Su(H) mediated Notch signalling and the role of different *her* genes during zebrafish somitogenesis

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Abstract

Somitogenesis is the key developmental process, which divides the vertebrate body axis into segmentally repeated structures. These structures are called somites. Somites derive from the unsegmented presomitic mesoderm (PSM) that flanks the notochord to both sides. A prepatterning process, taking place in the PSM, is necessary to allow the exact spatial and temporal formation of the somites. The prepatterning is achieved by a clock and wavefront mechanism. The clock consists of the Delta-Notch (D-N) pathway, building up a genetic circuit with several cyclically expressed *h/E(spl)/hey*-related genes while the wave front is created by a FGF gradient, showing its highest expression in the posterior PSM. Disturbance of the clock or the mediator of the wavefront (*her13.2*) results in a disruption of cyclic gene expression and posterior somite border formation, while anterior somites are still formed. On the level of Delta-Notch signalling it is not clear if the escaped anterior somites are formed due to redundancy, since there are at least four *notch* and four *delta* homologues in zebrafish. Furthermore it is not known if Notch signalling is transmitted via the canonical way through *Su(H)* during somitogenesis or if an alternative way is used.

Since there appears to be only one complete *Su(H)* homologue in zebrafish, the function of this gene was analyzed using morpholino oligonucleotides. The knockdown of *Su(H)* leads to a clear disruption of cyclic gene expression, comparable to effects in previously described D-N mutants. Beyond this, posterior somite defects were detected while anterior somites were still formed, implying that their formation is not due to redundancy between different *delta* or *notch* genes. Performing the *Su(H)* knockdown in the *fss/tbx24* mutant it could be shown that D-N signalling is necessary for the creation and synchronization of cyclic gene expression. These results clearly suggest that the canonical way of Notch signalling is used during somitogenesis.

To further specify the prepatterning process two newly identified *her* genes, *her11* and *her12*, were analyzed during somitogenesis. It turned out that both genes are dynamically expressed in the PSM and are differentially regulated by D-N signalling. Functional studies suggest that *her11* interacts with *her1* and *her7* and is involved in the fine tuning of cyclic gene expression while *her12* seems to be involved in somite border formation and cyclic gene expression.

It was recently shown that the D-N driven Her1 protein and the FGF activated Her13.2 protein form heterodimers in vitro. To proof a combinatorial function also in vivo, both genes were knocked down individually and in combination. The combined knockdown leads to distinct additional effects, namely the break down of cyclic gene expression right

from the start and a disruption of anterior somite formation. This suggests clearly a combinatorial role for both genes in vivo during early somitogenesis.

Zusammenfassung

Während der Somitogenese wird der sich entwickelnde Vertebraten Embryo in sich wiederholende mesodermale Einheiten unterteilt. Diese Einheiten werden als Somiten bezeichnet und vom präsomitischen paraxialen Mesoderm (PSM) abgegliedert, welches das Neuralrohr zu beiden Seiten flankiert. Damit dieser Prozess räumlich und zeitlich koordiniert abläuft, findet im PSM ein „Prepatterning"-Prozess statt. Dieses „Prepatterning" wird durch einen sogenannten „clock and wavefront" Mechanismus erreicht. Die „clock" besteht aus dem Delta-Notch Signalweg, welcher einen genetischen Regelkreis mit den zyklisch exprimierten *h/E(spl)/hey* Genen bildet, während die „wavefront" einen FGF Proteingradienten im PSM beschreibt. Unterbrechungen der „clock" oder des Übermittlers der „wavefront" (*her13.2*) führen zu gestörter zyklischer Genexpression im PSM sowie zu posterioren Somitendefekten. Die ersten vier bis acht Somiten sind davon nicht betroffen. Da im Zebrafisch mindestens vier *delta* und vier *notch* Homologe existieren, war bisher nicht klar, ob diese anterioren Somiten auf Grund von Redundanz in diesem Signalweg weiterhin gebildet werden. Des Weiteren war unklar, ob der D-N Signalweg während der Somitogenese wirklich über *Su(H)* vermittelt wird oder ob eine alternative Signaltransduktion stattfindet.

Da im Zebrafisch offensichtlich nur ein funktionelles Su(H) Gen existiert, wurde dieses Gen mittels Injektion von Morpholino Oligonukleotiden ausgeschaltet und seine Funktion analysiert. Der *Su(H)* „knockdown" führt zu einem Zusammenbruch der zyklischen Expression im PSM, direkt vergleichbar mit den Phänotypen von D-N mutanten Embryonen. Weiterhin wurden posteriore Somitendefekte entdeckt, während die anterioren Somiten gebildet wurden. Dies zeigt eindeutig, dass die intakten anterioren Somiten nicht durch Redundanz zwischen verschiedenen *delta* oder *notch* Genen zu erklären sind. Durch „knockdown" von Su(H) im *fss/tbx24* mutanten Hintergrund konnte gezeigt werden, dass der D-N Signalweg für die Entstehung und Synchronisation der zyklischen Expression im PSM verantwortlich ist. Zusammenfassend zeigen diese Experimente, dass der D-N Signalweg während der Somitogenese eindeutig über Su(H) vermittelt wird.

Um den Prozess des „Prepatterning" näher zu untersuchen, wurden zwei weitere, erst kürzlich identifizierte *her* gene, *her11* und *her12*, analysiert. Es stellte sich heraus, dass beide Gene dynamisch, jedoch unterschiedlich im PSM exprimiert sind und differentiell durch den D-N Signalweg reguliert werden. Die funktionelle Analyse zeigte, dass *her11* zusammen mit *her1* und *her7* an der Feinregulation der zyklischen Genexpression beteiligt

ist, wohingegen *her12* eine Funktion in der Somitengrenzbildung zu haben scheint und zusätzlich an der Regulation zyklischer Expression beteiligt ist.

Erst vor kurzem konnte gezeigt werden, dass das über D-N regulierte Her1 Protein und das über FGF aktivierte Her13.2 Protein in vitro Heterodimere bilden. Um zu prüfen, ob beide Proteine in vivo wirklich eine kombinatorische Funktion haben, wurden beide Proteine einzeln und in Kombination ausgeschaltet. Der kombinatorische „knockdown“ zeigte klare zusätzliche Effekte, wie eine sehr frühe Unterbrechung zyklischer Genexpression, sowie den Zusammenbruch der anterioren Somitogenese. Diese Experimente belegen eine kombinatorische Funktion für Her1 und Her13.2 in der frühen Somitogenese.

1. Introduction

The subdivision of the body axis during development into repeated structures/segments is a basic characteristic of many animal species ranging from invertebrates to man. This developmental process is known as segmentation in annelids and arthropods and is called somitogenesis in vertebrates. The subdivision of the body axis is not only visible during development; even in the adult animal one can recognize the principle of segmentation. Insects show a fusion of different segments to functional units, which form head, thorax and abdomen. In the adult vertebrate body segmentation is most obvious at the level of the vertebral column and its associated muscles, and also in the structure of the peripheral nervous system (PNS). Disruption of somitogenesis in humans results in severe defects of the spinal column. They include Klippel-Feil syndrome, spondylocostal dysostosis, Jarcho-Levin syndrome and Alagille syndrome (for review see Pourquié and Kusumi 2001). The defects range from generalized vertebral malformations and rib fusions (Jarcho-Levin syndrome) to regionalized malsegmentation (Klippel-Feil syndrome) or affect only one or two vertebrae (Alagille syndrome). For some of these defects the mutated genes have been identified. *Delta-like 3* is mutated in spondylocostal dysostosis/Jarcho-Levin syndrome and *Jagged1* (*Jag1*) is mutated in Alagille syndrome. Since these genes belong to the *Notch* pathway this provides evidence that the *Notch* pathway also controls somitogenesis in humans, as it has been shown for fish, chicken and mouse (discussed below). Thus somitogenesis is a crucial step during development to ensure the exact formation of such an important structure as the vertebral column.

Somitogenesis proceeds during vertebrate development as follows: the transient segments (somites) are sequentially added along the anterior-posterior axis of the embryo (for review see Saga and Takeda 2001; Maroto and Pourquié 2001; Rida et al., 2004). The somites derive from the unsegmented, mesenchymal, presomtic mesoderm (PSM), which flanks the notochord on both sides. There are three major phases of somitogenesis. First, the prepatterning of the unsegmented PSM and the establishment of the rostrocaudal polarity of the future somite (Stern and Keynes 1987; Aoyama and Amasoto 1988); second, the formation of the somitic border and third, the differentiation of the somites to generate the muscles and vertebrae of the trunk and tail (Tam and Trainor 1994).

Several models have been proposed to explain this complex scenario, including the wave gradient model (Flint et al., 1978), the reaction-diffusion type model (Meinhardt, 1982+1986), the cell-cycle model (Primett et al., 1988+1989; Stern et al., 1988), the wave-

cell polarisation model (Polezhaev, 1992+1995) and the clock and induction model (Schnell and Maini 2000). But the best fitting model seems still to be the clock and wavefront model, which was proposed even before the molecular components for the prepatterning of the PSM were identified (Cooke and Zeeman 1976; reviewed by Dale and Pourquié 2000). This model assumes that the prepatterning is achieved by an oscillator mechanism in combination with a wavefront activity. Evidence for this oscillator mechanism came through the identification of the *c-hairy1* gene (Palmerin et al., 1997), which is dynamically expressed in the chick PSM. Due to this cyclic expression, which progresses from the posterior to the anterior PSM, the cells in the chick embryo undergo several on and off phases of *c-hairy1* transcription before they become a somite. *c-hairy1* encodes a bHLH transcription factor, which is a homologue of the *Drosophila* pair-rule gene *hairy* (Ish-Horowicz et al., 1985). During the past few years various *hairy (h)* and *Enhancer of split (E(spl))* related genes were identified, which were named *Hes* in mouse, *her* in fish and *esr* in Xenopus; while *hey* genes in all species represent a subclass of bHLH genes, characterized by the presence of a C-terminal YRPW motif instead of the WRPW motif. These genes, showing also a dynamic expression in the vertebrate PSM include the *c-hairy2* and *Hey2* gene in chick (Jouve et al., 2000; Leimeister et al., 2000), *Hes1*, *Hes5*, *Hes7* and *Hey2* in mice (Jouve et al. 2000; Bessho et al. 2001b+2003; Leimeister et al., 2000; Dunwoodie et al. 2002), esr9 and esr10 in *Xenopus* (Li et al., 2003) and *her7* and *hey1* in medaka (Elmasri et al., 2004) (the zebrafish situation will be discussed in detail below). In addition to the genes of the *h/E(spl)* family the *Delta-Notch* pathway plays a major role in the prepatterning process and builds up a genetic circuit with the genes of the *h/E(spl)* family (for review see: Maroto and Pourquié 2000; Saga and Takeda 2001; Rida et al., 2004). A disruption of Delta-Notch signalling and thus of the oscillator leads in all investigated vertebrate species to posterior somite defects, while the anterior most four to seven somites seem to be unaffected (reviewed by Rida et al., 2004). The canonical model for *Notch* signalling assumes that after ligand binding (*Delta*/*Serrate*/*Jagged*) the *Notch* receptor is cleaved and the intracellular domain of *Notch* (NIC) is translocated to the nucleus (reviewed by Artavamis-Tsakonas et al. 1999). Once in the nucleus, NIC interacts with members of the CSL (CBF1, *Suppressor of Hairless*, Lag-1) family of transcription factors and activates target genes such as the *h/E(spl)* family genes. The CSL transcription factors have a dual role: when *Notch* signalling is inactive they act as a repressor, whereas during active *Notch* signalling they interact with NIC and promote activation of their target

genes. However, there is some evidence that there is also another pathway for transmitting the *Notch* signal, which is independent of the interaction with the CSL transcription factors. This alternative pathway may involve *Deltex*, a cytoplasmic adaptor protein that interacts with *Notch*, and there may also be a connection to the Wnt-signalling pathway (reviewed by Martinez Arias et al. 2002). Although the available evidence suggests that the prepatterning process of the PSM involves only the canonical *Notch* signalling pathway, this has not yet been tested in all consequence.

To date there is also molecular evidence for the existence of the postulated wavefront. The wavefront or determination front is positioned at a threshold level of FGF, which constitutes a regressing gradient showing its highest expression in the posterior PSM (reviewed in Saga and Takeda, 2001; Aulehla and Herrmann, 2004; Dubrulle and Pourquie, 2004). Above the threshold FGF keeps the cells in the posterior PSM in an undetermined state, while cells in the anterior PSM fall under the threshold level are determined to become somites, dependent on their phase of the oscillation cycle.

Furthermore there is recent evidence from studies in mice that the Wnt-pathway is involved in somitogenesis (Aulehla et al. 2003). It seems that the Wnt-pathway is necessary to set up the segmentation clock and acts upstream of the *Delta-Notch* pathway. But so far the role of the Wnt-pathway could only be shown in mice and it remains unclear if this pathway is also involved in somitogenesis in other species.

