fgfr1* that is the only FGF receptor expressed in the tailbud in medaka (Elmasri in preparation). In *dpk* mutants, *Fgfr1* transcription was significantly down-regulated in the formed somites. In addition to this, only low levels of *fgfr1* RNA were detectable in the anterior PSM. This finding indicates that the mutation in *dpk* might be located in the promoter region of the *fgfr1* and thus regulates its expression (Elmasri in preparation). Following this, we analyzed phosphorylated MAPK activity in *dpk* mutants to search for potential differences to wildtype embryos. Intriguingly, MAPK activity was completely absent in the tailbud of *dpk* embryos. Furthermore, we detected ectopically elevated levels of MAPK activity in the axial mesoderm. In contrast to this, phosphorylated MAPK activity remains constant in the tailbud of zebrafish *after eight* (*aei)* and *fused somite (fss)* mutants (Sawada et al., 2001). This indicates that the *dpk* mutant potentially represents the first somitogenesis mutant in teleost fish that shows defects in FGF signaling. Therefore, this makes *dpk* a good candidate to analyze regulation of phosphorylated MAPK activity in the tailbud and its exact function during somite formation. Finally, we investigated the expression of the two ligands *fgf3* and *fgf8* in *dpk* mutants. Interestingly, in *dpk* mutants, *fgf3* and *fgf8* transcript levels appeared normal and the gradient expression of *fgf8* in the tailbud seemed not to be affected. This further indicates that the *dpk* mutation affects a gene regulating *fgfr1* expression (Elmasri in preparation). Altogether, this suggests that *dpk* is a likely candidate for a mutant directly involved in FGF signaling. If this is the case, then *dpk* would be the first FGF signaling mutant with somitic defects in vertebrates.

### 3.4.2 *Schnelles ende* (*sne)* as a potential candidate mutant in the *fgfr1* gene

In the *fgf8* knockdown experiment, we observed a block of somite formation after the first 3-4 anterior somites were formed. This phenotype very much resembles those observed in the medaka *schnelles ende* (*sne*) mutant. *Sne* is a member belonging to the medaka class I somite mutants, in which tailbud and somite formation following the first 3-4 somites is impaired. The similarity in the phenotype between *fgf8* knockdown embryos and *sne* mutants gave us the first hint that probably a mutant FGF signaling component might be affected in *sne*. Interestingly, *sne* maps to a region of medaka linkage group (LG) 9 that is syntenic to a region of zebrafish LG 8 (Table 4). In zebrafish, *fgfr1* maps to this region in LG 8, thus opening the possibility that *sne* encodes the medaka FGFR1 receptor. To confirm this hypothesis, we are currently mapping medaka *fgfr1* on its LG using fluorescence *in situ* hybridization (FISH) method on chromosomes (Nanda et al., 2002). Finally, we have sequenced the *fgfr1* gene in the three available mutant alleles of *sne*, and we are currently searching for mutations in these alleles.

