her15, a novel gene with oscillating mRNA expression domains and its potential role in zebrafish somitogenesis

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Abbreviations

AP:	Alkaline Phosphatase
bp:	Base pairs
BSA:	Bovine serum albumine
ddNTP:	Dideoxynucleotidetriphosphate
DEPC:	Diethylpyrocarbonate
dNTP:	Deoxynucleotidtriphosphate
hpf:	hours post fertilisation
Hybmix:	Hybridization Mix
L	Litre
MO:	Morpholino antisense Oligomer
nt:	Nucleotide
ORF	Open reading frame
PBS:	Phosphate buffered saline
PBST:	Phosphate buffered saline + Tween-20
PCR:	Polymerase chain reaction
PFA:	Paraformaldehyde
PSM:	Pre Somitic Mesoderm
RACE:	Rapid amplification of cDNA ends
SDS:	Sodium Dodecyl Sulphate
RT:	Room temperature
s/som	Somites
SSC:	Sodium chloride/Sodium citrate
SSCT:	SSC + Tween-20
TAE:	Tris-Acetate EDTA (Electrophoresis Buffer)
wt:	Wildtype

Zusammenfassung

Die Somitogenese ist der entscheidene entwicklungsbiologische Prozess, welcher die Grundlagen für eine strukturierte Körperachse in Vertebraten und Cephalochordaten festlegt. Während der Somitogenese wird der Embryo in transiente, segmentale Strukturen (Somiten) unterteilt, welche später zu Muskeln und Wirbeln des Rumpfes und Schwanzes differenzieren. Somiten entstehen aus dem unsegmentierten, präsomitischen, paraxialen Mesoderm (PSM), in welchem ein komplexer Musterbildungsprozess abläuft. Für den koordinierten Ablauf dieses Prozesses ist ein Oszillator Mechanismus („Segmentation clock“) verantwortlich. Dieser beinhaltet eine wellenartige, von posterior nach anterior verlaufende Expression verschiedener Gene. Die konservierten Hauptkomponenten dieses Oszillator Mechanismus sind der Delta-Notch Signaltransduktionsweg sowie zahlreiche *hairy/(E(spl)-C)*-homologe Gene. Zwei der *hairy/(E(spl)-C)*-homologen Gene, *her1* und *her7*, spielen eine entscheidene Rolle während der Somitogenese im Zebrafisch. Offen bleibt jedoch die Frage, wieviele *her* Gene Hauptkomponenten des Oszillator Mechanismus sind und wie diese miteinander interagieren. Um diese Frage zu beatworten wurde eine Suche nach weiteren *hairy/(E(spl)-C)*-homologen Genen von Sieger et. al, (2004) im Zebrafisch durchgefürt. Es konnten so drei weitere *her* Gene mit oszillierender Expression im PSM identifiziert werden. Eines dieser Gene ist *her15*, das in dieser Arbeit charakterisiert wurde.

Das *her15* Gen zeigt im posterioren PSM eine oszillierende Expressionsdomäne, welche hauptsächlich in drei Phasen unterteilbar ist: eine breitere, eine intermediäre sowie eine punktartige Expression. Vergleichbar mit anderen *her* Genen zeigt auch *her15* eine streifenartige Expression im anterioren PSM. Diese Streifen sind jedoch im Gegensatz zu allen anderen *her* Genen in einem doppelt segmentalen Abstand exprimiert. Zusätzlich konnte gezeigt werden, dass einer der *her15* Streifen die posteriore Grenze des letzten Somiten markiert. Durch Morpholino „knock down“ Studien wurde gezeigt, dass die oszillierende *her15* Expression in gewisser Hinsicht vom Her7 Protein beeinflusst wird, jedoch völlig unabhängig von Her1 ist. Die oszillierende *her15* Expression im posterioren PSM zeigt gewisse Unterschiede zwischen der rechten und linken Seite des Embryos, welches ein klarer Hinweis darauf ist, dass das oszillierende Signal auf beiden Körperseiten autonom generiert wird. Missexpressionsstudien deuten auf eine mögliche Beteiligung von *her15* an der Bildung der Somitengrenzen, sowie eine mögliche Funktion

in der Regulation der oszillierenden Genexpression im PSM hin. Die Injektion von Morpholinos gegen *her15* zeigte jedoch weder Effekte auf die Bildung der Somitengrenzen noch auf die Expression verschiedener Gene des Delta-Notch Signalweges.

ZfChp ist das zweite Gen, welches in dieser Arbeit charakterisiert wurde. Dieses Gen entstammt einer Suche nach Kandidatengenen in der „NIH cDNA *in situ* expression database“. *ZfChp* zeigt eine dynamische, streifenartige Expression im intermediären PSM, einer Region in welcher die Zellen vom mesenchymalen in den epithelialen Zustand übergehen („Mesenchymal to Epithelial transition“-MET), und damit zu Somiten zu differenzieren. Das ZfChp Protein weist die molekulare Struktur einer zur Rho Familie gehörenden GTPase auf (basierend auf den konservierten Rho GTPase Domänen). Durch eine Mutantenanalyse konnte gezeigt werden, dass der Delta-Notch Signalweg die streifenartige Expression des *ZfChp* Gens im intermediären PSM postiv reguliert. Dies ist der erste molekulare Hinweis auf eine Verknüpfung des Musterbildungsprozesses im PSM und dem Übergang vom mesenchymalen in den epithelialen Zustand (MET).

Abstract

Somitogenesis is the key developmental process which lays down the framework for an organised body plan in vertebrates and cephalochordates. Somitogenesis divides the body axis into transient segmental structures called somites, which later give rise to muscles and vertebrae of the trunk and tail. Somites are generated from the unsegmented presomitic mesoderm (PSM) by an intricate process of prepatterning. Prepatterning is driven by a segmentation clock referred to as the presomitic mesoderm oscillator. This oscillator consists of certain gene members with oscillating mRNA expression compartments that sweep like a wave from the posterior to the anterior end of the embryonic PSM. The Delta-Notch pathway and various genes belonging to the *hairy-(h)* and *Enhancer of split* – [*E(spl)*] related family, are the core conserved components of this oscillator. *h/E(spl)* genes in zebrafish are commonly referred to as *her* genes. *her1* and *her7*, play very important roles in the regulation of somitogenesis. The open question is how many *her* genes are core components of the zebrafish presomitic mesoderm oscillator and how do they interact with one another? To answer this, an *in situ* screen for *h/E(spl)* genes in zebrafish was conducted by Sieger et al., (2004). Three new *her* genes with oscillating mRNA expression domains were identified and one of them is *her15*, which has been further characterized in this PhD thesis.

her15 mRNA is expressed as a distinct oscillatory posterior PSM domain which shows three primary phases, namely broad, intermediate and dot-like. Comparable to other *her* genes, *her15* also showed stripes in the anterior PSM but unlike others, these stripes were found to be expressed at double segmental distance. Additionally, the *her15* stripe was found to label the posterior border of the last somite. Morpholino gene knock down studies showed that the oscillating expression of *her15* is partly dependent on *her7* regulation, but independent of *her*1. Oscillating *her15* mRNA signals in the posterior PSM displays fluctuations with respect to left and right halves of the embryonic PSM, suggestive of the autonomy of both halves of the embryo in generating the signal. Misexpression studies suggest a prospective role for *her15* in the regulation of somite border formation and oscillatory gene expression in zebrafish PSM. Morpholinos against *her15* did not result in morphological border disruption, or in changes in mRNA expression of genes of the Delta-Notch pathway.

ZfChp, the second candidate gene which has been analyzed in the present thesis, came out of a screen of the NIH cDNA *in situ* expression database. *ZfChp* exhibits dynamic stripes of mRNA expression in the intermediate PSM region, a dynamic zone where tail bud mesenchymal cells undergo transition to epithelial state (mesenchymal to epithelial transition-MET), thus giving rise to somites. It the molecular signature of a Rho family GTPase with respect to conserved Rho GTPase domains. The Delta-Notch signaling pathway positively regulates the dynamic stripes of *ZfChp* in the intermediate PSM region. This provides the first molecular evidence supporting a link between prepatterning of the PSM and MET in zebrafish embryos.

1. Introduction

Designing a body plan is an architectural challenge. Both invertebrates and vertebrates have addressed this problem by first establishing repeated units of equivalent identity (segments), and later coordinating these motifs into regionally specialized and integrated structures. Formation of the anteroposterior (AP) axis of vertebrates occurs progressively in the wake of the rostral-to-caudal regression of the embryonic organizer-known as the node and Hensen's node in mammals and birds, and the Spemann organizer and the shield in amphibians and fishes, respectively. This regression movement results in the production of a variety of tissues, which includes the paraxial mesoderm that gives rise to head mesoderm anteriorly and to somites at the body level (Psychoyos and Stern, 1996, Tam et al., 2000). The most distinct feature of vertebrate mesoderm segmentation is the somite. Somites are transient segments of the paraxial mesoderm that are present in developing cephalochordates and vertebrates. The specific paraxial mesoderm region which gives rise to somites is called the presomitic mesoderm (PSM) and the process of somite formation is referred to as somitogenesis. Important landmarks in somitogenesis are periodicity, segmentation, epithelialization and differentiation. Somitogenesis begins soon after gastrulation and the process lasts until the number of somites characteristic of the species is reached. Somitic derivatives become later regionalized into different morphological domains-such as cervical, thoracic, lumbar and sacral regions as a result of the specific combination of Hox genes expressed in these domains. This body plan is highly variable within a given species indicating that the mechanism of segmentation and regionalization of the axis have to be tightly coordinated during development. Indeed cell fate in the immature somite is flexible and dependent on local environmental signals. Consequently, somitogenesis has generated considerable interest and the somite now serves as a paradigm for investigating how naive cells adopt identity.

Somites were first identified at the beginning of the last century, and much of our understanding of somite development comes from morphological observations and experimental manipulations in the avian embryo (Christ and Ordahl, 1995), and more

recently, from embryo culture and genetic studies in mice (Gossler and Hrabe de Angelis, 1997). The rapidly developing zebrafish model promises to unite these approaches.

1.2 Origin of the somitic territory in the vertebrate blastula

Studies on the amphibian model system have provided most of the details pertaining to the dynamic cellular interactions that lead to the formation of the paraxial mesoderm during gastrulation (Keller 2000). In *Xenopus* and zebrafish, the paraxial mesoderm arises from deep layers of symmetrical tissue, located on either side of the Spemann organizer, at the marginal zone. The Spemann organizer corresponds to the shield in zebrafish (Kimmel et al., 1990; Warga and Kimmel, 1990) (for review see (Pourquie, 2001) .

1.3 Convergence-extension gives rise to presomitic mesoderm

Morphogenetic movements in the gastrulating embryo lead to the establishment of the paraxial mesoderm, which consists of the PSM region. It is this PSM region which gives rise to somites. These morphogenetic movements are called convergence-extension movements. Both in zebrafish and *Xenopus*, the prospective paraxial mesoderm region converges towards the blastopore during gastrulation, where they undergo invagination and subsequent elongation along the antero-posterior axis. First, the head mesoderm involutes and actively migrates towards the anterior (Niehrs et al., 1994). This is followed by the involution of the somitic mesoderm regions, which progressively elongates along the medio-lateral axis by a process of medio-lateral intercalation of cells, which results in the formation of PSM. The segmentation of the PSM into somites then takes place at the rostral end (Shih and Keller, 1992; Wilson et al., 1989). Convergence-extension of the paraxial mesoderm is crucial for the antero-posterior elongation of the vertebrate body axis. It is widely accepted that axis elongation is driven by the organizer and its major derivative the notochord, which is also formed by similar convergence-extension movements. But the paraxial mesoderm plays a prominent role in axis elongation and this is supported by the observation that embryos which the lack the notochord are capable of

axis elongation, while those embryos which are deprived of paraxial mesoderm are not (Malacinski and Youn, 1982; Scharf and Gerhart, 1980).

In lower vertebrates like zebrafish, gastrulation proceeds until the closure of the blastopore. During this phase, the somitic mesoderm which involutes, gives rise to the anterior most mesoderm, *i.e*., the head mesoderm and the anterior somites. Additionally, convergence-extension movements give rise to the anterior region of the paraxial mesoderm. Production of the more posterior somitic mesoderm occurs by a different process which occurs at the end of the classical gastrulation period. In *Xenopus* and zebrafish, the 12 anterior most somites are formed as a result of involution occurring during gastrulation (Kanki and Ho, 1997; Keller, 2000).

Gastrulation comes to an end with the closure of the blastopore in lower vertebrates such as zebrafish, or complete regression of the primitive streak, as in the case of higher vertebrates, such as chick. Subsequently, caudal somites are produced from the tail bud, which is located at the caudal end of the embryo. The tail bud consists of an apparently homogeneous mass of cells, namely mesenchymal cells. In fish, somites caudal to the twelfth are produced from the tail bud. The trunk-tail boundary lies at the level of somite 17, which consists of 5 trunk somites and all of the tail somites (Kanki and Ho, 1997; Prince et al., 1998)

1.4 Somitogenesis and the Segmentation Clock

Vertebrates take their name from the segmented column of bones and joints that give rise to the main body axis. These elements, with their associated muscles, ribs and other connective tissue, derive from the somites which are blocks of mesodermal cells that form on either side of the central axis of the early embryo (Hirsinger et al., 2000). In all vertebrates, somites are generated sequentially from the caudal mesenchymal portion of the unsegmented paraxial mesoderm called the presomitic mesoderm (PSM) present at the tail end of the embryo. At the anterior end of the PSM, clefts appear, splitting off the successive blocks of somitic tissue; simultaneously, the embryo continues growing caudally, thereby maintaining the amount of PSM tissue approximately constant.

Accordingly, a new somite is generated in zebrafish every 30 mins; in the *Xenopus* every 40 mins, in the chick every 90 mins and in the mouse every 120 min.

Somites are laid down sequentially and this gives rise to a final spatially periodic pattern. This spatial periodicity reflects a temporally periodic process, an oscillation, at the growing end of the developing organism, where anterior and posterior halves of somites are generated alternately. The possibility of there being a biological and molecular clock driving vertebrate segmentation and somitogenesis was put forward in 1976 by Cooke and Zeeman. They presented theoretical evidence for a *'clock and wavefront'* model controlling the number of repeated structures formed during animal morphogenesis (as described in their own words). This abstract suggestion came to life after 30 years in 1997, with the discovery of certain genes whose mRNA expression oscillates at the tail end of the vertebrate embryo (Palmeirim et al., 1997). This ticking molecular clock is central to the process of segmentation of the vertebrate body. The first cycling or oscillating gene to be discovered in vertebrates was *c-hairy1* in chicken which is a homologue of the *Drosophila* pair rule gene *hairy* (Palmeirim et al., 1997). *hairy* in *Drosophila* regulates segmentation and is expressed as stripes in alternate segments numbering a total of 7 stripes in the embryo (Ish-Horowicz et al., 1985). The fact that the first cycling gene to be discovered is a homologue of the *Drosophila* pair rule segmentation gene, suggests that certain aspects of invertebrate and vertebrate segmentation have been conserved. An overview of somitogenesis or segmentation in zebrafish is presented in Figures 1 and 2.

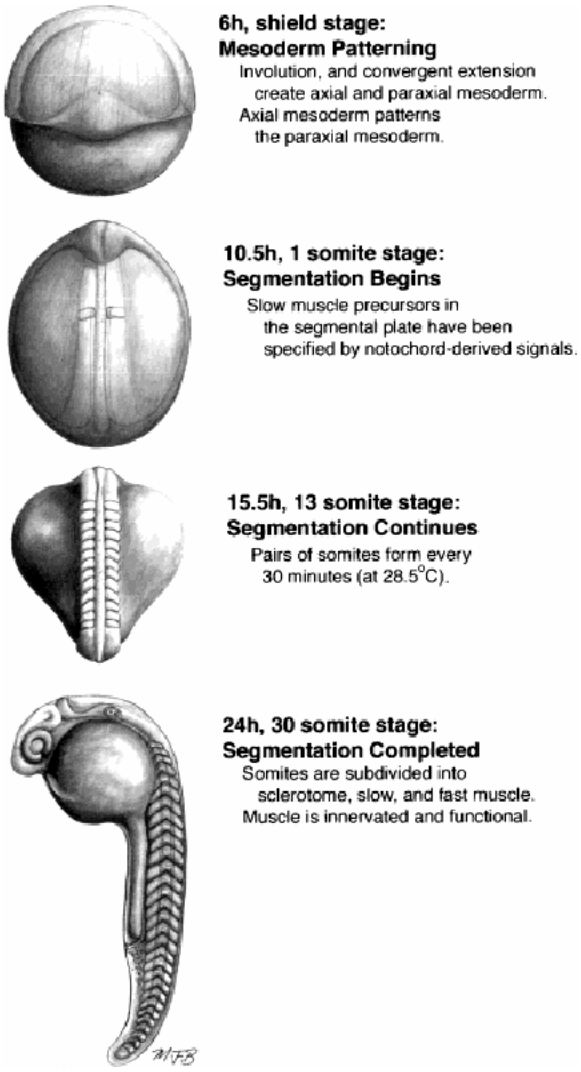


Figure 1. Temporal landmarks during zebrafish somitogenesis. This figure and legend has been taken from Stickney et al., (2000).

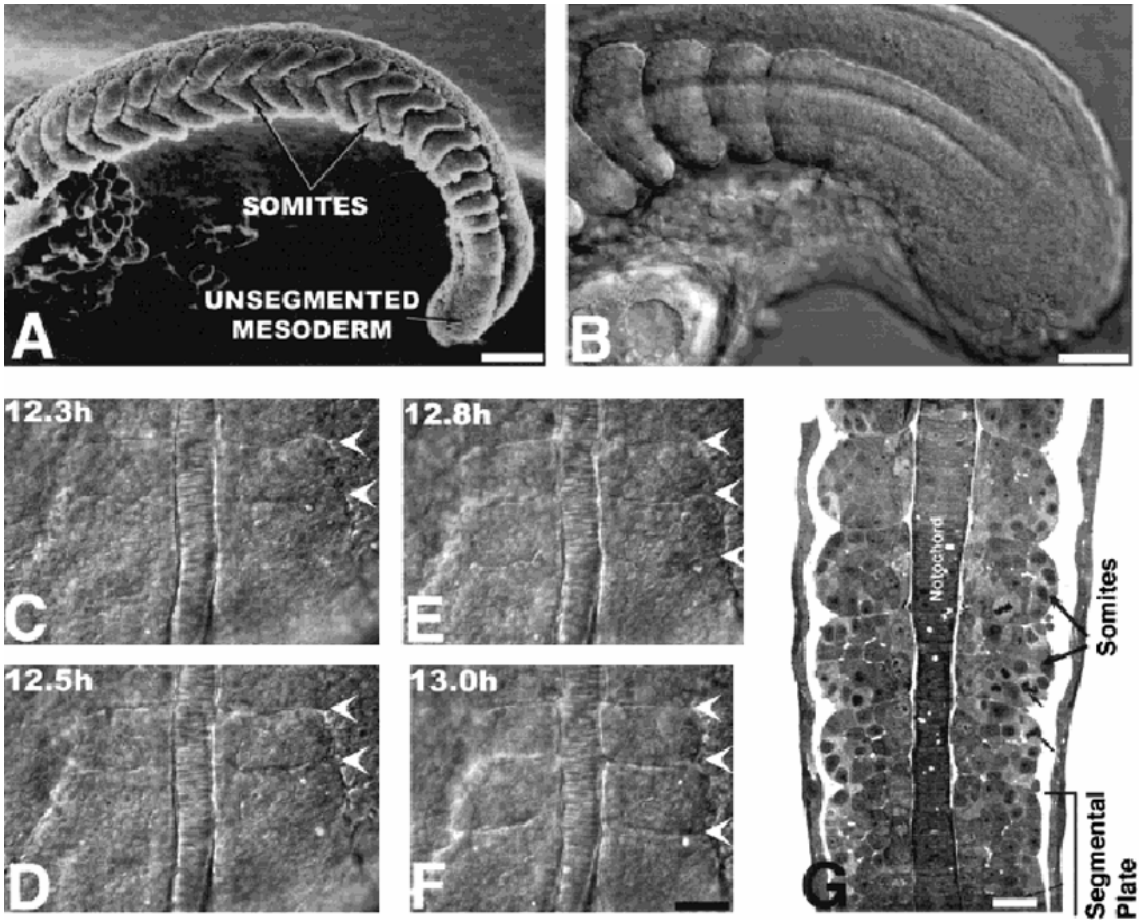


Figure 2. Overview of zebrafish somitogenesis. (A) Scanning electron micrograph of a 19 somite embryo. Shortly after somites form, they change from a cuboidal to a chevron shape. Reproduced from Waterman and McCarty (1977) with permission of Scanning Microscopy International. (B) Live, lateral view of somitogenesis in a 20 somite embryo. The notochord is out of focus, medial to the somites and presomitic mesoderm. (C-F) Time lapse views of an embryo undergoing somitogenesis, as observed from dorsal. The notochord, in the center, is flanked on either side by paraxial mesoderm. Arrowheads indicate the positions of somitic furrows. Somitic furrows are first visible in the lateral part of the paraxial mesoderm. (C) Six somites have formed; arrowheads bracket somite 6. (D) The furrow on the right side between somite 6 and the future somite 7 has begun to form in the lateral presomitic mesoderm. (E) The furrow between somite 6 and the future somite 7 is nearly complete on both sides and a new furrow between somites 7 and 8 has begun to form. (F) Somite 7 has fully separated from the presomitic mesoderm. (G) Horizontal section through a 20 somite embryo at the level of the notochord. Epithelial boundaries and loosely packed central cells are visible in several of the somites. Reproduced with permission, from Waterman (1969**).** Scale bars = 100 μm (A); 50 μm (B); 25 μm (C-F); 25 μm (G).This figure and legend has been taken from Stickney et al., (2000).

Research in the last couple of years has brought to light a significant number of genes with dynamic and oscillating mRNA expression domains which form the core of the molecular oscillator driving vertebrate somite segmentation. In the PSM, a particular subset of genes display oscillating mRNA expression, which switches on and off at the rate corresponding to the formation of one somite in each clock cycle (for review see Giudicelli and Lewis, 2004). The tempo for the entire process is set in the region where all the cells originate, namely, the posterior part of the PSM. The posterior PSM defines the periodicity of the entire process and hence this region behaves like a pace maker. One by one, each cohort of cells mature and pass from the posterior to the anterior part of the PSM. Eventually, at the anterior end of the PSM, each cohort of cells slow down its oscillation and become arrested in either an 'on' or 'off' state, according to its time of exit from the PSM. Cells arrested in the one state are stamped with the character assigned to the anterior part of the somite; while those arrested in the other, are stamped as posterior. Accordingly, phases of the oscillation cycle are recorded along the antero-posterior axis of the body, thus generating a pattern of gene expression, which appears to govern the physical process of segmentation, presumably through effects on cell-cell adhesion (Saga and Takeda, 2001).

As the oscillation gradually slows down in the anterior region of the PSM, anterior cells become retarded in their phase relative to the posterior cells. This gives rise to a spatio-temporal pattern of waves of expression that appear to sweep forward through the PSM tissue, prepatterning the somites. This phenomenon has been illustrated in oscillating expression of zebrafish *deltaC* shown in Figure 3. The maturation *wavefront* is the moving interface between the PSM region where, gene expression oscillates and the somitic mesoderm where oscillation is halted and overt differentiation begins.

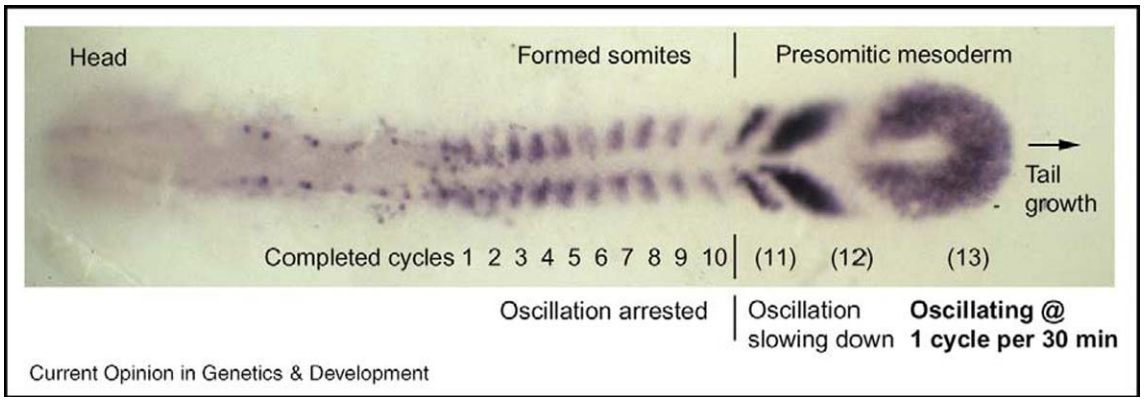


Figure 3. The oscillatory expression of *deltaC.* The periodic spatial pattern of somites represents the trace of a temporal oscillation of gene expression at the tail end of the embryo. In this figure, the pattern of expression of *deltaC*, coding for the Notch ligand DeltaC, is shown by *in situ* hybridization in a zebrafish embryo at the 10 somite stage; other oscillating genes show a similar pattern. Somites are formed sequentially as growth continues at the tail end of the embryo. In the posterior region of the presomitic mesoderm (PSM), the mRNA levels rise and fall with a period of 30 min (at 28°C). As cells emerge into the anterior PSM, the oscillation in each of them slows down, finally halting as somite formation begins. The prominent stripes in the anterior PSM corresponds to the cells that are still oscillating, but in different phases of their cycle; in the region of formed somites, oscillation has stopped, leaving cells arrested in different phases according to their time of exit from the PSM (Lewis, 2003). This figure and legend has been taken from Giudicelli and Lewis, (2004).

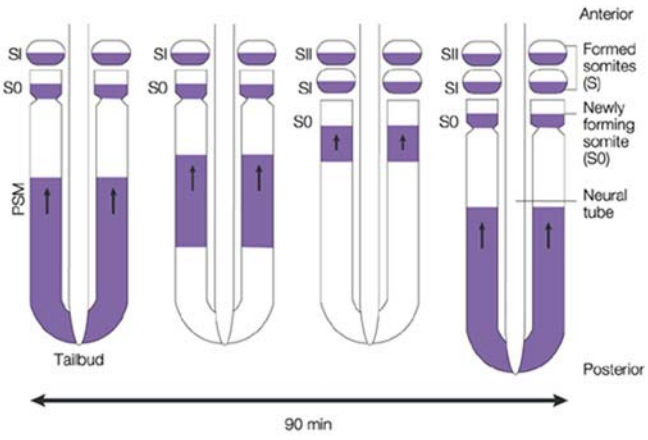


Figure 4: A model for the Presomitic Mesoderm Oscillator in chick embryos. It shows cyclic *c-haiy1* mRNA expression (chick *hairy* homologue) in the PSM. These oscillations are bilaterally synchronous, and appears as antero-posterior waves of expression that sweep across the PSM approximately every 90 min (see figure in which c-hairy1 expression is shown in purple), which equals one cycle of somite formation in the chick embryo (Palmeirim et al., 1997). This figure and legend have been taken from Saga and Takeda, (2001).

1.5 The Notch signaling pathway

The Notch signaling cascade is crucial for both the generation and regulation of the is shown in Figure 5 (for review see Weinmaster and Kintner, 2003). Genetic studies in mice have shown that inactivation of many components of the Notch pathway result in dramatic segmentation defects and severe impairment in the periodic expression of the cyclically expressed genes (Barrantes et al., 1999). The Notch pathway mutants in mouse and fish lose the dynamic expression of cyclically expressed genes, indicating that Notch signaling is required for their periodic expression, or is required to coordinate the oscillations between the PSM cells. It has therefore been proposed that the Notch pathway is either part of the core mechanism of the segmentation clock (Pourquie, 2000), or acts as a cofactor to synchronize the cyclical gene expression in neighboring cells (Jiang et al., 2000). A further potentially important role for the segmentation clock is to periodically activate Notch signaling in the rostral presomitic mesoderm, thereby generating the periodic formation of somite boundaries.

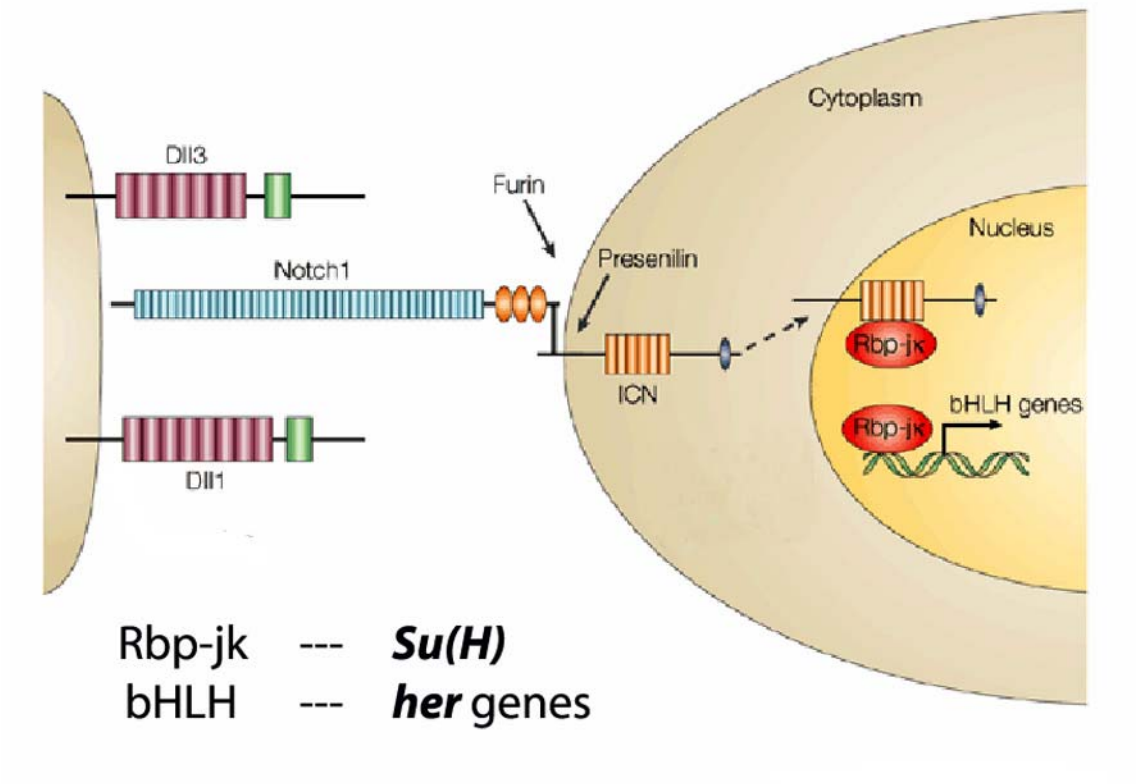


Figure 5: The Notch signaling pathway. The figure has been modified from Sawada and Takeda, (2001).

Genetic studies have shown that Notch signaling is necessary for somite formation and for periodic expression of the cyclically expressed genes (Pourquie, 1999). Periodic expression of genes of the Notch signaling pathway provide a link between the signaling cascade and the segmentation clock. Notch is a large transmembrane receptor, which is able to recognize two sets of transmembrane ligands, Delta and Serrate. Upon ligand binding, Notch undergoes a proteolytic cleavage at the membrane, leading to the translocation of its cytoplasmic domain into the nucleus, where together with the transcription factor *Su(h)/RBPjk*, it activates the transcription of downstream genes such as those of the Enhancer of split complex in the fly or *Her/Hes* in vertebrates (Artavanis-Tsakonas et al., 1999). In the fly, Notch signaling has been implicated in several distinct developmental processes such as lateral inhibition or boundary formation between compartments.

Notch pathway directs somitogenesis in vertebrates and other phyla. The mechanisms utilised during somitogenesis in vertebrates and segmentation in lower animals such as in an annelid, the leech and in an arthropod, the fruit fly *Drosophila*, exhibit gross differences, at both molecular and morphological levels. These differences have led many to conclude that metameric body plans evolved independently, at least three times over the course of animal evolution. However, leeches and flies both exhibit evolutionarily 'derived' modes of segmentation and the molecular mechanisms that underlie these may be unrepresentative of their phyla. In this context, recent work on the spider *Cupiennius salei* is of great interest, for spiders are chelicerates and thus represent a branch of the arthropods that diverged very early from the insect/crustacean lineage (Cook et al., 2001). Stollewerk et al., (2003) recently demonstrated that the similarity between vertebrate somitogenesis and opisthosomal segmentation in *Cupiennius salei*, is not merely morphological in nature. In both cases, the Notch signaling pathway is involved and thus provides the evolutionarily conserved link between somitogenesis in vertebrates and segmentation in lower animals.

All the cyclically expressed genes characterized until now can be grouped into four categories. The first one corresponds to the glycosyl-transferase, *Lunatic fringe*, in chick and mouse, which acts as a modulator of *Notch* affinity for its ligands *Delta* and *Serrate*

(Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). The second includes the zebrafish *deltaC*, one of the Notch ligands (Jiang et al., 2000). The third category is related to known or supposed direct downstream targets of Notch signaling, namely the *hairy/Enhancer of split* (*Hes/her*) family of transcription factors including *chairy1*, *chairy2, cHey2* in chick, *Hes1, Hes7* and *mHey2* in mouse as well as *her1*, *her7*, *her11*, *her12* and *her15* in zebrafish and *esr9* and *esr*10 in *Xenopus* (Bessho et al., 2001; Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2000; Jouve et al., 2000; Leimeister et al., 2000; Li et al., 2003; Oates and Ho, 2002; Palmeirim et al., 1997; Sawada et al., 2000; Sieger et al., 2004; Winkler et al., 2003). The fourth category consists of genes belonging to the Wnt-β catenin signaling pathway which includes *Axin2* and *Nkd1* (Aulehla et al., 2003; Ishikawa et al., 2004). All the genes implicated in the vertebrate segmentation oscillator mechanism and the corresponding references have been listed in Table1.