In zebrafish 2 *h/E(spl)* related genes, *her1* and *her7*, were studied intensively during the last years. Both genes show an oscillating expression in the PSM and are coexpressed, with the only difference that *her7* shows a weaker expression in the anteriormost PSM (Holley et al. 2000; Oates and Ho 2002; Gajewski et al. 2003). The analysis of a deletion mutant for *her1* and *her7* as well as Morpholino (Mo) knockdown studies suggest that Her1 and Her7 protein function is required for the exact prepatterning of the zebrafish PSM (Henry et al. 2002; Holley et al., 2002; Oates and Ho 2002; Gajewski et al., 2003). The loss of Her1 and Her7 protein in a deletion mutant (b567) seems to result in alternating weak and strong somite boundaries (Henry et al., 2002), which could not be confirmed by knockdown studies so far (Oates and Ho 2002; own unpublished observations). In addition, a disruption of rostrocaudal polarity within the somites was observed. By analysing the mutants of the *fused somite type* class, overexpression studies and Mo-knockdown experiments the involvement of the *Delta-Notch* pathway in zebrafish somitogenesis and in particular the control of *her1* and *her7* expression could be shown (Dornseifer et al. 1997;

van Eeden et al. 1998; Takke et al. 1999; Holley et al. 2000; Holley et al. 2002; Jülich et al., 2005b; Oates et al., 2005). Interestingly one of the Notch ligands, *deltaC*, shows an oscillating expression in the zebrafish PSM, which is highly similar to *her1* and *her7* expression, while the other involved ligand, *deltaD*, shows a likewise pattern but does not oscillate (Holley et al., 2000; Jiang et al., 2000; Oates and Ho 2002). The oscillating expression of *her1, her7* and *deltaC* is disrupted in *bea/deltaC, des/notch1* and *aei/deltaD* mutant embryos and posterior somite defects starting with somite 3-4, 5-7 or 7-8 respectively can be detected (van Eeden et al. 1996+1998; Holley et al. 2000; Holley et al. 2002; Jülich et al., 2005b). Furthermore a direct correlation between the disruption of cyclic gene expression and the onset of segmentation defects could be found. The earliest disruption of cyclic gene expression and somite border formation can be detected in *bea/deltaC* embryos followed by *des/notch1a* and *aei/deltaD* embryos (Jiang et al., 2000; Oates and Ho 2002; van Eeden et al., 1998). These findings show undoubtedly that *Delta-Notch* signalling is necessary for proper oscillation of *her1* and *her7*. Interestingly, it could be shown by Mo knockdown approaches that Her1 and Her7 feedback on their own transcription as well as on *deltaC* and *deltaD* transcription (Holley et al. 2002; Oates and Ho 2002; Gajewski et al., 2003). While previous studies suggested a negative feedback loop for Her1 and Her7 on their own expression (Holley et al. 2002; Oates and Ho 2002) recent data disprove this assumption. By using an intron probe for *her1* it turned out that former knockdown studies were misinterpreted because of a RNA stabilisation mediated by the Mo (Gajewski et al., 2003). The current view is that *her7* is required for initiating the expression in the posterior PSM, while *her1* is required to propagate the cyclic expression in the intermediate and anterior PSM. Thus instead of acting as repressors, a function, which undoubtedly can be deduced from the *her1* and *her7* amino acid sequence, both transcription factors rather seem to act formally as activators. Initial studies of the *her1* promotor further support a separate regulation in the posterior versus intermediate and anterior PSM (Gajewski et al., 2003). Nevertheless, these data lead to the conclusion that *her1* and *her7* and the *Delta-Notch* pathway built a genetic circuit, which is a core component of the oscillator in the zebrafish PSM. The involvement of two further *her* genes, *her4* and *her6*, in zebrafish somitogenesis could be shown recently (Pasini et al., 2004). These genes are expressed in a stripe like manner in the anterior PSM but do not cycle (Takke et al., 1999; Pasini et al., 2001). It seems in the moment that *her4* and *her6* are required for maintaining the synchronisation of cyclic gene expression during later

somitogenesis and are involved in somite border formation beyond somite 11 (Pasini et al., 2004).

A further transcription factor, which is necessary to maintain cyclic gene expression in the anterior PSM of zebrafish embryos is *tbx24* (van Eeden et al., 1998; Holley et al., 2000; Nikaido et al.; 2002). *fss/tbx24* mutant embryos show a disruption of anterior and posterior somites and fail to generate the anterior most *her1* stripe while posterior oscillations are apparently normal.

Sawada et al., (2001) could show that the wavefront in zebrafish is determined by an FGF gradient, showing its highest expression in the posterior PSM. Manipulating this gradient leads to an altered somite size and influences cyclic gene expression, proofing the functional role of FGF signalling during somitogenesis. Since the *fgf8* mutant *acerebellar* (*ace*; Reifers et al., 1998) shows only midbrain-hindbrain boundary defects and somites develop apparently normal, there might be functional redundancy in FGF signalling during zebrafish somitogenesis. This has been shown for example for *fgf8* and *fgf24* and their role in posterior mesoderm development, since it turned out that it is necessary to mutate both genes to inhibit this process (Draper et al., 2003). Recently it could be shown that the FGF pathway is linked via a *her* gene (*her13.2*) to the segmentation clock in zebrafish (Kawamura et al., 2005). *her13.2* shows a gradient like expression in the posterior PSM, is regulated via FGF signalling and the knockdown leads to a disruption of the oscillator (Kawamura et al., 2005). Thus *her13.2* is the first non-cyclic *her* gene involved in somitogenesis, which is independent of D-N signalling but nevertheless involved in oscillator control and posterior somite formation (Kawamura et al., 2005).

But not only members of the *h/E(spl)* family and of the Delta-Notch pathway play a role in zebrafish somitogenesis. Kawahara et al. 2005 reported the involvement of two genes, which were known from mammals to be involved in stress response during cell cycle control. These genes, *gadd45ß1* and *gadd45ß2*, are expressed as dynamic stripes in the anterior PSM of zebrafish embryos (Kawahara et al., 2005). Knockdown of both genes leads to a clear segmentation defect and a disruption in cyclic gene expression, showing their importance in this process (Kawahara et al., 2005). This reminds of the cell cycle model, which postulates that cells entering the PSM are synchronized in their cell cycle and somite border formation is only possible at a certain time point of the cell cycle (Primett et al., 1988; Stern et al., 1988). Thus the new findings in zebrafish show that it is

not only clock and wavefront driving somitogenesis, since the mechanism seems to be more complex and cell cycle control is a crucial aspect to coordinate somitogenesis.

Results from Aerne and Ish-Horowicz 2004 imply the importance of *receptor protein tyrosine phosphatase ψ* (*RPTPψ*) during somitogenesis. Protein tyrosine phosphatases have been shown to play a major role during neuronal development (for review see Stoker and Dutta 1998) but no hint for a role in somitogenesis was found before. *RPTPψ* is expressed uniformely during early zebrafish development with a slight increase in the somites (Aerne and Ish-Horowicz 2004). Interestingly the knockdown of *RPTPψ* leads to defects in posterior somitogenesis resembling the phenotype of the various Delta-Notch mutants (Aerne and Ish-Horowicz 2004). Furthermore *RPTPψ* morphants show a disruption and a strong decrease of cyclic gene expression in the PSM, leading to the conclusion that *RPTPψ* acts upstream or in parallel to Delta-Notch signalling (Aerne and Ish-Horowicz 2004).

Another factor, which is at least important for somite border formation is the forkhead transcription factor *foxc1a* (Topczewska et al., 2001). This gene does not seem to be involved in cyclic gene expression but interacts later with downstream genes like *mespb*, *ephrinB2*, *ephA4*, *notch5* and *notch6* to establish somite border formation (Topczewska et al., 2001).

Although several components of the somitogenesis oscillator have been investigated during the last years, there are still two main problems to be solved:

1) It is still not possible to build a model with the so far identified components, which is able to explain in detail how the oscillations travel from posterior to anterior. It turns out that there are still important players missing to explain this complex mechanism in the zebrafish PSM. Since *her* genes are known to form homo- and heterodimers, it is highly possible that further, so far not identified *her* or *hey* genes are involved. To solve this problem a search for *h/E(spl)/hey*-related genes in the third release of the zebrafish genomic sequence was performed and revealed the existence of at least 23 *h/E(spl)/hey*-related genes in zebrafish (M. Gajewski). Three out of fourteen newly identified *her* genes, namely *her11*, *her12* and *her15*, show an interesting expression in the PSM and thus are possible candidates. *her15* has been analysed recently but its role during somitogenesis is not completely understood at present (Shankaran 2005).

2) Anterior somite formation seems to require a different mechanism compared to posterior somite formation, which is specifically perturbed in single mutations of Delta-Notch genes (van Eeden et al., 1996+1998; Holley et al., 2000; Holley et al., 2002) or in single knockdowns of D-N dependent *her* genes like *her7* (Henry et al., 2002; Oates and Ho 2002; Oates et al., 2005). To date, the only clock gene, which shows a mild influence on anterior somites, when knocked down, is *her1*, which displays in the respective morphant slight morphological defects in the anterior borders (Henry et al., 2002). Another mutation in the *integrin5alpha* gene has recently been described, which shows a loss of anterior somitic borders while posterior somites stay intact (Jülich et al., 2005a; Koshida et al., 2005). But obviously, due to unperturbed cyclic gene expression, no direct relation to the D-N pathway seems to exist. Thus it remains unclear in the moment if anterior somite formation requires a completely different mechanism or if it is just more robust compared to posterior somitogenesis, as recent findings reveal. Oates et al. (2005) could show that a combined knockdown of *deltaC* and *her7* leads to a disruption of all somite borders except the first one and to disturbed oscillations right from the start. This implies that anterior somitogenesis is dependent on the oscillator and only the removal of two crucial components leads to a perturbation.

1.1 Aims of the thesis

The aim of this thesis was to further specify the role of the two main components of the somitogenesis oscillator, the Delta-Notch pathway and the *h/E(spl)/hey*-related genes.

Since it is still not clear whether Delta-Notch signalling is exclusively transmitted via the canonical CSL dependent pathway during zebrafish somitogenesis (discussed before), knockdown studies were performed for the zebrafish *Suppressor of Hairless (Su(H))* homologue and the influence on somite border morphology and cyclic gene expression was analysed.

As already discussed earlier, there are still important components missing to explain the nature of cyclic gene expression in the PSM. To investigate if further *her* genes are involved, the newly identified *her* genes *her11* and *her12* were studied. The expression of both genes was analysed in wildtype embryos and Delta-Notch mutant embryos and furthermore functional studies were performed to proof their role in somitogenesis.

Recent data reveal that anterior somitogenesis might just be more robust than posterior somitogenesis and no additional pathway might be required (Oates et al., 2005). Furthermore it could be shown that the FGF regulated gene *her13.2* interacts in vitro with *her1* (Kawamura et al., 2005). Since *her1* has been shown in the knockdown situation to have a mild influence on anterior somitogenesis, the question arises if an interaction with *her13.2* could enhance this phenotype. To answer this question and to test whether there is a synergistic role for *her1* and *her13.2* in vivo both genes were knocked down solely and in combination. These knockdown embryos were then analysed for additional effects on somite morphology and cyclic gene expression.

2. Materials

2.1 Buffers

Buffers and solutions, which are not mentioned separately, have been prepared according to Sambrook et al., 1989.

2.2 Primer

The used primers were synthesized by the company Metabion. The lyophylized primer was dissolved in an appropriate volume of H_2O to obtain a concentration of 100 μM. All used primers are listed in table1. Some primers were prolonged with the T3 and T7 promotor sequence with the aim to use obtained PCR products directly for in vitro transcription. The T3/T7 sequences are marked in small letters.