# 4. Zusammenfassung

Die Somitogenese stellt einen entscheidenden Prozess bei der Entwicklung von Wirbeltierembryonen dar. Somiten sind transiente Strukturen, die sich im Verlauf der Embryonalentwicklung zu Skelettmuskulatur, Dermis und Wirbelkörper differenzieren. Somiten entstehen in einem sich regelmäßig wiederholenden Zyklus aus Stammzellen des präsomitischen Mesoderms (PSM), einer Wachstumszone am caudalen Ende des Embryos. Ein wichtiger Bestandteil der Somitogenese ist ein molekularer Oszillator, das so genannte „Segmentierungs-Uhrwerk". Die periodische Segmentierung des präsomitischen Mesoderms wird reguliert durch eine Reihe komplexer Interaktionen von unterschiedlichen Signale wegen. Der Notch/Delta Signalweg spielt dabei eine zentrale Bedeutung, da hierbei Komponenten entdeckt wurden, die während der Somitogenese zyklisch im PSM exprimiert werden. Außer dem Notch/Delta Signalweg spielen auch ein FGF und Retinolsäure Gradient, sowie Wnt Signale eine wichtige Rolle bei der Somitogenese. Trotz mehrerer Mutagenese Screens im Zebrafisch wurden bislang keine Mutanten im FGF oder Wnt Signalweg entdeckt, die einen spezifischen Somiten Defekt besitzen. Die wurde durch eine funktionelle Redundanz unterschiedlicher Gene erklärt, die durch eine Duplikation im Genom von Teleostieren vor 350 Millionen Jahren enstanden ist. Da unterschiedliche Duplikate in verschiedenen Fischspezies existieren, wurde in den letzten Jahren ein grosser Mutagenese Screen bei Medaka (*Oryzias latipes*) in Kyoto, Japan durchgeführt. In meiner Arbeit habe ich neue Somitogenese Mutanten aus dieser Screen isoliert und Phänotypisch charakterisiert. Die neun isolierten Mutanten zeigten unterschiedliche Somiten Phänotypen. Einige Mutanten hatten wenige oder gar keine Somitengrenzen (z.B *bms* oder *sne*), andere hatten unregelmäßige Somiten Formen (z.B. *krz* oder *fsl*) oder unterschiedlich große Somiten (z.B *dpk*). Manche dieser Medaka wiesen Änlichkeiten Mutanten zu im Zebrafisch beschriebenen Somiten Mutanten auf. Die Mehrzahl der Mutanten zeigten jedoch Phänotypen, die bis jetzt noch nicht in Zebrafisch Screens gefunden worden. *In-Situ* Analysen mit Hilfe unterschiedlicher, neu isolierter Somitenmarker, wie z.B. *her7* einem Bestandteil des molekularen Oszillators, *mesp* einem anterior-posterioren Gen oder den Somitendifferenzierungsgenen *lfng* oder *myf5* erlaubten, die Medaka Mutanten in zwei unterschiedliche Gruppen zuzuordnen. Gruppe I zeigt Defekte in der Bildung der Schwanzknospe und der Musterbildung im PSM. Ein besonderes Beispiel dieser Gruppe ist die Mutante *doppelkorn* (*dpk*), die einen bislang nicht beschriebenen Somitenphänotyp besitzt. *In-situ* Analysen von *dpk* zeigten, dass zyklische Gene im anterioren PSM dieser Mutante nicht stabilisiert werden und auch A-P Polaritätsgene fehlerhaft reguliert werden.

Das deutet darauf hin, dass in der *dpk* Mutante ein Faktor der sogenannten „Wavefront“ betroffen sein könnte, der wichtig ist für die Regulation von oszillierenden Genen im anterioren PSM ist. Ich konnte zeigen, daß der wichtige Notch/Delta Signalweg in dieser Mutante nicht betroffen ist, weil alle unterschiedlichen zyklischen Gene, *her1*, *her5* und *her7*, eine normale dynamische initiation ihrer Expression zeigten. Gruppe II Mutanten zeigen Defekte bei der Bildung der Somitengrenzen und Epithelialisierung der Somiten trotz normales, Musterbildung im PSM. Solche Mutanten wurden bislang weder in Zebrafisch, noch in Maus oder Hühnchen gefunden. Deshalb sollten nach der molekularen Identifiezierung der mutierten Gene neue Faktoren erhalten werden, die vor allem für die Regulation später Somitogenese-phasen wichtig sind.