Table 1. Genes implicated in the vertebrate segmentation oscillator mechanism

	Notch signaling components	*hairy/E(spl)* related Transcription factors	Wnt/β-catenin pathway components
Cycling genes	**Mouse** ***Lfng*** [Aulehla et al; 1999] **Chicken** ***Lfng*** [McGrew et al., 1998] **Zebrafish** ***deltaC*** [Jiang, et al., 2000] **Xenopus** ***Xdelta2*** [Jen et al., 1997]	**Mouse** ***Hes1*** [Jouve et al; 2000] ***Hes7*** [Bessho et al; 2001] **Chicken** ***c-hairy1*** [Palmerim, et al., 1997] ***c-hairy2*** [Jouve et al., 2000] ***c-hey2*** [Leimeister *et al.*,2000] **Zebrafish** ***her1*** [Holley et al.,2000] [Sawada et al.,2000] ***her7*** [Oates &Ho,2002] [Henry et al.,2002] **Xenopus** ***esr4*** [Jen et al., 1999] ***esr5*** [Jen et al., 1999] ***esr9*** [Li et al., 2003] ***esr10*** [Li et al., 2003]	**Mouse** ***Axin2*** [Aulehla et al., 2003] ***Nkd1*** [Ishikawa et al., 2004] – – –
Gene functions required for oscillations	**Mouse** ***Cbf1*** [Morales et al., 2002] [Barrantes *et* al., 1999] ***Delta1*** [Jouve et al., 2000] ***Lfng*** [Dale et al., 2003; Serth et al., 2003] **Zebrafish** ***deltaD*** [Jiang et al., 2000] [Holley et al., 2000] ***mindbomb*** [Jiang et al., 2000] [Holley et al., 2000] ***notch1a*** [Holley et al., 2002] ***deltaC*** [Scott Holley, personal communication] ***Su(H)*** [Sieger et al., 2003]	**Mouse** ***Hes7*** [Bessho et al., 2001] [Bessho et al., 2003] **Zebrafish** ***her1/her7*** [Holley et al., 2002] [Oates & Ho,2002] [Henry et al., 2002] [Gajewski et al., 2003]	**Mouse** ***Wnt3a*** [Aulehla et al., 2003] **Zebrafish** **Receptor protein tyrosine phosphatase (RPTP ψ)** [Aerne et al.,2004]

1.6 Delayed negative feed back loops give rise to oscillations

In many biological clocks, such as the circadian rhythm, molecular oscillations rely on a delayed negative feed back loop. This implies that a particular component of the system is able to switch between active and inactive states and is auto inhibitory, so that in switching on it somehow sends itself a delayed signal to switch off, and in switching off it sends itself a delayed signal to switch on. The resulting system can then oscillate between the active and inactive states, with a cycle time determined by the delay in the feedback loop.

It has been suggested that the *Hes/her* genes, which behave as transcriptional repressors downstream of the Notch pathway, act by repressing Notch activity and thus directly participate in the generation of the transcriptional periodicity seen in the PSM cells. *Hes/her* genes have characteristic repressor domains which led to the conclusion that they behave as repressors in all situations. However, the mice mutant of the cycling gene *Hes1* does not display somitic defects or show disruption of the periodic expression of other cyclically expressed genes (Jouve et al., 2000; Ohtsuka et al., 1999). Recently a new member of the *Hes* family, *Hes7*, has been characterized in mouse. This gene acts as a repressor of its own transcription and is a target of Notch signaling (Bessho et al., 2003; Bessho et al., 2001). It is expressed in a periodic fashion in the PSM like other cycling genes and homozygous mice for a null allele of *Hes7* present strong segmentation defects. In these mice, expression of other cyclically expressed genes like *Hes* genes, *Hes1* or *mHey2* is lost, whereas *Lunatic fringe* remains expressed in the PSM but its expression is no longer dynamic (Bessho et al., 2001). Therefore, *Hes7* represents the first *Hes* gene shown to be involved in regulating the periodic expression of cyclically expressed genes in the mouse PSM.

Further evidence pointing to a pivotal role of *hes7* during mouse somitogenesis has been put forward in a recent publication which provides conclusive evidence that the instability of the Hes7 protein is crucial for sustained oscillation and for its function in the somite segmentation clock (Hirata et al., 2004). Mice were generated expressing a mutant version of Hes7 protein with a longer half-life (approximately 30 min as

compared to approximately 22 min for wild-type Hes7), but normal repressor activity. In these mice, oscillatory expression and somite segmentation became severely disorganized after a few normal cycles of segmentation. The observed effect could also be simulated mathematically using a direct auto repression model.

In the zebrafish embryo, the disruption of the expression of the two cyclically expressed *her* genes namely, *her1* and *her7* by morpholino antisense oligonucleotide injections as well as the misexpression of *her1* lead to a disruption of the dynamic expression of cyclically expressed genes (Gajewski et al., 2003; Holley et al., 2002; Oates and Ho, 2002; Takke and Campos-Ortega, 1999) and disruption of somite border formation. Remarkably, the sequences of *her1* and *her7* are much more closely related to that of *Hes7* than that of *Hes1*. Recent studies in zebrafish present a slightly different picture regarding the negative feed back loop of *her1* and its activity as an auto repressor. Based on morpholino gene knock down studies and exon probe *in situ* experiments, Holley et al., (2002) and Oates and Ho, (2002), have proposed that *her1* acts as a repressor on its own transcription. But a recent publication by Gajewski et al., (2003), has challenged this by using an intron probe to detect the nascent transcript of *her1*. The intron probe studies show that *her1* does not repress itself but instead acts as an activator for its own anterior-most PSM stripes. All these data, especially the studies in mouse, argue in favor of a central role for a subfamily of *Hes/her* genes in the mechanism of the oscillator.

1.7 A Model for the zebrafish somitogenesis oscillator

after eight (*aei*; *dld*), *deadly seven* (*des, notch1*), *fused somites* (*fss, tbx24*), *beamter* (*bea, deltaC*) and *white tail/mindbomb* (*wit,mib*) are the five genes that are necessary for normal somite formation which were isolated in the zebrafish genetic screen done in Tübingen (van Eeden et al., 1996). In zebrafish, oscillations in the expression of a *hairy*-related transcription factor, *her1* and the *notch* ligand *deltaC* precede somite formation. Scott Holley has proposed a model depicted in Figure 6, which suggests that *her1* and the *notch* pathway have cyclical functions at the center of the somitogenesis oscillator.

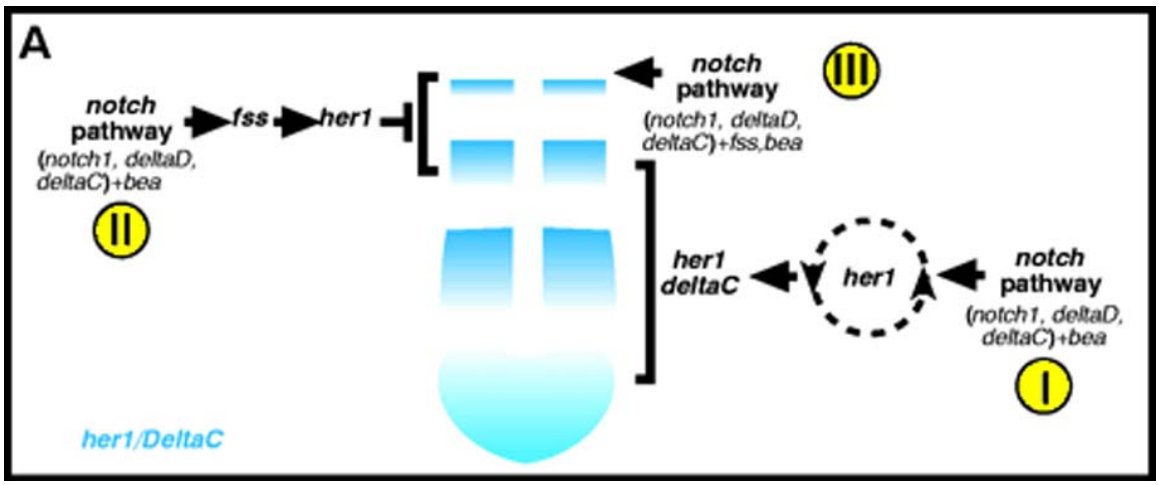


Figure 6: A summary of the genetic analysis of the functions of *her1* and the *notch* pathway during somitogenesis. (A) Anterior upwards. (I) The *notch* pathway and *bea* are required to generate the oscillating expression of *deltaC* and *her1* in the posterior and intermediate PSM. *Her1* probably functions within the oscillator and feeds back on the *notch* pathway to create the oscillating pattern of both *deltaC* and *her1*. (II) *fss* functions downstream of the *notch* pathway but upstream of *her1* in the anterior PSM. (III) Slightly later, the *notch* pathway, *bea* and *fss* function in the anterior most PSM/somitic mesoderm. The figure and legend has been taken from Holley et al; (2002).

1.8 Wnt/β-catenin signaling and the segmentation clock - upstream and downstream!

Wnt/β-catenin signaling cascade plays a major role in the segmentation clock and in the control of segmentation in mouse (Aulehla et al., 2003). *Axin2*, a negative regulator of Wnt signaling, shows oscillating mRNA transcription in the presomitic mesoderm (PSM) and the tail bud. Cyclic *Axin2* alternates with *Lfng* expression, a Notch pathway cycling gene, and occurs even when *Notch* signaling is impaired. In contrast, *Lfng* is down regulated in the posterior PSM when *Wnt3a* activity is lacking and does not show cyclic expression anymore. This implies that *Wnt3a* indirectly controls Notch signaling. Moreover, misexpression of *Axin2* in the PSM resulted in ectopic upregulation of *Lfng*, disrupting its cyclic expression pattern, and impairing the segmentation process.

Therefore, Notch signaling appears to act downstream of *Axin2*. In addition, it was shown that *Axin2* is a direct target of Wnt/β-catenin signaling in the PSM and acts downstream of *Wnt3a*, strongly suggesting that *Wnt3a* controls Notch signaling via *Axin2*. Furthermore, misexpression of *Axin2* in the PSM resulted in enlarged somites, while expression of *Wnt3a* from NIH3T3 cells transplanted on beads into the PSM of chick embryos had the opposite effect, the formation of smaller somites. There is also indirect evidence for a graded distribution of *Wnt3a* activity in the PSM. Thus, it can be concluded that *Wnt3a* plays a major role during mouse and chick segmentation process and lies upstream of the Notch signaling cascade.

A very recent publication had identified a novel gene *Nkd1*, which is a Wnt antagonist transcribed in an oscillatory manner (Ishikawa et al., 2004). The transcription of *Nkd1* is extremely down regulated in the PSM of *vestigial tail (vt/vt)*, a hypomorphic mutant of Wnt3a. *Nkd1* oscillations have a similar phase to *lunatic fringe (L-fng)* transcription and they are arrested in Hes7 (a negative regulator of notch signaling), deficient embryos. The results suggest that the transcription of *Nkd1* requires Wnt3a, and that its oscillation pattern depends on the function of Hes7. Previously Wnt3a has been postulated to be upstream of Notch signaling but the present study demonstrates that a Wnt-signal-related gene may also be regulated by Notch signaling.

A new model for vertebrate somitogenesis in which the clock and the gradient are joined together has been proposed (Aulehla and Herrmann, 2004). *Axin2* is the first gene found to be linked to both central components controlling segmentation, namely the transcriptional oscillator and the gradient. *Axin2* displays oscillating transcription with periodicity similar to the segmentation process and is a direct target of Wnt3a gradient. All other genes known to play a role in somitogenesis are either linked to the clock or the gradient, but not to both. Additionally, Wnt3a controls *Fgf8* expression in the tail bud and oscillating Notch activity in the PSM. Therefore, Wnt3a and the Wnt signaling cascade must play a central role in the segmentation clock and in the gradient controlling somitogenesis.

Compelling and direct evidence for the involvement of the Wnt/β-catenin signaling cascade in somitogeneis has only been obtained till date from mouse embryos. But, a recent paper from Aerne and Ish-Horowicz (2004), reveals a potential role for the non-canonical Wnt-signaling pathway, which is independent of β-catenin, playing a role in zebrafish somite formation. The paper unveils a new gene namely *receptor protein tyrosine phosphatase ψ* (*RPTPψ*), which is essential for normal functioning of the somitogenesis clock in zebrafish. *RPTPψ* gene knock down in zebrafish embryos result in severe disruption of somite border formation and a loss of cyclic gene expression. Impairing *RPTPψ* activity also interferes with convergent-extension during zebrafish gastrulation. Convergent-extension is a process of cell polarisation and intercalation that leads to lengthening and narrowing of the embryonic body during gastrulation and is regulated by the non-canonical Wnt signaling pathway. The authors state that a plausible explanation for the dual effect of *RPTPψ* on the somite oscillator and convergent extension is that changes in *RPTPψ* activity influences both adhesion and migration processes during convergent extension movements, and on Wnt-directed transcriptional regulation of the somite oscillator. This paper thus provides the first hint for a possible role for the non-canonical Wnt-signaling pathway in the regulation of zebrafish somitogenesis.

1.9 Mesenchymal to Epithelial transition during somitogenesis – a closer look!

In the growing vertebrate embryos for the first 24 hours, starting at around 10 hpf, mesenchymal cells in the tail bud region undergo epithelial transition and thus give rise to somites. This dynamic process is called MET-Mesenchymal to Epithelial transition. MET involves extensive cytoskeletal changes. In chick embryos these changes are mediated by members of the Rho GTPase super family, namely Rac1, and Cdc42 (Nakaya et al., 2004).

During somite formation, a palisade- like structure called intersomitic furrow is formed by the alignement of the PSM cells positioned on either side of the prospective intersomitic boundary (Fig 4A). In zebrafish, there is virtually no cell movement during this process (Wood and Thorogood, 1994), as opposed to the situation in chick embryos (Kulesa and Fraser, 2002). Nevertheless, transformation from a mesenchymal to epithelial morphology brings about changes in the boundary cells. Aspects of epithelialization characteristic of cells at somite boundaries are

(i) Acquisition of a columnar shape
(ii) Accumulation of molecules associated with adhesion complexes, such as β-catenin, at the apical pole of cells (Fig 4B)
(iii) Basally directed relocalization of cell nuclei towards the somite boundary (Fig 4C)
(iv) Apical relocalization of centrosomes (Figure 4D)

These changes are initiated simultaneously with, and not prior to, intersomitic boundary formation. The cells within the core of the somite remain mesenchymal, while the cells at the boundary become epithelialized.

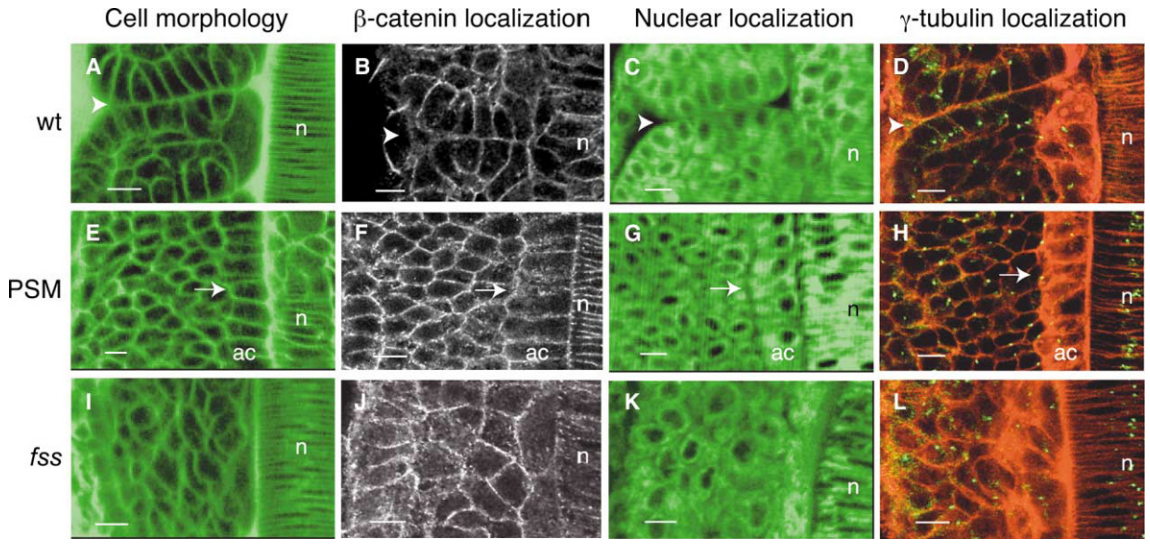


Figure 7 Cells Undergo Mesenchymal-to-Epithelial Transition at Somite Boundaries. (A–L) Dorsal views of the left-sided paraxial mesoderm of embryos labeled with Bodipy ceramide (which reveals cell morphology; [A], [E], and [I]) or with Bodipy 505-515 (which reveals nuclear position, [C], [G], and [K]) or immunostained for β-catenin ([B], [F], and [J]) or for γ-tubulin (which labels centrosomes) and stained with phalloidin (which labels actin) ([D], [H], and [L]). Anterior is oriented toward the top. (A–D) Cells at somite boundaries in wild-type embryos. The arrowheads point to the intersomitic boundary. (E–H) Cells in the presomitic mesoderm (PSM) of wild-type embryos. The arrows point to epithelial adaxial cells in which centrosomes are apically localized (H), as also seen in epithelial cells at somite boundaries (D). Centrosomes are randomly positioned in other PSM cells. (I–L) Cells in the somitic mesoderm of *fss*$^{-/-}$ embryos. n, notochord; ac, adaxial cells. The scale bars represent 10 μm. This figure and legend has been taken from Barrios et al., (2003).

In zebrafish embryos, Eph receptor/Ephrin signaling plays a significant role in driving the morphogenetic events associated with somite epithelialization. Numerous members of the Eph family of transmembrane receptor tyrosine kinases and other Ephrin ligands are expressed in a segmental pattern in the rostral presomitic mesoderm (Durbin et al., 1998) This pattern leads to the establishment of a receptor-ligand interface at each site of somite furrow formation. In the *fused somites* (*fss/tbx24*) mutant embryos, the cells in the paraxial mesoderm fail to undergo MET, as a result of which they fail to form intersomitic boundaries and epithelial somites. As the cells fail to epithelialize, they remain mesenchymal (Fig 4I), β-catenin appears localized homogeneously throughout the cell membrane (Fig 7J), nuclei remain at the centre of the cells (Figure 7K), and

centrosomes are distributed randomly within the cytoplasm (Figure 7L). The PSM cells in *fss* mutant displays the characteristics of mesenchymal morphology and not of epithelial morphology, hence they resemble the mesenchymal cells present in the core of the PSM in wild type embryos (Figures 7E-H). Additionally, cells with epithelial morphology can be seen in the PSM of wild type embryos, at sites where the paraxial mesoderm borders with the notochord, neural and surface ectoderm, and lateral plate (the arrows in the Figures 4E-H) (Barrios et al., 2003).

The *fused somites fss/tbx24* mutants are also characterized by the absence of Eph/Ephrin signaling interfaces. Restoration of the Eph/Ephrin signaling interface is capable of rescuing the formation of morphological distinct boundaries in the paraxial mesoderm of *fused somites* mutant embryos (Barrios et al., 2003; Durbin et al., 2000).

1.10 Somite Differentiation

Somites give rise to the axial skeleton and the skeletal muscle of the trunk. Zebrafish require large muscles to locomote through their relatively viscous aquatic environment. As the fish is supported by the buoyancy of water and their swim bladder, it has no use for the robust skeleton needed to support the terrestrial vertebrates. Hence zebrafish somite is predominantly mytome which gives rise to muscles as opposed to sclerotome from which the skeletal components such as vertebra and ribs are derived. Zebrafish sclerotome can be identified morphologically, shortly after somite formation, as a cluster of cells on the ventromedial surface of the somite which lies ventral to the myotome. *pax9* and *twist* are expressed in this cluster of mesenchymal cells (Morin-Kensicki and Eisen, 1997; Nornes et al., 1996), many of which will migrate dorsally to encircle the spinal cord and notochord, forming the vertebrae (Fig. 8C, F). In the adult, zebrafish muscle fibers can be subdivided into two broad classes (Bone, 1978). Slow muscle fibers, which are specialized for slow swimming and fast muscle fibers, used during bursts of rapid swimming. Slow muscle fibres are found in a wedge-shaped triangle on the lateral surface of the adult myotome whilst fast muscle fibers, are located in the deep portion of

the myotome. Slow fibers are smaller, darker, and more heavily vascularized than fast fibers (see review by Stickney et al., 2000).

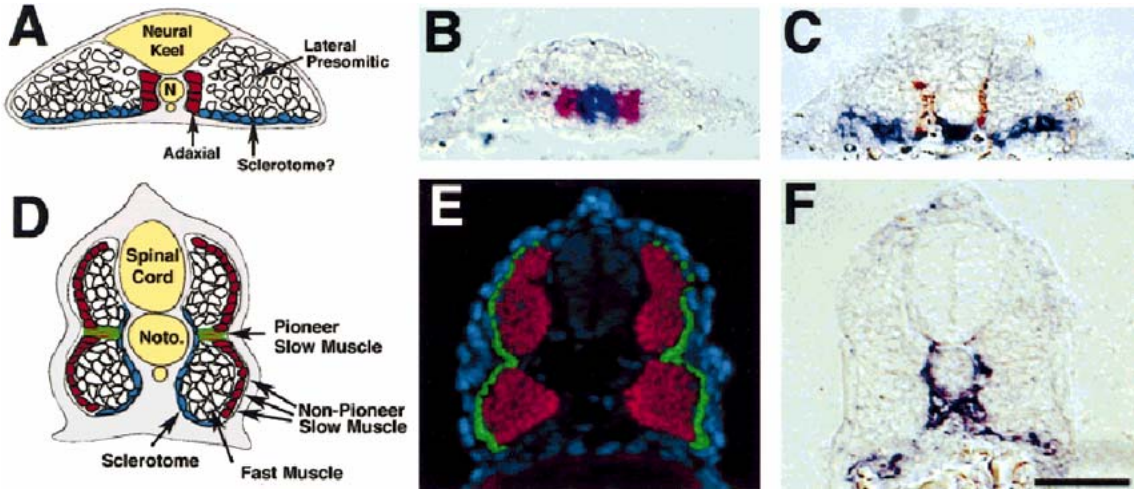


Figure 8. The four cell types of zebrafish somite. Depicted in this figure are the starting and final positions for cells expressing markers of sclerotome (*twist*), slow muscle (S58, F59) and fast muscle (ZM4). (A-C) Cross-sections of zebrafish embryos at 13 hr. (A) Schematic cross-section through the anterior psm, showing the relative positions of fast muscle precursors (lateral presomitic), *twist* expressing cells (sclerotome?), slow muscle precursors (adaxial). (B) Adaxial cells express *myoD* (red) while still adjacent to the *shh*-expressing notochord (blue) in the segmental plate. (C) *twist* expressing cells (blue) are initially ventral to the myotome and are separated from the notochord by the medial-most adaxial cells that give rise to slow muscle fibers (brown, F59). Dark blue staining directly beneath the notochord is *twist* labeling of the hypochord. (D-F) Cross-sections of zebrafish embryos at 24 hr. (D) Schematic cross-section through a 24 hr zebrafish embryo, showing the positions of the four characterized cell types. (E) Slow muscle cells (green) form a superficial monolayer whereas fast muscle cells (red) remain deep. (F) *twist* expressing cells (blue) at 24 hr are found ventral and medial to the myotome, such that expression is directly adjacent to the notochord and ventral spinal chord. Dorsal is to the top. Scale bar = 100 μm (B, C, E, F).This figure and legend has been taken from Stickney et al, (2000).

1.11 Medical relevance of somitogenesis studies

Spondylocostal dystostosis (SCD) is a term given to a heterogeneous group of disorders in humans, with severe axial skeletal malformation, characterized radiologically by multiple vertebral segmentation defects. Additionally, in these patients the ribs are frequently misaligned, with points of fusion and sometimes reduction in number.

Mutations in *Delta-like 3* (*DLL3)*, which codes for one of the Delta ligands for the Notch receptor in humans; as well as *MESP2*, a basic-helix-loop-helix transcription factor, have been shown to cause SCD in humans. Somitogenesis studies in lower vertebrates such as zebrafish and mouse, may someday provide detailed information facilitating better understanding and treatment of human SCD (Whittock et al., 2004).

1.12 Central Theme of Doctoral thesis

The objective of the present doctoral thesis was to identify new genes, which regulate the zebrafish segmentation clock or somitogenesis oscillator. The search was initiated by looking for genes which interact with or belong to the Notch pathway or Wnt/β-Catenin signaling pathway, as there is compelling evidence that both these pathways play significant roles in regulating somitogenesis in both zebrafish and mouse. To identify new candidates, I participated in two cDNA RNA *in situ* screens conducted the lab namely

(i) Screen for *hairy (h) and enhancer of split (E(spl))* family genes in zebrafish (Sieger, D et al., 2004)

(ii) Selective rescreening of the NIH zebrafish cDNA *in situ* expression database from the lab of Dr.Igor Dawid (Kudoh et al., 2001).

The experimental strategy was to select potential new genes involved in somitogenesis by analyzing the mRNA expression patterns in wild type zebrafish embryos and later in the somitogenesis mutants namely, *after eight (aei* /mutant for *deltaD), deadly seven (des/* mutant for *notch1a*), *beamter* (*bea/*mutant for *deltaC*) and *fused somites* (*fss/*mutant for *tbx24*). This would give a preliminary hint as to which among them would possibly function in regulating somitogenesis. All of these mutants encode genes belonging to the Notch signaling cascade, with the exception of *fused somite* (*fss/*mutant for *tbx24*) which codes for a T-box gene.

I focused on two candidate genes which came out of the screens namely *her15* and *ZfChp*. *her15* was analysed for its potential role as a novel component of the zebrafish presomitic mesoderm oscillator. *ZfChp*, on the other hand was examined for its role as a link between somite prepatterning and downstream processes such as somite border formation, as suggested by its mRNA expression pattern in the intermediate PSM region.

2. Materials

2.1 Buffers

Buffers and solutions have been prepared according to Sambrook et al., (1989).

2.2 Primers and RZPD clones

The primers used were synthesized by the company Metabion and are listed in the following table. The lyophilized primer was dissolved in the appropriate volume of water to obtain a concentration of 100µM.

Table2. Primers used

No	Name	Sequence from 5' to 3'	Hybridization Temperature
1	T3 Primer	AAT TAA CCC TCA CTA AAG GG	50°C
2	T7 Primer	TAA TAC GAC TCA CTA TAG GG	50°C
3	SP6 Primer	ATT TAG GTG ACA CTA TAG	50°C
	SP6 primer pCS2	CCC AAG CTT GAT TTA GGT GAC	50°C
	T7 primer pCS2	AAT ACG ACT CAC TAT AG	50°C
4	Zf Chp For-Cla1	ACA TCG ATA TGC CAC CTC AAA TGG AT	56°C
5	Zf Chp rev-Xho1	TAC TCG AGT CAG ATG AAG CAG AAG AA	56°C
6	T3-esr7up	AAT TAA CCC TCA CTA AAG GGC TCC TGC GTA TAT G	55°C
7	T7-esr7downrc	TAA TAC GAC TCA CTA TAG GGT CTC CAG AGC GGA G	55°C
8	H15overexp Cla1for	GCA TCG ATA TGG CTC CTG TGT ATA TGAC	58°C

9	H15overexp Xho1rev	TAA CTC GAG CTA CCA GGG TCT CCA GAG	58°C
10	H15traceFor Amp	GTG GGA AAG CTA ATC CTG AC	52°C
11	H15trace RevAmp	TGC TTG ATG TGT GTG TGC TG	52°C
12	H15 For 5'UTR check	ATC AGT GCA CGC TGA TGT TC	52°C
13	2H15 For 5'UTR check	GAT GCC TCT TCC ATT GTG TG	52°C
14	H15 Rev 5'UTR check	TGC TTG ATG TGT GTG TGC TG	52°C
15	T3-her1-29	AAT TAA CCC TCA CTA AAG GGT GTA TCG TCT TCT T	45°C
16	T7-her1-1037	TAA TAC GAC TCA CTA TAG GGT CTC CAC AAA GGC T	45°C

Table 3. RZPD clones corresponding to the ESTs from NIH cDNA *in situ* expression database and their cloning vectors

No	NIH cDNA *in situ* expression database	RZPD clone ID	Vector
1	Clone No 5116	IMAGp998D158968Q3	pSPORT1
2	Clone No 5096	IMAGp998E028969Q3	pSPORT1
3	Clone No 5144	IMAGp998A178977Q3	pSPORT1
4	Clone No 3259	IMAGp998K0510339Q3	pSPORT1
5	Clone No 2247	IMAGp998C028964Q3	pSPORT1
6	Clone No 5038	LLKMp964N1517Q2	pBK-CMV

2.3 Sequence of morpholinos used in gene knock down experiments

her1 morpholino -5′-AGT ATT GTA TTC CCG CTG ATC TGT C-3′

her7 morpholino – 5′-ATG CAG GTG GAG GTC TTT CAT CGA G-3′

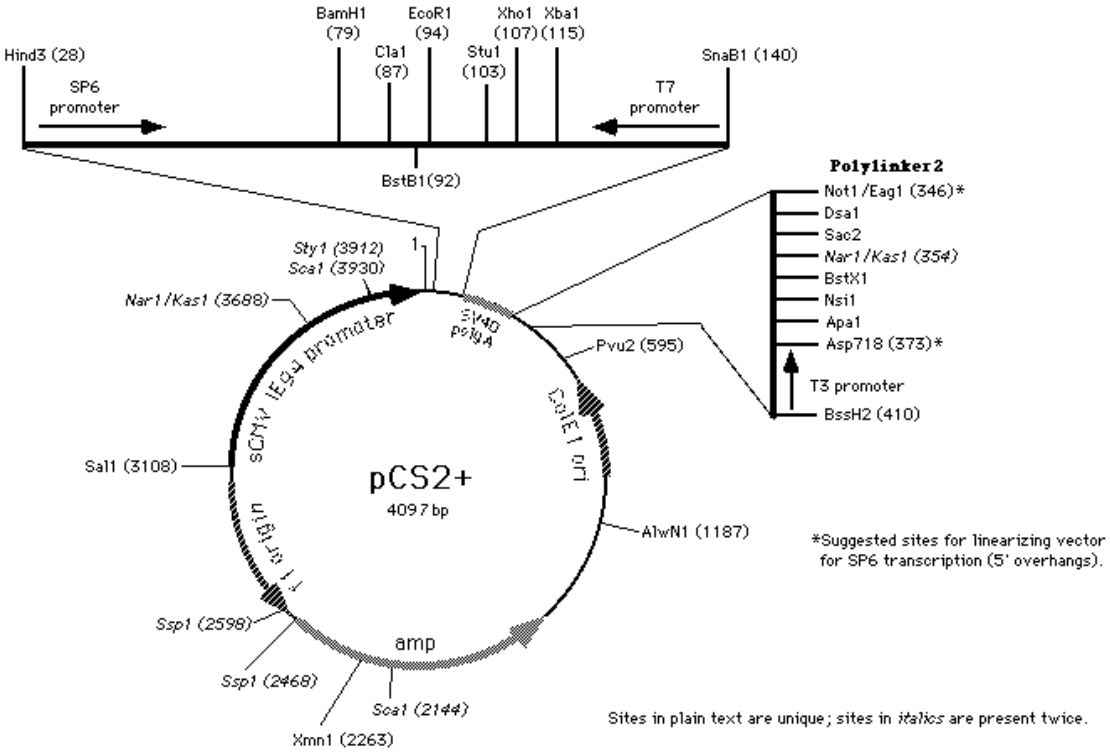
Su(H) morpholino – 5′-CAA ACT TCC CTG TCA CAA CAG GCG C-3′

ZfChp morpholino – 5′-ACC CTC TGC TTA CCC GAG AAG ACG T-3′

her15 morpholino – 5′-CAG GAG CCA TTG CTT CTT CAG GAG A-3′

2.4 Vector maps

pCS2+ is the standard vector used in zebrafish studies for misexpression experiments. As control for these functional studies, a modified pCS2+eGFP is used. pCS2+eGFP vector was constructed by cloning the Eco47III/XhoI-EGFP fragment from the pEGFP-C1 vector into the StuI/XhoI site of the CS2+ vector.



2.5 Computer system

The data acquisition as well as the picture formating and word processing were done using personal computers with operating systems Windows 2000 and Windows XP Professional.

2.6 Software

Acrobat Reader 5.0 (*Adobe*)
AxioVision 2.0.5.3 (*Zeiss*)
BioEdit 5.0.7 (*Tom Hall, Department of Microbiology, North Carolina State University*)
Entrez (*National Centre for Biotechnology Information = NCBI*)
Photoshop 6.0 (*Adobe*)
Vector NTI 6.0 (Info Max, Inc.)
Microsoft Office 2000 Premium

Moreover, the services of PubMed (*NCBI*), Blast (Altschul et al., 1997), Zebrafish EST Database (http://zfish.wustl.edu/) (*Washington University, St. Louis*) and ZFIN (www.zfin.org) (Sprague et al., 2001; Sparague et al, 2003) have been utilized and are acknowledged.

3.Methods

3.1 Zebrafish methods

3.1.1 Rearing of zebrafish and collection of embryos

3.1.1.1 Origin of the zebrafish

The zebrafish *Danio rerio* is a fresh water fish from the Ganges that belongs to the family *Cyprinidae*. The animals in our facility were acquired from pet shops in Cologne and Gottingen. These parent animals were then further bred.

3.1.1.2 Growth conditions

Starting from day2, zebrafish were grown in an aquarium, consisting of several serial 12L tank units, with a water temperature between 26 and 28°C (Mullins et al., 1994). The maximum extent of utilization of a unit amounted to 40 fish per liter. The aquarium was supplied continuously with fresh water, whereby daily 1/10 of the liquid volume was replaced by fresh water. One half of the fresh water was adjusted by means of an ion exchange resin to a total hardness between 6-10 degrees of hardness units; the other half was transmitted from a reverse osmosis plant. Within the aquarium, the water was circulated by a pump system. Suspended particles were sieved by integrated filter units from the water and the filtered water was sterilized afterwards by UV irradiation. The accumulation of toxic substances (e.g. nitrite) was prevented by using a bacterial filter. Feeding of the fish took place thrice daily. Beside the usual trockenfutter (Tetramin), Artemia and Bosmina were fed, in order to ensure balanced nutrition. The light and darkness rhythm was adjusted to 14 hours light and 10 hours darkness.

3.1.1.3 Zebrafish embryos

The collection of zebrafish embryos for various experiments like total RNA isolation and *in situ* hybridization took place in the morning, directly after the dark phase came to an end. The previous evening the male and the female zebrafish pair were put in plastic boxes with a divider that separated them. The males and females can be differentiated by the shape of the under belly. The under belly of the female fish will be rounder. These plastic boxes contained normal aquarium water and the bottom was lined with marbles to prevent the eggs from being eaten by either of the parents after they had been laid. The divider was removed in the morning as soon as the light phase had started. The fish were then allowed to mate for 30 min to 1 hr and the eggs were collected in petridishes containing autoclaved aquarium water. These embryos were then allowed to develop at 28.5°C in an incubator and the desired stages were collected.

Solutions

Autoclaved aquarium water

3.1.2 Dechorionisation and storage of zebrafish embryos

3.1.2.1 Mechanical dechorionisation of embryos

Embryos of the desired growth stage were fixed in 4% paraformaldehyde (PFA) in PBS (phosphate buffered saline). This fixation can take place for 2 hr at room temperature (RT) or overnight at 4°C. After fixation, the embryos were dechorionated with a pair of sterile, fine-pointed watch-makers forceps.

Solutions

<u>20X PBS</u>

2,76 M NaCl

50 mM KCl

160 mM Na_2HPO_4

50 mM KH_2PO_4

3.1.2.2 Storage of Embryos

The dechorionated embryos were then dehydrated by a series of methanol:PBST solutions (33% methanol/PBST, 66% methanol/PBST) and twice in 100% methanol. The incubation was for 10 min in each solution. After this, the embryos were stored at -20°C.