Table 1: used primer

Primer to generate PCR products for GFP-morpholino controls		
#	Name	Sequence from 5' to 3'
1	h12-cMo-for2	AGG AAT TCA TAC AAG CCT CTG CAC CAT CCA
2	h12-cMo-rev2	ATC CAT GGT CAT GTC TGT GCT CGA ACA GCT
Primer to generate PCR products for misexpression		
3	her12-XhoI-for	ATC TCG AGC TGT TCG AGC ACA GAC ATG G
4	her12-XbaI-rev	AGT CTA GAC TCA GGG TTG TCA GTC CAC A
Primer to generate PCR products for in vitro transcription		
5	T7-her1-1037	taa tac gac tca cta tag ggT CTC CAC AAA GGC T
6	T3-her1-29	aat taa ccc tca cta aag ggT GTA TCG TCT TCT T
7	T7-her7-1059	taa tac gac tca cta tag ggT GGA ATG TAC TGA T
8	T3-her7-45	aat taa ccc tca cta aag ggA CAT TTT CTG GAA T
9	DeltaC fw	AAA CGT AAC TGA AGG GTC CAA
10	DeltaC rv	Taa tac gac tca cta tag ggT CCG GGG GTT TAT TTA TTT G
11	DeltaD fw	GCC ATG GGA CGA CTA ATG ATA
12	DeltaD rv	taa tac gac tca cta tag ggC GTT GCT GTC GGT TTA CTT CA
13	T3-her1intron sense2	aat taa ccc tca cta aag ggT GTA TAA TTA ATG
14	T7-her1intron antisense2	taa tac gac tca cta tag ggC TGA ATT TAA ACA
15	Hey1-up	ATG AAG AGA AAT CAC GAT TTC AGC TCG TCG

16	Hey1-down	taa tac gac tca cta tag ggC CTG TAC GGC TTC
17	T3-her11-start	aat taa ccc tca cta aag ggA TCA AAA GAA GGC T
18	T7-her11-reverse	taa tac gac tca cta tag ggA TAA GAG GAA GCC
19	her12-for	ATG GCA CCC CAC TCA GCC ACA CTC GCC TCC
20	T7-her12-rev	taa tac gac tca cta tag ggT CTC CAG ACG GCC C
21	her13.2 up	CAG CAA CAC TCA CGA CGA GGA TAA TTA CGG
22	T7-her13.2 down	taa tac gac tca cta tag ggT CTC CAA ATG GAC
23	Spt fw	CGT GTG AAG CTC TGG ATG AT
24	Spt rv T7	taa tac gac tca cta tag ggA TTC GGT GGG AAG GT G ATG A
25	T3-myoD	aat taa ccc tca cta aag ggG TCG GAT ATC CCC TT
26	T7-MyoD	taa tac gac tca cta tag ggG TTT CCA GCA GTG GA
27	T7-tbx-for	taa tac gac tca cta tag ggG GCA TCG ATA CCA GCC AC
28	T3-tbx-rev	aat taa ccc tca cta aag ggC GGA GGG AAA GGA AAG GC

2.3 Cells and plasmids

For misexpression studies the pCS2+ vector was used, which is the standard vector for misexpression experiments in zebrafish and *Xenopus* (Turner and Weintraub 1994). As control for these functional studies and for GFP-morpholino control experiments, a modified pCS2+eGFP was used (kindly provided by the Lab of Prof. Campos-Ortega). Standard transformations were done using competent *XL10-Gold Ultracompetent cells* (Stratagene).

2.4 Morpholino oligonucleotides

The following morpholinos, obtained from the company Gene Tools, were used:

Su(H): *Su(H)*-5': 5'-CGC CAT CTT CAC CAA CTC TCT CTA A-3'

Su(H)-ORF: 5'-CAA ACT TCC CTG TCA CAA CAG GCG C-3'

Su(H)-5bm : 5'-CAA AGT TGC CTG TGA CAA GAG CCG C-3'

her 1 : 5'-AGT ATT GTA TTC CCG CTG ATC TGT C-3'

her 7 : 5'-ATG CAG GTG GAG GTC TTT CAT CGA G-3'

her11: *her11*-start: 5'-CAT TCG AGG ATA TGG GAA ACT GCT G-3'

her11-ORF: 5’-CGT CAT GTT GAA AGT CGG TGT GCT C-3’

her11-5bm: 5’-CGT GAT CTT GAA ACT CGG TCT GGT C-3’

hey1: 5’-GAC GAG CTG AAA TCG TGA TTT CTC T-3’

her12: *her12*-5’: 5’-CAT GTC TGT GCT CGA ACA GCT TGC T-3’

her12-ORF: 5’-AGG CGA GTG TGG CTG AGT GGG GTG C-3’

her12-c: 5’-CGA ATG CAT GTG ACA GGG AGG TCA T-3’

her13.2: her13.2: 5’-CAT ATT GCT GCA AGT TCA GGA CGC TT-3’

her13.2 MO1: 5’-TGC AGT TCA GGA CGC TTG AAT GGG-3’

her13.2 MO2: 5’-GGC AGA TGG TCG GCG GTT CAG TTC-3’

her13.2 MO1 + MO2 were kindly provided by Akinori Kawamura (Kawamura et al., 2005)

2.5 Computer system

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

2.6 Software

Acrobat Reader 5.0 (*Adobe*)

AxioVision 2.0.5.3 (*Zeiss*)

Entrez (*National Center for Biotechnology Information = NCBI*)

Photoshop 7.0 (*Adobe*)

Vector NTI 6.0 (Infor Max, Inc.)

Microsoft Office 2000 Premium

Furthermore the services of PubMed (*NCBI*), Blast (Altschul et al., 1997), the Zebrafish EST-Database (http://zfish.wustl.edu/) (*Washington University, St. Lois*) and ZFIN (www.zfin.org) (Sprague et al., 2001) have been used.

3.Methods

3.1 Zebrafish methods

3.1.1 Keeping and raising zebrafish

3.1.1.1 Origin of zebrafish

The zebrafish *Danio rerio* is a three to four centimetres long fresh water fish from the Ganges that belongs to the family *Cyprinidae.* Animals kept in the facility were obtained from pet shops in Cologne and Göttingen and were further bred.

3.1.1.2 Growth conditions

Starting with day2, zebrafish were kept in an aquarium, consisting of several serial 12 L tank units, at 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. Fish were fed thrice daily. Beside the usual fodder (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 embryos for various experiments took place in the morning starting with the light phase. The evening before the adult male and female fish were put into a plastic box, divided by a separator. The bottom of the box was filled with marbles with the aim to prevent the adult fish feeding their own eggs. In the morning the divider was removed at the designated time point, allowing the fish to mate and 30 minutes later the embryos were collected. Embryos were kept in petridishes with aquarium water before and after the

experiments and were allowed to develop until the desired stage in an incubator at 22.5 to 28.5°C.

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% Paraformaldehyd (PFA) in PBS (phosphate buffered saline). The fixation took place from two hours at room temperature (RT) to several days at 4°C. After fixation embryos were transferred to PBST (PBS + 0.1 % Tween-20) and the chorion was removed using fine-pointed watch-makers forceps.

Solutions:

20 × PBS:

2.76 M NaCl
50 mM KCl
160 mM Na_2HPO_4
50 mM KH_2PO_4

3.1.2.2 Storage of embryos

Embryos were stored in 4% Paraformaldehyd (PFA) at 4°C until they were used for further experiments.

3.1.3 In situ hybrdisation of whole embryos

In situ hybridisation by means of Digoxygenin labelled 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 labelled anti-sense RNA probes. The hybridised 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 hybridisation 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

hybridisations were carried out in the automated *InsituPro* machine (Abimed) (Plickert et al., 1997)

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

The ribosomes and other proteins, which are associated with mRNA molecules, can be denatured by heat treatment. The RNA molecule becomes more accessible for the labelled anti-sense mRNA probe in this way.
Embryos were transferred from 4% PFA to 1ml PBST in 1.5 ml eppendorf tubes, further incubated for 10 min in freshly boiled water and whirled every 2.5 min to prevent them from sticking together. Subsequently embryos were cooled down for 5 min on ice to prevent proteins from renaturing.

3.1.3.2 Treatment with acetanhydrid

Treatment with acetanhydrid leads to an inactivation of endogenous phosphatases. This helps to reduce background since phosphatase coupled antibodies will be used for the staining.
Embryos were washed twice for 10 min in PBST. Subsequently, PBST was replaced by a solution of 2.5µl acetanhydrid/ml in 0.1M triethanol amine (pH 7.8) and the embryos were incubated for 10 min. Following this, the embryos were washed 4 times for 10 min in PBST.

3.1.3.3 Prehybridisation

Embryos were first incubated in a solution of 50% PBST / 50% Hybridisation solution (Hybmix), which was replaced after 5 min by 100% Hybmix. The incubation in Hybmix was 1 hour at 65°C.

3.1.3.4 Hybridisation

The RNA probes were dissolved in a small volume of Hybmix (250µl). After the prehybridisation the pure Hybmix was replaced by the prepared probe/Hybmix solution, in which the embryos were incubated for 16h at 65°C (or at 50°C for the *her1* intron probe).

3.1.3.5 Washing steps

After hybridisation the embryos were incubated for 30 min in Hybmix at 65°C. 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 Antibody incubation

The embryos were first washed twice for 5 min and later once for 20 min with PBST at RT. Then the embryos were incubated for 10 min in Block I and subsequently in Block II for 60 min. Block II was afterwards replaced by an 1:2000 dilution of anti-Digoxygenin-AP F_{ab} fragments (Roche) in Block II. The incubation was carried out for 6h at RT, followed by eight times washing for 15 min with PBST.

3.1.3.7 Color substrate reaction

Embryos were incubated 2 times for 5 min in AP buffer and subsequently transferred to 24 well plates, in which the AP buffer was replaced by *BM-Purple*-solution (Roche). The color substrate reaction took place in the dark and was carried out from 30 min to several hours, depending on the target RNA. 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).
To stop the reaction *BM-Purple* was removed, the embryos were washed twice in PBST and afterwards fixed in 4% PFA.

3.1.3.8 Double in situ hybridisation

For double in situ hybridisation, both probes have to be added to the embryos at step 3.1.3.4 for hybridisation. In this case one probe was labelled with digoxygenin while the other was labelled with flourescein. The fluorescein labeled probe was visualized first. The antibody and color substrate reaction took place separately for the second probe, after the first probe reaction was completed. Subsequent to the first probe reaction, the embryos

were fixed in 4% PFA over night at 4°C. Then they were washed 2 times for 10 min 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 antibody incubation and color substrate reaction was performed according to paragraph 3.1.3.6 and 3.1.3.7.

3.1.4 Solutions for in situ Hybridisation

20 × PBS

2.76 M NaCl
50 mM KCl
160 mM Na_2HPO_4
50 mM KH_2PO_4

1 × PBST

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 Albumine) in PBST
Block II: 0.2 % BSA / 5 % sheep serum (heat inactivated) in PBST

AP-buffer

100 mM Tris pH 9,5

50 mM $MgCl_2$

100 mM NaCl

0.1 % Tween-20

5 mM Levamisol

Hybmix

50 % Formamid

5 × SSC

1 % *Boehringer Block* (Roche)

1 mg/ml Yeast RNA (Roche)

100 µg/ml Heparin

1 × Denhards

0.1 % Tween-20

0.1 % Chaps

5 mM EDTA

3.1.5 Analysing embryos after in situ hybridisation

3.1.5.1 Analysing whole-mount embryos

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

3.1.5.2 Analysing flat-mount embryos

To generate flat-mount preparations, the embryos were transferred over an ascending glycerol gradient into 99% glycerol on a glass slide. First the yolk was removed using a sharp needle, then the PSM was cut from the embryo and remaining yolk cells were removed with a lash. Then the embryos were transferred into a fresh drop of glycerol using a preparing needle. The embryos were orientated with a lash and covered with a glass 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). Flat preparations were partly provided by Irene Steinfartz and Eva Schetter.

3.2 Molecular biology protocols

3.2.1 Polymerase chain reaction (PCR) (Mullis et al., 1986)

3.2.1.1 PCR with double stranded (ds) DNA as template

To perform the PCR the machines *Mastercycler Grandient* und *Mastercycler Personal* (Eppendorf) were used.

Components for the PCR:
For one reaction 1-100ng template DNA were used. Furthermore 200nM sense Primer, 200nM antisense Primer, 200µM Desoxynucleotidtriphosphate (dNTP)-mixture (Sigma) and 1 × reactionbuffer were added. The end volume of the reaction was 50µl. 0.5µl of the Ampliqon Taq DNA Polymerase (5units/µl) were required for each reaction.
PCR conditions:

(1) 2 min @ 95°C for Denaturation
(2) 15 sec @ 95°C
(3) 30 sec @ 50°C for primer annealing (temperature depending on the used primer)
(4) 1 min @ 72°C for DNA synthesis (time depending on product length; 1 min for 1kb)
(5) 29 repeats of steps 2-4
(6) 5 min @ 72°C for elongation
(7) hold @ 16

Using ds DNA as template PCRs were performed for *her11* (p17+p18), *her12* (p19+20), *her13.2* (p21+p22), *her1* exon (p5+p6), *her1* intron (p13+p14), *her7* (p7+p8), *hey1* (p15+p16), *spt* (p23+p24), *deltaC* (p9+p10), *deltaD* (p11+p12), *myoD* (p25+p26) and *tbx24* (p27+p28) to generate the required DNA template for in vitro transcriptions (primer sequences are listed in table1 in the materials section). The obtained PCR products were then run on an agarose gel (3.2.2) and extracted following the protocol of the Rapid Gel Extraction Kit (Marligen).

3.2.1.2 PCR with first strand synthesis as template

The used first strand synthesis was produced with RNA from somitogenesis stage embryos. RNA was isolated from 100 embryos using the μMacs mRNA isolation kit (Miltenyi Biotec) according to the protocol. The *SuperScript First-Strand Synthesis System for RT-PCR* (Gibco BRL) and an oligo dT-primer were used for the first strand reaction (set up according to kit protocol).

The *her12* coding sequence was amplified (PCR conditions see 3.2.1.1) with p3 and p4 and first strand synthesis as template (primer sequences are found in table1-materials section). The obtained PCR product, and in parallel the pCS2+ vector, was digested with XhoI and XbaI (NEB), purified as described (3.2.2) and extracted using the Rapid Gel Extraction Kit (Marligen). Afterwards the digested product and the plasmid were ligated (ligation see 3.2.6). The ligation was then used to transform *XL10 Gold ultracompetent cells* (Stratagene)(transformation see 3.2.7).