# 5. Summary

Somites are repeated epithelial segments that are generated in a rhythmic manner from the presomitic mesoderm (PSM) in the embryonic tailbud. Later, they differentiate into skeletal muscle, cartilage and dermis. Somitogenesis is regulated by a complex interplay of different pathways. Notch/Delta signaling is one of the pathways well characterized in zebrafish through mutants affected in its different components. Previous work in mouse, chicken and zebrafish has shown that also additional components are required during somitogenesis, most importantly through an FGF and Retinoic acid (RA) gradient, as well as Wnt signaling. However, no zebrafish mutants with defects in these pathways showing specific somite malformations are described. This was explained by functional redundancies among related genes that have resulted from a whole genome duplication which occurred in a teleost fish ancestor 350 million years ago. As distinct duplicates exist in different teleost species, a large scale mutagenesis screen in the medaka (*Oryzias latipes*) has been performed successfully in Kyoto, Japan. I analyzed nine of the isolated medaka mutants that show variable aspects of somitic phenotypes. This includes a complete or partial loss of somite boundaries (e.g. *bms* and *sne*), somites with irregular sizes and shapes (e.g. *krz* and *fsl*) or partially fused and enlarged somites (e.g. *dpk*). Although some of these medaka mutants share characteristics with previously described zebrafish somite mutants, most of the mutants represent unique phenotypes, not obtained in the zebrafish screens. *In-situ* hybridization analyses with marker genes implicated in the segmentation clock (e.g. *her7*), establishment of anterior-posterior (A-P) polarity (e.g. *mesp*) and differentiation of somites (e.g. *myf5*, *lfng*) revealed that the medaka mutants can be separated into two classes. Class I shows defects in tailbud formation and PSM prepatterning, and lateron somite boundary formation was impaired in these mutants. A unique member of this class with a novel phenotype is the *doppelkorn* (*dpk*) mutant that has single fused or enlarged somites. This phenotype has not been reported till now in zebrafish somite mutants. *In-situ* analyses on *dpk* showed that stabilization of the cyclically expressed somitogenesis clock genes must be affected in this mutant. This is accompanied by a disrupted regulation of A-P polarity genes like *mesp*. This suggests that *dpk* is a mutant deficient in the wave front, which is necessary for the down-regulation of oscillating genes in the anterior PSM. Furthermore, as the initiation of oscillation of all three cyclic *her* genes was unaffected in *dpk* embryos, I could exclude that this mutant in affected in the Notch/Delta pathway. Another mutant that belongs to this class is the *samidare* (*sam*) mutant. Morphologically, *sam* mutants are similar to zebrafish *after eight* (*aei*). In both cases, the first

7-9 somites are formed properly, but after this somite formation ceases. Different to the situation in *aei, sam* mutant embryos presented an additional defect in the mid-hindbrain boundary (MHB) region. Similar MHB defects were described in the zebrafish *fgf8* mutant *acerebellar* (*ace*). In *ace* zebrafish mutant, somites were only slightly defective, although FGF signaling has been shown to be important for somite formation in chicken, mouse and zebrafish. This was explained by functional redundancy between *fgf8* and *fgf24* ligands in the tailbud of zebrafish. Thus, it is interesting to suggest that the *sam* mutant, based on the parallel defects in somites and MHB, is a potential member of the FGF signaling pathway muatnts. It was shown that FGF plays a crucial role during MHB formation in medaka. In addition, I showed that *fgf8* acts non-redundantly during tailbud formation and somitogenesis in medaka. Furthermore, I showed that FGF signaling regulates somite size also in medaka and that *fgfr1* is the only FGF receptor expressed in the tailbud and somites. In class II medaka somite mutants, PSM prepatterning appears normal, whereas A-P polarity, boundary formation, epithelialization or the later differentiation of somites appears to be affected. Such mutants have not been isolated so far in zebrafish, mice or chicken. Therefore, medaka class II somite mutants seem to be a novel group of mutants that opens new perspectives to analyze A-P polarity regulation, determination and boundary formation in the presence of a normally functioning clock in the PSM. Identifying the encoding genes for all analyzed medaka somite mutants will contribute to the understanding of the molecular interactions of different signaling pathways involved during somitogenesis, and is expected to result in the identification of new components.