3.1.3 *In situ* hybrdization of whole embryos with digoxygenin labeled RNA probes

In situ hybridization by means of Digoxygenin labeled probes is a non-radioactive procedure, which makes it possible, to determine the spatial expression of mRNA (Tautz and Pfeifle, 1989). The embryos were incubated with digoxygenin labeled anti-sense RNA probes. The hybridized probes were then detected immunochemically, by means of alkaline phosphatase (AP) conjugated anti-digoxygenin Fab fragments, whereby the enzymatic conversion of specific substrates resulted in the production of colored precipitates.

For *in situ* hybridization of zebrafish embryos the protocol by Schulte-Merker et al., (1992) was followed with slight modifications. The Proteinase K treatment was replaced by heat treatment and the composition of some of the solutions was modified. All *in situ* hybridizations were carried out in the automated, *InsituPro* machine (Abimed) (Plickert et al., 1997)

3.1.3.1 Heat treatment of zebrafish embryos (personal communication from Gajewski,M)

The ribosomes and other proteins, which are associated with mRNA molecules, can be denatured by heat treatment. The molecule becomes more accessible for the labeled anti-sense mRNA probe in this way.

The embryos which had been stored after dehydration in methanol at -20°C were first rehydrated by a series of methanol: PBST solutions in the reverse order to that which has been described in section 3.1.2.2 and later transferred into PBST. The rehydrated embryos were then incubated in a 1.5 ml eppendorf containing 1ml of PBST. The eppendorf was then incubated for 10 min in boiling water and whirled every 2.5 min to prevent them from sticking together. Following this, the embryos were cooled down for 5 min on ice, in order to prevent renaturing of the proteins. Subsequently, the embryos were fixed for 20 min in 4% PFA in PBS.

3.1.3.2 Treatment with acetanhydrid

Incubation with acetanhydrid is carried out to inhibit the endogenous alkaline phosphatase activity. This procedure can reduce nonspecific background staining as in the case of use of alkaline phosphatase linked antibodies. The embryos were washed 2 times for 10 min with PBST. Subsequently, PBST was replaced by a solution of 2.5µl acetanhydrid/ml in 0.1M triethanol amine (pH 7.8), where the embryos were incubated for 10 min. Following this, the embryos were washed 4times for 10 min in PBST.

3.1.3.3 Prehybridization

The embryos were incubated first in a solution of 50%PBST/50% hybridization solution (hybridization solution is called hybmix). This was replaced after 5 min by hybmix, in which the embryos were incubated for 1 hr at 65°C.

3.1.3.4 Hybridization

For hybridization, embryos were incubated in hybmix containing antisense RNA probe for 16 hrs at 65°C. The antisense RNA probes were taken up in a small volume of hybmix (usually a concentration of 20-100ng of probe in 1ml hybmix was used).

3.1.3.5 Washing steps

After removing the probe, the embryos were incubated for 30 min at 65°C in hybmix. Subsequently and successively the embryos were washed 2 times for 30 min with a solution of 50% hybmix/50% 2xSSCT and 2times for 30 min with 0.2xSSCT at 65°C.

3.1.3.6 Incubation in antibody

After 2 times for 5 min and later 20 min of incubation in PBST at RT, the embryos were then incubated for 20 min in block I and thereupon 60 min in block II-solution. Block II was removed and replaced by a 1:2000 dilution of anti-DIG alkaline phosphatase linked antibody (Roche) in block II. This incubation was carried out for 4 hr at RT. Subsequently the embryos were washed 6 times for 10 min in PBST.

3.1.3.7 Color substrate reaction

The embryos were incubated 2 times for 5 min in AP buffer and then transferred into 24-well flat bottomed plates. The AP buffer was removed and replaced by BM Purple AP substrate (Roche). The color substrate reaction took place in the dark. Depending on the target mRNA concentration in the embryos and the concentration of the antisense probe used, the reaction took between 30min to several hours. For stopping the color substrate reaction, BM purple AP substrate was removed and the embryos were washed 2 times for 5 minutes with PBST. Then the embryos were fixed for 20 min in 4% PFA in PBS, in order to inactivate the alkaline phosphatase. To obtain a fluorescent red signal, BM Purple AP substrate and AP buffer were substituted by Vector Red kit (Linaris) and Histoprime Buffer pH 8.2 (Linaris).

3.1.3.8 Double *in situ* hybridization

For double *in situ* hybridization, both probes have to be added to the embryos at step 3.1.3.4 for hybridization. The antibody and color substrate reaction takes place separately for the second probe, after the first probe reaction is completed. Normally, if one probe is labeled with a fluorescien label and the other with a digoxygenin label, then the fluorescien labeled probe is visualized first. After the first probe reaction, the embryos were fixed in 4% PFA over night at 4°C. Then they were washed 2 times for 10 monutes each with PBST. Following this, they were incubated twice in a 0.1M Glycine solution containing 0.1% Tween 20, for 15 minutes. In the next step, they were washed twice for 10 minutes in PBST and then incubated in 4% PFA for 20 minutes. This was followed up with two 10 minute washes in PBST. Once the washes were completed, the embryos were incubated in Block I for 10 minutes and later in Block II for 1 hour. Next, the embryos were incubated for 6 hours in the antibody of the second probe to be visualized. After 6 hours, the antibody solution was removed and the embryos were subject to 8 PBST washes lasting 15 minutes each the first five times and then1 hour each. Extensive PBST washes are required to remove the excess antibody and thus reduce the probability

of unspecific background staining in the embryo. Finally, the embryos were subject to color substrate reaction as described in section 3.1.3.7.

Solutions for *in situ* hybridization

20 × PBS

2,76 M NaCl
50 mM KCl
160 mM Na_2HPO_4
50 mM KH_2PO_4

1 × PBST (used in all washing steps)

1 × PBS
0,1 % Tween-20

20 × SSC

3 M NaCl
0,3 M NaCitrat
pH 4,7

2 × SSCT

2 × SSC
0,1 % Tween-20

0,2 × SSCT

0,2 × SSC
0,1 % Tween-20

Blocking Solutions

Block I: 0,2 % BSA (Bovine Serum Albumin) in PBST

Block II: 0,2 % BSA / 5 % Sheep-Serum

AP-Buffer

100 mM Tris pH 9,5

50 mM $MgCl_2$

100 mM NaCl

0,1 % Tween-20

5 mM Levamisol

Hybmix

50 % Formamide

5 × SSC

1 % *Boehringer Block* (Roche)

1 mg/ml Helper RNA (Roche)

100 µg/ml Heparin

1 × Denhards

0,1 % Tween-20

0,1 % Chaps

5 mM EDTA

3.1.4 Analysis of embryos after *in situ* hybridization

3.1.4.1 Analysis by whole-mount preparations

The embryos were transferred into 4% methylcellulose (sigma), on a hollow grinding slide and bought into a suitable position using a fine needle. The embryos were then analyzed using a stereomicroscope (MZFLHIII; Leica) and photographed with a digital camera (Axiocam, Zeiss).

3.1.4.2 Analysis by flat-mount preparations

For making flat preparations, the embryos were transferred over an ascending glycerol gradient into 100% glycerol on a glass slide. The embryo was crushed and stripped of yolk using a pair of fine-pointed watchmaker's forceps. The yolk was removed entirely from the region of interest, namely the presomitic mesoderm using a preparing needle. After this, the embryo was transferred into a fresh drop of glycerol using a preparing needle and the remaining yolk cells were removed with a lash. The embryo was oriented using the preparing needle on the glass slide and then covered with a cover slip. The analysis of the preparation took place with a microscope (Axioplan 2, Zeiss) and the embryos were photographed with a digital camera (Axiocam, Zeiss). Irene Steinfartz and Eva Schetter provided the flat preparations.

3.2 Molecular biology protocols

3.2.1 RZPD clones

The NIH zebrafish cDNA *in situ* Expression Database was screened and the clones of interest were ordered from RZPD (Resourcezentrum für Genomforschung). These clones are listed in Table 2.

These zebrafish EST sequences had been ligated into vectors like pSPORT and pCAM which carried different antibiotic resistance clones and had been cloned into *E.coli* cells.
On arrival, these clones were streaked onto LB amp plates containing the antibiotic of interest and allowed to grow overnight at 37ºC (Ampicillin-50µg/ml and Kanamycin – 50µg/ml). Subsequently, single colonies were selected from this plate and inoculated into LB medium containing the antibiotic of interest. *E.coli* culture maintenance was according to standard protocols (Sambrook *et al.*, 1989).

3.2.2 Diatamaceous earth plasmid DNA miniprep

1.5 ml of over-night bacterial culture (*E.coli*) was transferred into an eppendorf and the cells were pelleted by centrifugation for 30 sec. The supernatant was then removed by aspiration. To the tube, 200µl of Solution I was added and the pellet was resuspended completely by vortexing. Following this, 200µl of Solution II was added and each eppendorf tube was inverted gently 10 times. Subsequently 200µl of Solution 3 was added to each tube and inverted gently for 10 times. The cell debris was then precipitated by centrifugation for 5 min. During the centrifugation, a filter column (Bio rad) was inserted into a 2ml eppendorf tube. The lysate obtained after centrifugation was transferred into filter columns. To this was added 200µl of DNA binding matrix and mixed well by pipeting up and down 5 times. This was then centrifuged for 30s and the flow through was discarded. 500µl of wash buffer was added two times and centrifuged 30s and 2 min. Each time the flow through was discarded. The filter column was then transferred into a clean 1.5 ml eppendorf tube and 100µl of elution Buffer (10mM Tris pH 8.0) was added. This was centrifuged for 1 min and the samples were stored at -20°C.

Solutions

Stock Concentration	**Volume**	**Final Concentration**
LB Medium		
Peptone 140	10 g	
Yeast extract	5 g	
NaCl	10 g	
H_2O	to 1000 ml	

Sterilize by autoclaving

Cell Resuspension Buffer (Solution 1)

1 M Glucose	50 ml	50 mM
1 M Tris-Cl pH 8.0	25 ml	25 mM
0.5 M EDTA pH 8.0	20 ml	10 mM
RNase A (10 mg/ml)	2 ml	20 μg/ml
H_2O	903 ml	
	1000 ml	

Alkaline Lysis Solution (Solution 2)

10 N NaOH	20 ml	0.2 N
10% SDS	100 ml	1%
H_2O	880 ml	
	1000 ml	

Neutralization Solution (Solution 3)

Guanidine-HCl	506 g	5.3 M
3M KOAc pH 5.0	233 ml	0.7 M
H_2O	to 1000 ml	

Binding Matrix

Guanidine-HCl	253 g	5.3 M
1M Tris pH 8.0	10 ml	20 mM
H_2O	to 500 ml	

Then add:

Diatomaceous earth (Sigma D-3877)	75 g	0.15 g/ml

Wash Buffer

1 M Tris-Cl pH 8.0	20 ml	20 mM
0.5 M EDTA pH 8.0	4 ml	2 mM
5 M NaCl	40 ml	0.2 M
100% EtOH	500 ml	50%
H_2O	436 ml	
	1000 ml	

3.2.3 Quantification of DNA by Spectrophotometric determination

For quantification of amount of DNA, reading is taken at a wavelength of 260 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An O.D of 1 corresponds o approximately 560µg/ml of double-stranded DNA. The readings were taken using a Biophotometer (Eppendorf).

3.2.4 Sequencing of DNA

The sequencing of DNA took place according to the Dideoxy Chain Termination method (Sanger et al., 1997). With the help of the terminator ready reaction mix (Amersham/Pharmacia), a linear amplification was accomplished. Besides dNTPs, this mix also contains Dideoxynucleotidetriphosphate (ddNTPs), for each of the four different dNTPs with a specific flurophore attached. Synthesis is initiated at a specific site on the sequencing template, as determined by the annealed sequencing oligonucleotide primer. The synthesis reaction is terminated by the incorporation of a dideoxynucleotide analog (ddNTP) that will not accept further elongation of the synthesized strand. The incorporation of one ddNTP during the extension phase causes premature termination of polymerization.

3.2.4.1 Sequencing Reaction

The reaction mix contained 2µl DNA (0.1-0.25ng/µl), 2µl primer (3µM) and 2µl terminator ready reaction mix and the total volume were made up to 10µl using water. The primers used for sequencing are given in Table 2 in the Materials section.

3.2.4.2 Reaction Profile

Initial denaturation of DNA took place at 96°C for 1 min. At the beginning of a cycle, there is 10 seconds of denaturation at 96°C. The annealing of the sequencing primer took place for 15 seconds at 50°C (The annealing temperature depends on the sequence of the primer used). The extension was accomplished at 60°C for 4 min. The cycle was repeated 25-30 times.

3.2.4.3 Cleaning of the reaction products

The sequenced product DNA was transferred into a sephadex G50 matrix column (Amersham/ Pharmacia) and centrifuged for 2 min at 1500xg. The flow through was discarded. The column was then transferred into a clean, labeled eppendorf tube. 10µl of water was added and then the DNA was dried completely using a vacuum concentrator (DNA *Mini*; vacuum dry). The dried pellet was dissolved in 1.5µl of formamide loading buffer and loaded onto a sequencing gel.

3.2.4.4 Analysis

The analysis of the sequencing reactions was carried out on an *ABI3700* (Applied Biosystems / Perkin Elmer) machine.

3.2.5 Linearisation of plasmid DNA by restriction digestion

The sequencing reactions were carried out to make sure that the RZPD clones obtained contained the EST of interest. The RZPD clones will have the EST sequence cloned into the polylinker site of an appropriate transcription vector, which contains adjacent to the polylinker a promoter for SP6, T7 or T3 polymerase. For the synthesis of 'Run Off' transcripts the plasmid is linearised by a suitable restriction enzyme. Restriction enzymes creating 5' overhangs are used. 3' overhangs should be avoided.

3.2.5.2 Restriction enzyme digestion of plasmid DNA

The reaction consisted of plasmid DNA 1µl (1.0µg), restriction enzyme 10X buffer 2µl, restriction enzyme 1µl (10 units/µl) and 16µl of water that made the total volume of the reaction 20µl. This was incubated at 37°C for 2 hr.

3.2.5.3 Phenol-Chloroform extraction

The standard way to remove proteins from nucleic acid solutions is to extract first with phenol: chloroform and then with chloroform. To the restriction enzyme digestion, is added equal volume of phenol-chloroform and mixed together until an emulsion forms. It is then centrifuged at 12,000g for 15s, at room temperature. Using a pipette, the aqueous phase is transferred into a fresh eppendorf tube and the interface along with the organic phase is discarded. Subsequently, an equal volume of chloroform is added to the sample and the centrifugation step and the transfer of the aqueous phase is repeated.

3.2.5.4 Ethanol Precipitation

To the sample obtained is added 1/10 volume of Na.acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol. This was then incubated at -20°C for 30 min and then centrifuged at 12,000xg for 20 min. The pellet thus obtained was washed with 500µl of 70% ethanol and centrifuged at 12,000xg for 5 min. The pellet was then air dried for 5 min and dissolved in 10 to 20µl of water.

3.2.6 Agarose gel electrophoresis

This was carried out for the electrophoretic separation of DNA according to size. Agarose gels of concentration 0.8% to 1.2% were cast in 1X TAE buffer (Tris-Acetate EDTA). The electrophoresis was carried out in a horizontal flat bed gel apparatus using 1X TAE as buffer, according to standard protocols (Sambrook et al., 1989). The DNA was dissolved in gel loading buffer before loading in the gel. To be able to visualize the DNA bands, 4µl of ethidium bromide solution (10µg/ml) was added to 100ml of the melted agarose gel. Ethidium bromide will intercalate into DNA and will fluoresce on absorption of UV light. The DNA was visualized using on UV-Transilluminator (Image Master VDS; Amersham/Pharmacia) and photographed.
DNA size standards used: 2-Log ladder (NEB)

Solutions

Ethidium Bromide Solution

1g of Ethidium Bromide dissolved in 100ml of H_20.

50X TAE Buffer

242g Tris base

57.1 ml glacial acetic acid

100ml 0.5M EDTA

6X Gel Loading Buffer

0.25% Bromophenol Blue

0.25% Xylene Cyanol FF

30% Glycerol

(Stored at room temperature)

3.2.7 Invitro transcription for the production of *in situ* probes

The production of labeled antisense RNA probes for *in situ* hybridization was made using the polymerase T7 or T3 (Roche), according to the promoter present at the 3' end of the coding region. The probes were labeled using Digoxygenin RNA labeling mix (Roche). The transcription reaction consisted of 500ng DNA, 1µgl of 10X labeling mix, 1µl of 10X transcription buffer (containing 60mM $MgCl_2$), 0.5µl of RNase inhibitor (40 U/µl, Roche, Mannheim) and 1µl RNA polymerase (20U/µl). DEPC water (DEPC=Diethylpyrocarbonate, Rnase free water) was added to the reaction to give a final volume of 10µl. The transcription reaction was incubated for 2 hr at 37°C and stopped by addition of 1µl of RNase free 0.2M EDTA. Subsequently the RNA transcripts were purified by ethanol precipitation as described in section 3.2.6.4. The RNA pellet obtained was dissolved in a mixture of 20µl of formamide and 20µl of DEPC water and stored at -20°C. The probe was analyzed by loading 5% of the reaction in a 1% agarose gel.

3.2.8 Preparation of total RNA and cDNA from zebrafish embryos

Total RNA was prepared from 150-200 somitogenesis stage embryos namely, 8-12 somite, using the protocol and solutions from the Perfect RNA Eukaryotic Mini Kit

(Eppendorf). This RNA was then used to make cDNA by first strand synthesis. The initial reaction consisted of 1ng-1µg total RNA, 1µl oligo dT (0.5 µg/µl stock) and water to make up the total volume to 17.75µl. The reaction was incubated for 5 min @ 70°C and then for 5 min on ice. Following this, 5µl M-MLV 5X reaction buffer,1.25µl of dNTP's(10mM stock), 1µl M-MLV RT(H-)(50-100 units) (RT=Reverse Transcriptase enzyme) and autoclaved water to a maximum volume of 25µl. The reaction was then incubated for 60 min @ 42°C and finally for 15 min @ 70°C.

3.2.9 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify a specific region of DNA, in order to produce enough DNA fragments for cloning and analysis. *ZfChp* and *her15* full length sequences amplified by PCR using the following primer sets namely

(a) Zf Chp For-Cla1 and Zf Chp rev-Xho1

(b)H15overexp Cla1for and H15overexp Xho1rev.

The 50µl PCR cocktail consisted of 1-10ng of template DNA, 300nM of primer1 and primer2, 2.6 Units of Taq polymerase enzyme, 5µl of 10X PCR buffer containg $MgCl_2$, additional 2.5µl of $MgCl_2$ and water to make up the final volume.

The standard reaction conditions for fragments less than 3 kb:

(1) 2 min @ 95°C for Denaturation

(2) 15 sec @ 95°C

(3) 30 sec @ 50°C for DNA synthesis

(4) 2 min @ 72°C

(5) 29 repeats of steps 2-4

(6) 5 min @ 72°C for elongation

(7) hold @ 16°C

The amplified DNA, namely the PCR product, was then run on an agarose gel and then extracted following the protocol and solutions of Rapid Gel Extraction Kit (Marligen). The gel extracted purified DNA was then digested with the appropriate restriction enzymes to generate overhangs which can be ligated. The restriction enzyme digestion

was carried out as explained previously. The digested fragments are then purified using protocol and solutions from Rapid Gel Extraction Kit (Marligen).

3.2.10 Ligation

Adequate concentrations of the vector and insert, namely either in 1:1 or 1:3 ratio, was taken and incubated overnight at 16°C. The ligation mix consists of digested insert DNA, digested vector DNA, 10x Ligation Buffer –1 µl, Ligase enzyme –1µl (400,000 units/ml concentration) and finally autoclaved water to a final volume of 10µl.

3.2.11 Cloning

Following over night incubation at 16°C, the ligation mix was transformed into competent cells to enable cloning of the insert namely *ZfChp* and *her15* full length into the vector pCS2+ .The cloned constructs can then be used for RNA misexpression studies in zebrafish embryos. The Vector map of pCS2+ is given in the Materials section.

3.2.11.1 Preparation of competent cells

5 ml of LB Tet was inoculated with one colony from *XL1blue* strain of *E.coli* cells which had been streaked out on an LB plate. The LB Tet was allowed to grow over night at 37°C. The next day the culure was diluted to 1:500 in 250ml SOB medium. This was grown at 18°C until OD 600=0.6 was reached. One the specific optical density was reached, the cells were kept on ice for 10 minutes and then spun at 2500xg (5000 rpm in a Sorvall GSA or 3000 rpm ina Beckman J-6B centrifuge) for 10 min at 4°C.The cells were then resuspended gently in 20 ml of ice cold TB solution. Following this, the cell suspension was transferred into two 15 ml falcon tubes and the volume was estimated. DMSO was added to a final concentration of 7% of the estimated volume a nd mixed by gentle inversion. It was then placed on ice for 10 min .The suspension was later aliquoted into 1.5 ml pre cooled, autoclaved, eppendorf tubes and immediately quick frozen using liquid nitrogen. Finally the aliquots of competent cell were stored at -80°C.

3.2.11.2 Transformation

For transformations, an aliquot was removed from the -80°C and thawed on ice. Once the cells are completely thawed, they are mixed with 5-10µl of ligation mix and incubated on ice for 20 min. It was then heat shocked at 42°C for exactly 2 min. After heat shock, the cells were immediately cooled down on ice for 10 min and then diluted with 1 ml of LB medium. This was allowed to grow for 1 hour at 37°C. Later, the culture was spun down at 4000 rpm for 2 min to obtain a pellet and three quarters of the medium was removed. The pellet was then redissolved in low volume of the remaining medium and plated in different concentrations on LB plates carrying the appropriate antibiotic resistance. The LB plate was then incubated over night at 37°C.

The following day, the LB plate was checked for positive transformants showing antibiotic resistance and verified using insert DNA specific PCR and sequencing. The sequencing is done to verify the accuracy of the cloned insert. This accurate clone is the then used for synthesising large quantities of capped mRNA for injections into 1 -2 cell stage zebrafish embryos.

3.3 Preparation of capped mRNA for zebrafish injections

The capped mRNA was made using the protocol and solutions from the commercial mMessage kit (Ambion).

3.4 Synthesis of morpholinos

The appropriate sequences for morpholino design were selected from the full length sequences of the gene candidates and sent to the company Gene tools for synthesis. The sequences of all the morpholinos used in experiments, have been listed in the Materials section.

3.5 Injection of zebrafish embryos

Zebrafish embryos of 1-2 cell stage were injected with varying concentrations of morpholino ranging from 0.5 mM to 1 mM .The injection solution additionally contains 0.1 M KCL and 0.2% Phenol red. Injections were carried out using FemtoJet®, and a Micromanipulator (Eppendorf).

In the case of RNA misexpression/ Gain of function experiments, capped mRNA of concentrations ranging from 50ng to 500ng was injected in zebrafish embryos of 1-2 cell stage. GFP mRNA synthesised using the pCS2+eGFP was injected as control, in the same or higher concentrations as compared to the candidate gene, to enable the proper verification of morphological and *in situ* effects obtained.

3.6 Confocal imaging

The confocal sections were made using Leica TCS SP2 confocal microscope.

3.7 Eppon embedding and sectioning

Eppon embedding and sectioning were carried out according to protocols described in Nüsslein-Volhard and Dahm R, (2002).

4. Results

4.1 *hairy (h)* and *enhancer of split (E(spl))* related genes in zebrafish

In all vertebrates known to date, the cyclically expressed genes in the PSM are those which belong to the Delta-Notch signaling pathway and *hairy (h)* and *enhancer of split (E(spl))* family, with the only exceptions being *Axin2* and *Nkd.1* in mouse (Aulehla et al., 2003; Ishikawa et al., 2004; Rida et al., 2004). Efforts to identify cyclically expressed Wnt signaling pathway genes in zebrafish by us and other research groups have been unsuccessful. For a better understanding of the process of somitogenesis in zebrafish, an attempt was made to identify the total number of *hairy (h)* and *enhancer of split* [*E(spl)*] genes and to classify their mRNA expression in the posterior PSM as cycling or non cycling. An analysis of the latest release of the zebrafish genome namely version 3.1x (85% complete) was carried out and a total of 25 *her* genes were discovered (Sieger et al., 2004). Out of the 25, of which several are already known, there are at least 6 cyclically expressed genes (Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2000; Holley et al., 2002; Oates and Ho, 2002; Winkler et al., 2003). One of the candidates which came out of this screen is *her15*, which has been analysed and the data presented in this PhD thesis.

4.1.1 Molecular nature of *her15*

her15 is a member of the *hairy (h)* and *enhancer of split (E(spl))* gene family, in zebrafish, commonly referred to as *her* genes. It belongs to the mouse *Hes5* subfamily which includes *her2*, *her4* and *her12* in zebrafish (Sieger et al., 2004). It has the characteristic protein domains of *her* genes which include conserved basic-Helix I-Loop-Helix II, Orange domain and a WRPW motif at the C-terminus (for review see Davis and Turner, 2001). The protein consists of 149 amino acids. An alignment with other related members of the same family such as zebrafish *her12*, mouse *Hes5* and *Xenopus esr9* and

esr10, shows a certain degree of sequence conservation (Figure 9) (Table 4) (*her12*-Sieger et al, manuscript in preparation; (Dunwoodie et al., 2002; Li et al., 2003).

Table 4. Protein sequence comparison of *her15* with other mouse *Hes5* homologues

	Identity	similarity
her15 to *her12*	47.1%	58.7%
her15 to mouse *Hes5*	41.1%	50.6%
her15 to *Xenopus esr9*	37.2%	46.2%
her15 to *Xenopus esr10*	41%	50%

4.1.2 Analysis of *her15* mRNA expression domains

Expression of *her15* mRNA was examined by whole-mount *in situ* hybridization. *her15* mRNA transcripts appear first during late epiboly stages namely 60% to 70% epiboly. It is visible as stripes of cells labeling the marginal zone, and also on either side of the dorsal midline (white arrows point to the expression domains in Figure 10B). The dorsal midline is itself devoid of *her15* transcripts (white asterisk * in Figure 10B-C). As gastrulation proceeds further, at around 80% epiboly, the *her15* mRNA transcripts on either side of the dorsal mid line extend slightly towards the anterior/animal pole (white arrows in Figure 10C). On comparison to the fate map of a 60% epiboly zebrafish embryo, (Woo et al., 1997 and Grinblat et al., 1998), it can be seen that the *her15* cells label the precursor cells which will later give rise to somites and notochord (Figure 10A-C) (note that the embryo views are dorsal and the embryo in the fate map has a lateral view).

Starting at the tail bud stage, *her15* mRNA appears in the prospective mesencephalic region, as an initially ‘V’-shaped expression domain (black asterisk in Figure 11D-E). *her15* is also expressed in the primordium of the trigeminal ganglion (trg) (black arrows in Figure 11D-E). Two neuronal expression domains referred to as lateral stripe (ls) and

intermediate stripe (is) also appear at this stage. A third stripe of neuronal expression called medial stripe (ms) appears at 3-6 somite stage along the midline. *her15* displays elaborate and complex mRNA expression domains distributed throughout the embryo during somitogenesis stages.

Starting at the tail bud stage, *her15* mRNA shows cyclical/oscillating expression in the extreme posterior region of the PSM/tail bud. This dynamic expression domain has 3 primary phases namely, broad, intermediate and dot-like (Figure 10D-E, Figure 11A-C and Figure 12A-C). Whole-mount views show clearly only the broad and dot-like oscillating/cycling mRNA expression phases of *her15*. The intermediate phase is clearly visible only in the flat-mounts from bud stage to 3-6 somite stages. This is due to the fact that PSM tissue is broad only when the embryo is younger. As the embryo grows older namely 6-8 somite stage and 10-12 somite stages, the PSM tissue becomes narrower and hence only the broad expression phase and dot-like expression phase are clearly visible. The intermediate phase of *her15* mRNA expression is present during older stages namely 6-8 somite and 10-12 somite stages. But it cannot be clearly resolved at the mRNA *in situ* level by whole-mount or flat-mount preparation and photography, due to the narrowing of the PSM tissue. In addition to the broad expression domain in the posterior PSM, *her15* also shows stripe-like mRNA expression domains in the anterior PSM.

This cycling expression domain of *her15* in the posterior PSM is different compared to mRNA expression domains observed in other cycling genes like *her1* and *her7* (Gajewski et al., 2003; Holley et al., 2000; Muller et al., 1996; Oates and Ho, 2002). *her1* and *her7* in zebrafish, have an independent broad mRNA expression compartment in the posterior PSM, which has a ‘U’ shape and two broad stripes in the anterior PSM which appear to bud off from this U-shaped posterior domain. Both genes show oscillation in the broad expression domain in the posterior PSM and in the stripe-like expression domains in the anterior PSM and hence they give the impression of mRNA expression sweeping across the PSM like a wave from posterior to the anterior during somitogenesis (Figure 12D-G). In *her15*, there is oscillation in the broad domain in the posterior PSM which has a round or oval shape, and there are no stripe-like expression domains which are budding off

from this broad posterior domain. The stripe-like expression domains of *her15* are located more anterior in the PSM tissue, when compared to that of *her1* and *her7* and hence *her15* oscillation does not give the impression of a wave sweeping across the PSM, from posterior to anterior (compare Figure 12A-C with Figure 12D-E). The broad mRNA expression domain of *her15* appears to pulsate in the posterior PSM of embryos and hence we have referred to it as 'micropulsing' *her15* mRNA expression.

To a large extent, *her15* mRNA expression compartments during the first 24-27 hours are comparable to *her4* (Takke et al., 1999); *deltaD* (Hans and Campos-Ortega, 2002), *her2* (von Weisäcker, 1994) and *her12* (Sieger et al., manuscript in preparation) in zebrafish, to *esr9* and *esr10* in *Xenopus* (Li et al., 2003) and to *Hes5* in mouse (Dunwoodie et al., 2002). The stripe-like expression of *her15* as well as the oscillating posterior domain persists in late somitogenesis stages such as 12-14 somite and 16-18 somite stages (white asterisk * in Figure 13D-E). *her15* mRNA is expressed dynamically in the notochord and neural tube during early somitogenesis stages and later becomes restricted to the notochord during the 12-14 somite stage, 16-18 somite stage (black arrows in Figure 13D-E). In the 24-27 hour old zebrafish embryo, *her15* mRNA shows strong expression domains distributed along the entire embryo (Figure 13F).

Figure 9. Protein architecture of *her15*

The characteristic domains of *h/E(spl)* genes can be seen in the alignment and they are basic Helix I loop Helix II, Orange domain and WRPW motif. The sequences have been aligned using BioEdit Sequence Alignment Editor Version 5.0.6. *Dr-Danio rerio, Mm-Mus musculus* and *Xl-Xenopus laevis*

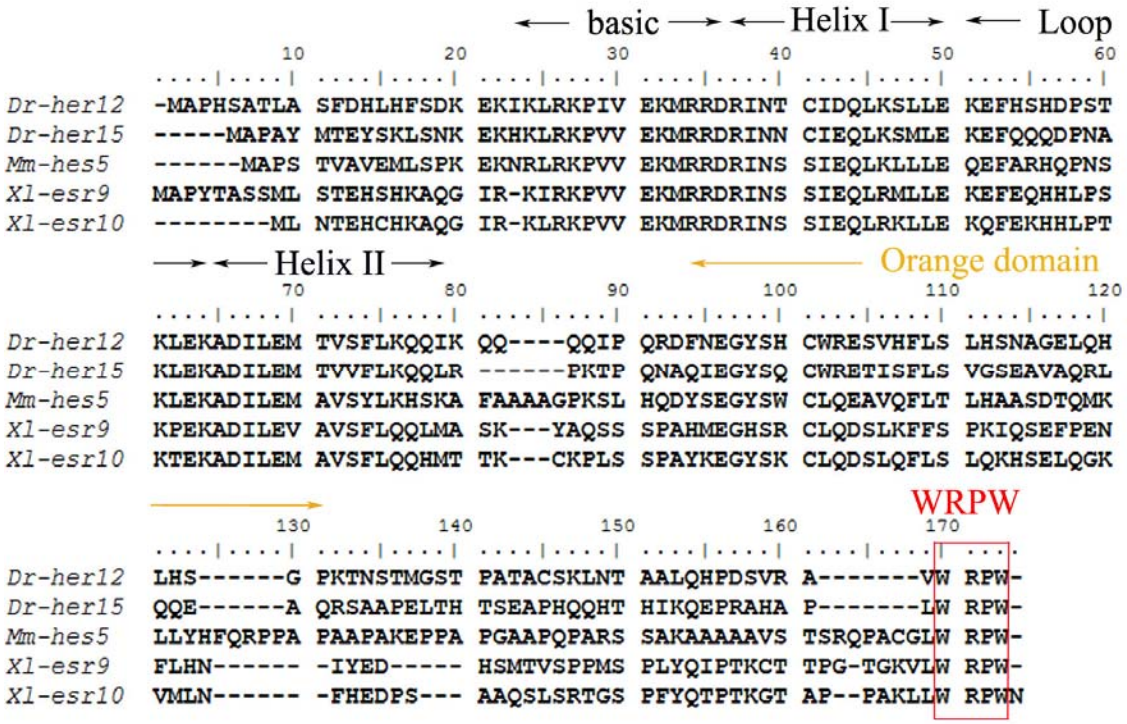


Figure 9. Protein architecture of *her15*

Figure 10. *her15* mRNA expression pattern during late gastrulation and somitogenesis stages.

(A) Fate map of zebrafish embryo which shows the future cell fates of the *her15* mRNA expression domains during epiboly stages. Note that the fate map shows the lateral view of an embryo with the dorsal side on the extreme right. (B) Embryo at 60% epiboly showing *her15* mRNA expression at the marginal zone on either side of the dorsal midline (marked by white arrows). The midline itself is devoid of *her15* mRNA transcripts (white asterisk). (C) 80% epibloy embryo showing *her15* expression on both sides of the midline, which has expanded slightly towards the anterior/animal pole (white arrows). In this stage also, the dorsal midline is devoid of *her15* transcripts (white asterisk). Note that the embryo views are dorsal and the embryo in the fate map has a lateral view. On comparison with the fate map, it can be seen that *her15* labels the precursor cells of somites and notochord (D) Oscillating broad expression of *her15* in the posterior PSM representing broad cycling phase (marked by white asterisk *). (E) Dot-like expression domain of *her15* in the posterior PSM representing the reduced cycling phase (marked by white asterisk *). Both (D) and (E) are 3 somite stage embryos and together show the broad and dot-like phases of cycling *her15* mRNA expression in the posterior PSM. Whole-mount views show clearly, only the broad and dot-like cycling mRNA expression phases of *her15*. The intermediate phase is clearly visible only in the flat-mounts. All embryos are in dorsal view and whole-mounts. Figure1 and the legend have been taken from Hammerschmidt et al., (2004).