3.2.1.3 PCR with genomic DNA as template

To amplify the *her12* 5'UTR (p1+p2) for the morpholino control experiments, genomic DNA was used as template for the PCR (PCR conditions see 3.2.1.1; primer seq. see materials table1). Genomic DNA was isolated from 24h old embryos like specified in chapter 3.2.14. The obtained PCR product, and in parallel the *eGFP*-pCS2+ vector, was digested with EcoRI and NcoI (NEB), purified as described (3.2.2) and extracted using the Rapid Gel Extraction Kit (Marligen). Afterwards the digested product and the plasmid were ligated (ligation see 3.2.6). The ligation was then used to transform *XL10 Gold ultracompetent cells* (Stratagene) (transformation see 3.2.7).

3.2.2 Agarose gel electrophoresis

To separate nucleic acids depending on their size agarose gels were used. The amount of agarose was between 0.8 % und 2 % in 1 × Tris-Acetat Electrophoresis (TAE)-buffer. 1/6 6 × loading-buffer II was added to the samples and the gel was run in a horizontal flat bed gel chamber filled with 1 x TAE. To visualize nucleic acids Ethidiumbromide was added to the melted gel (4μl Ethidiumbromide-solution (10μg/ml) in 100 ml) and the gels were analysed and pictured on a UV-Transiluminator (*Image Master VDS*; Amersham/Pharmacia).

Used DNA ladders:

2-Log DNA Ladder (NEB)

50bp DNA 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

15% Ficoll

(Stored at room temperature)

3.2.3 Extraction of PCR fragments from agarose gels (Gelextraction)

The fragment of interest was cut from the gel using a scalpel and transferred into a 1.5 ml eppendorf. The further extraction was done using the Rapid Gel Extraction Kit (Marligen) according to the manual.

3.2.4 Restriction enzyme digestion of DNA

The total volume of the reaction was 50µl. Depending on the following experiment 1-5µg of DNA were digested. Furthermore the reaction consisted of 1/10 reaction buffer and 10U of the desired restriction enzyme and was incubated for 1-2h at 37°C. Afterwards the DNA was cleaned doing a gel extraction (3.2.3) or a phenol-chloroform extraction (3.2.5).

3.2.5 Phenol-Chloroform Extraction and Ethanol precipitation

The standard way to remove proteins from nucleic acid solutions is to extract first with phenol: chloroform and then with chloroform. To the DNA protein mixture an equal volume of phenol-chloroform was added and mixed together until an emulsion forms (Vortex, 30 sec). It was then centrifuged at 13000g for 60s, at RT. Using a pipette, the aqueous phase was transferred into a fresh eppendorf tube and the organic phase was discarded. The phenol-chloroform extraction was repeated twice. Subsequently, an equal volume of chloroform was added to the sample, mixed (Vortex, 30 sec) and centrifuged at 13000g for 60s. Afterwards the aqueous phase was transferred to a new eppendorf tube. Subsequently an ethanol precipitation was performed to concentrate the DNA:
1/20 Vol 5M NaCl and 2.5 Vol Ethanol (100%) were added to the solution. The DNA was precipitated for ≥10h at -20°C. This was followed by a centrifugation step at 13000g for 20 min. The pellet was washed using 70 % Ethanol and again centrifuged at 13000g for 5 min. Then the Ethanol was removed and the pellet was allowed to dry for 5 min before it was resuspended in H_2O.

3.2.6 Ligation

20-40ng of the vector were used for the ligation and the amount of the insert-DNA was adjusted to a molar ratio between 1:1 to 3:1 to the vector. Additionally the ligation reaction contained 1/10 ligation buffer (NEB), 1 µl Ligase enzyme (400,000 units/ml concentration) and finally autoclaved water to a final volume of 10µl. The reaction was incubated overnight at 16°C and was used the next day to transform bacteria cells (3.2.7).

3.2.7 Transformation of bacteria cells

The ligation mix was transformed into *XL10 Gold ultracompetent cells* (Stratagene) according to the manual. Following this, different concentrations of the transformed bacteria were plated on LB plates carrying the appropriate antibiotic resistance (100µg/ml). The LB plate was then incubated over night at 37°C.

3.2.8 Growing *Escherichia coli*

E. coli was grown according to existing protocols (Sambrook et al., 1989).

3.2.9 Minipreparation of plasmid DNA

Using sterile tips single clones were picked from the bacteria plate and transformed to 3-5 ml LB-medium containing the required antibiotic in a concentration of 50µg/ml. The culture was incubated overnight at 37°C. The next morning the plasmid preparation was performed using the *Wizard Plus Minipreps DNA Purification system* (Promega) according to protocol.

3.2.10 Sequencing of DNA

The sequencing of DNA took place according to the dideoxy chain termination method (Sanger et al., 1977). The reaction was performed using the terminator ready reaction mix (Amersham/Pharmacia) with slight modification to their protocol:
Every sequencing reaction contained 2µl DNA (0.1- 0.25ng/µl), 2µl Primer (3µM) and 2µl *Terminator Ready Reaction Mix*, at a total volume of 10µl.

Reaction profile:
Initial denaturation of DNA took place at 96°C for 1 min. At the beginning of a cycle, there was 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.

Purification and analyses:
After the reaction the volume was adjusted to 20µl. The further cleaning and the analysis of the reaction were performed in the Sequencing Facility (Institute for Genetics, Cologne) according to their standards. The obtained sequence files were analysed using Vector NTI 6.0 (Infor Max, Inc.)

3.2.11 in vitro transcription to produce in situ probes

To produce labelled RNA probes the T3 or T7 polymerase (Roche) was used, depending on the promotor present on the template DNA. The probes were either labelled using the *Digoxygenin-RNA Labeling Mix* or the *Flourescein-RNA Labeling Mix* from Roche.
The transcription reaction contained 200-500ng DNA, 1µl 10 × Labeling Mix, 1µl RNA Polymerase (20 U/µl), 1µl 10 × Transcription-buffer (contains 60 mM $MgCl_2$) and 0.5µl

RNase Inhibitor (40 U /µl, *Roche,* Mannheim). The total volume was adjusted to 10µl with H_2O_{DEPC} (DEPC = Diethylpyrocarbonat). The reaction was incubated at 37°C for 2h and subsequently stopped by adding 1µl RNase free 0,2M EDTA. Purification of the transcripts was done by ethanol precipitation according to Roche protocol. The RNA pellet was resolved in a mixture of 20µl H_2O_{DEPC} and 20µl Formamide and stored at -20°C.

3.2.12 Injection of morpholino oligonucleotides and mRNA into zebrafish embryos

3.2.12.1 Preparation of capped mRNA for zebrafish injections

Capped mRNA for the misexpression of *her11, her12, GFP* and for the *her12-GFP* morpholino control RNA was made using the mMessage mMachine kit (Ambion) according to the manual.

3.2.12.2 Morpholino design

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 are listed in the Materials section. Morpholinos were delivered lyophilized and were immediately diluted in H_2O. The concentration of this stock solution was 3µM.

3.2.12.3 Injection of zebrafish embryos

Zebrafish embryos were injected in the 1-2 cell stage into the yolk directly under the first cell(s). Embryos were put in a row on a dark agarose plate (1% Agarose in H_2O containing activated carbon), the water was removed and embryos were injected immediately using FemtoJet® and a Micromanipulator from Eppendorf. The used capillaries (Hildenberg) were pulled using a *Sutter P9 Micropipette Puller* (Sutter) (pulling conditions: heat 537, pull 100, velocity 100, time 150). The concentration of the morpholino was between 0.3 and 1.2 mM, and additionally 0.1 M KCL and 0.2% Phenol red were added to the injection solution. mRNA was injected in range of 200-800ng/µl in a solution containing 0.1 M KCL and 0.2% Phenol red. After the injection the embryos were transferred into a petridish with aquarium water and incubated to the desired stage.

3.2.13 Epon embedding and sectioning

Epon embedding and sectioning were done by Irene Steinfartz and carried out according to protocols described in Nüsslein-Volhard and Dahm R, (2002).

3.2.14 Isolation of genomic DNA

Genomic DNA was isolated from 24h old embryos. 100 embryos were transferred into a 1.5ml eppendorf, the water was removed and 990µl DNA-extraction buffer as well as 10µl Proteinase K (Roche) were added. The tube was mixed (Vortex, 10 sec) and incubated for 3h at 55ºC. Every 45 min the tube was mixed again (Vortex, 10 sec). Afterwards the DNA was precipitated by adding 1Vol of 100% Ethanol. The tube was inverted several times and the DNA was pulled out using a glass pipette and transferred to a fresh 1.5ml eppendorf containing 70% Ethanol. Subsequent the Ethanol was removed the DNA was dried for 5-10 min at RT. Then the DNA was solved in 500µl low TE.

Buffer and Solutions:

DNA-extractionbuffer:
10 mM Tris pH 8,2
10 mM EDTA
200 mM NaCl
0.5 % SDS

low TE:
10 mM Tris pH 8,0
0.1 mM EDTA

3.2.15 Quantification of DNA by Spectrophotometric determination

To quantify the amount of DNA a Biophotometer (Eppendorf) was used. Reading was taken at a wavelength of 260/280 nm according to the manual.

4. Results

4.1 The role of *Suppressor of Hairless* in Notch mediated signalling during zebrafish somitogenesis

To test whether Notch signalling is indeed exclusively mediated via the canonical CSL dependent pathway during zebrafish somitogenesis, the zebrafish *Suppressor of Hairless (Su(H))* homologue was analysed. Analysis of the genome sequence implies the existence of only one functional *Su(H)* homologue in zebrafish, which was shown to be ubiquitously expressed during zebrafish somitogenesis in my diploma thesis (Sieger 2002). Provided that Notch signalling is mediated via this homologue, a knockdown should influence somite border morphology and cyclic gene expression.

4.1.1 *Su(H)* knockdown leads to defects in somite formation

Two different Morpholino oligonucleotides (Mo) specific to *Su(H*) were injected, one complementary to the 5'UTR (5'Mo) including the AUG and the other one designed against the region downstream of the AUG (ORF-Mo). Both Mos have the same influence on the expression patterns of all the genes that were examined (see Tab.2 and respective Figure Legends) and both disturb somite patterning in the same way.

Table 2: Effects of *Su(H)*-Mo injections on cyclic gene expression and somite morphology

Treatment	Conc. (mM)	Total no. of embryos	No. of experiments	Cyclic gene expression*		Somite phenotype**	
				wt(%)	disrupted(%)	wt(%)	7±2(%)
Uninject.	-	422	4	100	0	100	0
ORF-Mo	0.3	138	2	8	92	9	91
	0.6	143	2	2	98	3	97
	0.9	214	3	1	99	1	99
5'Mo	0.3	95	1	92	8	93	7
	0.6	88	1	90	10	92	8
	0.9	112	2	27	73	29	71
	1	215	7	16	84	20	80
Control injections:							
5-bm Mo	0.3	78	2	100	0	100	0
	0.6	113	2	99	1	100	0
	0.9	98	2	98	2	100	0

*Cyclic gene expression was monitored by *her1* and *her7* in situ hybridisations in 10s stage embryos.
**The somite phenotype, characterised by appearance of only the 7±2 anteriormost somites, was observed in living embryos at the 14s stage under a dissection microscope.

Embryos injected with the ORF-Mo (ORF morphants) resemble the *notch1a* mutant *deadly seven* during the first 20 hours of development (Fig.1 C, H). These embryos develop 7±2 somite borders, which are not properly arranged in comparison to the control embryos (compare Fig.1 E, J with Fig.1 D, I). Beyond these anterior somites further somitic tissue is generated but somite borders are missing (Fig.1 C, E, H, J). At the end of somitogenesis the ORF-Mo injected embryos exhibit a more severe phenotype than the *des* mutant. The ORF morphants show a curved trunk and tail phenotype as it has been observed after overexpression of a DNA-binding mutant of the *Xenopus Su(H)* homologue in zebrafish (Fig.1 L, P, R; Lawson et al. 2001). In addition ORF morphants are shorter than the comparable control embryo and lack posterior trunk pigmentation (compare Fig.1 O, Q with Fig.1 P, R). They survive up to 200 hpf, show severe defects in blood vessel formation and develop a large heart oedema (data not shown). Almost the same phenotype has been observed for the zebrafish mutant *mindbomb*, which codes for a defective E3 ubiquitin ligase (Itoh et al. 2003; Jiang et al. 1996). Since this ubiquitin ligase is necessary for the endocytosis of Delta the mutation leads to a breakdown of *delta* dependent Notch

signalling. This suggests that the phenotype observed here for the ORF morphants is typical for defective Notch signalling in zebrafish.