# 6. Abbreviations

| | |
|---|---|
| A | anterior |
| A-P | anterior-posterior |
| *ace* | *acerebellar* |
| *aei* | *after eight* |
| *bea* | *beamter* |
| *bfe* | *before eight* |
| BMP | Bone morphogene protein |
| *bms* | *bremser* |
| *C-hairy1* | *Chicken hairy 1* |
| cM | Centi morgan |
| *con* | *chameleon* |
| *Con* | *Control* |
| *des* | *deadly seven* |
| DNA | dioxyribonucleicacid |
| *dpk* | *doppelkorn* |
| *D. rer* | *Danio rerio* |
| *eng2* | *engrailed 2* |
| ENU | Ethyl-nitroso-urea |
| *E (spl)* | *Enhancer of split* |
| Fig | Figure |
| FISH | Fluorescence *in situ* hybridization |
| *FGF* | Fibroblast growth factor |
| *Fgfr1* | *Fibroblast growth factor receptor 1* |
| *Fgfr3* | *Fibroblast growth factor receptor 1* |
| *Fgfr4* | *Fibroblast growth factor receptor 1* |
| *fgf3* | *Fibroblast growth factor 3* |
| *Fgf8* | *Fibroblast growth factor 8* |
| *fgf24* | *Fibroblast growth factor 24* |
| *fn* | *fibronectin* |
| *fsl* | *fussel* |
| *fss* | *fused somites* |
| *h* | *hairy* |
| *her1* | *hairy enhancer of split related 1* |
| hpf | hours post fertilization |
| *itga5* | *integrinα5* |
| *kas* | *knollnase* |
| Kb | kilobasespair |
| *krz* | *kurzer* |
| LG | linkage group |
| *lia* | *limabsent* |
| lfng | lunatic fringe |
| MAPK | Mitogen- activated protein kinase |
| Mbp | Mega basepair |
| MEK | medaka enhancer kinase |
| MHB | midbrain-hindbrain boundary |
| *mhey1* | *mouse hey1* |
| *mib* | *mindbomb* |
| Min | Minutes |
| *mlb* | *masterblind* |

| | |
|---|---|
| NICD | Notch intracellular domain |
| *noi* | *no-isthmus* |
| *O. lat* | *Oryzias latipes* |
| *opf* | *orgelpfeifen* |
| P | posterior |
| *Pac* | *parachute* |
| *par* | *paraxis* |
| *Papc* | *paraxial protocadherin* |
| *pll* | *planlos* |
| PSM | presomitic mesoderm |
| Raldh2 | retinaldehyde dehydrogenase |
| RA | Retinoic acid |
| RNA | Ribonucleicacid |
| RT | Room temperature |
| S-II | somite-2 |
| S-I | somite-1 |
| S0 | somite 0 |
| SI | somite 1 |
| SII | somite 2 |
| *sam* | *samidare* |
| *slb* | *silberblick* |
| *shh* | *sonic hedgehog* |
| *sne* | *schnelles ende* |
| *spt* | *spadetail* |
| *Su (H)* | *suppressor-of-Hairless* |
| *syu* | *sonic-you* |
| *Tbx24* | *T-box 24* |
| *ubo* | *u-boot* |
| Wt | wildtype |
| *Yot* | *you-too* |
| *zlk* | *zahnluecke* |

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# Publications


1. Fischer, A., Leimeister, C., Winkler, C., Schumacher, N., Klamt, B., **Elmasri, H**., Steidel, C., Maier, M., Knobeloch, K.-P., Amann, K., Helisch, A., Sendtner, M., and Gessler, M. 2002. *Hey* bHLH Factors in Cardiovascular Development. **Cold Spring Harbor Symposia on Quantitative Biology**, 67, 63-70.

2. Winkler, C., **Elmasri, H**., Klamt, B., Volff, J.N., Gessler, M., 2003b. Characterization of *hey* bHLH genes in teleost fish. **Developmental Genes and Evolution**. 213: 541-553.