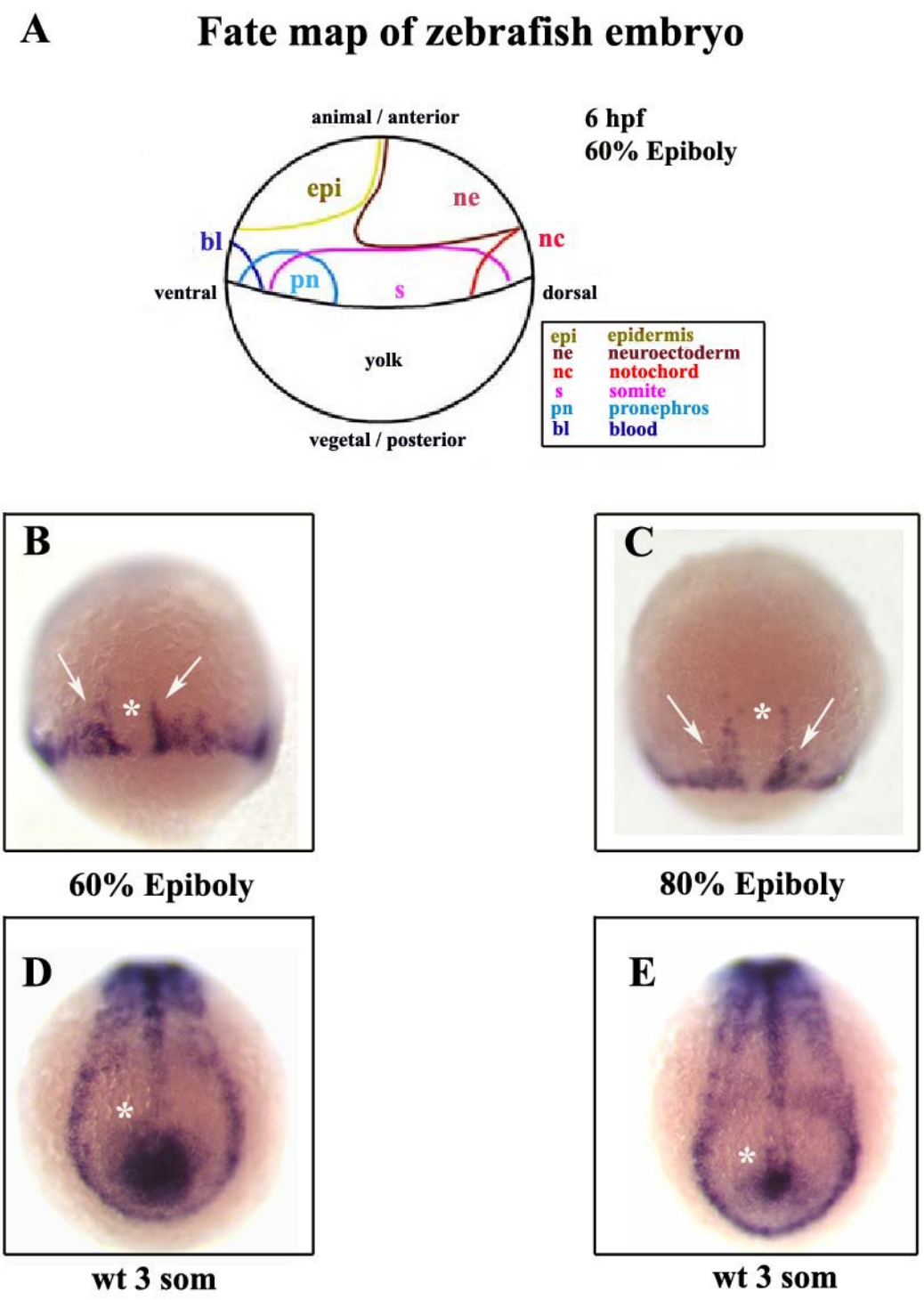


Figure 10. *her15* mRNA expression pattern during late gastrulation and 3 somite stages

Figure 11. *her15* in bud stage and 3-6 somite stage

(A-C) Bud stage wild type embryos showing the three cycling phases of *her15*, namely I, II and III as indicated. Cycling phase I corresponds to the broad mRNA expression phase, cycling phase II corresponds to the intermediate mRNA expression phase and cycling phase III corresponds to the dot-like mRNA expression phase. (D Bud stage embryo showing two neuronal stripe-like expression domains which are called intermediate stripe (is) and lateral stripe (ls). (E) 3-6 somite stage embryo showing a third stripe of *her15* expressing cells, extending across the midline and called the medial stripe (ms). *her15* transcription in the primordium of the trigeminal ganglion (trg) is marked with black arrow heads and expression in the prospective mesencephalic region is shown by black asterisk *. Embryo views are dorsal, anterior to the top and flat-mounted.

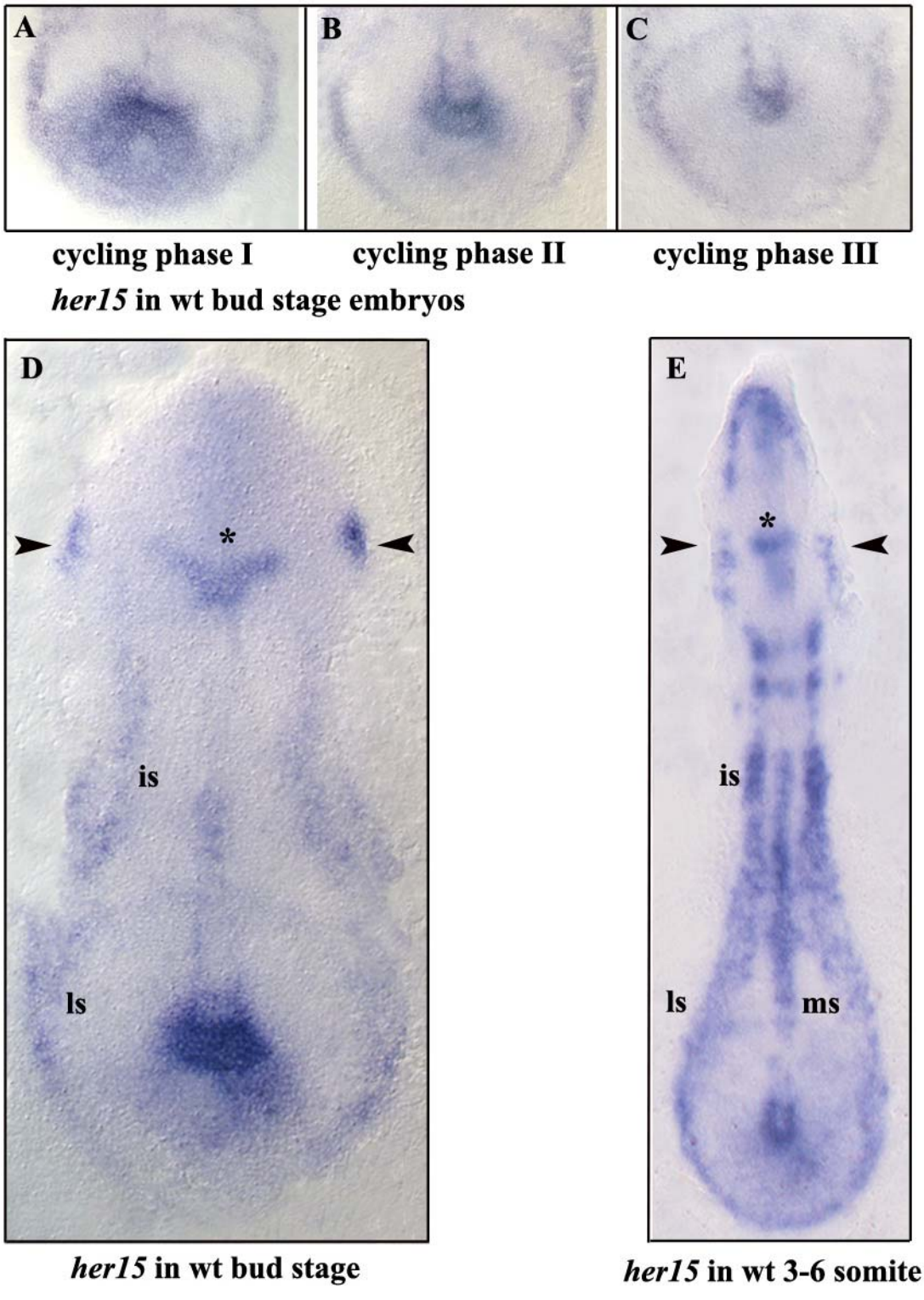


Figure 11. *her15* in bud stage and 3-6 somite stage

Figure 12. *her15* is dynamically expressed in the posterior PSM

(A) *her15* cycling phase I, broadest expression domain in the posterior PSM, (B) *her15* cycling phase II, intermediate expression domain, (C) *her15* cycling phase III, dot-like expression domain which is the smallest. (A-C) Embryos are 3-6 somite stage. (D-G) Embryos showing the different cycling phases of *her7* It can be seen that oscillatory *her15* expression is restricted to the posterior PSM and is micropulsing, whilst, *her7* expression sweeps across the PSM like a wave from posterior to anterior. (D-G) Embryos are 6-10 somite stages and have been kindly provided by Gajewski, M. Embryo views are dorsal, anterior to the top and flat-mounted.

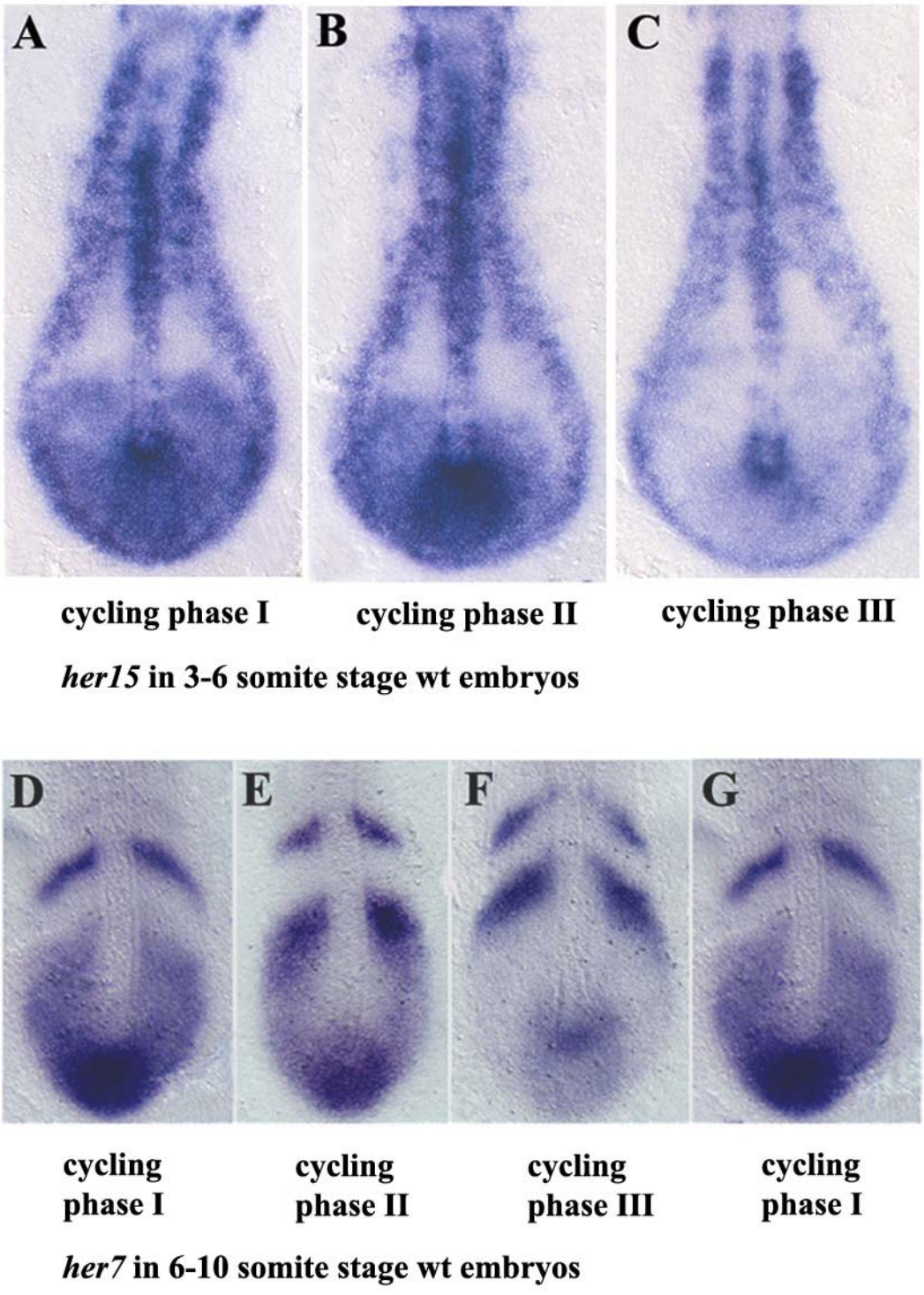


Figure 12. *her15* is dynamically expressed in the PSM

Figure 13. *her15* is expressed throughout somitogenesis

(A) (A-F) Embryos showing the complex and widely distributed *her15* mRNA expression domains during somitogenesis. The age of the respective specimens is indicated in the picture. The asterisk * points to the stripes of *her15* during late somitogenesis stages namely 12-14 somite and 16-18 somite stage. (D) Embryo shows two pairs of stripes, while (E) shows only one pair of stripes. Zebrafish has a total of 30-33 somite pairs which are generated in the first 24 hours. (A) Dorsal view, (B) dorsal and medial view, (C-F) lateral views.

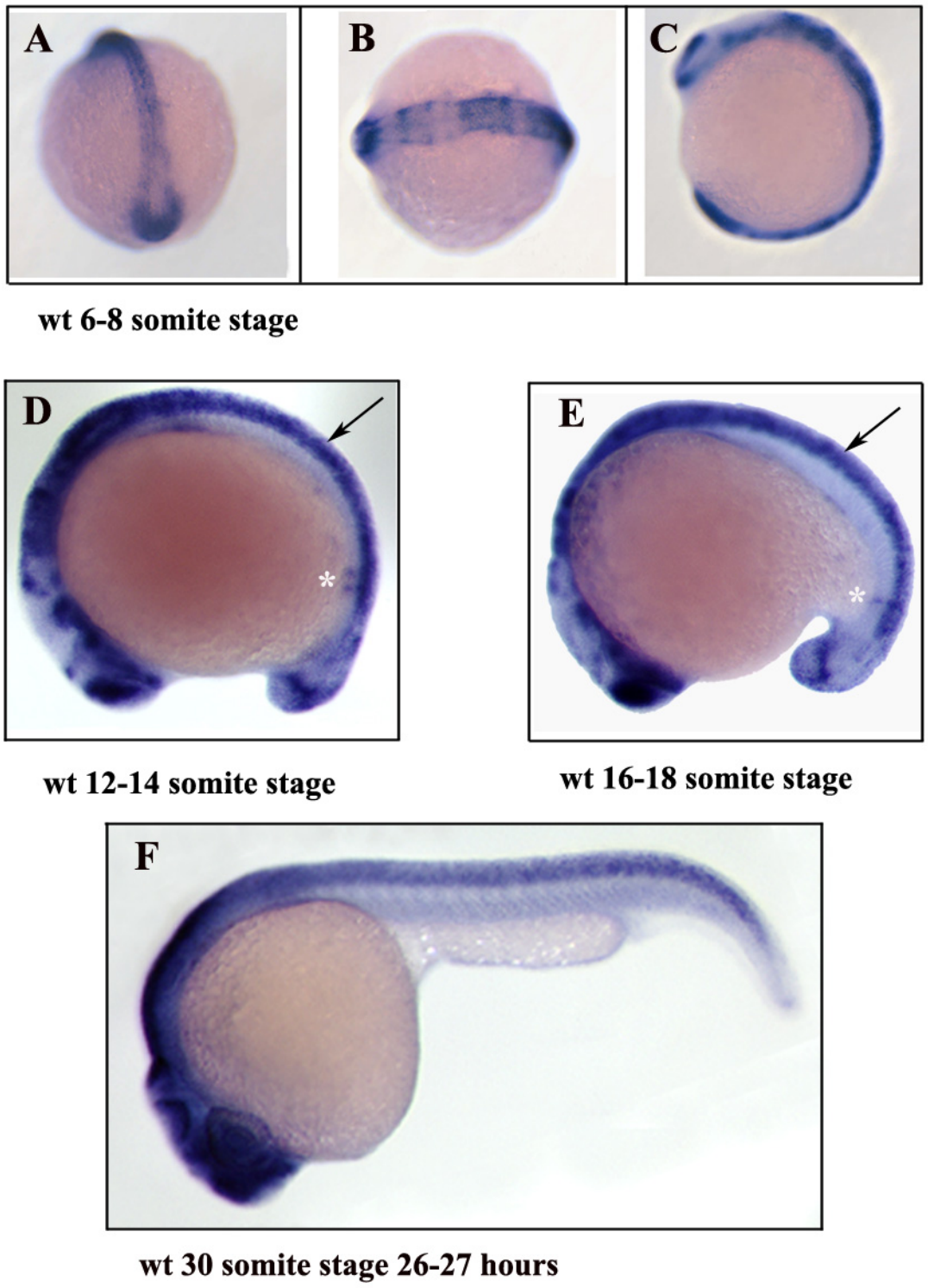


Figure 13. *her15* is expressed throughout somitogenesis

4.1.3 The stripes of *her15*

her15 mRNA shows stripe-like expression domains in the anterior PSM. The 'stripe-like expression domains of *her15*' will be hence forth referred to as '*her15* stripes' for the sake of simplicity. These stripes are first visible at around 3-6 somite stage and later in 6-8 somite stage, but are very faint and barely visible at the flat-mount level. The posterior domain of *her15* is still oscillating at 6-8 somite stage and is posterior to the strong *myoD* stripes (Figure 14A-D). At around 10-12 somite stage, the stripes become very prominent, varying between one pair of stripes on either side of the notochord or two pairs of stripes (Figure 14E-F). *her7,* is another *her* gene in zebrafish which oscillates and has stripes. On comparison with *her7*, the *her15* stripes are very thin (compare Figure 14E and 14F to Figure 14G).

To determine the exact location of the *her15* stripes in the anterior PSM, a double *in situ* hybridization was carried out with *myoD*. *myoD* is a myogenic basic helix-loop-helix (bHLH) transcription factor. *myoD* is expressed in the posterior of all formed somites starting at around 10.5 hours to 12 hours. It is also expressed in two pairs of less intense stripes in the anterior PSM, caudally to the last formed somite, i.e., in the posterior of the prospective somites S0 and S-I (Weinberg et al., 1996). Thus, the total number of *myoD* stripes seen in the embryo, correspond to the number of somites + 2. The stripes shown in the somites are prominent and broad. The two stripes present caudally to the formed somites are faint and thin. There is a decrease in *myoD* transcript level in a somite, 3-4 hours after its formation. Hence, by the time 12-13 somites are formed in a 14.5 hour embryo, the first 1-4 somites show less *myoD* transcript and this illustrates the transient expression of the gene in any one somite. *myoD* is also expressed in the adaxial cells bordering the notochord.

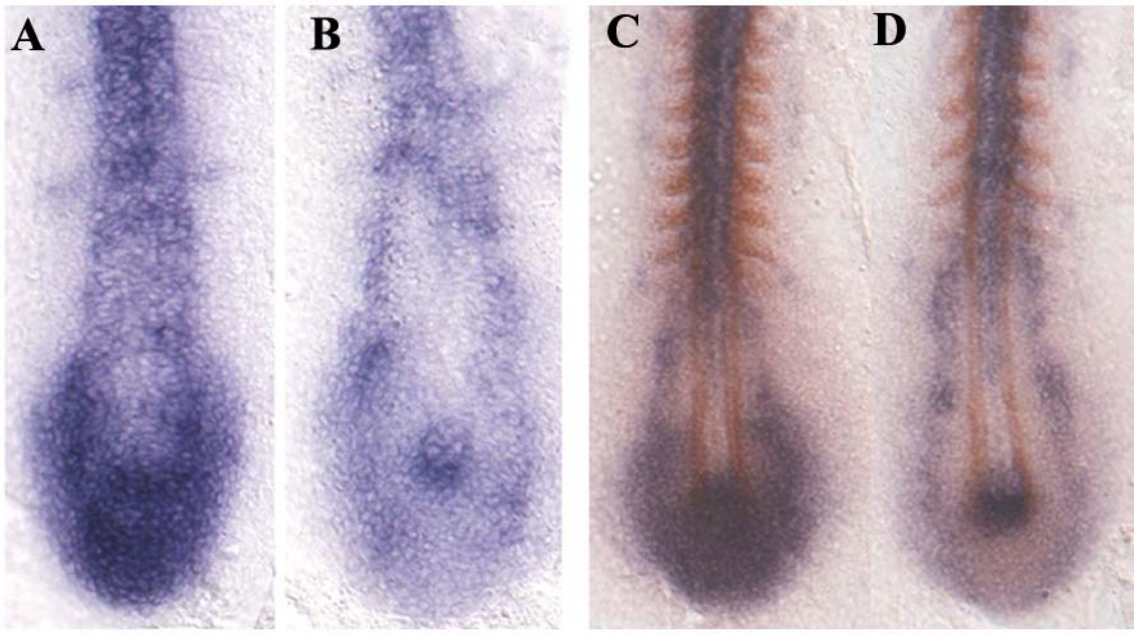
By double *in situ* hybridization, it was revealed that the stripes of *her15* are positioned at the posterior border of S-I and SI somites (Figure 15). S0 is the somite which is in the process of epithelialization and S–I is the somite which will be subject to epithelialization after S0. SI is the somite which has completed epithelialization (see review Vertebrate

Somitogenesis by Pourquie, 2001 for somite nomenclature guidelines). *her15* and *myoD* expression overlap in the SI somite. This indicates that *her15* labels the cells in the posterior border of the just formed somite (SI) and the somite which will be formed next but one (S-I) (Figure 15).

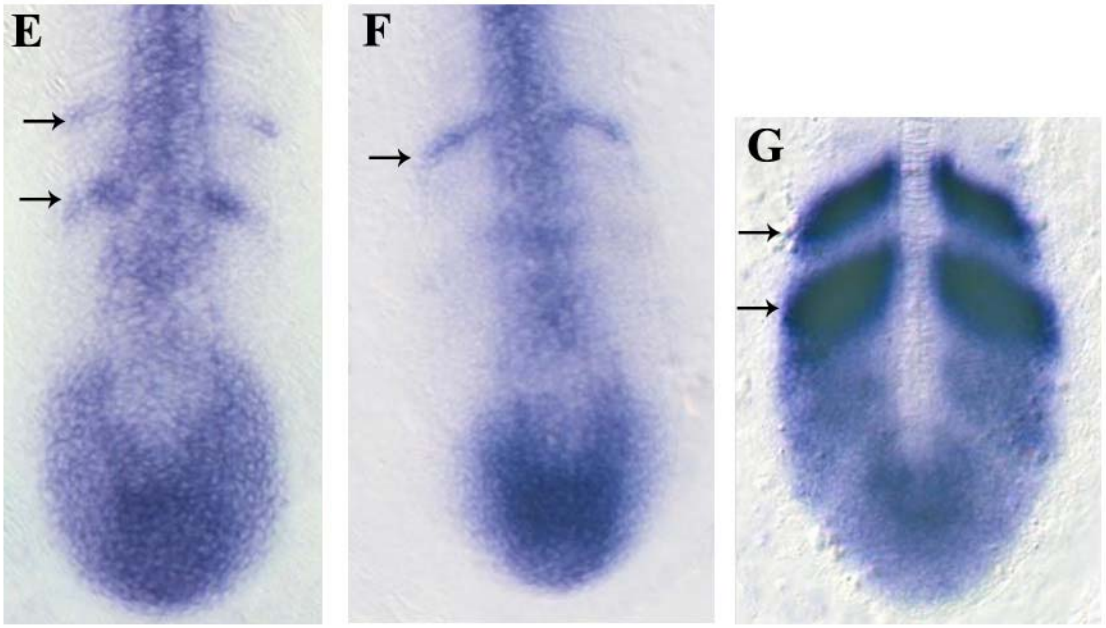
To further examine the exact location of the *her15* stripes in the anterior PSM, a series of embryos (n=17), were flat mounted and analyzed by measuring the distance between the borders of the already formed somite and the first or second *her15* stripe (Table 5). The embryos show different number of stripes for *her15* namely either one pair or two pairs. One embryo which uniquely showed three pairs of stripes was also included in the analysis. The analysis revealed that *her15* when present as two sets of stripes showed a double segmental distance. Other *her* genes in zebrafish like *her1* and *her7* show two pairs of stripes but these stripes are sweeping across the PSM and vanishing at S0 (Holley et al., 2000; Oates and Ho, 2002). The stripes of *her15* are thinner when compared to those of *her7* (Figure 14, compare E, F with G). The double segmental stripe distance is unique for *her15* and till date has not been reported for other *her* genes in zebrafish. Since 1 or two pairs of *her15* stripes are visible in different embryos, decay and initiation of transcription must be fast and indicate high dynamic oscillation at a specific position within the PSM instead of the wave like behavior of *her1* and *her7*. As control for the *her15* experiment, which showed double segmental distance, the stripes of *her4* (Takke et al., 1999), were analyzed in the same fashion and they were found to show only single segmental distance in the PSM (Table 6).

Figure14: *her15* mRNA expression during somitogenesis stages.

(A-D) Embryos showing oscillating *her15* expression in 6-8 somite stage. (A)Embryo showing *her15* mRNA expression in posterior PSM-broad domain, and in the notochord. (B)Embryo showing *her15* mRNA expression in posterior PSM- the dot-like expression profile and in the notochord. (C-D) Embryos showing *her15* mRNA in blue and *myoD* in red, the broad and dot-like expression domains respectively. It is clear that the broad expression domain of *her15* lies posterior to the stripes of *myoD* in the somites. (A-D) All embryos are wild type 6-8 somite stage. (E) Embryo showing two stripes of *her15* expression in anterior PSM (labeled with black arrows) and in the notochord. (F) Embryo showing only one set of stripes. (E-F) Embryos are 10-12 somite stage when the stripes of *her15* are clearly visible. (G) Embryo showing *her1* expression with arrows pointing to the two stripes in 8-12 somite stage. The stripes of *her15* are thin, while those of *her1* are thicker. Embryo views are dorsal anterior to the top and flat-mounted.



***her15* in blue in *myoD* in red in 6-8 somite wt embryos**



***her15* in 8-12 somite wt embryos**

***her1* in 8-12 somite wt embryo**

Figure 14. *her15* mRNA expression during somitogenesis stages.

Figure 15. The stripes of *her15*

(A) An embryo in 10-12 somite stage double-labeled with *her15* and *myoD in situ* probes. *her15* is seen in blue/black and *myoD* in red/orange. *myoD* stripes are visible at the posterior border of somites, S0, SI, SII and SIII. *her15* and *myoD* stripes overlap at the posterior border of SI. *her15* stripes are visible at the posterior border of somites, S-I and SI, and hence it is expressed at double segmental distance. Embryo views are dorsal, anterior to the top and flat-mounted.

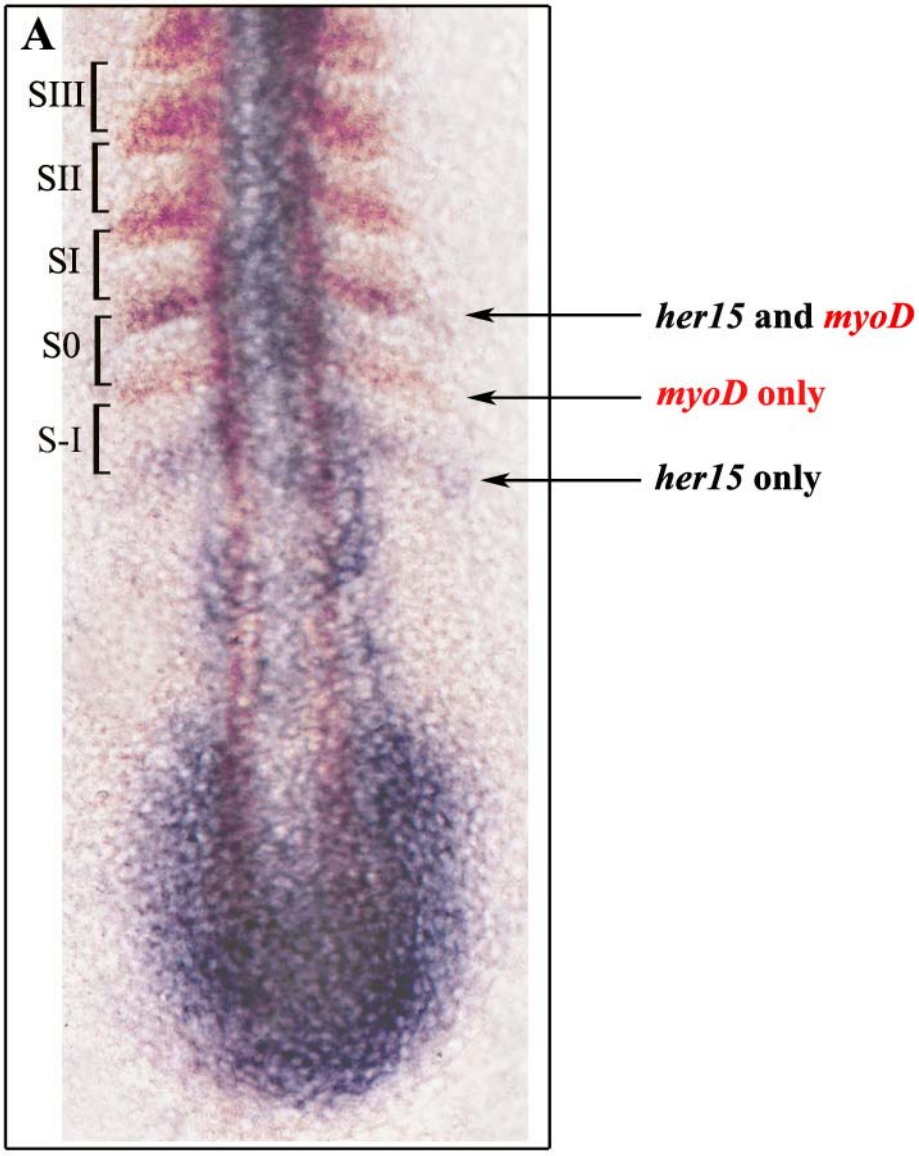


Figure 15. The stripes of *her15*

Tables 5. Analysis of *her15* stripes and *her4* stripes

her15	1 stripe		2 stripes			3 stripes			
No	Control (c)	c to 1	c	c to 1	1 to 3	c	c to 1	1 to 2	1 to 3
1	1.76/1.83 **1.795**	1.59/1.66 **1.625** **0.905**							
2			1.69/1.73 **1.71**	1.55/1.69 **1.62** **0.947**	3.35/3.25 **3.30** **1.93**				
3						1.59/1.76 **1.625**	1.69/1.55 **1.605** **0.987**	1.31/1.38 **1.345** **0.838**	3.10/3.46 **3.28** **2.01**
4	**1.73**	**1.8** **1.04**							
5			1.94/2.14 **2.04**	2.08/2.05 **2.065** **1.01**	4.06/4.16 **4.11** **2.014**				
6			1.91/1.98 **1.945**	1.76/1.91 **1.835** **0.943**	3.5/3.6 **3.55** **1.825**				
7			1.73/1.8 **1.765**	1.8/1.8 **1.8** **1.019**	3.67/3.46 **3.565** **2.019**				
8	1.94/1.76 **1.85**	1.76/1.98 **1.87** **1.011**							
9			1.83/1.83 **1.83**	1.73/1.76 **1.745** **0.954**	3.3/3.25 **3.255** **1.778**				
10			1.45/1.31 **1.38**	1.34/1.38 **1.36** **0.985**	2.72/2.54 **2.63** **1.905**				
11			1.62/1.59 **1.605**	1.66/1.59 **1.625** **1.012**	2.86/3.32 **3.09** **1.925**				
12			1.83/1.73 **1.78**	1.59/1.83 **1.71** **0.961**	3.7/3.49 **3.595** **2.019**				
13			**1.76**	**1.59** **0.903**	**3.46** **1.966**				
14			1.76/1.76 **1.76**	1.69/1.69 **1.69** **0.960**	3.1/3.1 **3.1** **1.761**				
15	1.52/1.48 **1.50**	1.41/1.38 **1.395** **0.93**							
16	1.83/1.87 **1.85**	1.98/1.76 **1.87** **1.011**							
17	1.83/1.94 **1.885**	1.87/1.91 **1.89** **1.00**							
avg		**0.983**		**0.969**	**1.91**				

her4 No	2 stripes			3 stripes			
	Control (c)	c to 1	1 to 2	c	c to 1	1 to 2	1 to 3
1[1]	0.68/0.72 **0.70**	0.62/0.72 **0.67**	0.73/0.67 **0.70**				
2				0.64/0.77 **0.705**	0.67/0.69 **0.68**	0.72/0.75 **0.735**	1.28/1.52 **1.4**
3[1]	0.68/0.63 **0.655**	0.66/0.63 **0.645**	0.75/0.8 **0.775**				
4[3]	0.69/0.69 **0.69**	0.66/0.6 **0.65**	0.69/0.59 **0.64**				
5[3]	0.75/0.64 **0.695**	0.61/0.59 **0.6**	0.75/0.78 **0.765**				
6[1]	0.73/0.66 **0.695**	0.63/0.73 **0.68**	0.71/0.73 **0.72**				
7[2]	0.77/0.72 **0.745**	0.74/0.67 **0.705**	0.66/0.75 **0.705**				
8[2]	0.77/0.69 **0.73**	0.69/0.72 **0.705**	0.9/0.8 **0.85**				
9				0.67/0.77 **0.72**	0.72/0.61 **0.665**	0.75/0.72 **0.735**	1.3/1.23 **1.265**
10[2]	0.69/0.8 **0.745**	0.55/0.65 **0.6**	0.76/0.76 **0.76**				
avg	**0.707**	**0.657**	**0.739**	**0.7125**	**0.673**	**0.735**	**1.33**

Measurements were done on both sides of the embryo and are represented in normal font .Bold font shows the average measurement.

The stripe measurement was normalized to control.

Avg = average over all samples of a given type.

Control is penultimate formed somite (SII)

C to stripe 1 is the ultimate somite (SI)

Consequently, the anterior most stripe is always at the posterior border of SI

Second stripe corresponds to posterior border of S-I

3 subtypes of 2 striped embryos:

1: both stripes same intensity

2: both stripes have same intensity, but some weak staining in between

3: posterior stripe is broader than anterior stripe and appears to be fading towards the tip.