However, embryos injected with the 5'Mo (5' morphants) show morphologically a much stronger effect, which is similar to the respective *RBP-Jκ* null-mutant in mouse (Oka et al. 1995). The embryos are always developmentally retarded and drastically reduced in size when compared to their wildtype counterpart and the ORF morphant (Fig.1). As a consequence of the reduction in size, these embryos show an incomplete rotation around the yolk after 16 hpf (Fig. 1 B). Measuring the length of the knockdown embryos after 16 hpf shows that they have only approximately 2/3 of the length of a comparable wildtype embryo. The 5' morphants form, like the ORF morphants, only 7±2 somites, which show segmental defects and are irregularly arranged when compared to their wildtype complement (Fig.1 B, G). After 16 hpf distinct areas of degenerating cells can be detected in the head of the embryo (Fig.1 B) and during the next hours of development degenerating cells can also be observed in the trunk and tail (data not shown). Since *RBP- Jκ*$^{-/-}$ mutant mice embryos show also distinct areas of cell degeneration and none of the control injections leads to this effect one might assume that the effect is specific to the knockdown of *Su(H)*. However, it is not possible to exclude that this is a toxic effect caused by Mo injection (for review see Heasman 2002). After the formation of the first 7±2 somites the embryos essentially stop growing and it appears that no further somitic tissue is generated. The embryos develop for a few more hours and die after approximately 35-60 hpf.

To analyse the identity of the mesodermal tissue generated in *Su(H)*-knockdown embryos in situ hybridisations for *MyoD* were performed. The somites express *MyoD* in *Su(H)* morphants, indicating that muscle cells are still forming (Fig.1 N). However, while *MyoD* expression is normally restricted to the posterior parts of newly formed somites (Weinberg et al. 1996), this distinct expression is disturbed in the *Su(H)*-knockdown embryos when compared to wildtype embryos of the same stage (Fig.1 M, N). This suggests that not only the somite borders are missing beyond the anterior somites but that there is also disturbance of the A-P polarity of the somitic tissue.

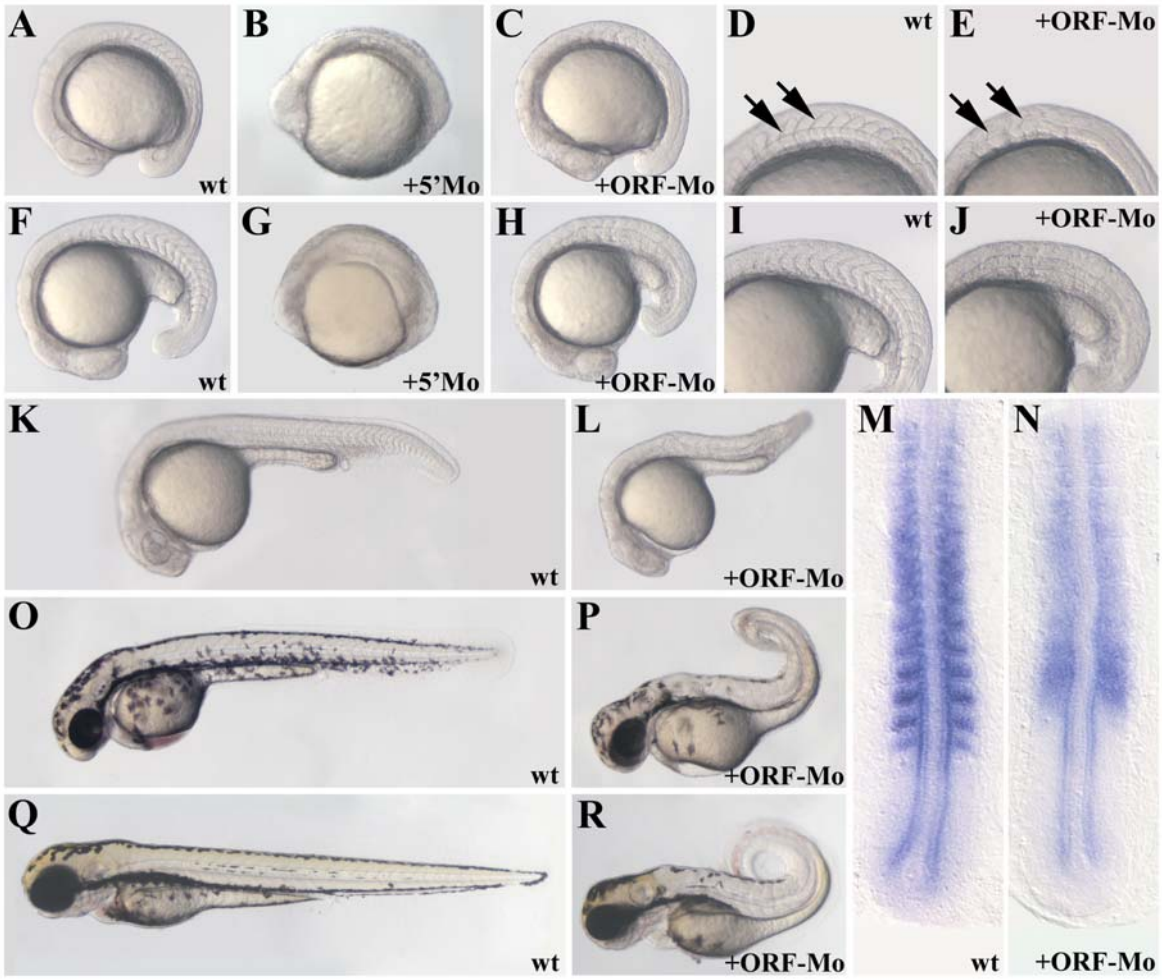

Fig. 1:

Influence on morphology after *Su(H)* morpholino injections and effects on *MyoD* expression. (A), (F), (K), (O), (Q) wildtype embryos at 16, 18, 28, 45, 75 hpf, respectively. (B), (G) *Su(H)*-5'Mo injected embryos at 16, 20 hpf, respectively. (C), (H), (L), (P), (R) *Su(H)*-ORF-Mo injected embryos at 16, 18, 28, 45, 75 hpf, respectively. (D), (I) higher magnification of the somites in wildtype embryos at 16 and 18 hpf, respectively. (E), (J) higher magnification of the somites in ORF-Mo injected embryos at 16 and 18 hpf, respectively. Arrows in (D), (E) indicate anterior somite borders in wildtype embryos and ORF morphants, respectively. (A)-(L), (O)-(R) lateral view, dorsal to the top. (M) wildtype embryo at 16 hpf stained for *MyoD*, (N) *Su(H)*-ORF-Mo injected embryo at 16 hpf stained for *MyoD* (0.9mM, 2 experiments, n=131, 98% affected). (M), (N) dorsal view, anterior to the top, flat mounted embryos.

4.1.2 Cyclic gene expression is disturbed in *Su(H)*-knockdown embryos

To analyse whether Notch signalling in the segmentation clock is mediated via *Su(H)* the expression of different oscillating genes of this pathway was analysed in *Su(H)* knockdowns. The effects described below were observed with the same high penetrance in the 5' morphants and ORF morphants and were not observed in control injections (for details see Tab.2 and respective Figure Legends).

Wildtype embryos show for *her1* and *her7* a cyclic expression in a U-shaped domain in the posterior PSM and two to three pairs of stripes in the intermediate and anterior PSM. *Su(H)*-knockdown leads to a disruption of this dynamic expression for both genes. In *Su(H)* morphants a diffuse expression for *her1* and *her7* was observed throughout the whole PSM with an area of stronger expression in the anterior PSM (Fig.2 C, D, G, H), which is comparable to *her1* and *her7* expression in *des/notch1a* and *aei/deltaD* mutant embryos (van Eeden et al. 1998; Oates and Ho 2002; Fig.2 B, F). Analysis of *aei/deltaD*; *fss/tbx24* and *des/notch1a;fss/tbx24* double mutants had shown that the activation for *her1* in the anterior PSM is due to the function of the *fss/tbx24* gene, which acts independently of the *Delta-Notch* pathway (van Eeden et al. 1998; Holley et al. 2000; Nikaido et al. 2002). To ascertain whether the observed activation of *her1* and *her7* in the anterior expression domain in *Su(H)* morphants is also dependent on the function of the *tbx24* gene, *Su(H)*-Mo injections were performed in *fss* embryos. It was previously shown that *her1* shows still a cyclic expression in the posterior PSM of *fss* embryos while its expression in the most anterior stripe is lost (van Eeden et al. 1998; Nikaido et al 2002 and Fig.2 J). In situ hybridisation for *her7* in *fss* embryos shows the same pattern as for *her1* (compare Fig.2 J with Fig.2 N) suggesting that both bHLH genes are equally regulated by *fss/tbx24*. *Su(H)*-Mo injections in *fss* embryos lead to a breakdown of cyclic *her1* and *her7* expression in the posterior PSM of these embryos (Fig.2 K, L, O, P). Both genes show now only a diffuse expression in the posterior PSM with no signs of cyclic expression, indicating that Notch signalling is necessary for the initiation of oscillation in the *fss/tbx24* mutant. The stronger activation in the anterior PSM observed in *Su(H)* morphants is also lost after *Su(H)*-knockdown in *fss* embryos. This indicates that the activation of *her1* and *her7* in the anterior PSM after *Su(H)*-knockdown is indeed due to the function of *fss/tbx-24*. However, the weak expression in the posterior U-shaped domain is still present, probably due to activation by tissue specific activators (Fig.2 K, L, O, P).

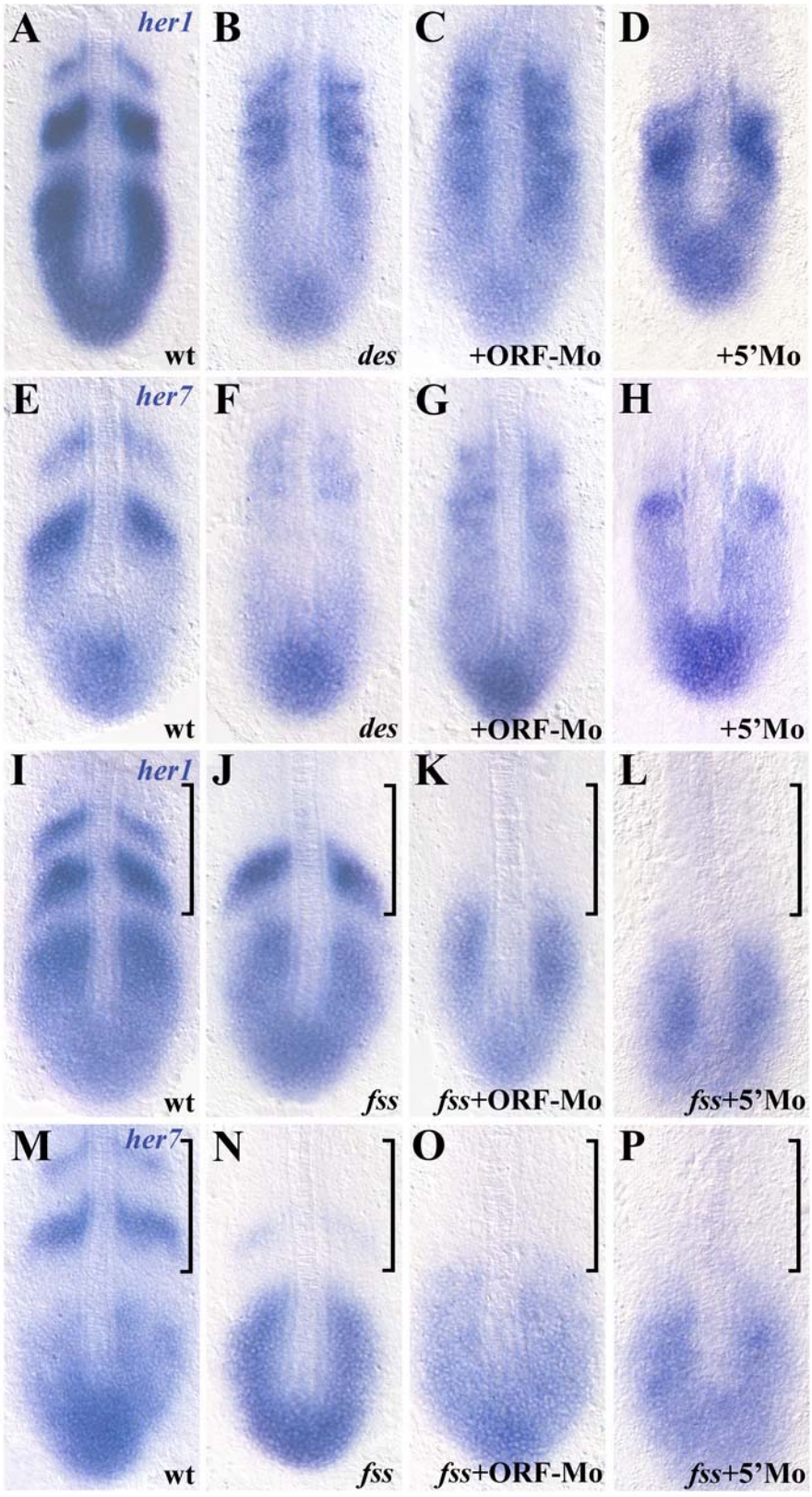

Fig.2 (legend on next page)

Fig. 2:
Effects of *Su(H)* morpholino injections in wildtype and *fss/tbx24* mutant embryos on the expression patterns of *her1* and *her7*. All embryos are between the 9-10 somite stage. (A), (I) wildtype expression of *her1*. (E), (M) wildtype expression of *her7*. (B), (F) *her1* and *her7* expression, respectively, in *des/notch1a* mutant embryos. Cyclic *her1* and *her7* expression is disrupted in ORF morphants (C) and (G), respectively, as well as 5' morphants (D) and (H), respectively (concentration of the Mos and number of injected embryos see Tab.2). (D), (H) the PSM is slightly shorter because of the reduction of size in 5' morphant embryos. Note a slightly stronger basal expression of *her1* and *her7* in *Su(H)* morphants compared to *des/notch1a* mutant embryos (compare Fig.2 B with 2 C, D and Fig.2 F with 2 G, H). (J), (N) *her1* and *her7* expression, respectively, in *fss/tbx24* mutant embryos. Cyclic *her1* and *her7* expression is disrupted after ORF-Mo injection in *fss* embryos (K), (O) respectively (*fss*+ORF-Mo *her1*: 0.9mM, 2 experiments, n=87, 97% affected; *fss*+ORF-Mo *her7*: 0.9mM, 2 experiments, n=76, 96% affected) and after 5'Mo injection in *fss* embryos (O), (P) respectively (*fss*+5'Mo *her1*: 1mM, 1 experiment, n=45, 95% affected; *fss*+5'Mo *her7*: 1mM, 1 experiment, n=41, 93%, affected).
Brackets in (I)-(P) mark the region in the intermediate and anterior PSM where the oscillating stripes are observed. Note a loss of the anterior *her1* and *her7* stripe in *fss* embryos (J), (N) respectively and a complete loss of *her1* and *her7* stripes in *Su(H)* morphants (K), (L) and (O), (P) respectively. Only a weak basal *her1* and *her7* expression can be detected in the posterior PSM of *Su(H)* morphant *fss* embryos (K), (L) and (O),(P) respectively. (L), (P) the PSM is slightly shorter because of the reduction of size in 5' morphant embryos. (A)-(P) dorsal view, flat mounted embryos, anterior to the top.