3. **Elmasri, H**., Liedtke, D., Luecking, G., Volff, J.N., Gessler, M., Winkler, C., 2004. *her7* and *hey1*, but not *lunatic fringe* show dynamic expression during somitogenesis in Medaka (*Oryzias latipes*). **Gene Expression Patterns**. 4: 553-9.

4. **Elmasri, H**., Winkler, C., Liedtke, D., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Henrich, T., Hirose, Y., Yasuoka, A., Yoda, H., Watanabe, T., Deguchi, T., Iwanami, N., Kunimatsu, S., Osakada, M., Loosli, F., Quiring, R., Carl, M., Grabher, C., Winkler, S., Del Bene, F., Wittbrodt, J., Abe, K., Takahama, Y., Takahashi, K., Nishina, H., Kondoh, H., and Furutani-Seiki , M., 2004. Mutations affecting somite formation in the Medaka (*Oryzias latipes*). **Mechanism of Development.** 121: 659-71.

5. Kitagawa, D., Watanabe, T., Saito, K., Asaka, S., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Yasuoka, A., Deguchi, T., Yoda, H., Hirose, Y., Henrich, T., Iwanami, N., Kunimatsu, S., Osakada, M., Winkler, C., **Elmasri, H**., Wittbrodt, J., Loosli, F., Quiring, R., Carl, M., Grabher, C., Winkler, S., Del Bene, F., Katada, T., Nishina, H., Kondoh, H., and Furutani-Seiki , M., 2004. Genetic dissection of the formation of the forbrain in Medaka (*Oryzias latipes).* **Mechanism of Development.** 121: 673-85.

6. Loosli, F., Del Bene, F., Quiring, R., Rembold, M., Martinez-Morales, J-R., Carl, M., Grabher, C., Iquel, C., Krone, A., Wittbrodt, B., Winkler, S., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Henrich, T., Deguchi, T., Hirose, Y., Iwanami, N., Kunimatsu, S., Osakada, M., Watanabe, T., Yasuoka, A., Yoda, Winkler, C., **Elmasri, H**., H., Kondoh, H., and Furutani-Seiki, Wittbrodt, J., 2004. Mutations affecting retina development in Medaka (*Oryzias latipes*). **Mechanism of Development.** 121: 703-14.

7. Furutani-Seiki ,M., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Yoda, H., Deguchi, T., Hirose, Y., Yasuoka, A., Henrich, T., Watanabe, T., Iwanami, N., Kitagawa, D., Saito, K., Asaka, S., Osakada, M., Kunimatsu, S., **Elmasri, H**., Winkler, C., Ramialison, M., Loosli, F., Quiring, R., Carl, M., Grabher, C., Winkler, S., Del Bene, F., Shinomiya, A., Kota, Y., Yamanaka, T., Okamoto, Y., Takahashi, K., Todo, T., Abe, K., Takahama, Y., Tanaka, M., Mitani, H., Katada, T., Nishina , H., Nakajima, N., Wittbrodt, J., and Kondoh , H. A., 2004. A systematic genome-wide screen for mutations affecting organogenesis in Medaka (*Oryzias latipes).* **Mechanism of Development.** 121: 647-58.

8. Winkler, C. and **Elmasri, H.,** 2005. Somitogenesis in laboratory fish models. In: Somitogenesis. Ed: Neil V. Whittock; Research Signpost, Kerala, India. In press.

9. Gajewski, C., **Elmasri, H.,** Girschick, M., Sieger, D., Winkler, C. Comparative analysis of *her* genes during fish somitogenesis reveals a tetrapod mode of oscillation in Medaka. **Submitted.**

10. **Elmasri, H.,** Carl, M., Gajewski, M., Wittbrodt, J., Winkler, C. Non-redundant activity of *fgf8* during somite formation in medaka (*Oryzias latipes*). In preparation.

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