4.1.4 *her15* oscillation in the posterior PSM is a target of the Delta-Notch signaling pathway

The *hairy (h)* and *enhancer of split (E(spl))* family genes which number around 25 in zebrafish (Sieger et al., 2004), play prominent roles in regulating both neurogenesis and somitogenesis and are known targets of the Notch signaling pathway. In particular, somitogenesis in zebrafish is known to be regulated by *notch1*, *deltaD* and *deltaC* and their differential activity on *her1, her7, her4, her6* and *her11* (Takke and Campos Ortega, 1999; Holley et al., 2000; Holley et al., 2002; Henry et al.,2002; Oates and Ho, 2002; Gajewski et al.,2003; Pasini et al., 2004, Sieger et al., 2004). The activity of the Notch signaling cascade on downstream target genes of the *hairy (h)* and *enhancer of split (E(spl))* family is mediated through *Su(H)* (Sieger et al., 2003). To investigate whether *her15* is also regulated by Notch signaling, its mRNA expression pattern was analyzed in the somitogenesis mutants namely *after eight* (*aei*; mutant for *deltaD*), *deadly seven* (*des;* mutant for *notch1*) and *beamter* (*bea;* mutant for *deltaC*, Scott Holley personal communication). The cyclic expression of *her15* in the posterior PSM was affected in these mutants, as there was a total absence of cyclic expression and only a residual dot-like expression was visible (Figures 16 and 17). The mutant embryos were obtained from crosses of homozygous pairs and all the embryos in the particular experimental batch showed the total absence of cyclic expression (100% effect). The number of embryos analysed in each case were as follows:

- for *aei/deltaD* mutant, n=69, of somite stages 3-12, from 5 independent experiments
- for *bea/deltaC* mutant, n=40, of somite stages 3-12, from 4 independent experiments
- for *des/notch1* mutant, n=87, of somite stages 3-12, from 4 independent experiments.

Thus the Delta-Notch pathway is required explicitly for the cycling (dynamic) mRNA expression of *her15*. It is to be noted the effect of the Delta-Notch pathway on *her15*

expression, is not uniform, with strongest down regulation of *her15* seen in *aei/deltaD* mutant embryos compared to the *bea/deltaC* mutant embryos. This would suggest differential regulation of *her15* by various zebrafish Delta genes. The neural expression compartments in the embryo seem unaffected and this might be due to the function of other Delta-Notch genes in zebrafish (for review, see Stickney et al., 2001). Additionally, the striped expression compartment of *her15* in the anterior PSM is lost in the mutant embryos of 10-12 somite stage (data not shown). This suggests that the stripe generation relies on active and full functionality of the Delta-Notch signaling pathway or is a hint to a Delta-Notch independent function.

To test whether the regulation of *her15* activity is fully dependent on the Notch signaling pathway, *her15* mRNA expression was analysed in *Su(H)* knockdown embryos (Sieger et al.,2003). In these embryos, an overall down regulation of *her15* mRNA transcripts could be observed (Figure 18). The cycling expression of *her15 i*n the tail bud was lost and a very faint signal resembling a crescent shaped moon was visible. The embryos were injected with a concentration of 0.6mM *Su(H)* morpholino solution, capable of bringing about total gene knock down (Sieger et al, 2003). The number of embryos analysed were n=21, of somite stages 3-10, from 3 independent experiments and all the embryos showed overall down regulation of *her15* mRNA transcripts and loss of cycling expression in the tail bud (100% effect). In all the Notch pathway mutants and additionally the *Su(H)* morpholino knock down embryos, the stripes of *her15* in the anterior PSM, at 10-12 somite stage were lost (data not shown). Thus one can conclude that the Notch pathway is required for the complete activation of *her15*, in particular the cycling mRNA expression compartments in the posterior PSM. There is residual expression of *her15*, visible in the regions of the brain and notochord in the zebrafish embryos following *Su(H)* morpholino knockdown. The residual expression domains throughout the embryo can be explained as being due to the activity of local activators which are switched on, in the absence of the repressor *Su(H)* (Sieger et al., 2003). In contrast, the expression of *her15* in a particular brain region is not affected in the *Su(H)* morpholino knock down embryos and hence seems independent of the Delta-Notch signaling (black arrows in Figure 18A-B). Thus *her15* expression in this particular brain region is similar to *her5* in the mid brain hind

brain boundary (MHB). The MHB in zebrafish is an active proliferation zone for neuronal stem cells and this region appears to be independent of Delta-Notch signaling. This aspect has been exemplified in the case of *her5* which is specifically expressed in the MHB and is not a target of Delta-Notch signaling (Geiling et al., 2004).

4.1.5 *her15* dynamics in the posterior PSM is independent of *fss/tbx24*

The fused somite (*fss*) mutant in zebrafish codes for a T-box gene namely *tbx24* and is the only one somitogenesis mutant which does not code for a Notch pathway gene (Nikaido et al., 2002). This mutant is characterized by the total absence of visible somite borders during the first 24 hours of somitogenesis. Some rudimentary borders do however appear after 24 hours, in the paraxial tissue, which is explained as resegmentation induced by late signals from the notochord and adaxial cells. The horizontal myoseptum and muscle differentiation is not affected in the *fss/tbx24* mutant (van Eeden et al., 1996). In *fss/tbx24* mutant, the lack of intersomitic borders and epithelial somites is also accompanied by lack of Eph receptor/Ephrin signaling interfaces, which when restored rescues most aspects of somite morphogenesis in the mutant (Durbin et al., 2000; Barrios et al., 2003). *fss/tbx24* mutants fail to generate the stripes of *her1* and *her7* in the anterior PSM while the cyclic expression in the posterior PSM remains unaffected (van Eeden et al., 1996, Holley et al, 2000, Oates and Ho, 2002).

To analyse the oscillatory behavior of *her15* in 3-8somite stage embryos of the *fss* mutant, embryos were collected from crosses of homozygous pairs. The number of embryos analysed were n=30, of somite stages 3-10, from 2 independent experiments *her15* mRNA oscillations in the posterior PSM are not affected in *fss/tbx24* mutant (Figure 17, D-F). All the 3 cycling phases were visible in the pool of embryos analysed. The stripes of *her15* which are clearly visible at 10-12 somite stage in the anterior PSM are lost in the *fss* mutant (data not shown). The rest of the expression compartments of *her15* which are mainly neuronal, remain unaffected in *fss/tbx24* mutant. Hence, *her15*

cycling in the posterior PSM is independent of *fss/tbx24*, while the stripes in the anterior PSM are positively regulated by *tbx24*.

Figure 16. *her15* is a target of Notch signaling

(A-B) Wild type embryos of 3 somite stage, (A) showing the broad cycling phase and (B) showing the dot-like cycling phase. (C-E) Notch pathway mutant embryos showing the absence of a broad oscillating signal for *her15*. In these mutants only residual expression was observed. Note also that among the mutants, the effect on *her15* is not uniform; with stronger down regulation of *her15* seen in *aei/deltaD* mutant embryos compared to the *bea/deltaC* mutant embryos. This would suggest differential regulation of *her15* by various zebrafish Delta genes. Arrows point to the oscillating domain in the case of the wild type embryos and to the minimal residual expression, in the case of the mutants. Embryo views are dorsal, anterior to the top, whole-mounts.

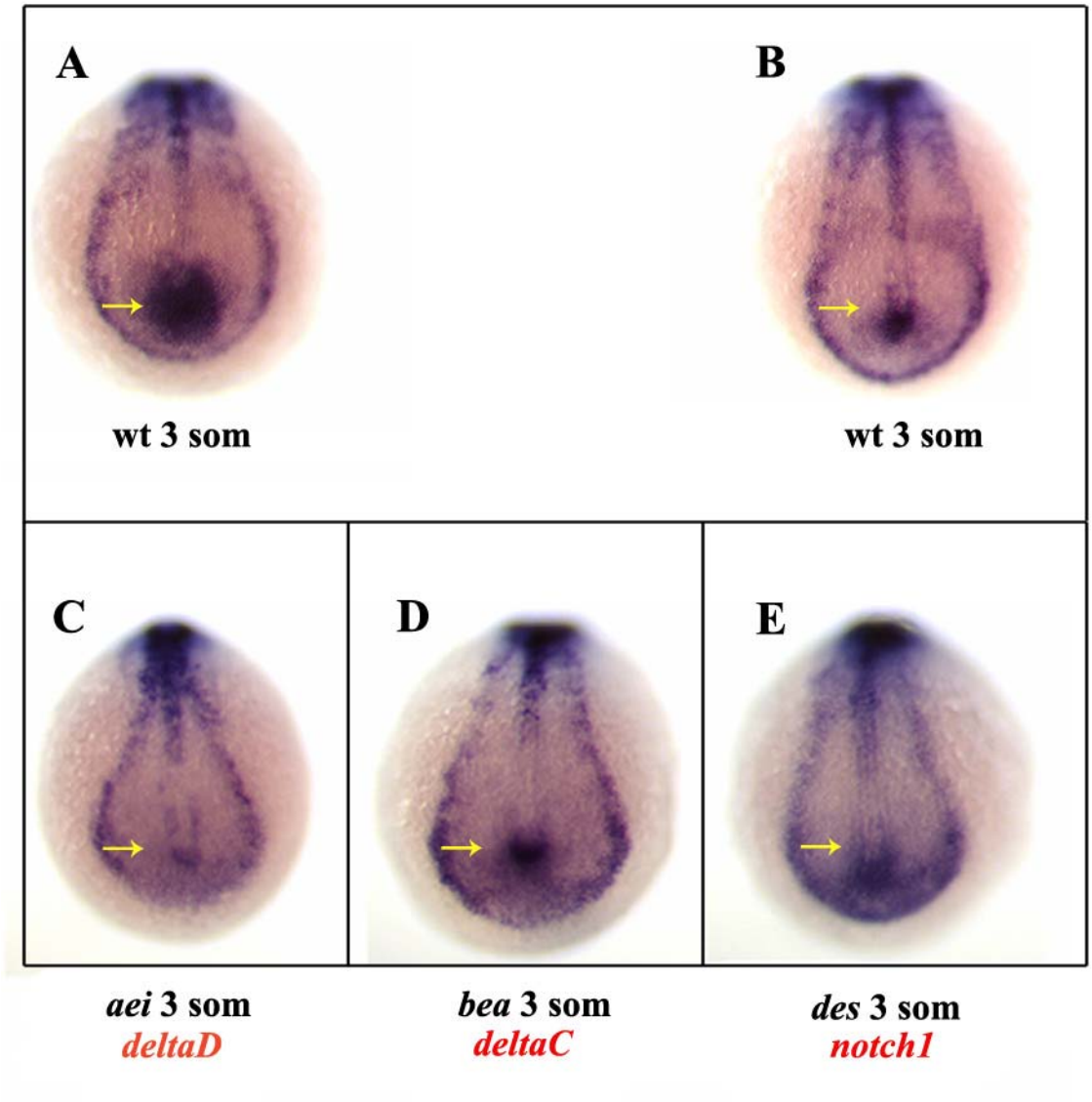


Figure16: *her15* is a target of Notch signaling

Figure 17: *her15* in Delta-Notch pathway somitogenesis mutants

(A-C) 3 somite stage Notch pathway mutants showing down regulation of *her15* mRNA signal in the posterior PSM coupled with the loss of cyclic behavior. Note that the down regulation is more pronounced in the *aei/deltaD* compared to the *bea/deltaC* mutant embryos. pointing to differential regulation by zebrafish Delta genes. Embryo views are dorsal, anterior to the top and flat-mounted.

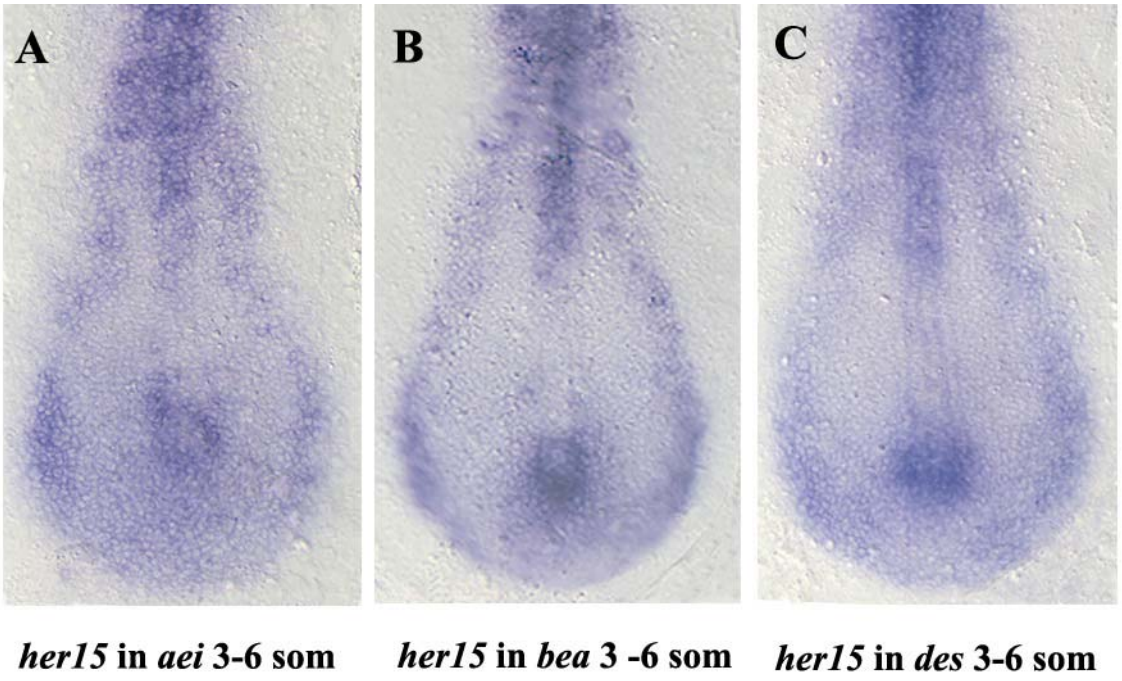


Figure 17. *her15* in Delta-Notch pathway somitogenesis mutants.

Figure 18: *her15* is activated by Delta-Notch signaling

(A) *her15* in wild type 3 somite stage embryo showing all the expression domains in the embryo. The arrow points to the *her15* signal in a particular brain region which is independent of Delta-Notch. (B) *her15* in *Su(H)* morpholino knockdown embryos showing severe down regulation of mRNA signal and absence of oscillatory expression. The mRNA signal of *her15* in the particular brain region appears unaffected in *Su(H)* morpholino knock down embryos compared to the wild type (wt) (black arrow). The asterisk * labels the posterior PSM domain in both embryos.

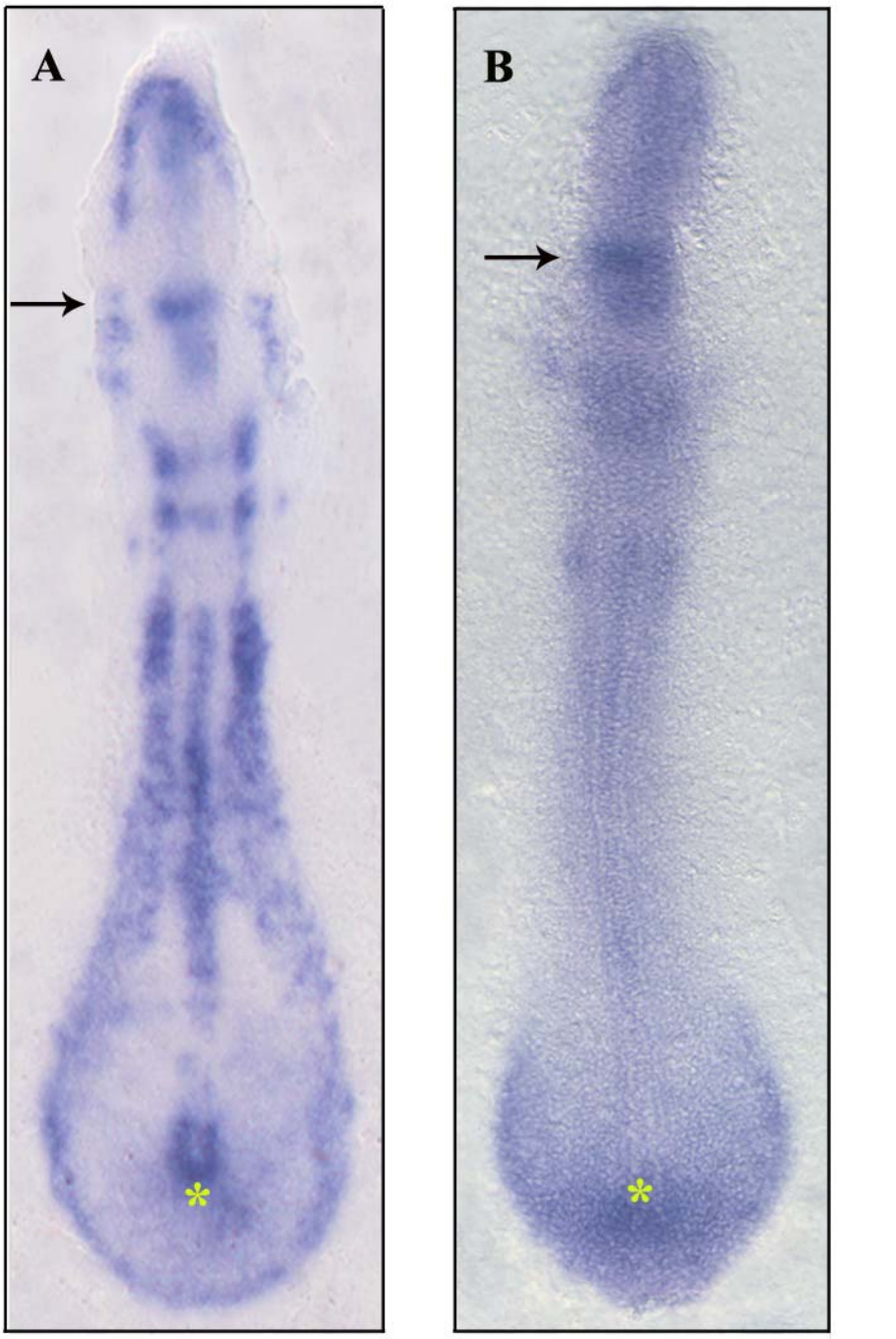


Figure 18: *her15* is activated by Delta-Notch signaling

Figure 19. *her15* in *fss/tbx24* mutant embryos

(A-C) *fss* mutant embryos showing all 3 cycling phases of *her15*. The cycling domain of *her15* mRNA expression in posterior PSM is independent of *fss/tbx24* activity. Embryo views are dorsal, anterior to the top and flat-mounted.

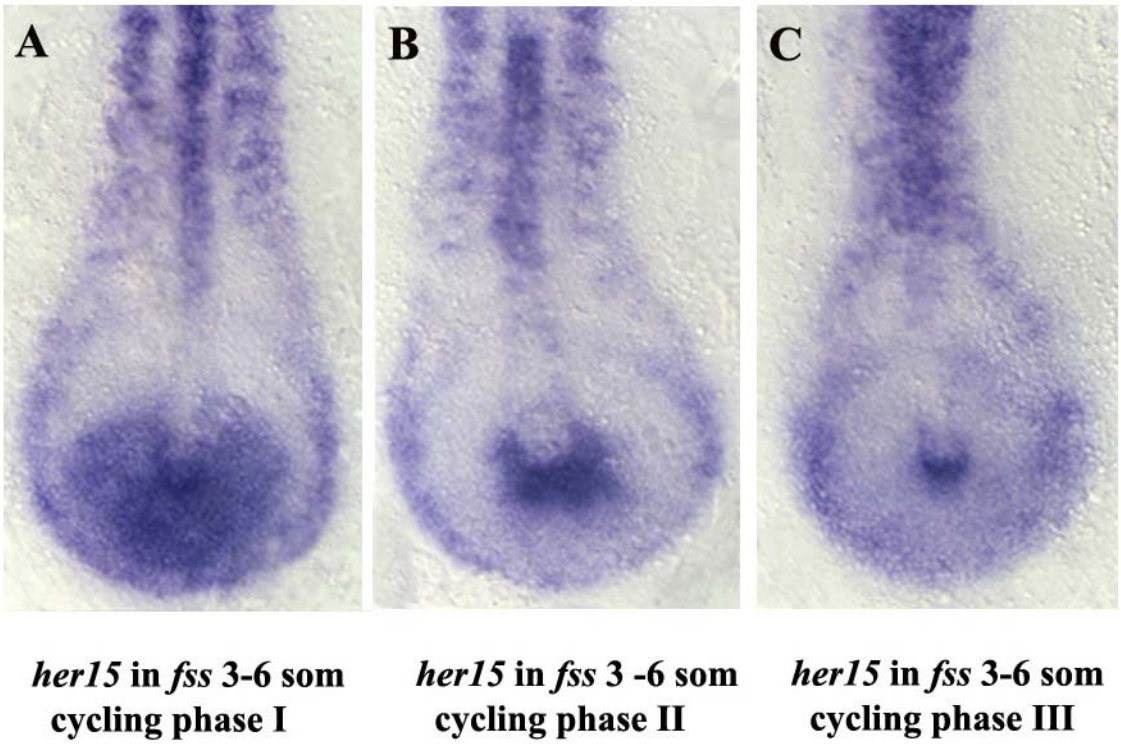


Figure 19. *her15* in *fss/tbx24* mutant embryos

4.1.6 Early and late somitogenesis

The first somites in zebrafish, approximately 3, seem to be regulated independent of the Notch signaling cascade. The known Notch related somitogenesis mutants in zebrafish namely *aei/deltaD* and *des/notch1*, have the first 7±2 somite borders intact. The absence of somite borders occur in these mutants only after the first 7±2 somite borders are properly formed (van Eeden et al., 1996). The only mutant in this class which shows an exception is *bea/deltaC*, in which only the first 4 somite borders are formed, following which there is severe disruption. Knock down of the whole signaling pathway by *Su(H)* morpholino injection shows the strongest phenotype, namely the loss of somite borders after 3 somites are formed (Sieger et al., 2003).

In the case of cycling/oscillating *deltaC* mRNA expression, disruption of the cycling expression domain correlates to the onset of morphological failure in somite border formation. This is exemplified by the observance that in the mutant *bea/deltaC*, the break down of oscillating *deltaC* expression occurs at the 4 somite stage whilst in *aei/deltaD*, it is observed at a later stage namely around 6±2 somite stage. This phenomenon has been explained by the authors, stating that the function of Notch signaling during somite segmentation is to keep the oscillations of neighboring presomitic mesoderm cells synchronized (Jiang et al., 2000). But there are exceptions to this. *her1* and *her7* in zebrafish, which on knock down exhibit the strongest negative effects on cyclical gene expression and somite border formation, show disruption in their cycling mRNA expression domains in bud stage *aei/deltaD*, *des/notch1* and *bea/deltaC* mutant embryos, i.e., the gradual degradation in the integrity of the cyclic expression domain boundaries presage the appearance of defective morphological boundaries (Oates and Ho, 2002). Likewise, *her15* oscillating mRNA expression in the posterior PSM is present in wild type embryos (Figure 11A-C), but disrupted in *bea/deltaC* and *Su(H)* morpholino knockdown embryos at the bud stage prior to the appearance of morphologically defective somite borders (Figure 20A-B). Therefore, the disruption in the oscillating mRNA expression compartment of *her15*, as well as that of *her1*, *her7* and *her11* (Sieger

et al., 2004), occurs earlier than morphological somite border disruption, as the signal is disrupted at an earlier embryological stage, the bud stage.

Figure 20. *her15* in *bea* and *Su(H)* knock down, bud stage embryos

(A) *bea* bud stage embryo showing disruption of *her15* cycling/oscillation in posterior PSM. (B) *Su(H)* morpholino knock down bud stage embryo showing disruption of *her15* oscillation in posterior PSM. The asterisk* marks the disrupted oscillating mRNA expression domain in the posterior PSM. The disruption in the oscillation of *her15* does not have any relation to the time point at which morphological boundaries are disrupted in the two different classes of somite mutants shown in (A) and (B).

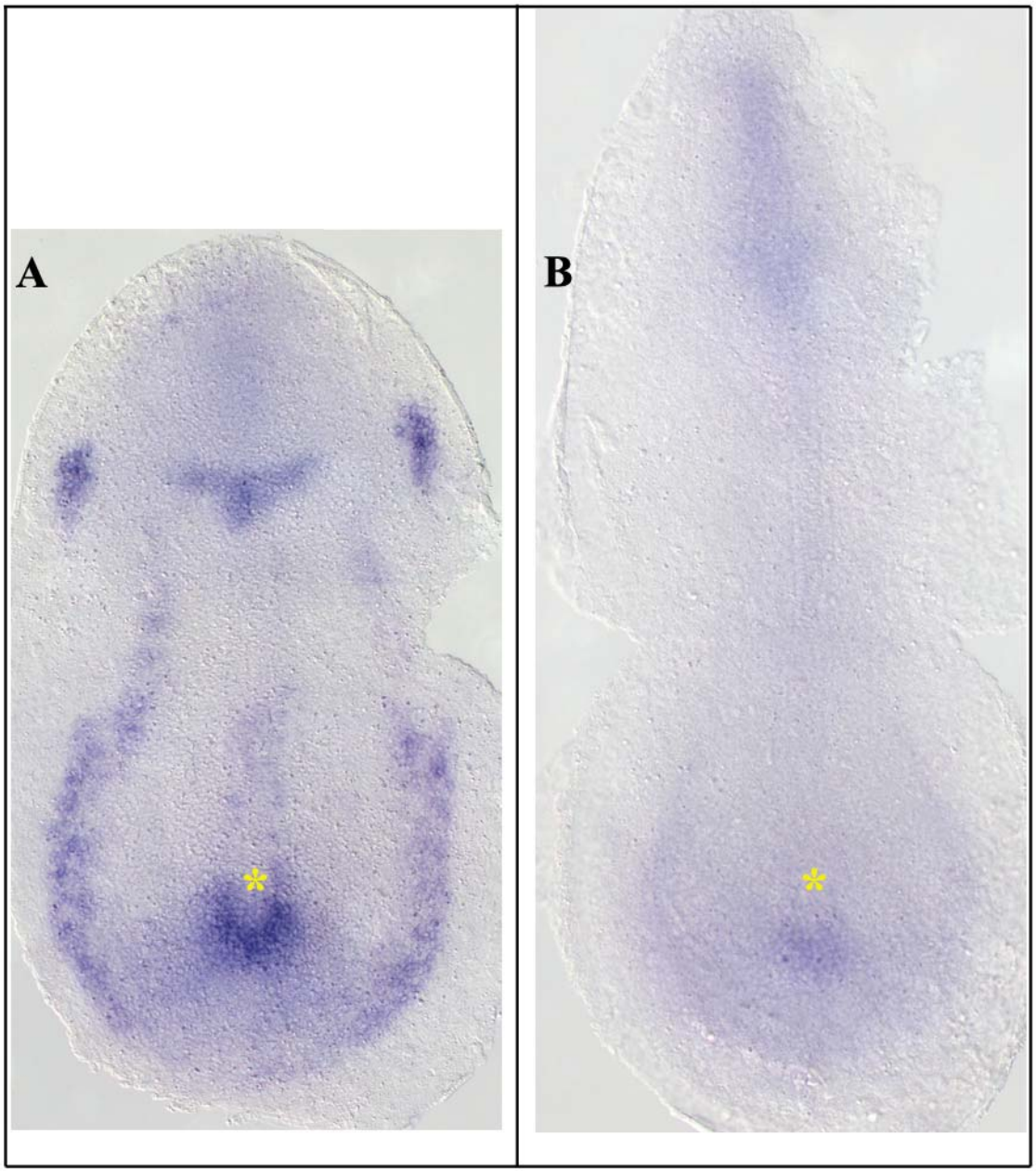


Figure 20. *her15* in *bea* and *Su(H)* knock down, bud stage embryos

4.1.7 *her15* oscillation in the posterior PSM is independent of *her1* and partially dependent on *her7*

her1 and *her7* morpholino knockdown in zebrafish embryos causes severe disruption of both oscillatory mRNA expression and somite border formation. *her1* and *her7* are expressed in overlapping domains in the presomitic mesoderm which is suggestive of a redundant function. But surprisingly, the severity of the knock down of *her1* and *her7* individually, is compounded in the double knock down situation (Oates and Ho, 2002; Henry et al., 2002; Holley et al., 2002). In mouse the situation is far less complicated as only *Hes7* mutants show a somite phenotype as opposed to the *Hes1* mutant. Recently, it has been established that mouse Hes7 protein is crucial for sustained oscillation and for its function in the somite segmentation clock (Hirata et al., 2004) and this supports the role of *Hes7* as the key regulator of mouse somitogenesis. Zebrafish *her1* and *her7* are more closely related to mouse *Hes7*, than to mouse *Hes1*. Thus in zebrafish, the mouse Hes7 ancestor seems to be duplicated. Both genes have retained their function in somitogenesis. In contrast, the duplicated mouse Hes1 homologues in zebrafish (*her6* and *her9*) either play no role (*her9*) or do not show cyclic expression (*her6*) (Leve et al., 2001; Gajewski et al., 2002; Sieger et al., 2004; Pasini et al., 2004) To examine whether there is a cross regulation by the mouse *Hes7* homologues, *her1* and *her7* to *her15*, mRNA expression was analysed in *her1* and *her7* morpholino knock down embryos.

In *her1* morpholino knockdown embryos, the three *her15* oscillating/cycling expression domains in the posterior PSM remain undisturbed (Figure 21A-C). In *her7* morpholino knock down embryos, however, *her15* mRNA does not show the typical three cycling phases as observed in wild type embryos. The visible expression domains in the PSM were the intermediate and dot-like cycling phase. The broad cycling phase of *her15* is invisible in embryos following *her7* morpholino knock down (Figure 21D). *her1* and *her7* morpholino solutions were injected in 0.6mM concentrations to achieve complete gene knock down (Gajewski et al.,2003). The number of embryos analysed for *her1* morpholino injections were n=44, of somite stages 3-10, from 2 independent experiments

and for *her7* morpholino injections were n=52, of somite stages 3-10, from 3 independent experiments.

The stripes of *her15* in the anterior PSM are unaffected in the early stages such as the 3-6 somite stage in both the *her1* and *her7* morpholino knock down embryos. In 10-12 somite stage embryos, the stripes appear to be much broader and stabilized than in the wild type situation. Moreover, always only one stripe is visible and never two stripes (data not shown). This suggests that *her15* mRNA oscillating expression in the posterior PSM is independent of *her1* activity and partly dependent on *her7* activity, while the stripes of *her15* in the anterior PSM are regulated by both *her1* and *her7*.

Figure 21. *her15* mRNA expression following *her1* and *her7* gene knockdown

(A-C) *her1* morpholino knock down embryos of 3-6 somite stage have all the 3 phases of *her15* oscillation. (D) *her7* morpholino knock down embryo having only the intermediate oscillating phase of *her15*. *her15* oscillation in the posterior PSM is independent of *her1* and partly dependent on *her7*. Embryo views are dorsal, anterior to the top and flat-mounted.

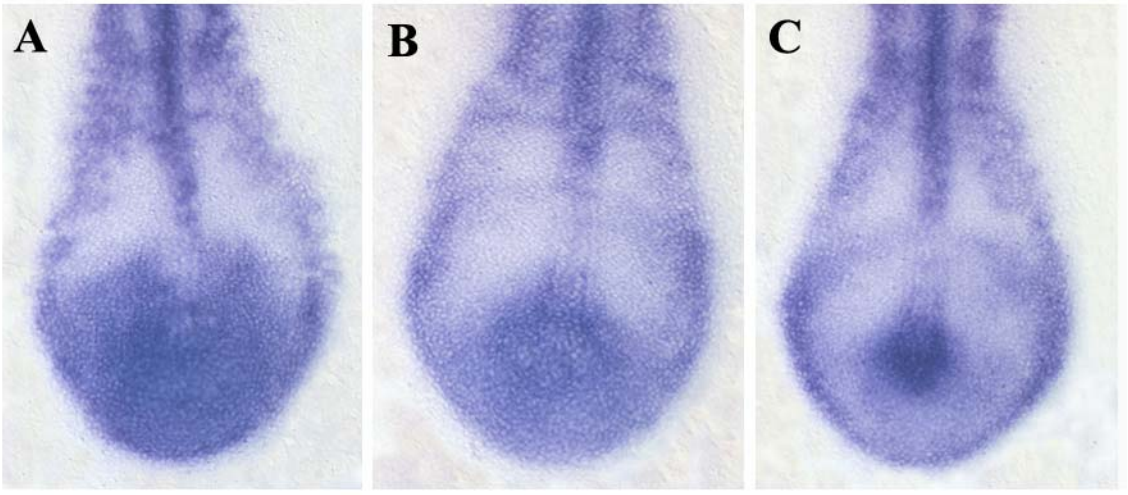


Figure 21. *her15* mRNA expression following *her1* and *her7* gene knockdown

4.1.8 Micropulsing *her15* domain is uncovered by left-right asymmetric and dynamic mRNA expression

The micropulsing domain of *her15* mRNA in the posterior PSM, during 3-12 somite stages, exhibit left-right assymetry in the distribution of the mRNA in flat mounted embryos of 3-6 somite stage and in transverse sections in 10-12 somite stage embryos (Fgure 22A). The transverse sections are from the extreme posterior of the PSM, caudal to the position of the notochord. The mRNA signal shows differential distribution on both left and right halves of the section (Figure 22C).

It is known that Notch activity induces Nodal expression and mediates the establishment of left-right asymmetry in vertebrate embryos. This is a conserved feature of Notch activity in vertebrates ranging from fish to mammals and is mediated through Notch-responsive elements in the Nodal promoter (Raya et al., 2003).

The argument is not for a possible role of *her15* in generation of left-right asymmetry in zebrafish embryos, but that the observed asymmetry is suggestive of the dynamic nature of *her15* expression and the autonomy of cells from the left and right parts of the posterior PSM concerning this process. Such an asymmetry in mRNA distribution for an oscillatory *hairy* and *E(spl)* related family gene has been reported for *esr9*, an orthologue of *her15* in *Xenopus* (Li Y et al., 2003). Both *her15* and *esr9* show sequence conservation to mouse *Hes5* (Figure 9).

Figure 22. *her15* mRNA shows asymmetric distribution on left-right halves of the embryo

(A) *her15* in 9 different embryos showing left right asymmetry in 3-6 somite stages. (B) *her15* in 10-12 somite stage embryo shown to understand the exact embryonic location of the section (C) Transverse section (cross section) from the extreme tip of the tail bud posterior to the notochord (posterior PSM region), showing the unequal distribution of *her15* mRNA signal on the left and right halves of the section. The section is 6µm in thickness. In figure (A) Embryo views are dorsal, anterior to the top and flat-mounted. In figure (B), embryo view is dorsal, anterior to the left and flat-mounted. The asterisk corresponds to the posterior oscillating domain of *her15* and the red line shows the approximate region from where the given section has been cut. In figure (C), the red line shows the prospective midline. Note that (B) and (C) are not the same embryo, as the embryo that was sectioned was not photographed prior to sectioning. Embryo (B) has comparable embryonic age and *her15* mRNA expression domain, to the sectioned embryo (C).