4.1.3 Cyclic gene expression during early somitogenesis stages

Since the first 7±2 somites form in *des/notch1*, *aei/deltaD* and *Su(H)*-Mo injected embryos it might be possible that another pathway is responsible for the generation of the anterior somites. To test if cyclic gene expression is dependent of *Notch* signalling during early somitogenesis stages *Su(H)*-Mo injected embryos were fixed in the 3 somite stage and in situ hybridisations for *her1* and *her7* were performed. In these embryos cyclic expression of *her1* and *her7* is affected after *Su(H)*-knockdown although less than in the 10 somite stage embryos (compare Fig.3 B, C, E, F with Fig.2 C, D, G, H). *Su(H)*-Mo injection leads to a diffuse *her1* and *her7* expression in the whole PSM of 3 somite stage embryos but still with a visible stripe activation. This suggests that early cyclic gene expression is differently regulated than later cyclic gene expression.

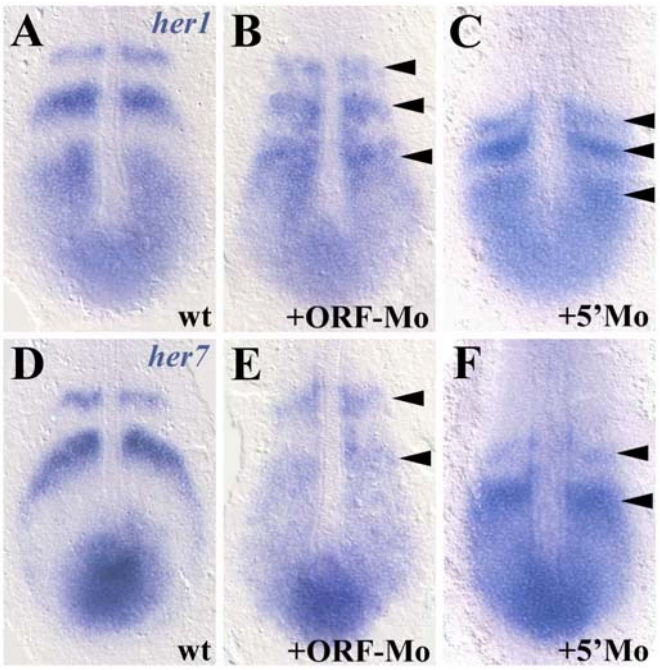

Fig. 3:

her1 and *her7* expression after *Su(H)*-knockdown in 3 somite stage embryos. (A), (D) expression of *her1* and *her7*, respectively, in wildtype embryos. After *Su(H)*-ORF-Mo injection *her1* (B) (0.9mM, 2 experiments, n=92, 91% affected) and *her7* (E) (0.9mM, 2 experiments, n=86, 89% affected) show a diffuse activity in the whole PSM with distinct areas of stripe like expression in the intermediate and anterior PSM. The same expression for *her1* (C) and *her7* (F) is observed after *Su(H)*-5'Mo injection (wt+5'Mo *her1*: 1mM, 1 experiment, n=67, 95% affected; wt+5'Mo *her7*: 1mM, 1 experiment, n=75, 93% affected). Note a slightly shorter PSM in 5' morphants (C), (F) because of size reduction of the whole embryo. Arrowheads in (B), (C), (E), (F) point out the stripe like expression in the intermediate and anterior PSM of *Su(H)* morphants. (A)-(F) dorsal view, flat mounted embryos, anterior to the top.

4.1.4 Expression of *deltaC* and *deltaD* after *Su(H)*-knockdown

It has previously been shown that *her1* and *her7* have a feedback loop on *deltaC* and *deltaD* expression (Holley et al. 2002; Oates et al. 2002; Gajewski et al. 2003). The question now is, whether *Su(H)*-knockdown and the observed change in *her1 and her7* expression have also an influence on *deltaC* and *deltaD* transcription. In wildtype embryos *deltaC* and *deltaD* expression consists of two paired stripes in the anterior PSM and an expression domain in the posterior PSM (Dornseifer et al., 1997; Smithers et al., 2000), with the difference that *deltaC* is cyclically expressed but *deltaD* seems not (Jiang et al. 2000; Holley et al. 2002). Furthermore *deltaC* is expressed in the posterior part of newly formed somites whereas *deltaD* is expressed in the anterior part of the somite. *Su(H)*-Mo injections lead to a clear disruption of *deltaC* and *deltaD* expression. After knockdown of

Su(H) both genes show a weak expression in the whole PSM and a broad expression domain appears in the anterior PSM (Fig.4 B, D). The *deltaD* expression in the newly formed somites is absent in *Su(H)* morphants but, surprisingly, *deltaD* expression is now ectopically expressed in the developing neural tube (compare Fig.4 C with Fig.4 D). *deltaC* expression is also affected in the somites of *Su(H)*-Mo injected embryos, which show only a weak residual staining when compared to the wildtype counterpart (compare Fig.4 A with Fig.4 B).

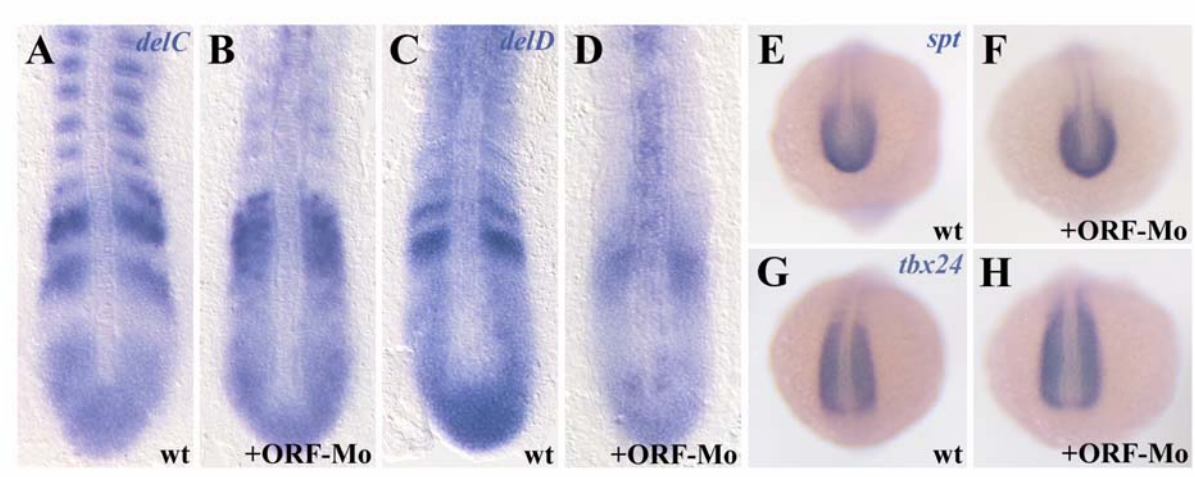

Fig. 4:
deltaC, deltaD, tbx24 and *spt* expression after *Su(H)*-ORF-Mo injection in 9-12 somite stage embryos. (A), (C), (E), (G) wildtype expression of *deltaC*, *deltaD*, *tbx24* and *spt*, respectively. (B), (D) *deltaC* and *deltaD* expression respectively in *Su(H)*-ORF-Mo injected embryos (wt+ORF-Mo *deltaC*: 0.9mM, 2 experiments, n=106, 94% affected; wt+ORF-Mo *deltaD*: 0.9mM, 2 experiments, n=78, 93% affected). (F), (H) *tbx24* and *spt* expression respectively in *Su(H)*-ORF-Mo injected embryos (wt+ORF-Mo *tbx24*: 0.9mM, 1 experiment, n=45, 99% show wildtype expression; wt+ORF-Mo *spt*: 0.9mM, 1 experiment, n=53, 100% show wildtype expression). (A)-(D) dorsal view, flat mounted embryos, anterior to the top. (E)-(H) dorsal view, posterior downwards.

4.1.5 *Su(H)*-knockdown does not affect *tbx-24* and *spt* expression

The expression of *tbx-24* and *spt* should be independent of the Delta-Notch pathway (Holley et al. 2002; Nikaido et al. 2002; Griffin et al. 1998) and one should therefore expect no effect of a *Su(H)* knockdown on the expression of these genes. Indeed, after *Su(H)*-Mo injection, *tbx-24* is still expressed in the intermediate and anterior PSM as in the wildtype situation (Fig.4 G, H). Similarly, the posterior expression of *spt* shows no change

after *Su(H)*-knockdown (Fig.4 E, F). Thus, the primary specification of the posterior PSM is apparently not affected by *Su(H)* knockdown.

4.2 *her11* is involved in the somitogenesis clock in zebrafish

The complex mechanism of prepatterning in the PSM can not be explained with the so far known components of the Delta-Notch pathway and the members of the *hairy (h)* and *Enhancer of split (E(spl))* family genes only. More components are necessary to understand the nature of cyclic gene expression in the PSM (Holley et al., 2002; Oates et al., 2002; Gajewski et al., 2003). A screen of the zebrafish genomic sequence revealed the existence of at least 23 *h/E(spl)/hey*-related genes (M.Gajewski). Four newly identified genes, namely *her11* (this chapter), *her12* (results 4.3), her13.2 (Kawamura et al. 2005, results 4.4) and *her15* (Shankaran 2005), show expression in the PSM and thus are possible interaction partners for *her1* and *her7*. In this chapter the analysis of *her11*, a paralogue of *her1*, is presented. Furthermore *hey1* was analysed, since it is the only *hey* gene (distinction *her-hey* genes see introduction p.2) expressed in the zebrafish PSM (Winkler et al., 2003) and shows a comparable, but slightly distinct expression to *her11*.

4.2.1 *her11* is synexpressed with *her1* and *her7* stripes in the intermediate and anterior PSM

her11 expression becomes first visible during epiboly in two different consecutively appearing domains (Fig.5). At around 75% epiboly *her11* transcripts are first detected in a head domain, where one broad stripe appears, which demarcates the prospective midbrain-hindbrain region at the anterior end of the embryo (Fig.5 A). During further development until the bud stage this stripe becomes V shaped and at around the six- to ten-somite stage only one transverse band of *her11* expression remains at the MHB (Fig.5 B–D). In embryos older than ten-somite stage, *her11* starts to be additionally expressed dorsally in the midbrain as a longitudinal stripe, which is connected to the transverse band (Fig.5 D). Thus the *her11* expression pattern in the midbrain-hindbrain region is very similar to *her5* expression (von Weizäcker 1994; Geling et al. 2003). But *her11* is also expressed in the posterior region of the embryo. One pair of stripes emerges anterior to the epibolic margin at 80–90% epiboly, in a region which gives rise to the first somites (Fig.5 B). At around bud stage another pair of stripes becomes visible, again anterior to the epibolic margin, while the former stripe is displaced to a more anterior position (Fig.5 C). From bud until mid-somitogenesis stages two or three pairs of stripes are detected in the PSM, which seem to be dynamically expressed (Fig.5 E–G). In embryos older than 10- to 12-somite stage, only two stripes are visible in the PSM. In addition, *her11* is expressed in the anterior half

of the four to six most caudal somites during the somitogenesis phase. Double staining with the *her1* and *her11* probes shows that the PSM expression of these two genes overlaps. All specimens analysed (n =45) showed a combination of the *her1* and *her11* expression pattern (Fig.5 H, I). In particular, the striped expression in the intermediate to anterior PSM was always visible with clear interstripe regions devoid of staining (Fig.5 I) and the width of the *her11* domains in the PSM decreased from posterior to anterior in a similar manner to *her1* and *her7* domains (compare Fig.5 F, G with H). Thus, the only difference in the PSM was the characteristic posterior *her1* and *her7* expression domain, which was missing for *her11*. Since *her7* also overlaps with *her1* (Oates and Ho 2002; Gajewski et al. 2003) all three of these genes are synexpressed in a stripe-like fashion in the intermediate to anterior PSM, although slight shifts with respect to each other cannot be excluded, as may be the case for *her1* and *her7* (Oates and Ho 2002).