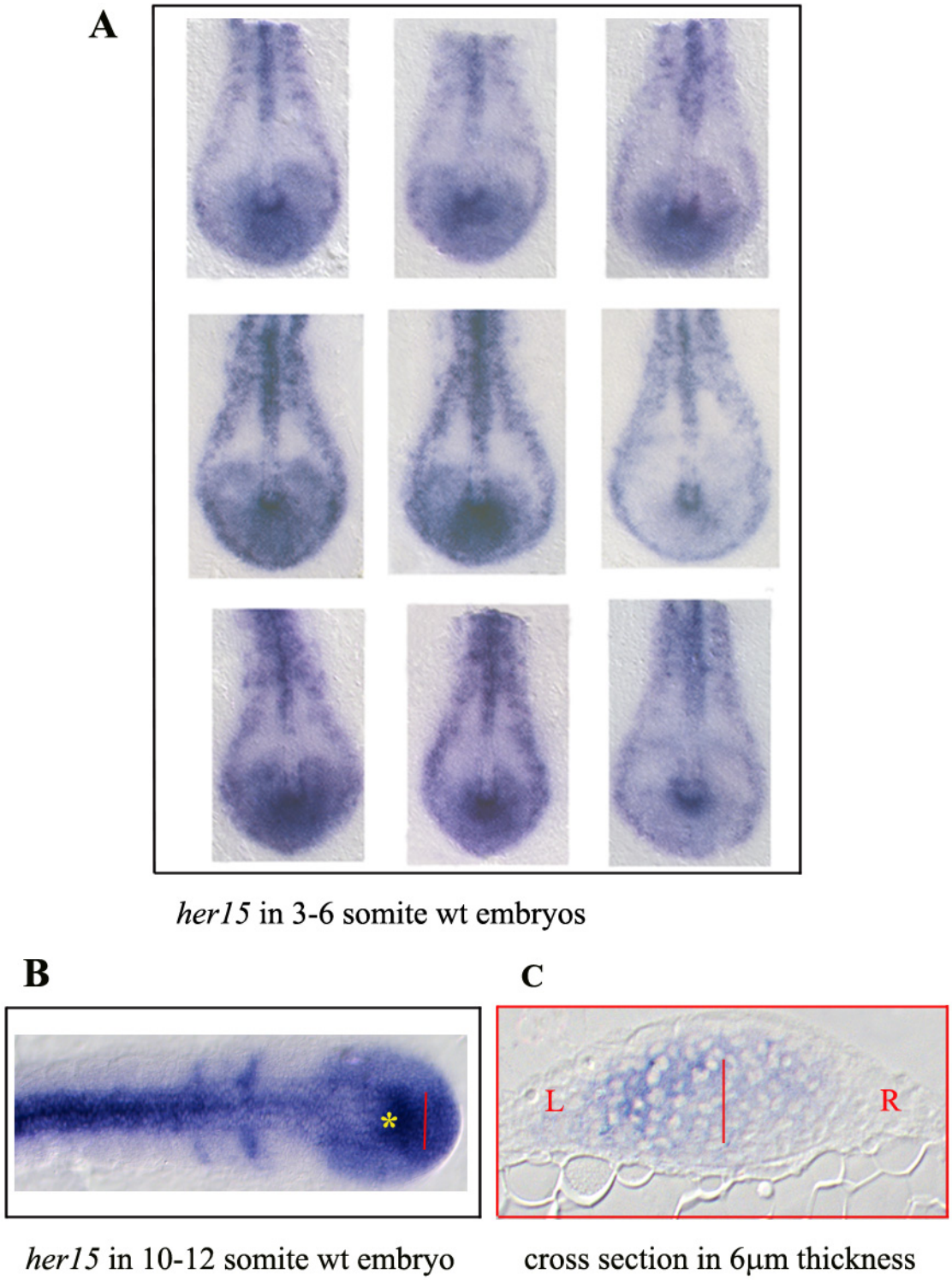


Figure 22. *her15* mRNA shows asymmetric distribution on left-right halves of the embryo

4.1.9 Confocal and microtome sectioning of *her15* stained embryos for details pertaining to the micropulsing posterior PSM domain

To examine the micropulsing expression of *her15* in the posterior PSM of zebrafish embryos, somitogenesis stage embryos of 6-10 somite stage were labeled with fluorescent *her15 in situ* probe, flat mounted and subjected to confocal sectioning in the frontal plane. The logic behind the experiment was to see if the *her15* mRNA signal was distributed in both dorsal and ventral halves of the posterior PSM region. This would imply a separate micropulsing mRNA expression in the posterior PSM, which could then drive forward the oscillating gene expression of genes such as *her1* and *her7* and thus drive the somitogenesis molecular clock. *her15* only oscillates in the posterior PSM and does not show the sweeping mRNA wave from the posterior to the anterior PSM, exhibited by *her1* and *her7*. The confocal images did not give a conclusive picture but was suggestive of a possible presence of *her15* signal in both ventral and dorsal halves of the posterior PSM region in zebrafish embryos (Figure 23, I). But sagittal sections from a 3-6 somite stage wild type embryo *in situ* hybridized with *her15* probe, showed the uniform distribution of *her15* mRNA signal in both dorsal and ventral halves of the posterior PSM (Figure 23, III). The distribution of *her15* signal in the ventral half of the embryo, or in other words, the posterior PSM wall (Griffin and Kimelman, 2002), would suggest that *her15* may play a role in maintaining the cells in the posterior PSM in the mesenchymal state prior to subsequent epithelialization during somitogenesis. At the moment, this is preliminary data and more experiments are needed in this direction.

Figure 23. Confocal and sagittal sections of *her15* micropulsing domain

In Figure (I), (A-T) Confocal sections of 6-8 somite stage embryos stained with *her15* carrying a fluorescent vector red label. The sections are 4-5µm in thickness and have been scanned in the xyz plane. Section (A) is dorsal most section and Section (T) is the ventral most section. The experiment was done to check if *her15* mRNA signal extended from the dorsal to the ventral side of the embryos and the results gave a hint that it might be the case. Embryo views are dorsal, posterior to the top and flat-mounted. (II) 3 somite stage embryo labelled with *her15* to show the specific cycling phase used to make sections. (III) Sagittal section from 3 somite stage embryo clearly showing the uniform expression of *her15* from dorsal to ventral halves in the posterior PSM. In the lower middle panel of the figure there is a small drawing, explaining the sectioning planes.

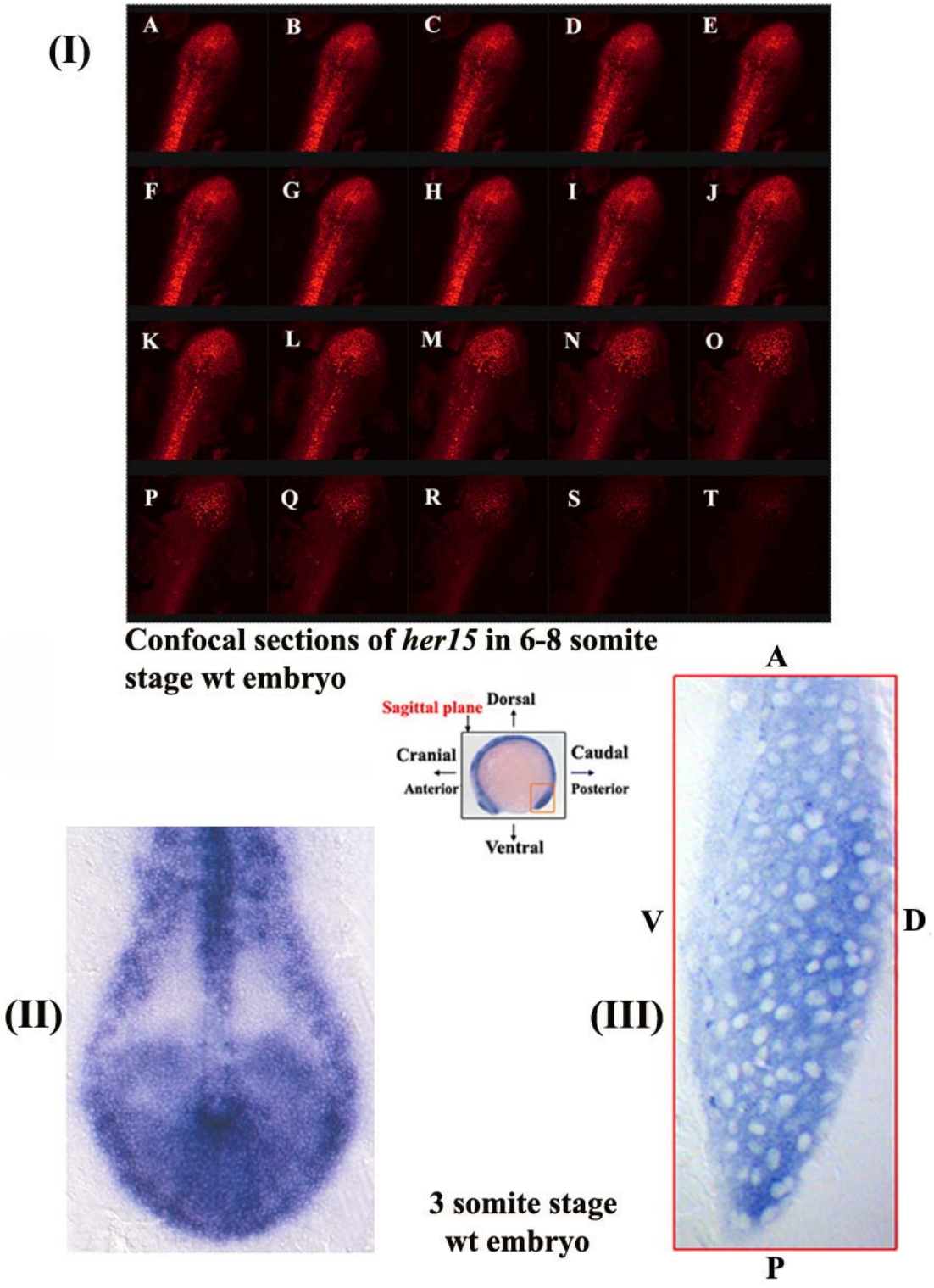


Figure 23. Confocal and sagittal sections of *her15* micropulsing domain

4.1.10 Functional analysis of *her15*

Initial *in situ* expression analysis studies in the wild type embryos and somitogenesis mutants described above, is suggestive of a possible functional role for *her15* in zebrafish somitogenesis. To explore this possibility, two experimental approaches were employed.

4.1.10.1 *her15* morpholino knockdown approach

Morpholino oligonucleotides were designed to specifically knock down the function of *her15* in zebrafish embryos. Morpholino oligonucleotides function by blocking translation of their target mRNA's (for review see Sumanas and Larson, 2002). The 25 base pair sequence selected for morpholino design consists of the last 15 bases of the 5'UTR and the first 10 bases of the open reading frame starting at Methionine.

The *her15* morpholino was injected in varying concentrations from 0.6mM to 1mM and the embryos were checked for morphological irregularities in somite formation the following day. The embryos were later fixed for *in situ* analysis of *her15* mRNA knockdown effects on gene candidates, which are known to play prominent roles in zebrafish somitogenesis namely *her1, her7, deltaC, deltaD, myoD, mespa, mespb* etc. Both at the morphological level and at the *in situ* level, no effects could be observed.

4.1.10.2 *her15* misexpression approach

The full length coding sequence of *her15* was cloned into pCS2+ vector (Figure 24A). The vector has a CMV promoter sequence 5' to the *her15* insert and SV40 polyA signal at the 3'end. This construct was used to prepare capped mRNA for injections into 1-2 cell stage zebrafish embryos. Varying concentrations were used ranging from 50ng/µl to 250ng/µl. As with the morpholino approach, both the morphological effects on live embryos and effects on RNA expression of two candidate genes involved in somitogenesis namely *her1* and *deltaC* were analysed.

her15 capped mRNA in concentrations ranging from 50ng/µl to 500ng/µl were injected in 1 to 2 cell stage zebrafish embryos. As control experiment, pCS2+eGFP mRNA was also injected in equal or higher concentrations. At *her15* mRNA concentration of 100ng/µl, 30% of injected embryos showed morphological somite border defects (Figure 24B-C). A total of 100 embryos were injected and analyzed in two independent experiments. Among the rest 70%, were present 45% showing defects during gastrulation and 25% were wild type looking embryos. The number of gastrulation defective embryos increased when higher concentrations of *her15* mRNA were injected. At eGFP concentration of 100-115ng/µl, 8% of injected embryos showed morphological somite border defects. A total of 161 embryos were injected and analyzed in two independent experiments.

The 30% of embryos showing morphological somite border defects following *her15* mRNA injection were fixed in 4% paraformaladehyde and processed for RNA *in situ* hybrization studies. In wild type embryos both *her1* and *deltaC*, are expressed as two paired stripes in the anterior PSM and a broader expression domain in the posterior PSM. Both genes are cyclically expressed (Takke and Campos Ortega, 1999; Holley et al, 2000; Smithers et al., 2000; Jiang et al., 2000; Holley et al., 2002). Moreover, *deltaC* is expressed in the posterior half of formed somites. The mRNA expression of *her1* and *deltaC* were analyzed and found to be disrupted in *her15* mRNA injected embryos. Both *her1* and *deltaC* exhibited loss of oscillating expression and de-repression of the mRNA signals in the interstripe regions in the anterior PSM (Figure 25A-D, black arrows). Furthermore, *her15* mRNA injected embryos showed disruption of the stripes of *deltaC* mRNA expression in the posterior half of somites (Figure25C-D, marked by black bracket)

The disruption in the *her1* mRNA expression following *her15* misexpression of *her15* mRNA, points to a disruption of somitogenesis clock/oscillator regulating somite border formation. One can argue that misexpression of a member of the *hairy* and *E(spl)* gene family can exert this effect rather unspecifically, but *her7*, *her9* and *her11*, on misexpression do not cause disruption of somite border formation or oscillating gene

expression (Pasini et al., 2004, Sieger and Gajewski - unpublished results).Misexpression studies imply a functional role for *her15* in regulation of cyclic gene expression and somite border formation in zebrafish. Thus, *her15* seems to be a core element of the somitogenesis clock.

Figure 24. *her15* misexpression-live embryo views

(A) Shows a schematic depiction of *her15* misexpression construct preparation and injection. The full length coding sequence of *her15* was cloned into the plasmid vector pCS2+, which is the commonly used zebrafish vector for misexpression experiments. (B) Shows a GFP RNA injected embryo (100-115 ng/µl) with normal somite borders while (C) shows *her15* RNA injected embryo (100 ng/µl) with disrupted somite borders. The white arrows point to somite borders in both embryos and both embryos are 8-12 somite stage. (B-C) live embryos have been photographed in methyl cellulose. Embryo views are dorsal, anterior to the top.

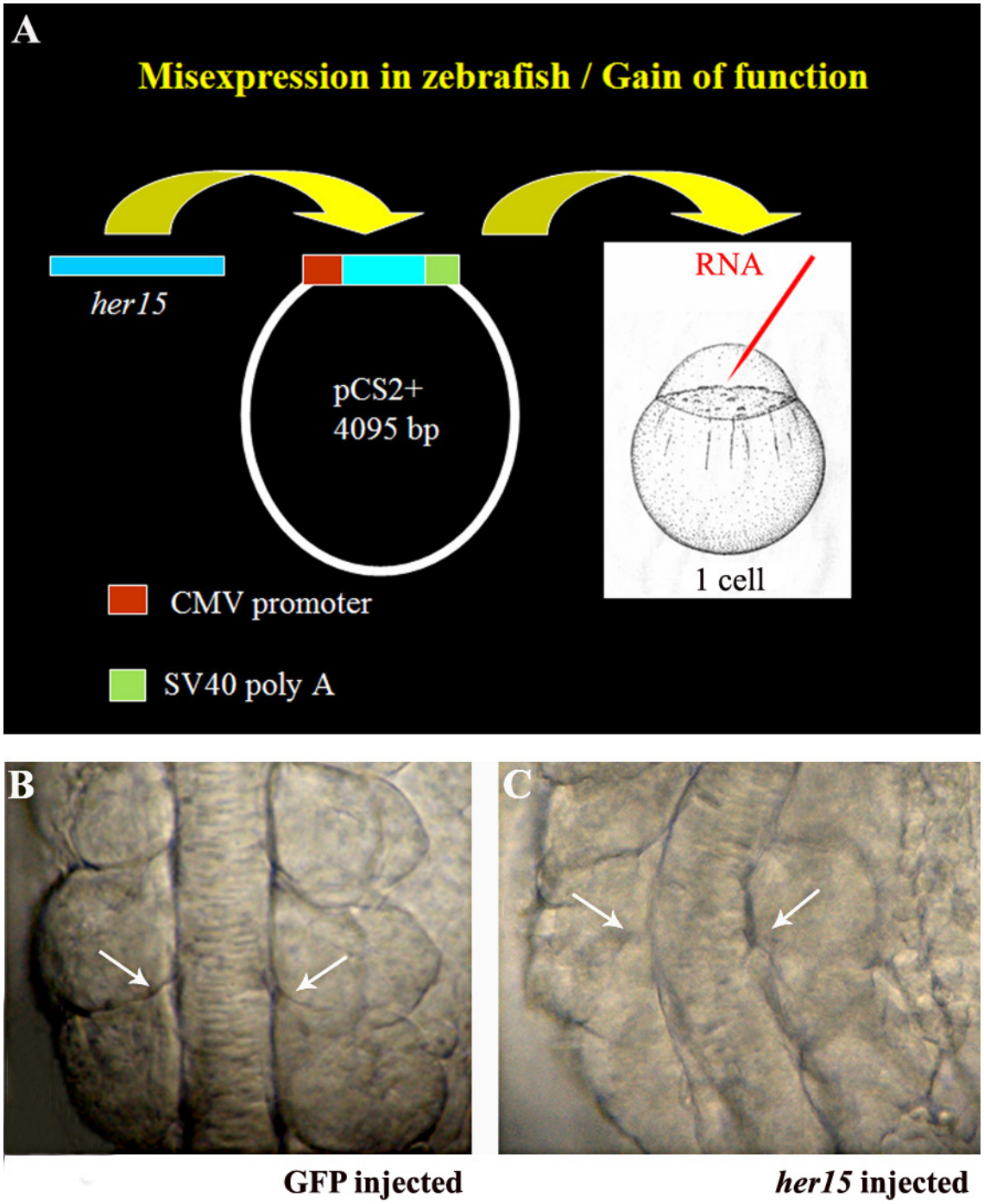


Figure 24: *her15* misexpression-live embryo views

Figure 25. *her1* and *deltaC* are disrupted after *her15* misexpression

(A) Embryos showing *her1* pattern after GFP RNA (115 ng/µl) injection. The pattern looks comparable to what can be seen in wild type embryos. The arrow points to the interstripe region in the anterior PSM showing interstripe repression. (B) Embryo showing disrupted *her1* pattern after *her15* RNA injection (100ng/µl). The arrow points to the loss of interstripe repression in the anterior PSM region. (C) Embryo showing *deltaC* pattern after GFP RNA injection (100-115 ng/µl).The arrow points to the interstripe region in the anterior PSM The bracket marks the expression of *deltaC* in the formed somites. (D) Embryo showing disrupted *deltaC* pattern following *her15* RNA injection (100ng/µl). In this case, the arrow points to the loss of interstripe repression in the anterior PSM. The bracket shows the disruption of *deltaC* signal in the formed somites. Embryo views are dorsal, anterior to the top and flat-mounted.

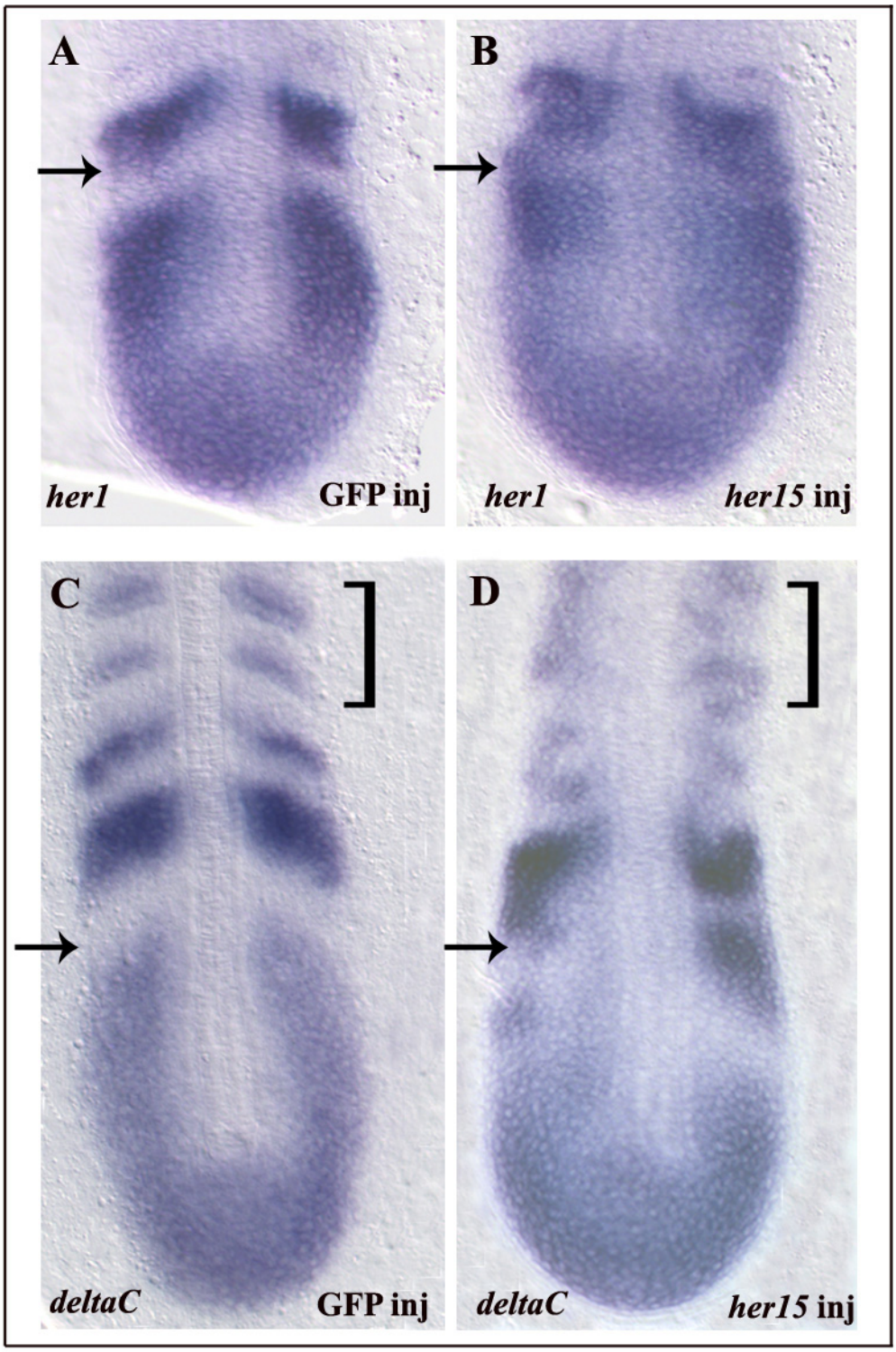


Figure 25. *her1* and *deltaC* are disrupted after *her15* misexpression

4.2 Selective Screening of the NIH Zebrafish cDNA *in situ* Expression Database

The lab of Dr. Igor Dawid has carried out a screen for genes expressed in differential patterns, during embryogenesis in zebrafish (Kudoh et al., 2001). This screen involved *in situ* hybridization of over 2,500 randomly picked cDNA clones from a normalized library from early somitogenesis stages. About 350 of these clones were considered suitable for further study by partial sequence analysis and radiation hybrid mapping. The results of this screen are available online at http://zf.nichd.nih.gov/pubzf/.

The NIH zebrafish cDNA *in situ* expression database was selectively rescreened for ESTs that showed the most promising RNA expression patterns in the regions of the tail bud, PSM and the developing somites in zebrafish embryos of growth stage 3-14 somites. Seven ESTs were selected for further analysis and study which were 3259, 5096, 5116, 5144, 2247, 5038. The original ESTs published in the website were not available and so other available ESTs were selected by sequence similarity and purchased from RZPD (Resourcezentrum für Genomforschung). The RZPD clones corresponding to the ESTs from the Igor Dawid's NIH *in situ* expression database are given in Table 2 in the Materials section. The numbers assigned to each clone are the original numbers which have been assigned in Igor Dawid's NIH zebrafish cDNA expression database.

The primers used for sequencing the ESTs have been given in Table 1. After sequencing, the accuracy of the clones were ascertained by RNA *in situ* hybridization. The *in situ* expression pattern was analyzed in wild type embryos of stages 90% epiboly, 10-14somites and 18-21 somites. The *in situ* expression was also analyzed in different homozygous mutants available in zebrafish for the *Notch* pathway genes such as *after eight* (*aei*; *dld*), *deadly seven* (*des, notch1*), *fused somites* (*fss, tbx24*) and *beamter* (*bea, deltaC*). These four of the genes that are necessary for normal somite formation, which were isolated in the zebrafish genetic screen conducted in Tübingen (van Eeden et al., 1996; Jiang et al., 1996).

Among the seven clones analyzed, clone no 3259 was selected for further study. I will explain in detail the results obtained so far with 3259 namely, *ZfChp* and analysis of the rest of the five gene candidates can be found in the appendix (Section 8).

4.2.1 Clone 3259 – *ZfChp*, molecular nature and protein architecture

In an EST screen for potential zebrafish genes expressed in the presomitic mesoderm during early development, we have been able to isolate *ZfChp*. The genomic organization of *ZfChp* consists of 3 exons. *ZfChp* codes for a 235 amino acid protein. Prosite analysis revealed that *ZfChp* had the classical Rho GTPase domains and hence could be classified as a Rho GTPase member. Phylogenetic analysis and blast analysis revealed that *ZfChp* belonged to the *Chp* class of Rho family GTPases (Figure 26). An alignment has been presented comparing *ZfChp* to human and rat *Chp*. Black shows identity while grey boxes show similarity. *ZfChp* shows sequence conservation to both Human *Chp* and Rat *Chp*, especially in certain regions which are capable of switching Rho GTPase family members into constitutively active or dominant negative forms. These specific residues are present at amino acid positions 38 and 43 respectively in *ZfChp*.

```
                    10         20         30         40         50         60
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Human  Chp     MPPRELSEAE PPPLRAPTPP PRRRSAPPEL GIKCVLVGDG AVGKSSLIVS YTCNGYPARY
Rat    Chp     MPPRELSEAE PPPLPASTPP PRRRSAPPEL GIKCVLVGDG AVGKSSLIVS YTCNGYPSRY
Zf     Chp     MPPQMDYFYH ESRVPSVCLE --QDEELLEP AISCMLVGDG AVGKTSMIVS YTTNGYPTDY

                    70         80         90        100        110        120
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Human  Chp     RPTALDTFSV QVLVDGAPVR IELWDTAGQE DFDRLRSLCY PDTDVFLACF SVVQPSSFQN
Rat    Chp     RPTALDTFSV QVLVDGAPVR IELWDTAGQE DFDRLRSLCY PDTDVFLACF SVVQPSSFQN
Zf     Chp     KQTAFDVFSG QVQVDGTPVR IQLMDTAGQE EFDEFRSLSY AHTDVFLLCF SVVNPTSFQN

                   130        140        150        160        170        180
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Human  Chp     ITEKWLPEIR THNPQAPVLL VGTQADLRDD VNVLIQLDQG GREGPVPQPQ AQGLAEKIRA
Rat    Chp     ITEKWLPEIR THNPQAPVLL VGTQADLRDD VNVLIQLDQG GREGPVPEPQ AQGLAEKIRA
Zf     Chp     ITKKWIPEIR ECNPSSPIIL VGTQSDLVLD VNVLIDLDR- YKVKPVCSSR ARSLSEKIRA

                   190        200        210        220        230
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|...
Human  Chp     CCYLECSALT QKNLKEVFDS AILSAIEHKA RLEKK--LNA KGVRTLSRCR WKKFFCFV
Rat    Chp     CCYLECSALT QKNLKEVFDS AILSAIEHKA RLEKK--LNA KGVRTLSRCR WKKFFCFV
Zf     Chp     AEYVECSALT QKNLKEAFDA AIFAAIKHKA RKAKKRRLSD RRTKAFSKCS WKKFFCFI
```

Figure 26. Phlogenetic analysis of *ZfChp* and other Rho GTPases

(A) All the sequences used for the phylogeny have been taken from NCBI. Except for *ZfChp* and yeast *Cdc42*, all the rest are human Rho family GTPases. *ZfChp* is closely related to human *Chp* and human *Wrch1*. The sequence of *ZfChp* has been deposited in Gen Bank with the accession number AY314756. Sequence alignment and phylogenetic tree calculation was performed with Clustal X (Thompson et al., 1997) and tree drawing done with Tree view (Page, 1996)

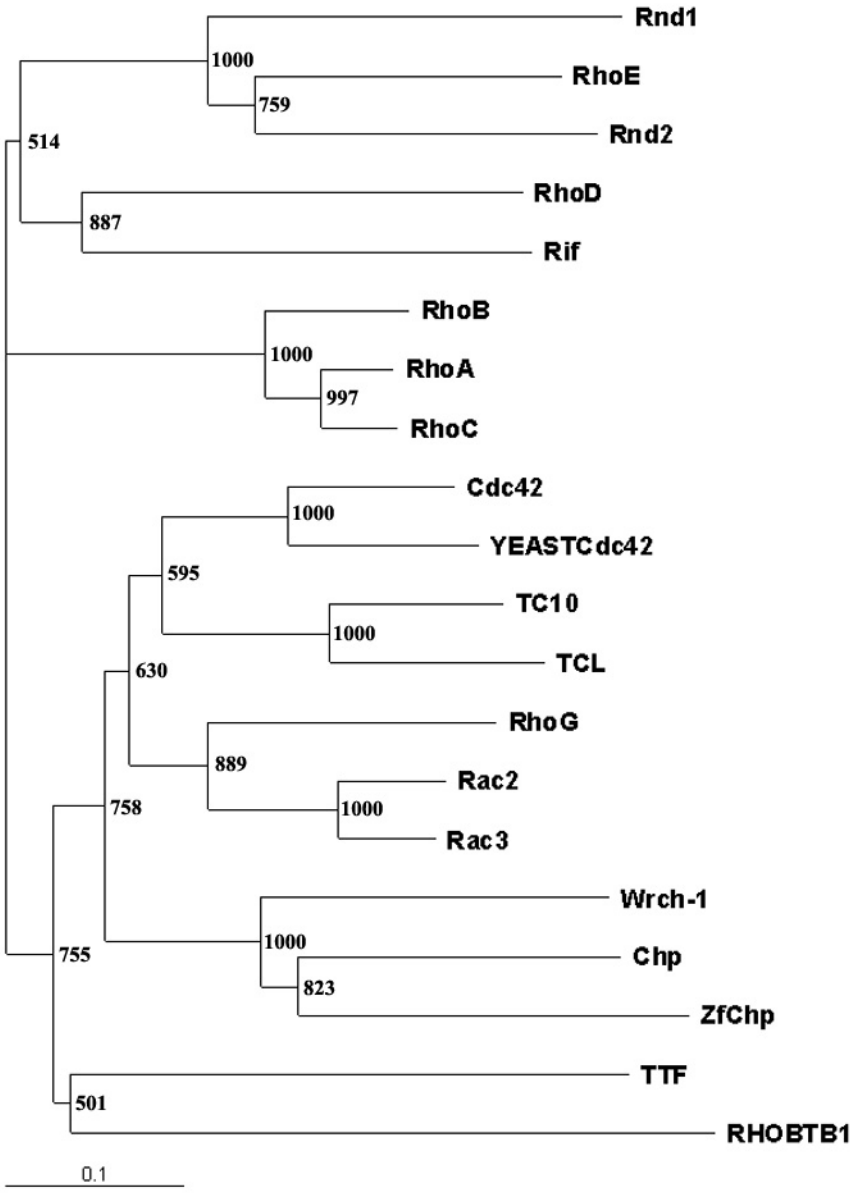


Figure 26. Phylogenetic analysis of *ZfChp* and other Rho GTPases

4.2.2 General properties of Rho family GTPases

Rho GTPases are molecular switches that regulate a variety of cellular processes, which include cytokinesis, cytoskeletal reorganization, membrane transport pathways, microtubule dynamics, transcription factor activity, cell adhesion, endocytosis, cell polarity, asymmetric cell divisions and cell cycle progression. These small, monomeric GTPases, which include Rho, Rac and Cdc42, cycle between active, GTP-bound and inactive GDP-bound forms and they hydrolyze GTP to GDP. In the 'on' (GTP) state, GTPases recognize target proteins and generate a response until GTP hydrolysis returns the switch to the 'off' state. This basic molecular switch mechanism is highly conserved and gene duplication and divergence has allowed expansion of the Rho GTPase family to continue late into evolution. Although these proteins have been known to control an amazingly diverse range of cellular functions, one general role is in the establishment of polarity and of polarized structures through dynamic regulation of the actin cytoskeleton. (Wherlock and Mellor, 2002; review by Etienne-Manneville and Hall 2003).

4.2.3 *ZfChp* mRNA is maternal and ubiquitously expressed

ZfChp messenger RNA transcripts are maternally deposited and this can be verified by *in situ* hybridization experiments in zebrafish embryos younger than 4 and1/2 hours. In zebrafish embryos, it is around 4 and 1/2 hours into development that zygotic transcription begins embryonic genes is referred to as Mid blastula transition (Kane and Kimmel, 1996). Any mRNA product detected in embryos before 4 and 1/2 hours which includes the 1 cell and 4 cell stage embryos, depict maternal mRNA deposition. *ZfChp* does not have a spatially restricted expression pattern during the first 16 hours of development (Figure 27 A-D). The most interesting feature is the presence of high mRNA expression in stripes of cells located on either side of the neural tube in the intermediate presomitic mesoderm region. This striped expression pattern is dynamic and the number of stripes varies between one or two pairs (Figure 27F-G)

4.2.4 Mapping of *ZfChp*

To exploit the possibility of mutants being present for *ZfChp* from the many zebrafish mutagenesis screens conducted in numerous labs, radiation hybrid panel mapping for the gene was done in the zebrafish RH mapping facility. This facility is located in the lab of Dr. Leonard Zon at Children's Hospital, Harvard University, Boston, U.S.A. As part of the ongoing zebrafish genome project, a comprehensive Radiation Hybrid map is being created using available EST sequences and genetic markers (Hukreide et al., 1999). This map can be used to identify genes contributing to mutant phenotypes and for comparative genomics. The RH maps offer a better resolution than available genetic maps and can also place the gene of interest in context to many other type of markers including ESTs, genes, BAC ends and other sequences. *ZfChp* was mapped to Linkage Group 20 which corresponds to chromosome 20 of the zebrafish genome and to EST zeh0300_T51 (Figure 28). But unfortunately, there are no available zebrafish mutants corresponding to this particular map location.

4.2.5 *ZfChp* is a target of Notch signaling

ZfChp is faintly expressed throughout the embryo with stronger stripe-like expression domains in the intermediate PSM in wild type embryos. The striped mRNA expression domain of *ZfChp* in the intermediate presomitic mesoderm is specifically lost in the Notch pathway zebrafish mutants namely *after eight (aei, deltaD), deadly seven (des, notch1), beamter (bea, deltaC)* and *fused somite (fss, tbx24).* It is only the stripes in the intermediate PSM region, which are lost in the zebrafish Notch pathway mutants while the expression domains of *ZfChp* in the other regions of the embryo remain unaffected, and is similar to the wild type situation. In all the mutants, it was observed that the presomitic mesoderm component of the *ZfChp* expression is either disturbed or expression is reduced or even lost completely (Figure 29). Instead of stripes, a broad expression is observed at least in *aei* and *bea,* whereas in *fss,* stripe expression is absent. In *Su(H)* morpholino knockdown embryos, as well as in *des,* stripe expression seems disturbed and greatly reduced. This suggests strongly that *ZfChp* is a target of the Notch

signaling pathway which is responsible for its stripe expression and dynamic expression. *tbx24* is obviously needed, either directly or indirectly, for activating *ZfChp*.

Figure 27. *ZfChp* mRNA is maternal and ubiquitously expressed

(A-D) Embryos of varying growth stages showing the ubiquitous distribution of *ZfChp* mRNA. The growth stages have been labeled beneath each embryo. The presence of *ZfChp* mRNA in 1 cell stage and 4 cell stage show that there is maternal contribution. (E-G) Embryos showing *ZfChp* mRNA distribution during 10-12 somite stage. There is ubiquitous expression in the PSM accompanied by an upregulated signal, visible as a pair of stripes. These stripes are dynamic because in a population of embryos, there are those which have one pair of stripes as can be seen in (F) and two pairs of stripes as is the case in (G). (I) Embryo showing *ZfChp* mRNA distribution during 18-21 somite stage. The asterisk* points to a strong signal in the eye and the black arrow points to the stripes in the PSM. Embryo views are lateral in A, C, D, I and dorsal in B. Anterior to the top in A, C, D, F, G. Anterior to the left in I. In E, both anterior and posterior are to the right. (A-E) and (I) are whole mounts and (F-H) are flat-mounted.

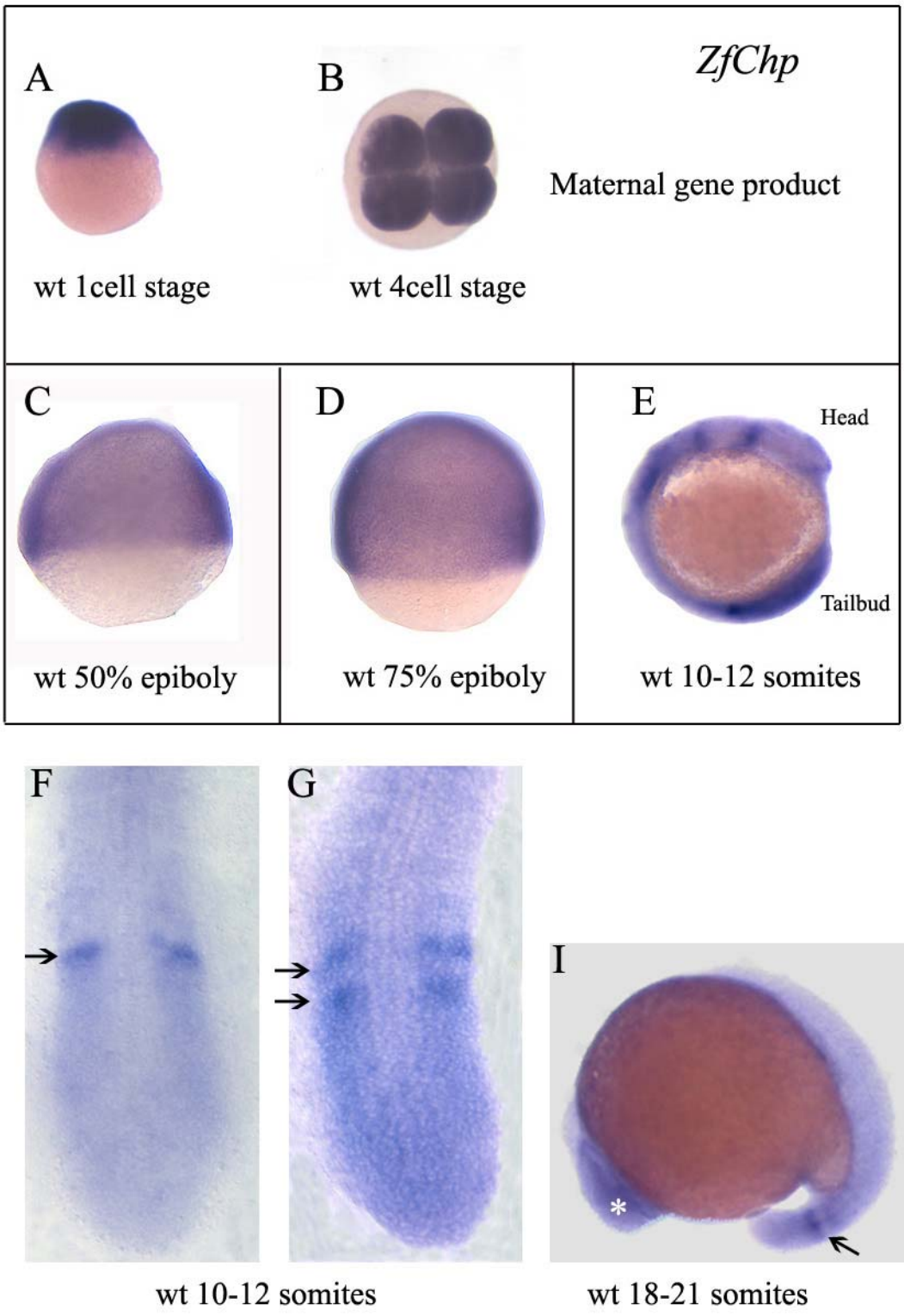


Figure 27. *ZfChp* mRNA is maternal ubiquitously expressed

Figure 28. Mapping of *ZfChp*

ZfChp **was mapped at the Zebrafish RH mapping facility, which is located in the lab of Dr.Leonard Zon at Children's Hospital, Harvard University , Boston, U.S.A.** ***ZfChp*** **was mapped to Linkage Group 20 which corresponds to chromosome 20 of the zebrafish genome and to EST zeh0300_T51, which has been marked in a red rectangle.**

Figure 28. Mapping of *ZfChp*

Figure 29. *ZfChp* is a target of the Notch signaling.

(A-C) and (E) Somitogenesis mutant embryos showing the loss of *ZfChp* stripes. The black arrows point to the region in the PSM where the stripes should appear normally in wild type embryos. As can be seen clearly, there are no visible stripes in the mutants and there is just ubiquitous distribution of the mRNA signal. Instead of stripes, a broad expression is observed at least in *aei* and *bea,* whereas in *fss*, stripe expression is absent. D) *Su(H)* morpholino knock down embryo also showing the loss of *ZfChp* stripes in the PSM region. In *Su(H)* morpholino knockdown embryos, as well as in *des*, stripe expression seems disturbed and greatly reduced. All embryos are 10-12 somites. Embryo views are dorsal, anterior to the top and flat-mounted.

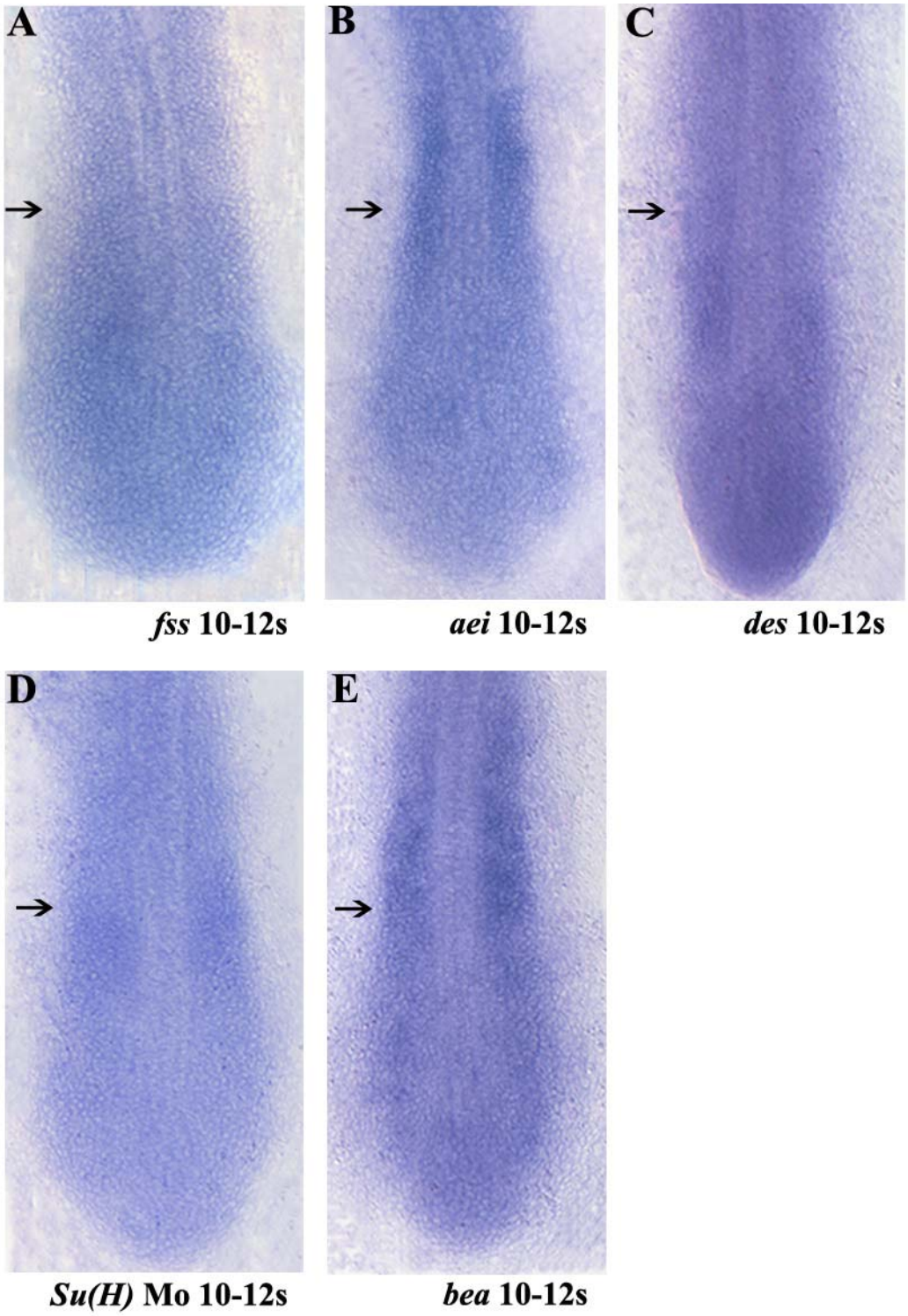
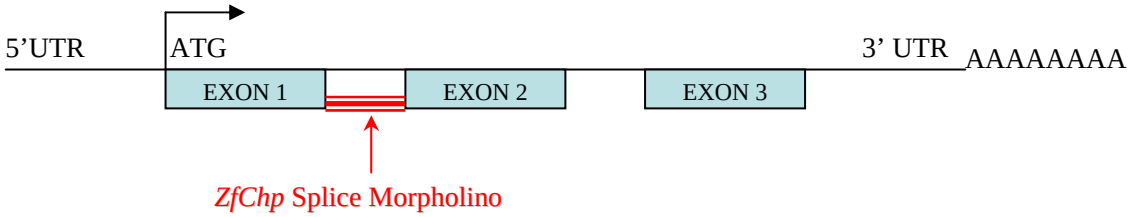


Figure 28. *ZfChp* is a target of the Notch signaling.

4.2.6 Functional analysis-*ZfChp* morpholino gene knockdown

To check for a functional role for *ZfChp* in zebrafish somitogenesis, morpholino oligonucleotides were designed to specifically knock down the function of *ZfChp* in zebrafish embryos. Morpholino oligonucleotides function by blocking translation of their target mRNA's (for review see Sumanas and Larson, 2002). To avoid the ambiguity associated with the presence of alternate 5' UTR's during morpholino target sequence selection, a Splice Morpholino was designed for *ZfChp* which targets the exon-intron border between the first and second exon.

***ZfChp* Splice Morpholino Design**



The *ZfChp* Splice Morpholino was injected in varying concentrations from 0.6mM to 1mM and checked for morphological effects the proceeding day. The embryos were later fixed for *in situ* analysis of *ZfChp* mRNA knockdown effects on gene candidates which are known to play prominent roles in zebrafish somitogenesis namely *her1, her7, deltaC, deltaD, myoD, mespa, mespb* etc. Both at the morphological level and the *in situ* level, no effects could be observed.

4.2.7 Functional analysis- *ZfChp* misexpression

The full length coding sequence of *ZfChp* was cloned into pCS2+ vector, capped mRNA prepared and injected as described in the Methods section. Varying concentrations were used, ranging from 50ng/µl to 250ng/µl. As with the morpholino approach, both the morphological effects on live embryos and *in situ* effects on candidate genes involved in somitogenesis were analyzed but unfortunately there were no clear, observable effects.

5. Discussion

Somite segmentation in vertebrate embryos is controlled by a molecular clock in the form of a transcriptional oscillator that operates in the presomitic mesoderm (PSM). Most of the genes implicated in the somitogenesis oscillator belong to the Notch signaling cascade (see review (Rida et al., 2004), the exceptions being *Axin2*, *Nkd1* in mouse which are Wnt pathway genes and *receptor protein tyrosine phosphatase ψ* (*RPTPψ*), in zebrafish (Aerne and Ish-Horowicz, 2004; Aulehla et al., 2003; Ishikawa et al., 2004). The *h/E(spl)* family of Notch target genes are transcription factors and important regulators of the somitogenesis oscillator. They are called *her/Hes/esr* genes and a subset of them is characterized by oscillating mRNA expression domains in the PSM, which include *her1*, *her7*, *her11*, *her12* and *hey-1* in zebrafish, *c-hairy1* and *c-hey2* in chick, *Hes7* and *Hes5* in mouse and *esr9* and *esr10* in *Xenopus* (Holley et al., 2000; Holley et al., 2002,Oates and Ho, 2002; Henry et al. 2002, Gajewski et al., 2003, Sieger et al, 2004, *her12*-Sieger et al., manuscript in preparation, Winkler et al., 2003; Palmerim et al., 1997; Leimeister et al., 2000; Li et al., 2003; Jouve et al., 2000; Bessho et al., 2001). They are called *her/Hes/esr* genes and are conserved components in all vertebrate somitogenesis models studied namely, chick, mouse, zebrafish and *Xenopus* (for review see Pourquie, 2000). But there are species specific differences in the recruitment of the different oscillating *h/E(spl)* related genes to the somitogenesis process and this has been reflected in the phylogentic relationships between them (Prince et al., 2001; Leve et al., 2001; Gajewski and Voolstra, 2002; Gajewski et al., 2003). A genome wide search for *h/E(spl) related* genes in zebrafish conducted by Sieger et al., (2004), resulted in three new oscillating genes, one of which is *her15*. *her15* has oscillating mRNA expression domains and that this feature is evolutionarily conserved in its homologues in mouse and *Xenopus* (Dunwoodie et al., 2002; Li et al., 2003), is strongly suggestive of a functional role in somitogenesis. The results obtained till date have been discussed in this perspective.

5.1 *her15* is a novel oscillating *her* gene in zebrafish

her15 is a novel component of the zebrafish presomitic mesoderm oscillator. The most interesting feature is the oscillating/cycling expression compartment in the tail bud region (posterior PSM). This expression compartment which is referred to as "micropulsing," is truly unique among the published zebrafish *hairy (h)* and *enhancer of split (E(spl)) related* genes. The cyclically expressed genes in zebrafish namely *her1* and *her7*, have an independent broad mRNA expression compartment in the posterior PSM, which has a 'U' shape and two broad stripes in the anterior PSM which appear to bud off from this U-shaped posterior domain (Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2000; Holley et al., 2002; Oates and Ho, 2002; Takke and Campos-Ortega, 1999). *her1* and *her7*, during the different cycling phases, give the impression of a wave moving from the posterior to the anterior PSM. In *her15*, there is only oscillation in a broad domain in the posterior PSM which has a round or oval shape, and there are no stripes which are budding off from this posterior domain (Figure 13). Hence, one does not have the impression of a wave moving from the posterior to anterior PSM, but more of a micropulsing mRNA expression domain restricted to the posterior PSM. This micropulsing domain, which is round or oval shaped exhibits changes in its surface area and goes through a broad phase where maximum surface area is seen, an intermediate phase and a dot-like phase where the mRNA expression seems to be restricted to a very small area in the posterior PSM surrounding the base of the notochord. The differentially expressed cycling *h/E(spl) related* genes in zebrafish give an idea of the compartmentalisation of the cycling mRNA expression compartments and also supports the data which has come out of the promoter analysis of *her1* (Gajewski et al., 2003).

On observing this micropulsing posterior domain of *her15* in detail in numerous embryos, it gives the impression that there could be a "Starting pulse" in the tail bud, which then drives the cycling mRNA expression wave of genes such as *her1* and *her7* forward, such that they sweep across the PSM, similar to a wave starting at the posterior and then moving towards the anterior. If this proves to be the case in the future, both *her15* and *her12* in zebrafish, would be potential candidates involved in this 'Starting pulse'.

Data available from somitogenesis studies in a variety of vertebrate models suggest that the Notch signaling cascade is a key regulator of the somitogenesis process (for reviews see (Pourquie, 2001; Saga and Takeda, 2001). An obvious conclusion would be that the activity of a potential somitogenesis candidate gene has to be regulated by the Notch signaling cascade. In agreement to this, *her15* mRNA expression analysis in the Delta-Notch class of somitogenesis mutants (namely *aei/deltaD, bea/deltaC* and *des/notch1*), have shown that *her15* oscillations in the posterior PSM is dependent on full functionality of the Notch signaling cascade. Full functionality has to be stressed due to the presence of multiple *notch* and *delta* genes in zebrafish (for review see (Stickney et al., 2000) and hence the high redundancy in gene activity which has to be expected as a result of a gene duplication event, which occurred during the evolution of the teleost lineage (Hoegg et al., 2004; Venkatesh, 2003). The *Su(H)* gene is the nuclear target of the Notch signaling cascade (Oka et al., 1995; Sieger et al., 2003). *her15* mRNA analysis in *Su(H)* morpholino gene knock down embryos have shown that *her15* is activated by the Notch signaling cascade. *her15* being a target of the Notch signaling cascade and its mRNA showing oscillatory expression, supports a potential role in the somitogenesis process.

To investigate the role of *her15* in the regulation of oscillatory gene expression in the PSM and somite border formation, functional analysis of *her15* was carried out by means of RNA misexpression studies and morpholino gene knock down approaches. *her15* RNA misexpression studies led to visible abnormal somite borders, coupled to the disruption of *her1* and *deltaC* mRNA signals in the PSM region and somites. This suggests a role for *her15* in somite border formation and regulation of oscillatory gene expression of *her1* and *deltaC*. Disruption of *deltaC* mRNA following *her15* misexpression may also point to a possible feed back loop exerted on the upstream Notch signaling cascade. *her15* morpholino geen knock down studies did not cause disruption of somite borders or oscillatory gene expression

5.1.1 Is *her15* a component of the zebrafish somitogenesis oscillator or just an output of the clock?

To be a component of the zebrafish somitogenesis oscillator, functional analysis of a candidate gene must lead to disruption of oscillatory gene expression in the posterior PSM region, coupled with defects in somite border formation and feed back regulation of the upstream Notch signaling cascade. These functional studies are largely carried out by means of mRNA misexpression and morpholino gene knock down analysis. Analysis of *her1*, *her7*, *her4* and *her6*, in zebrafish have shown that they are crucial for the regulation of the zebrafish somitogenesis oscillator (Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2002; Oates and Ho, 2002; Pasini et al., 2004), as they bring about the desired effects. Among them, *her1* and *her7* show oscillating mRNA expression domains in the posterior PSM, while *her4* and *her6* do not. A feed back loop on Notch signaling cascade would classify *her15* as a component of the zebrafish somitogenesis oscillator, while just the disruption of *her15* mRNA expression in Notch mutants would make *her15* an output of the somitogenesis clock. Misexpression data has to be coupled with morpholino gene knock down studies to provide evidence for a prominent functional role in somitogenesis. *deltaC* is a ligand of the Notch signaling cascade and acts upstream of *her15*. Disruption of *deltaC* following *her15* misexpression suggests that *her15* exhibits feed back regulation on the Notch cascade. It may also be the case that, the disturbance to *her1* by *her15* misexpression is responsible for the *deltaC* disruption as it is known that *her1* exhibits feed back regulation on the Notch pathway. Hence, *her15* may be affecting *deltaC* directly through a feed back loop, or indirectly through *her1*. *her15* misexpression studies support a potential role in the somitogenesis oscillator, but *her15* morpholino gene knock down experiments did not lead to a penetrant somitic phenotype.

According to the guide lines from Gene Tools Inc., morpholino oligonucleotides are usually designed against the 5’ UTR region of a chosen gene or against the start Methionine. In the case of *her15*, only one morpholino has been tested and this is directed against last 15 bases of the 5’UTR and the following first 10 bases from the open reading frame. A lack of penetrant phenotype in the *her15* morpholino studies cannot be

directly concluded as due to gene redundancy due to the fact that for certain genes, injection of two morpholinos simultaneously, one against the 5'UTR and one against the start Methionine (both not overlapping), is required to achieve complete knock down. This is the case when genes have alternative 5'UTR regions. Experiments to look for alternate 5 UTR regions for *her15* have not proven successful; nevertheless, one cannot exclude this possibility. In addition, the efficiency of the available *her15* morphlino to knock down *her15* transcripts has to be tested by invivo or invitro experiments (Hans et al., 2004; Sumanas and Larson, 2002). Hence there is a lack of conclusive evidence for primary involvement of *her15* in the regulation of the zebrafish somitogenesis oscillator. With the present data set, it can only be concluded that *her15* is indeed oscillating and is an output of the zebrafish somitogenesis oscillator. This may be due to the redundancy in gene activity generated by the presence of three other related genes in zebrafish namely *her2*, *her4* and *her12* (zebrafish mouse *Hes5* homologues(Sieger et al., 2004). In conclusion, the available data suggests that *her15* is a novel gene with oscillating mRNA expression domains and a potential role in zebrafish somitogenesis.

5.1.2 The stripes of *her15* are expressed at double segmental distance in the anterior PSM

The dynamic stripes of *her15* which are expressed in a double segmental distance at the posterior borders of somites, S-I and SI, is a unique feature when compared to the other *hairy and E(spl)* family genes in zebrafish known to date. This striped expression of *her15* is positively regulated by the Notch-signaling cascade, *fss/tbx24*, *her1* and *her7*. The double segmental feature is reminiscent of *her15*'s similarity to the *hairy* pair rule gene in *Drosophila*. *hairy* in *Drosophila* is expressed in every alternate segment formed and is seen as 7 stripes (Ish-Horowicz et al., 1985). The pair rule mechanism in *Drosphila* which directs segmentation is different from somitogenesis in zebrafish but the double segmental stripe feature displayed by *her15* may be a conserved mRNA expression feature between *her15* in zebrafish and *hairy* in *Drosophila*.

5.1.3 *her15* mRNA is expressed in the posterior wall of the PSM

The PSM is continuously formed from the marginal epiblast cells during gastrulation and from the dorso-medial region of the tail bud (the posterior wall) later on (Kanki and Ho, 1997). In a publication by Griffin et al, 2002, the authors have looked at the posterior PSM of wild type zebrafish embryo in great detail and discovered that there is a gradual progression in gene expression (Figure 30). Prospective PSM progenitors in the posterior wall express genes such as *notail (ntl)* and *wnt8* (Griffin et al, 1998; Schulte-Merker et al., 1992; Kelly et al, 1995). When cells leave the posterior wall, they activate expression of PSM marker genes such as *mesogenin*, which is excluded from the most distal cells in the tail bud. *mesogenin* expression overlaps significantly with *ntl* and *wnt8* in the distal region of the PSM and this specific region, where overlap can be seen is called the ‘maturation zone’ (the yellow zone in the schematic representation in Figure 29). In the maturation zone, the PSM cells remain in the mesenchymal state. The dorsal to ventral distribution of *her15* mRNA in the posterior PSM, visible after sagittal sections, is suggestive of *her15* being expressed in the posterior wall of the PSM. This expression points to the possibility that *her15* may play a role in maintaining the somite progenitor cells in the posterior PSM/tail bud in the mesenchymal state prior to MET.

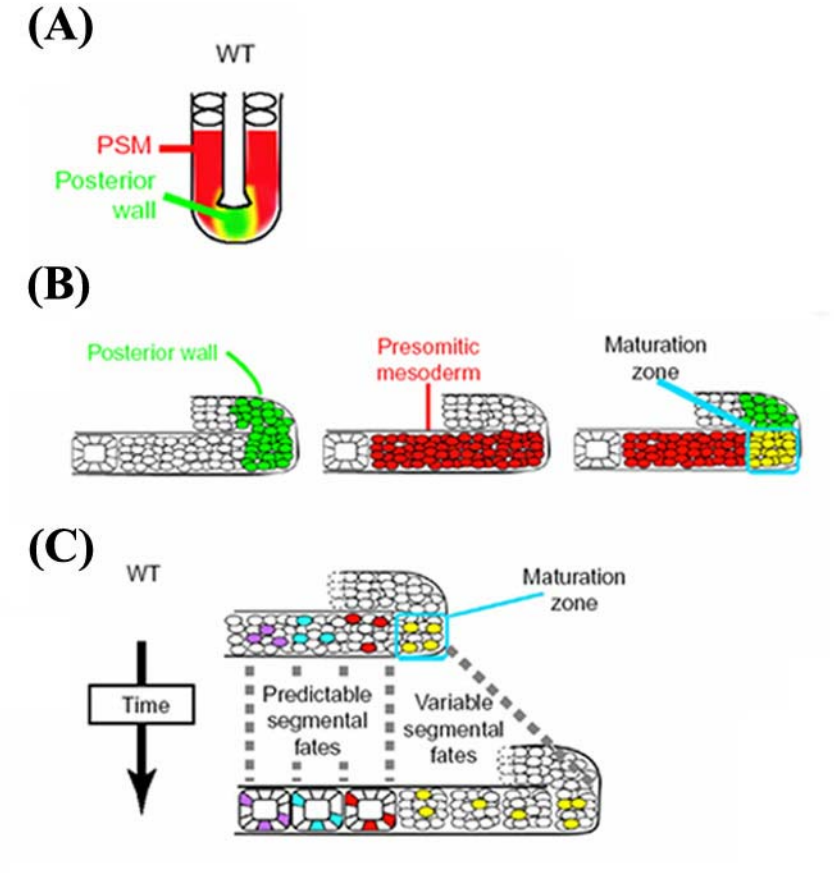


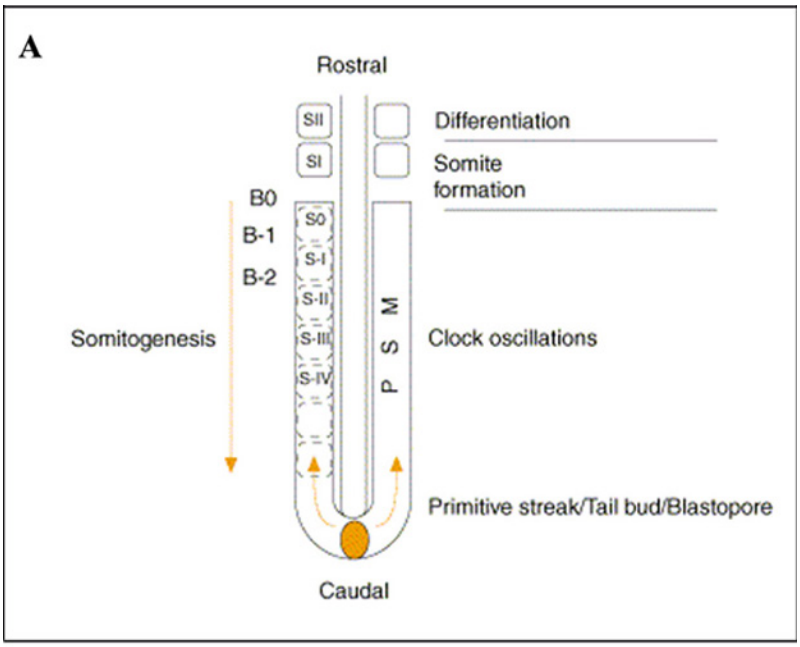
Figure 30. Gradual progression of gene expression in the posterior PSM wall of wild type zebrafish embryos. (A) Posterior PSM showing *Ntl* (green in schematic representation) and *mesogenin* (red in schematic representation) co-expressed only in the most distal PSM (bracket; yellow in schematic representation). (B) Schematic representations summarizing gene expression patterns in the PSM; rostral is left, dorsal uppermost. As progenitors move from the margin or posterior wall into the PSM, they switch off expression of posterior wall markers (for example,*ntl*, *wnt8;* green) and switch on expression of PSM markers (for example,*PAPC*, *mesogenin*; red). There is a region of overlap in expression (yellow) in PSM cells closest to the margin (the maturation zone). (C) A summary of single-cell labeling experiments to analyse segmental fates within the PSM. Cells are labeled with a lineage tracer at time zero and their segmental fate are assessed after variable time. PSM cells labeled at a distance from the margin have a predictable fate and tend to contribute to the same somite as their neighbours. In contrast, cells labelled in the maturation zone (yellow) have variable segmental fates and contribute to somites over a large area of the embryo. This indicates that cells reside in the maturation zone for variable periods of time. While resident in the maturation zone, cells move, or are passively carried, distally with the rest of the tail bud. The Figures (A-C) and the legends have been taken from Griffin et al., (2002).

5.1.4 *her15* shares conserved mRNA expression domains with other mouse *Hes5* homologues in chick, mouse and *Xenopus.*

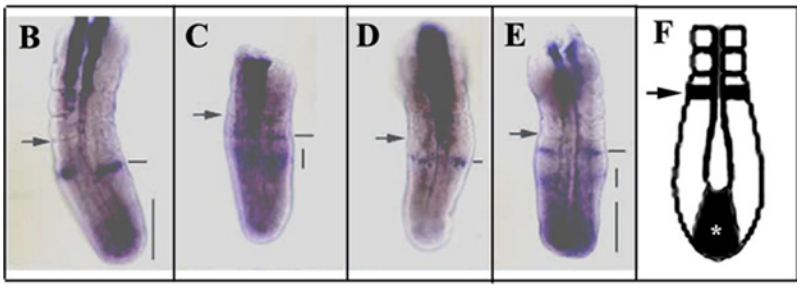
The homologues of *her15* in mouse and *Xenopus* are *Hes5* and *esr9* and *10* respectively. The micropulsing domain in the posterior PSM region, as well as the stripes in the anterior PSM are conserved between *her15* in zebrafish, mouse *Hes5* and *Xenopus esr9* (Figures 31 and 32) (Dunwoodie et al., 2002; Li et al., 2003). The micropulsing domain of *her15* is expressed in the posterior PSM region, which extends from the posterior of the notochord to the final edge. In chick and mouse, there is supporting evidence that this region demarcates a specific zone, which has the potential to give rise to pluripotent stem cells (Figure 31, A) (Pourquie and Tam, 2001; Cambray and Wilson, 2002; Liu, et al., 2004). But in zebrafish, to date, no stem cell populations have been identified in the tail bud region. Nevertheless, the location of the *her15* oscillating domain in the posterior PSM and pluripotent stem cell regions in chick and mouse embryos are comparable at the embryological level.

A phylogenetic analysis of all known *h/E(spl)* family genes in zebrafish, show that there are to date, 4 *Hes5* homologues namely *her2*, *her 4*, *her12* and *her15* (Sieger et al., 2004). All theses genes show comparable neurogenesis and somitogenesis mRNA expression domains distributed throughout the embryo. Among them, *her2* and *her4* (von Weisäcker, 1994; Takke et al., 1999) are very similar in expression but not oscillating, while *her12* and *her15* are oscillating in the posterior PSM. From the published data and our independent findings, *her15* appears to have overlapping expression domains with *her4*, in a small domain in the posterior PSM, at the base of the notochord, and in certain neuronal expression domains such as the lateral stripe, medial stripe and intermediate stripe (refer to Figure 11, for *her15* expression domains). The Notch ligand, *deltaD*, a somitogenesis and neurogenesis gene, (Hans et al, 2003), is also expressed in these embryonic regions. But the oscillating *deltaC*, also a Notch ligand, is only expressed in the posterior PSM and in the somites (Jiang et al, 2000). This is suggestive of possible differential regulation of *her15* and perhaps, *her4*, by the two Notch ligands, *deltaC* and *deltaD. deltaD* might regulate *her15* and *her4* expression domains in the lateral stripe,

medial stripe, intermediate stripe and posterior PSM region, where all three genes appear to have over lapping mRNA expression domains, while *deltaC* would regulate *her15* and *her4*, only in the posterior PSM.



Model of chick embryo showing the primitive streak



***Hes5* in wt mouse embryos of age 10.5 dpc**

Figure 31. A model of the chick embryo showing the primitive streak and oscillating expression domains of mouse *Hes5* (A) Model of the chick embryo showing the location of the primitive streak in the posterior PSM. There is compelling evidence that the primitive streak has the ability to generate pluripotent stem cell populations. This model has been taken from Pourquie and Tam, (2001). (B-E) Oscillating mRNA expression domains of *Hes5* in wild type (wt) mouse embryos of age 10.5 dpc. It consists of a cycling domain in the posterior PSM and stripes in the anterior PSM. A tight band of *Hes5* expression is marked with a horizontal line; broader caudal domains of expression are marked with a vertical line. The black arrows indicate stripes. *Hes5* is also strongly expressed in the neural tube. The Figures (B-E) and legend,

have been modified from Dunwoodie et al., (2002). (F) Schematic representation of mouse *Hes5* mRNA expression compartments. The black arrow points to the stripe and the white asterisk * marks the oscillating mRNA expression domain in the posterior PSM. This picture has modified from Barrantes et al., (1999).