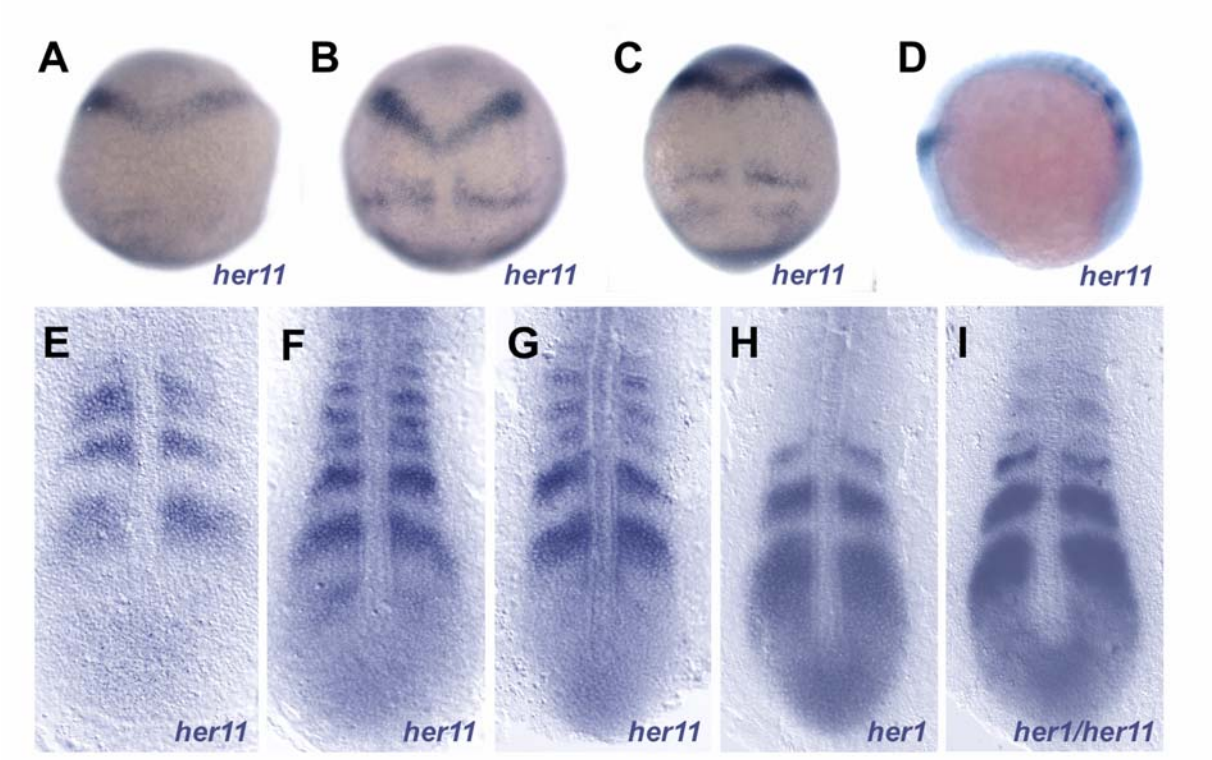

Fig. 5 (legend on next page)

Fig. 5:
Expression pattern of *her11* in different developmental stages (A-G). (A) 75% epiboly, (B) 90% epiboly, (C) bud stage, (D) 8 somite stage, (E) 3 somite stage, (F) 6 somite stage, (G) 10 somite stage; (H) *her1* in situ in a 8 somite stage, (I) *her1-her11* double insitu in a 8 somite stage embryo. (A)-(C) dorsal view; (D) lateral view, dorsal to the top; (E)-(I) flat mounted embryos, anterior to the top.

4.2.2 Delta-Notch signalling is required to regulate *her11* expression in the PSM

The bHLH genes *her1* and *her7* are controlled by the Delta-Notch pathway genes *notch1a, deltaC and deltaD* (Holley et al. 2000, 2002; Henry et al 2002; Oates and Ho 2002; Gajewski et al. 2003; Jülich et al., 2005b), which are mediated by Su(H) (see results 4.1). To investigate whether *her11* is also regulated by Delta-Notch signalling, its expression pattern was examined in the fused somite-type mutants *aei/deltaD*, *bea/deltaC* and *des/notch1a*. The cyclic expression of *her11* in the PSM is disrupted in the three mutants (Fig.6). Instead of two or three stripes of *her11* expression, a broad zone in the intermediate to anterior PSM is observed, in which all cells express *her11* homogeneously, suggesting that interstripe repression is lost in these mutants. However, some cyclic expression is still retained, since one can distinguish two different phases, one in which the *her11* expression domain in the intermediate PSM shows a clear anterior border with a gap towards the somite expression (Fig.6 C, E, G), and the other without such a gap (Fig.6 B, D, F). This residual cyclic expression is lost in *Su(H)* morphants (Fig.6 H), suggesting that it is dependent on the Delta-Notch pathway, but not via *deltaC, deltaD* or *notch1a*. Thus, it seems possible that an additional Notch gene is specifically involved in this cycling aspect of *her11*, or that a Notch-signalling-independent effect of *Su(H)* has been uncovered.

It is assumed that the specification of the first few somites in zebrafish is regulated differently from the trunk somites. This is most evident in *aei* and *des* where the first 7±2 somitic borders are still formed, while the remainder of the somitic borders are severely disrupted (van Eeden et al. 1996). In *bea* mutants, only the first four somitic borders are formed. This is also observed in the *Su(H)* morphants, where these first somites are also much less affected (see results 4.1). The morphological failure of somite border formation coincides with the disruption of cyclic gene expression, since *deltaC* expression is already disturbed in three- to four-somite stage *bea* embryos but appears normal in *aei* or *des* embryos of the same age (Jiang et al. 2000). The expression of *her11* is also differentially affected in these early somitogenesis stages. While *aei* and *des* mutants at the three- to four-somite stage show almost no effect on *her11* expression in the first stripes (Fig.6 K, L, M), *bea* mutants and *Su(H)* morphants lead to a broad domain (Fig.6 N, O) indicating that

their gene products are required for proper *her11* expression from the third somite stage on. No alterations in *her11* expression could be observed in *bea* or *Su(H)* morphant embryos in stages earlier than three somites indicating that *her11* underlies a different regulation before this time.

fss/tbx24 codes for a T-box gene (Nikaido et al. 2002) and is thus the only mutant of the fused somite class, which does not encode a gene belonging to the Delta-Notch pathway. In this mutant somitic border formation is completely disturbed (van Eeden et al. 1996) and a failure of the generation of the *her1* and *her7* stripes in the anterior PSM is observed. Cyclic expression of *her1* and *her7* in the posterior PSM is not affected in *fss* (Holley et al. 2000; results 4.1). This is similar for *her11*. Only the posteriormost one or two *her11* stripes are formed in *fss* mutants while the more anterior stripes and the half-segmental expression of *her11* in the somites is absent (Fig.6 I). An additional *Su(H)* knockdown in the *fss* mutant background leads to a broad uniform domain in the intermediate to anterior PSM (compare Fig.6 I with J), which is in contrast to the absence of *her1* and *her7* expression in the same mutant combination (results 4.1).

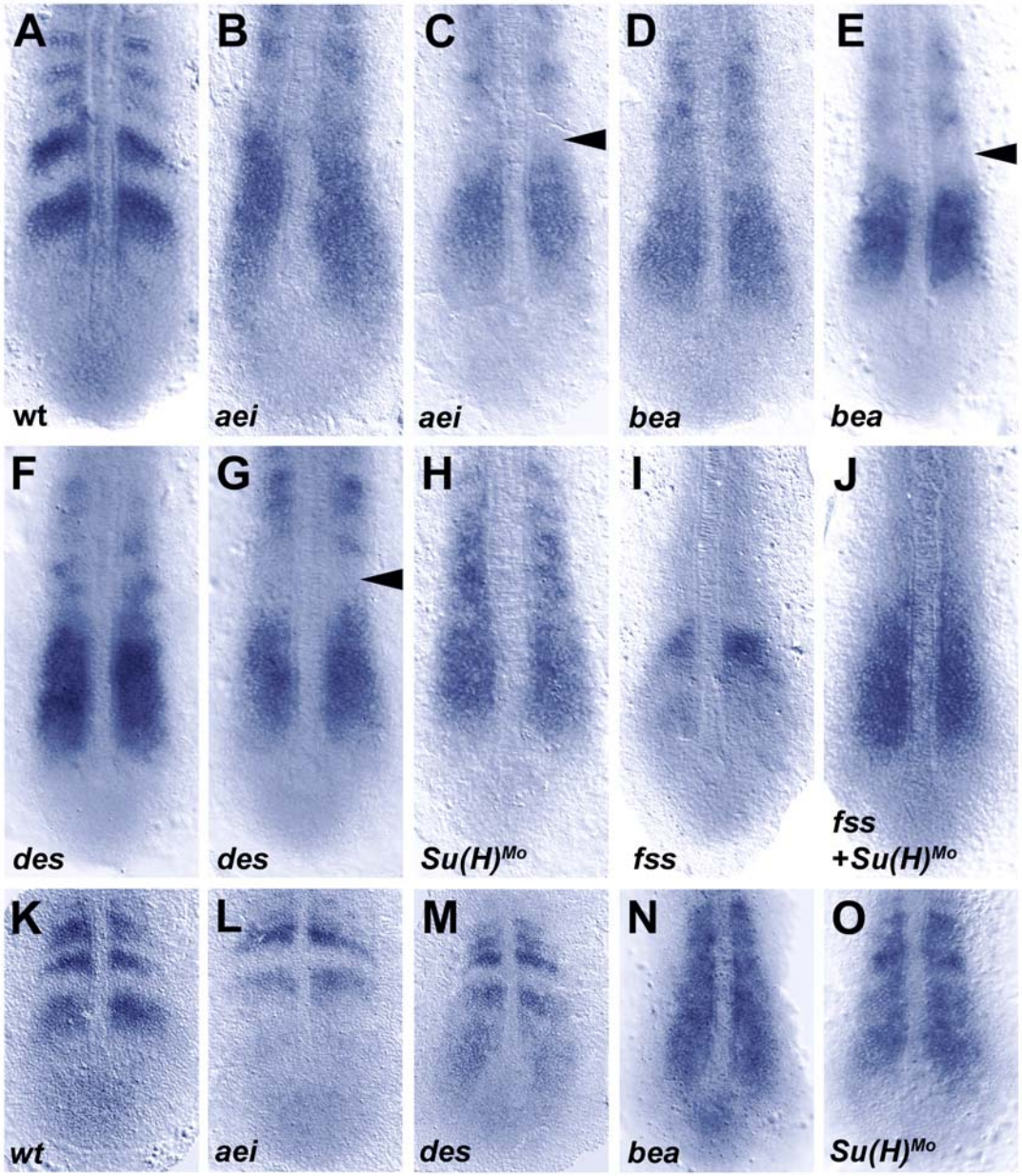

Fig. 6:

Expression of *her11* in the different *fss*-type mutants and Su(H) morphants. (A),(K) *her11* expression in the wildtype, (B),(C),(L) in the *aei* mutant, (D),(E),(N) in the *bea* mutant, (F),(G),(M) in the *des* mutant and in Su(H) morphants (Su(H)-ORF-Mo, 0.9mM, 2 experiments, n=94, 96% affected) (H),(O). (F) *her11* expression in the *fss* mutant and after additional knockdown for Su(H) in the *fss* mutant background (Su(H)-ORF-Mo, 0.9mM, 2 experiments, n=74, 95% affected) (G). (A)-(O) flat mounted embryos, anterior to the top; (A)-(J) 8-10 somite stage embryos, (K)-(O) 3-4 somite stage embryos.

4.2.3 Striped expression of *her11* in the PSM is cooperatively regulated by Her1 and Her7

To test the influence of *her1* and *her7* on the regulation of the *her11* stripes, *her11* expression was analysed in the respective morphants. The used *her1* and *her7* morpholinos were desribed and tested previously (Gajewski et al. 2003). In both, *her1* and *her7* morphants, the cyclic expression of *her11* in the intermediate to anterior PSM is disrupted (Fig.7 B, C). Interestingly, the residual expression levels of *her11* appear to be lower in *her7* morphants than in *her1* morphants, indicating a differential effect, similar as was found for the crossregulation between *her1* and *her7* on each other (Gajewski et al. 2003). The half-segmental expression of *her11* persists in both morphants with only weak signs of disruption. However, after combined knock down of both Her1 and Her7, the *her11* transcripts are homogeneously distributed in the PSM and in the area were the somites should normally be formed (Fig.7 D), probably as a consequence of loss of any segmentation. Thus, in contrast to the remaining cyclic expression of *her11* in the PSM of *aei, bea* and *des* mutants (Fig.6 B–G), *her11* expression does not show any residual dynamic behaviour in the *her1* or *her7* morphants. In fact, loss of cyclic expression of *her11* in *her1* and *her7* double morphants is observed as early as the expression starts, i.e. from 80% to 95% epiboly stages onwards including the regulation in the first somites (Fig.7 G, H). Thus, the cyclic expression of *her11* depends critically on *her1* and *her7* throughout development.