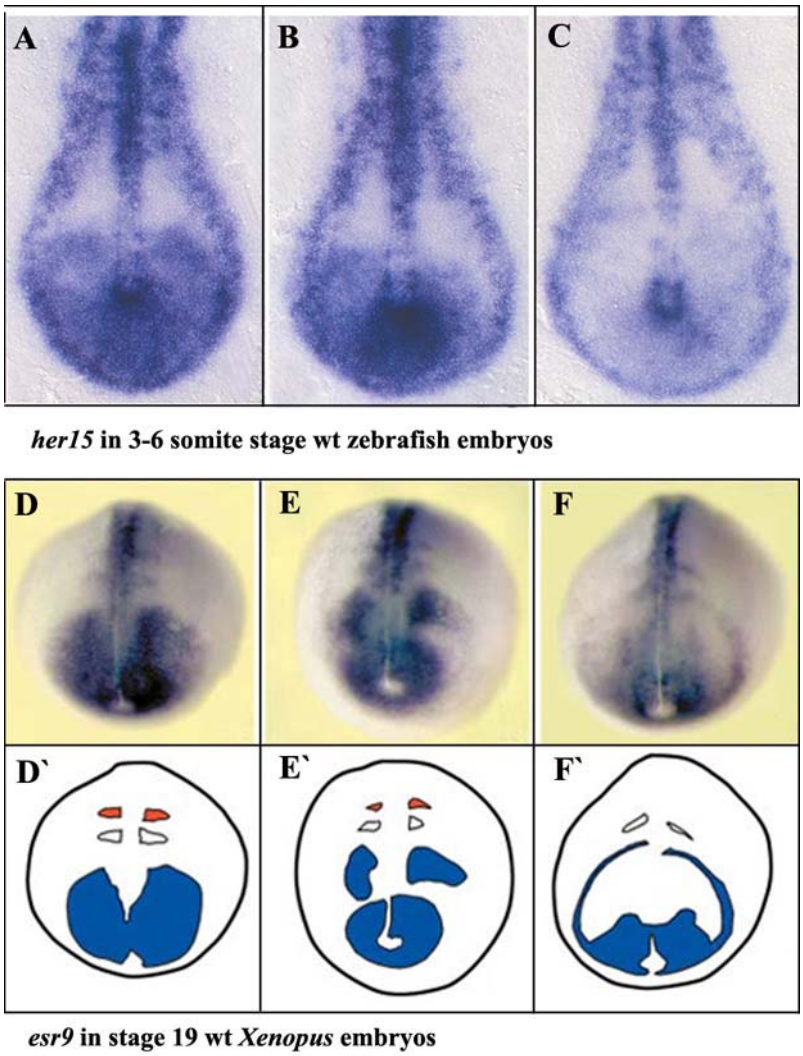


Figure 32. Comparison of mRNA expression domains of mouse *Hes5* homologues in zebrafish and *Xenopus*. (A-C) Zebrafish embryos showing the 3 cycling phases of *her15*. (D-F) *Xenopus* embryos showing *esr9* expression in 3 cycling phases. In addition to the oscillating domain in the posterior PSM, stripes can be seen in the anterior PSM. (D`-F`) Schematic representations of the *esr9* cycling phases in *Xenopus* embryos. Blue labels the oscillating domain in the posterior PSM while red marks the stripes in the anterior PSM. Embryo views are dorsal, anterior to the top. (A-C) are flat-mounted and (D-F) are whole-mount views. The pictures (D-F) and (D`-F`) have been taken from Li et al., (2003).

5.1.5 Synergistic interactions between *Hes1* and *Hes5* providing a possible explanation for the lack of penetrant phenotypes in mouse

her15 in zebrafish is a mouse *Hes5* homologue. Mouse *Hes1* and *Hes5* have oscillating mRNA expression domains in the PSM but the *Hes1* and *Hes5* null embryos have no somitic defects. Mouse *Hes7* also oscillates and the knock out shows a somitic phenotype. *Hes5* knock out mouse as such does not show any obvious defect in neurogenesis or somitogenesis. However, synergistic effects between *Hes1* and *Hes5* have been reported in mouse with respect to neurogenesis (Cau et al., 2000; Hatakeyama et al., 2004), but not somitogenesis. It has been shown in mouse that basic helix-loop-helix genes *Hes1* and *Hes5* are essential effectors for Notch signaling, which regulates the maintenance of undifferentiated cells (Artavanis-Tsakonas et al., 1999; Gaiano and Fishell, 2002; Hitoshi et al., 2002; Honjo, 1996; Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999; Selkoe and Kopan, 2003). Reports also mention that *Hes1* and *Hes5* play an important role in maintenance of neural stem cells (Cau et al., 2000; Nakamura et al., 2000; Ohtsuka et al., 2001; Tomita et al., 1996). In the mouse, complementary expression patterns have been observed for *Hes1* and *Hes5* in wild type embryos and compensatory expression patterns for *Hes1* in the *Hes5* null embryo and vice-versa. *Hes1;Hes5* double mutant embryos show defects which are more specific to the nervous system than those of Notch-mutant mice (Conlon et al., 1995; Hamada et al., 1999; Swiatek et al., 1994). A recent publication by Hatakayama et al., (2004), report that triple knock out embryos of mouse *Hes1, Hes5* and *Hes3* show that these genes are essential for the generation of brain structures of appropriate size, shape and cell arrangement by maintaining neural stem cells and controlling the timing of neural stem cell differentiation. Cell differentiation becomes greatly accelerated in the absence of *Hes* genes, which leads to the depletion of radial glial cells and the resultant disorganization of the structural integrity of the nervous system.

5.1.6 Mouse *Hes7, Hes5 and Hes1* homologues in zebrafish

h/E(spl) family genes in zebrafish which have an oscillating domain in the posterior PSM can be grouped into two classes namely mouse *Hes7* class consisting of *her1* and *her7* and mouse *Hes5* class consisting of *her12* and *her15* (Sieger et al., 2004). In zebrafish the mouse *Hes7* ancestor seems to be duplicated, and both genes have retained their function in somitogenesis. *her1* and *her7* in zebrafish have only the PSM mRNA expression compartments Both *her12* and *her15* show complex neuronal and brain expression domains coupled to an oscillating posterior PSM domain (*her12*-Sieger et al., manuscript in preparation). These complex oscillating mRNA expression domain of mouse *Hes5* homologues are comaparable in zebrafish, mouse and *Xenopus* (Figures 30 and 31). It is unclear why such a complex mRNA expression pattern exists in the case of mouse *Hes5* homologues in zebrafish, in comparison to mouse *Hes7* homologues. What is questionable is the lack of penetrant phenotypes in the independent gene knock down experiments carried out with oscillating *Hes5* in mouse and its homologue *her15* in zebrafish. Genes with such widespread and complex mRNA expression patterns are either suggestive of a possible independent functional role or may have no specific function due to redundancy in the system and compensatory or overlapping mRNA expression domains from related gene family members. The duplicated mouse *hes1* homologues in zebrfaish (*her6* and *her9*) either play no role in somitogenesis (*her9*) or do not show oscillating expression (*her6*) (Gajewski and Voolstra, 2002; Leve et al., 2001; Pasini et al., 2004; Sieger et al., 2004)

The *Hes* gene situation in mouse appears highly complicated. In zebrafish, gene duplication has led to a larger number of *her* genes (Gajewski and Voolstra, 2002; Hoegg et al., 2004; Sieger et al., 2004; Venkatesh, 2003) and greater probability of redundant gene activity. Obviously, what is seen in mouse is the complex function of one gene. Gene duplication not only gives rise to more genes, but also paves the way for subfunctionalization of gene activity (Force et al., 1999). *her1* and *her7* show differential effects on somitogenesis following morpholino gene knock down studies (Gajewski and Voolstra, 2002), even though they are duplicated genes of mouse *Hes7*. *her12* and *her15*,

are both mouse *Hes5* homologues in zebrafish, which are oscillating, sharing similar expression domains throughout the embryo, but at the same time dynamically and differentially expressed in a wide variety of tissues which include posterior and anterior PSM, notochord and neural tube. *her1* and *her7*, are the two *her* genes in zebrafish which show the strongest disruption of somitogenesis on morpholino gene knock down studies. In the situation that an individual gene candidate does not show a phenotype following gene knock down approaches, one can consider injecting different combinations of morpholinos to check how the many *her* genes in zebrafish regulate one another during somitogenesis. In addition to morpholinos, zebrafish transgene technology also has to be exploited to generate new deletion lines. A deletion line for both mouse *Hes7* homologues in zebrafish namely *her1* and *her7* (Henry et al., 2002), is available at the moment but unfortunately triple knock out experiments have proved difficult in this line. Nevertheless, deletion mutants for oscillating genes in zebrafish with possible additional morpholino gene knock down experiments, remain a definite future possibility for somitogenesis studies.

5.2 Clone-3259-*ZfChp*

Prepatterning of the PSM, establishment of rostro-caudal polarity and mesenchymal to epithelial transition occuring during somite border formation are important landmarks in somitogenesis. *ZfChp*, a candidate which came out of the screen of the NIH cDNA *in situ* expression database has been studied for a potential role in these processes.

5.2.1 *ZfChp* and its Rho family GTPase domains

ZfChp was chosen as one of the candidate genes for further study from the NIH cDNA *in situ* expression database because it showed a very dynamic striped mRNA expression domain in the intermediate presomitic mesoderm region. These stripes of *ZfChp* mRNA expression are not visible in the Delta-Notch class of somitogenesis mutants or in the *Su(H)* morpholino knock down embryos, which suggest that the particular striped expression compartment is positively regulated by the Delta-Notch signaling cascade.

ZfChp is a Rho family GTPase and there are no previous reported instances of a molecule of this family showing a dynamic expression component in the intermediate presomitic mesoderm or anywhere else in an embryo.The specific strip-like expression pattern in the intermediate presomitic mesoderm also presents the possibility that *ZfChp* may play a role in mesenchymal to epithelial transition (MET) which occurs in this region. *ZfChp* has the characteristic Rho GTPase domains and Rho GTPases namely *Rac1* and *Cdc42* have been implicated in cytoskeletal changes associated with MET in chick embryos (Nakaya et al., 2004). However, *Rac1* and *Cdc42* in chick embryos are ubiquitously expressed without local transcript amount differences. MET universally involves Rho GTPases and there is a high probability that the same or comparable situation exists in zebrafish.

5.2.2 *ZfChp* as a possible link between somite prepatterning and MET in zebrafish embryos.

It is known that Notch signaling plays a prominent role in prepatterning, establishment of rostro-caudal polarity and MET (Mesenchymal to epithelial transition) during somitogenesis (see vertebrate somitogenesis review by Pourquie O, 2001) but to date there was no gene candidate which could provide the molecular evidence for a link between the processes of prepatterning and MET. The expression pattern of *ZfChp* in the intermediate PSM region as dynamic stripes, its molecular nature as a Rho family GTPase capable of inducing cytoskeletal changes and it being a target of the Notch signaling pathway, taken together provide the first evidence that supports a link between prepatterning and MET in zebrafish embryos.

5.2.3 Proposal for functional analysis of *ZfChp*

Initial misexpression and morpholino gene knock down studies of *ZfChp* did not provide convincing results for a prominent role in zebrafish somitogenesis. As is normally the case in morpholino functional studies, efficiency of *ZfChp* protein knock down has to be

validated by in-vivo or in-vitro studies without which we cannot conclude whether the lack of an obvious phenotype was due to inefficiency of the morpholino or gene redundancy in the system. In addition to the misexpression and gene knock down studies, one can exploit the inherent potential of *ZfChp* to behave as a Rho GTPase, namely, a molecular switch, and employ new functional assays which make use of constitutively active and dominant negative versions of the molecule.

5.2.4. *ZfChp* dominant negative and constitutively active constructs

The appropriate mutatable molecular sites in the sequence are Guanine at position 38 and Threonine at position 43. For a constitutively active mutant, Guanine has to be replaced by Valine. For a dominant negative version, Threonine has to be replaced by Aspargine. The amino acid replacements can be done by PCR based single nucleotide mutagenesis approach.

G38V T43N

```
                    10        20        30        40        50        60
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Human  Chp     MPPRELSEAE PPPLRAPTPP PRRRSAPPEL GIKCVLVGDG AVGKSSLIVS YTCNGYPARY
Rat    Chp     MPPRELSEAE PPPLPASTPP PRRRSAPPEL GIKCVLVGDG AVGKSSLIVS YTCNGYPSRY
Zf     Chp     MPPQMDYFYH ESRVPSVCLE --QDEELLEP AISCMLVGDG AVGKTSMIVS YTTNGYPTDY

                    70        80        90       100       110       120
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Human  Chp     RPTALDTFSV QVLVDGAPVR IELWDTAGQE DFDRLRSLCY PDTDVFLACF SVVQPSSFQN
Rat    Chp     RPTALDTFSV QVLVDGAPVR IELWDTAGQE DFDRLRSLCY PDTDVFLACF SVVQPSSFQN
Zf     Chp     KQTAFDVFSG QVQVDGTPVR IQLMDTAGQE EFDEFRSLSY AHTDVFLLCF SVVNPTSFQN

                   130       140       150       160       170       180
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Human  Chp     ITEKWLPEIR THNPQAPVLL VGTQADLRDD VNVLIQLDQG GREGPVPQPQ AQGLAEKIRA
Rat    Chp     ITEKWLPEIR THNPQAPVLL VGTQADLRDD VNVLIQLDQG GREGPVPEPQ AQGLAEKIRA
Zf     Chp     ITKKWIPEIR ECNPSSPIIL VGTQSDLVLD VNVLIDLDR- YKVKPVCSSR ARSLSEKIRA

                   190       200       210       220       230
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|...
Human  Chp     CCYLECSALT QKNLKEVFDS AILSAIEHKA RLEKK--LNA KGVRTLSRCR WKKFFCFV
Rat    Chp     CCYLECSALT QKNLKEVFDS AILSAIEHKA RLEKK--LNA KGVRTLSRCR WKKFFCFV
Zf     Chp     AEYVECSALT QKNLKEAFDA AIFAAIKHKA RKAKKRRLSD RRTKAFSKCS WKKFFCFI
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Constitutively Active G38V - Glycine to Valine
Dominant Negative T43N – Threonine to Aspargine

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Finally, I would like to summarize the major findings of my PhD thesis. The present research findings support a potential role for both *her15* and *ZfChp* in zebrafish somitogenesis. *her15* plays a role in somite border formation and is an output of the zebrafish somitogenesis oscillator. The *her15* stripes which are expressed in double segmental distance in the anterior PSM, is a unique feature of *her15* when compared to other *her* genes, and points to conserved mRNA expression domains between *her15* and *drosophila* pair rule gene *hairy*. *ZfChp* studies have provided the first evidence for a possible molecular link between prepatterning and MET (mesenchymal to epithelial transition) in zebrafish embryos. It is also the only known Rho GTPase having dynamic stripe-like mRNA expression domains in the intermediate PSM region.

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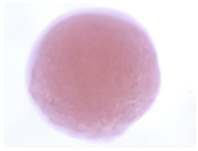
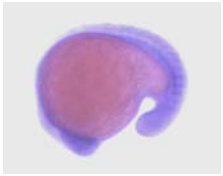
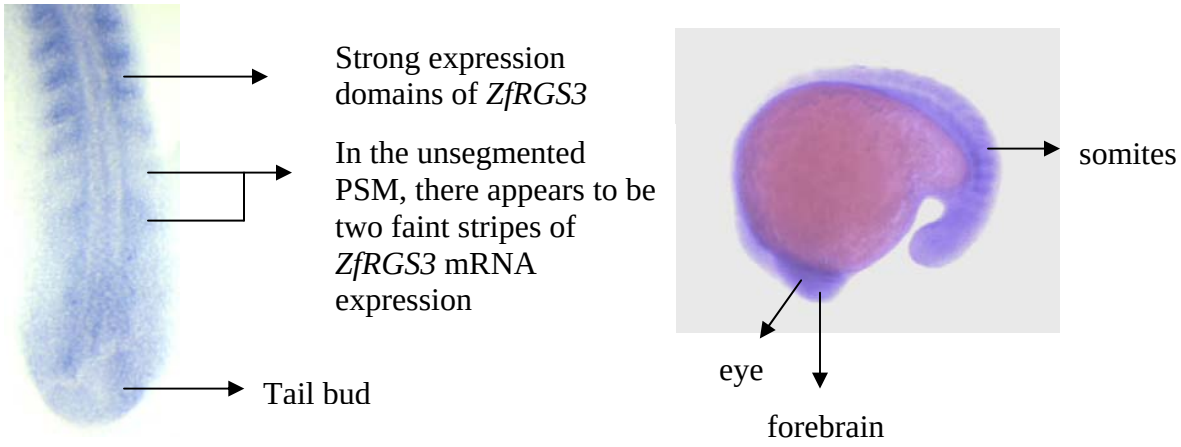
7. Selective Screening of the NIH zebrafish cDNA *in situ* expression database- Analysis of 5 candidates

7.1 Clone 5096

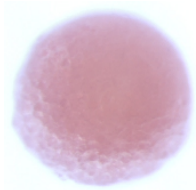
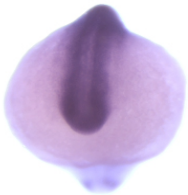
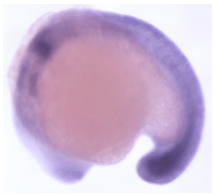
Wild type 90% Epiboly	Wild type 10-14 somites	Wild type 18-21 somites	*aei* 10-14 somites	*fss* 10-14 somites
 not expressed			Similar to wild type expression	strong expression domain only in the head , rest down regulated

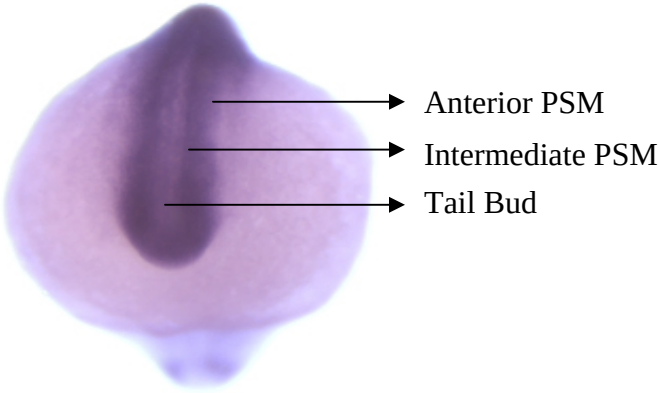
The RZPD clone that has sequence similarity to Clone 5096 has the ID IMAGp998E028969Q3 and this clone was used for RNA *in situ* probe preparation and expression analysis. This clone has been identified as coding for *RGS3*. RGS3 functions as a regulator of G-protein signaling (Gilman, 1987; Hamm and Gilchrist, 1996; Neer, 1995). The wild type 10-14 somite stage is a flat-mounted embryo. As can be seen from the above pictures of whole mount embryo namely 18-21 somite stage, the gene is expressed throughout the embryo in the somites, eye and brain regions. The *aei (deltaD)* shows the wild type expression pattern, while it is down regulated in *fss (tbx24)* mutant embryos. This gene does not show a spatially restricted expression pattern.

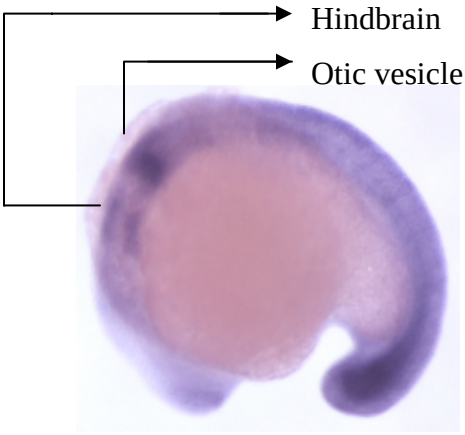


7.2 Clone 5116

The RZPD clone that has sequence similarity to Clone 5116 has the ID IMAGp998D158968Q3 and this clone was used for RNA *in situ* probe preparation and expression analysis. As can be seen from the above pictures of whole mount embryos, namely 10-14 somite satge and 18-21 somies stage, the gene is expressed in the tail bud, anterior PSM, intermediate PSM, otic vesicle and the hindbrain. In the *aei (deltaD)* and *fss (tbx24)* mutant embryos, clone 5116, shows the same mRNA expression pattern as the wild type. This gene does not show a spatially restricted expression pattern.

Wild type 90% Epiboly	Wild type 10-14 somites	Wild type 18-21 somites	*aei* 10-14 somites	*fss* 10-14 somites
 Not expressed			Similar to wild type expression	Similar to wild type expression

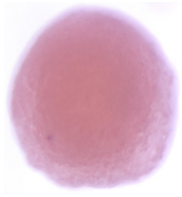
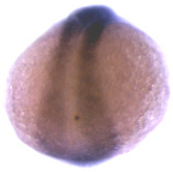
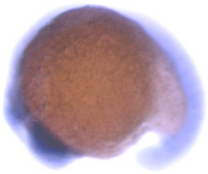


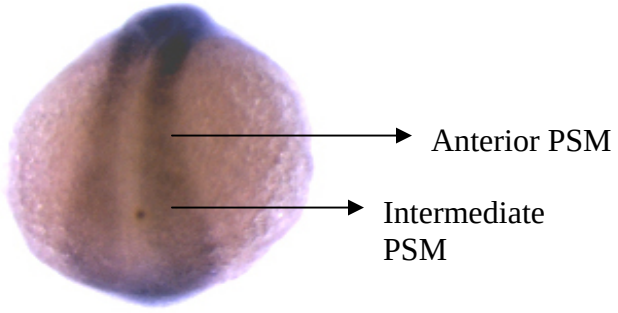


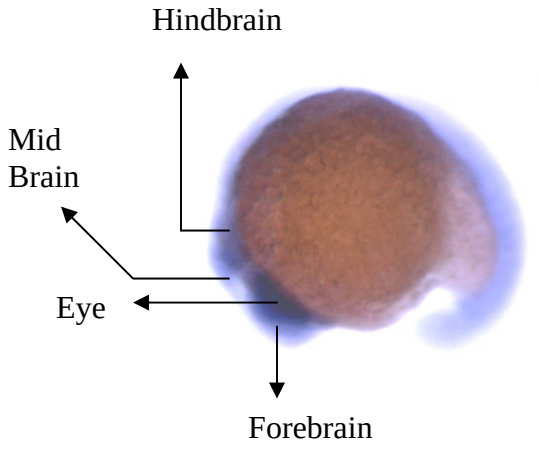
When this *in situ* screen was started, there was no data available through blast searches to identify this gene. The last update of the zebrafish sequencing project, which was released on 3rd April 2003, has provided no further information.

7.3 Clone 5144

The RZPD clone that has sequence similarity to Clone 5144 has the ID IMAGp998F228953Q3 and this clone was used for RNA *in situ* probe preparation and expression analysis. This EST codes for the gene *c-myc*, which is a nuclear target gene of the Wnt signaling pathway. As can be seen from the pictures of whole mount embryos, the gene is expressed strongly in the anterior PSM and intermediate PSM during the 10-14 somite stages. Later in the 18-21somite stage, the expression domain becomes stronger in the eye, forebrain, mid brain and hindbrain, with a weak basal expression throughout the embryo. The *aei (deltaD)* and *fss (tbx24)* mutant embryos show the same expression pattern as the wild type. *c-myc* does not show a spatially restricted expression pattern.

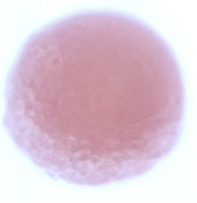
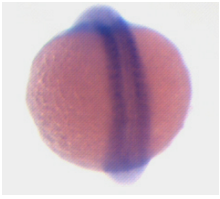
Wild type 90% Epiboly	Wild type 10-14 somites	Wild type 18-21 somites	*aei* 10-14 somites	*fss* 10-14 somites
 not expressed			Similar to wild type expression	Similar to wild type expression

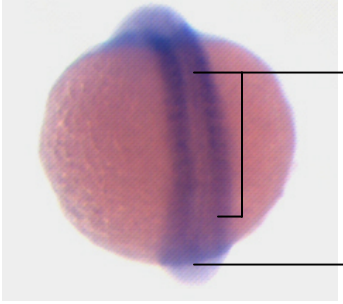


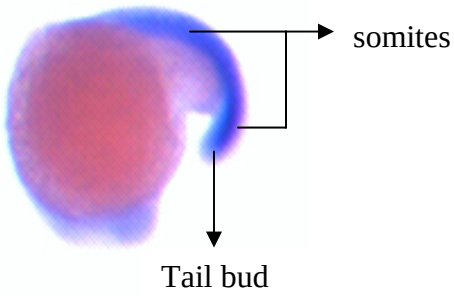


7.4 Clone 2247

The RZPD clone that has sequence similarity to Clone 5144 has the ID IMAGp998C028964Q3 and this clone was used for RNA *in situ* probe preparation and expression analysis. This EST codes for *myf-5* which is a Basic Helix Loop Helix protein involved in myogenesis. As can be seen from the pictures of whole mount embryos, the gene is expressed strongly in somites during the 10-14 somite stages. Later in the 18-21 somite stage, the stronger expression in the somites persists but one can also observe a weak basal expession throughout the embryo. The *aei (deltaD)* and *fss (tbx24)* mutant embryos show the same expression pattern as the wild type. This gene does not show a spatially restricted expression pattern. Unfortunately during the course of my experiments, a publication came out describing the functional role of *myf-5* in zebrafish (Chen and Tsai, 2002).

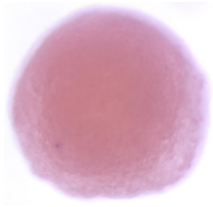
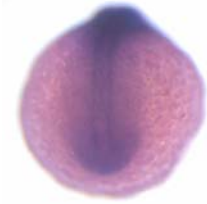
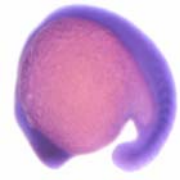
Wild type 90% Epiboly	Wild type 10-14 somites	Wild type 18-21 somites	*aei* 10-14 somites	*fss* 10-14 somites
 not expressed			Similar to wild-type expression	Similar to wild type expression

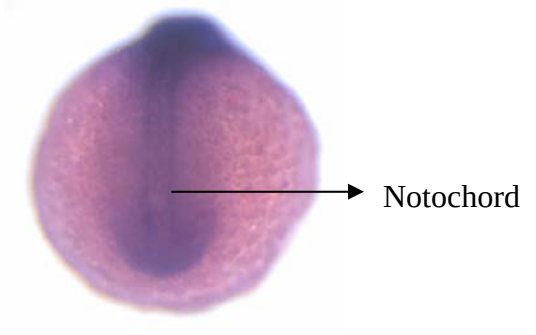


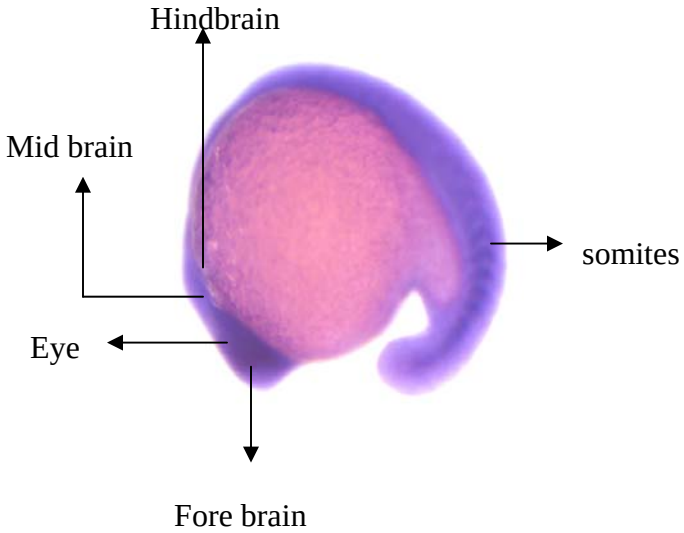


7.5 Clone 5038

The RZPD clone that shows sequence similarity to Clone 5144 has the ID LLKMp964N1517Q2 and this clone was used for RNA *in situ* probe preparation and expression analysis. As can be seen from the pictures of whole mount embryos, the gene is expressed strongly in somites, the notochord, different brain compartments and in the eyes in the 10-14 somite stage. Later in the 18-21 somite stage, the same expression domains persist. The *aei (deltaD)* and *fss (tbx24)* mutant embryos show the same expression pattern as the wild type. This gene does not show a spatially restricted expression pattern.

Wild type 90% Epiboly	Wild type 10-14 somites	Wild type 18-21 somites	*aei* 10-14 somites	*fss* 10-14 somites
 not expressed			Similar to wild type expression	Similar to wild type expression





During the course of this *in situ* screen there was no data available through blast searches to identify the gene corresponding to Clone 5038.The last update of the zebrafish sequencing project which was released on 3rd April 2003, did not give any further information.

7.6 GenBank entry for her15

```
LOCUS       AAT11018                 149 aa    linear   VRT 12-AUG-2004
DEFINITION  Her15 [Danio rerio].
ACCESSION   AAT11018
VERSION     AAT11018.1  GI:47112752
DBSOURCE    accession AY576277.1
KEYWORDS    .
SOURCE      Danio rerio (zebrafish)
  ORGANISM  Danio rerio
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi;
            Cypriniformes; Cyprinidae; Danio.
REFERENCE   1  (residues 1 to 149)
  AUTHORS   Sieger,D., Tautz,D. and Gajewski,M.
  TITLE     her11 is involved in the somitogenesis clock in zebrafish
  JOURNAL   Dev. Genes Evol. 214 (8), 393-406 (2004)
REFERENCE   2  (residues 1 to 149)
  AUTHORS   Shankaran,S.S., Sieger,D., Tautz,D. and Gajewski,M.
  TITLE     The mouse Hes5 homologs, her12 and her15, are cyclically
expressed
            in the zebrafish PSM
  JOURNAL   Unpublished
REFERENCE   3  (residues 1 to 149)
  AUTHORS   Gajewski,M., Shankaran,S.S., Sieger,D. and Tautz,D.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-MAR-2004) Department of Evolutionary
Genetics,
            Institute for Genetics, Weyertal 121, Cologne 50931,
Germany
COMMENT     Method: conceptual translation supplied by author.
FEATURES             Location/Qualifiers
     source          1..149
                     /organism="Danio rerio"
                     /db_xref="taxon:7955"
     Protein         1..149
                     /product="Her15"
                     /name="bHLH transcription factor of the
hairy/enhancer of
                     split family"
     CDS             1..149
                     /coded_by="AY576277.1:1..450"
ORIGIN
        1 mapaymteys klsnkekhkl rkpvvekmrr drinncieql ksmlekefqq
qdpnakleka
       61 dilemtvvfl kqqlrpktpq naqiegysqc wretisflsv gseavaqrlq
qeaqrsaape
      121 lthtseaphq qhthikqepr ahaplwrpw
//
```

7.7 GenBank entry for ZfChp

```
LOCUS       AAQ83831                 235 aa    linear   VRT 25-SEP-2004
DEFINITION  ras family GTPase [Danio rerio].
ACCESSION   AAQ83831
VERSION     AAQ83831.1  GI:34979444
DBSOURCE    accession AY314756.1
KEYWORDS    .
SOURCE      Danio rerio (zebrafish)
  ORGANISM  Danio rerio
            Eukaryota;   Metazoa;   Chordata;   Craniata;   Vertebrata;
Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi;
            Cypriniformes; Cyprinidae; Danio.
REFERENCE   1  (residues 1 to 235)
  AUTHORS   Shankaran,S.S. Jr., Gajewski,M. III and Tautz,D. V.
  TITLE     Zf Chp, A Ras family GTPase
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 235)
  AUTHORS   Shankaran,S.S. Jr., Gajewski,M. III and Tautz,D. V.
  TITLE     Direct Submission
  JOURNAL    Submitted (04-JUN-2003) Institute for Genetics, University
of
            Cologne, Weyertal 121, Cologne, NRW 50931, Germany
COMMENT     Method: conceptual translation supplied by author.
FEATURES             Location/Qualifiers
     source          1..235
                     /organism="Danio rerio"
                     /db_xref="taxon:7955"
     Protein         1..235
                     /product="ras family GTPase"
     CDS             1..235
                     /gene="chp"
                     /coded_by="AY314756.1:53..760"
ORIGIN
        1   mppqmdyfyh   esrvpsvcle   qdeellepai   scmlvgdgav   gktsmivsyt
tngyptdykq
       61   tafdvfsgqv   qvdgtpvriq   lmdtagqeef   defrslsyah   tdvfllcfsv
vnptsfqnit
      121   kkwipeirec   npsspiilvg   tqsdlvldvn   vlidldrykv   kpvcssrars
lsekiraaey
      181 vecsaltqkn lkeafdaaif aaikhkarka kkrrlsdrrt kafskcswkk ffcfi
//
```

7.8 The accession numbers of the sequences used to make the phylogenetic tree for ZfChp

gi|11177004|dbj|BAB17851.1| Rnd1 [Homo sapiens]

gi|1839517|gb|AAB47133.1| RhoE [Homo sapiens]

>gi|20379120|gb|AAM21120.1|AF498973_1 small GTP binding protein RhoD [Homo sapiens]

>gi|27469695|gb|AAH41791.1| RHOBTB1 protein [Homo sapiens]

>gi|5731800|emb|CAB52602.1| cell division cycle 42 (GTP binding protein, 25kDa) [Homo sapiens]

>gi|50263042|ref|NP_036381.2| ras-like protein TC10 [Homo sapiens]

>gi|20379128|gb|AAM21124.1|AF498977_1 small GTP binding protein TCL [Homo sapiens]

>gi|16508170|gb|AAL17966.1| Rho family GTPase Chp [Homo sapiens]

>gi|15077780|gb|AAK83340.1|AF378087_1 Wrch-1 [Homo sapiens]

>gi|51338611|sp|P84095|RHOG_HUMAN Rho-related GTP-binding protein RhoG

>gi|88546|pir||B34386 GTP-binding protein rac2 - human

>gi|2500200|sp|Q15669|RHOH_HUMAN Rho-related GTP-binding protein RhoH (GTP-binding protein TTF)

>gi|10952526|gb|AAG24952.1|AF239923_1 Rho family small GTPase [Homo sapiens]Rif)

>gi|10835049|ref|NP_001655.1| ras homolog gene family, member A [Homo sapiens]RhoA)

>gi|2507301|sp|P52198|RHON_HUMAN Rho-related GTP-binding protein RhoN (Rho7) (Rnd2)

>gi|34979444|gb|AAQ83831.1| ras family GTPase [Danio rerio](ZfChp)

>gi|4757764|ref|NP_004031.1| ras homolog gene family, member B [Homo sapiens]RhoB)

>gi|28395033|ref|NP_786886.1| ras homolog gene family, member C [Homo sapiens]RhoC)

>gi|2326206|gb|AAC51667.1| Rac3 [Homo sapiens]

>gi|2507302|sp|P19073|CC42_YEAST Cell division control protein 42

7.9 The accession numbers of the sequences used to make the alignment of *her15* with *her12, Hes5, esr9* and *esr 10*

>gi|34481703|emb|CAE46483.1| enhancer of split related 9 [Xenopus laevis]

>gi|34481704|emb|CAE46484.1| enhancer of split related 10 [Xenopus laevis]

>gi|3913838|sp|P70120|HES5_MOUSE Transcription factor HES-5 (Hairy and enhancer of split 5)

>gi|45387663|ref|NP_991182.1| hairy-related 12 [Danio rerio]

>gi|47112752|gb|AAT11018.1| Her15 [Danio rerio]

Erklärung

"Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen -noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Diethard Tautz betreut worden."

Köln, den 14.12.2004 Sunita Sathy Shankaran

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Ort, Datum Unterschrift