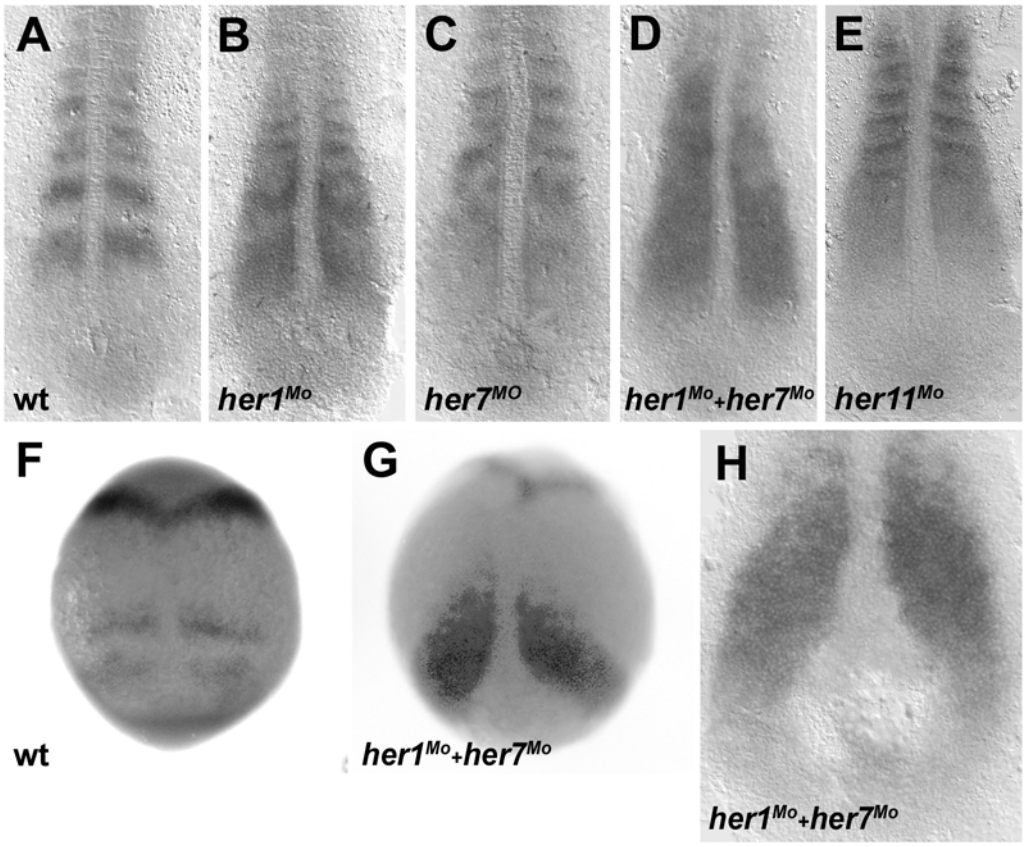

Fig. 7:

Influence of *her1* and *her7* on *her11* expression. (A),(F) *her11* expression in wildtype embryos, (B) and (C) *her11* expression in *her1* morphants (*her1*-5'Mo, 1mM, 2 experiments, n=118, 86% affected) and *her7* morphants (her7-5'Mo, 0.5mM, 2 experiments, n=122, 91% affected), respectively. (D),(G) and (H) disrupted *her11* expression after *her1* and *her7* double-knockdown (her1-5'Mo(0,5 mM)+her7-5'Mo(0,5mM), 2 experiments, n=70, 96% affected). (E) stabilized *her11* mRNA after *her11* Morpholino injection (*her11*-startMo and *her11*-ORF-Mo, 1mM, 2 experiments, n=102, 98% affected). (A)-(E),(H) flat mounted embryos, anterior upwards; (F),(G) dorsal view, anterior to the top. (A)-(E) 8-10 somite stage embryos, (F)-(H) bud stage embryos.

4.2.4 The regulation of cyclic *hey1* expression in the PSM

The only known member of the *hey* gene family that is expressed in the zebrafish PSM comparable to *her11*, namely *hey1*, is expressed in the interstripe regions of *her1-her7-her11* in the intermediate and anterior PSM (Winkler et al. 2003; Fig.8 A). In addition, *hey1* is transcribed in the posterior half of the somites. Thus, *hey1* expression domains complement the *her11* expression domains in the somites and the rostral half of the PSM. Winkler et al. (2003) have studied *hey1* expression in *aei* mutants and found a stripe disruption, but residual cyclic expression, very similar to the pattern observed for *her11*, i.e. with or without a gap between a homogeneous PSM domain and the first somitic expression (compare Fig.6 B, C). This observation can be confirmed (not shown), but

intriguingly, this residual cyclic expression of *hey1* is not observed in *bea* and *des* mutants (Fig.8 E, F), which is in contrast to *her11* (compare Fig.6 D-G). In both *bea* and *des* mutant embryos, *hey1* expression starts diffusely in the anterior PSM and merges with the expression in the altered somites without any signs of weaker expression in between (Fig.8 E, F). The same pattern is observed in *Su(H)* morphants (Fig.8 G). A further difference in the regulation of *her11* and *hey1* is observed in the *fss* mutant. While half segmental expression of *her11* is completely absent in the somites of *fss* embryos, *hey1* shows a strong, homogeneous expression in the somitic tissue of these embryos (compare Fig.6 I with Fig.8 H), indicating that *fss* specifically affects the formation of the anterior halves of the somites, probably mediated by *mespb* (Sawada et al. 2000). *hey1* expression is also differentially affected in *her1* and *her7* morphants. Knocking down Her1 leads to weak ectopic expression of *hey1* in the PSM interstripes and a broadened expression in the most anterior somites (Fig.8 B). This is in line with the observation of Henry et al. (2002) that *her1* is specifically required for anterior somite formation. In *her7* morphants one can observe a full disruptive effect on the *hey1* expression in the PSM, indicating that *her7* plays a prominent role in cyclic *hey1* regulation (Fig. 8C). The double morphants show perturbed *hey1* expression not only in the PSM but also in the formed somites (Fig.8 D).

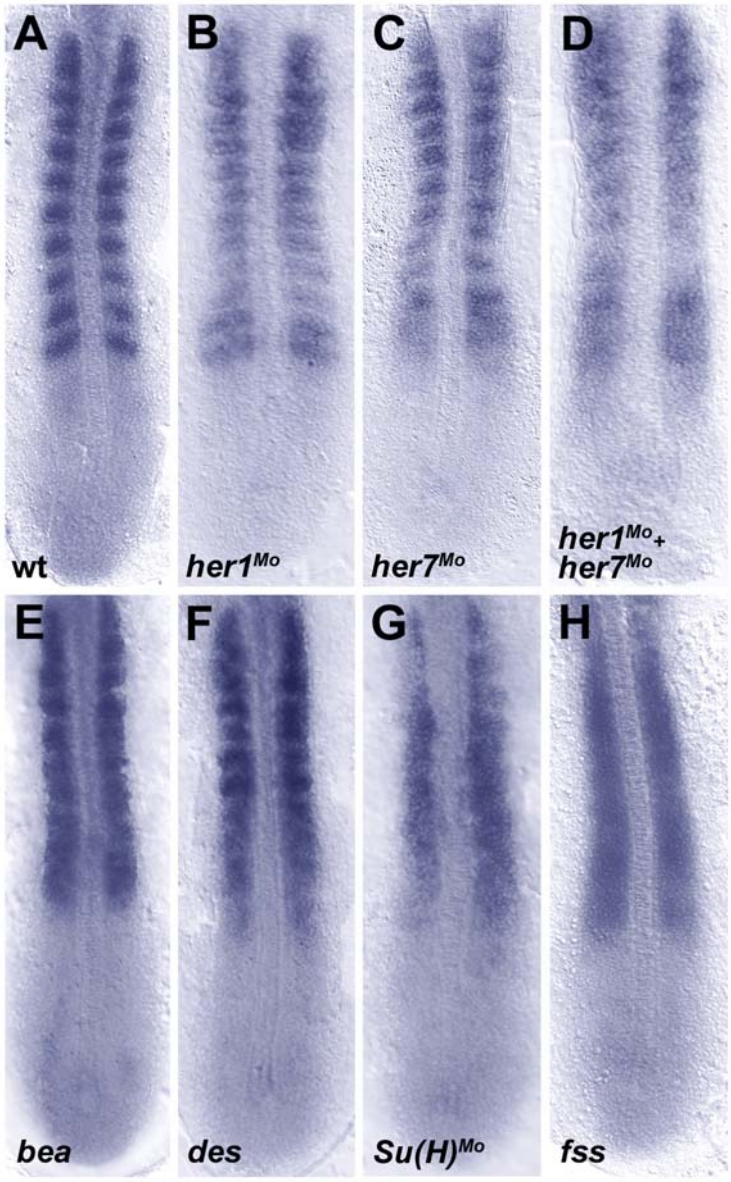

Fig. 8:
Regulation of *hey1* expression during somitogenesis. (A) *hey1* expression in the wildtype, (B),(C) and (D) altered *hey1* expression in *her1* (her1-5'Mo, 1mM, 2 experiments, n=68, 96% affected), *her7* (her7-5'Mo, 0.5mM, 2 experiments, n=65, 92% affected) and *her1/her7* (her1-5'Mo(0,5 mM)+her7-5'Mo(0,5mM), 2 experiments, n=55, 90% affected) double morphants, respectively. (E),(F) *her11* expression in *bea* and *des* embryos, respectively. (G) disrupted *hey1* expression in *Su(H)* morphants (*Su(H)*-ORF-Mo, 0,9mM, 2 experiments, n=95, 98% affected), (H) *hey1* expression in *fss* mutant embryos. (A)-(H) flat mounted embryos, anterior to the top. All embryos are between the 10 and 12 somite stage.

4.2.5 A role for *her11* and *hey1* in *her1* and *her7* stripe regulation?

Morpholino mediated knockdown was used to examine the functional role of *her11*. Two different Mos were used (see Materials and methods) and a five base mismatch Mo was applied as a control. The five base mismatch Mo did not have any effect on somite morphology and gene expression. In both the *her11*-start as well as in the *her11*-ORF morphants, *her11* transcript was detected in all somites and distributed over the whole width of a somite compared to the half-segmental expression in wildtype embryos (Fig.7 E). However, since the somitic stripes are still visible, this broadened expression of *her11* can be interpreted as an increase in transcript stability, analogous to the effects seen for *her1* and *her7* (Oates and Ho 2002; Gajewski et al. 2003). Thus, one might infer that the Mos bind effectively to the *her11* transcripts and presumably also inhibit their translation. Still, expression of *her1, her7, deltaC, deltaD, mespa* and *mespb* were not found to be significantly affected in the *her11* morphants, even when injected at high concentrations (1 mM) or with simultaneous injection of both Mos at 0.6 mM each (data not shown). The same result was obtained with injecting Mos against *hey1*. The *hey1* transcripts become stabilized in the *hey1* morphants, but the expression patterns of *deltaC, her1, her7, mespa* and *mespb* were not changed (data not shown).

This apparent lack of effect in the *her11* and *hey1* morphants may be due to redundancy caused by the earlier expressed *her1* and *her7* genes. Therefore it was specifically tested whether effects caused by the knockdown of *her1* or *her7* may be enhanced by co-injection of *her11* or *hey1* Mos. For *hey1* no difference was found (data not shown), but for *her11* there is indeed a difference with respect to the regulation of *her1* and *her7* on themselves. Knocking down Her11 together with Her1 leads to a specific perturbation of the residual cyclic *her1* expression in the posterior PSM, which is observed in *her1* morphants. These morphants show her1 expression in a U-shape (Fig.9 D), or a U with one stripe (Fig.9 E; compare also Gajewski et al. 2003). This latter stripe resolution is not found in the embryos co-injected with the *her11* Mo. Instead a U-shaped domain is found with either narrow or broad arms of strong expression in equal frequencies (Fig.9 G, H; two experiments: broad/small domain observed in 30/33 specimens, respectively). *her7* expression, on the other hand, is not different in the double morphants when compared to the *her1* morphant situation alone (data not shown). Contrariwise knocking down Her11 together with Her7 leads to a change in the *her7* expression pattern compared to the *her7* morphant alone (Fig.9 F, I), while the *her1* expression pattern shows no additional effects (data not shown). In *her7* morphants the cyclic *her7* expression is perturbed displaying

expression throughout the PSM with a higher expression level at the posterior end and a broad expression domain in the anterior end (Fig.9 F). This anterior domain is lost in double morphants (Fig.9 I). Thus, the observed effects hint to different cooperative functions for Her11 together with Her1 in intermediate and together with Her7 in intermediate to anterior PSM.

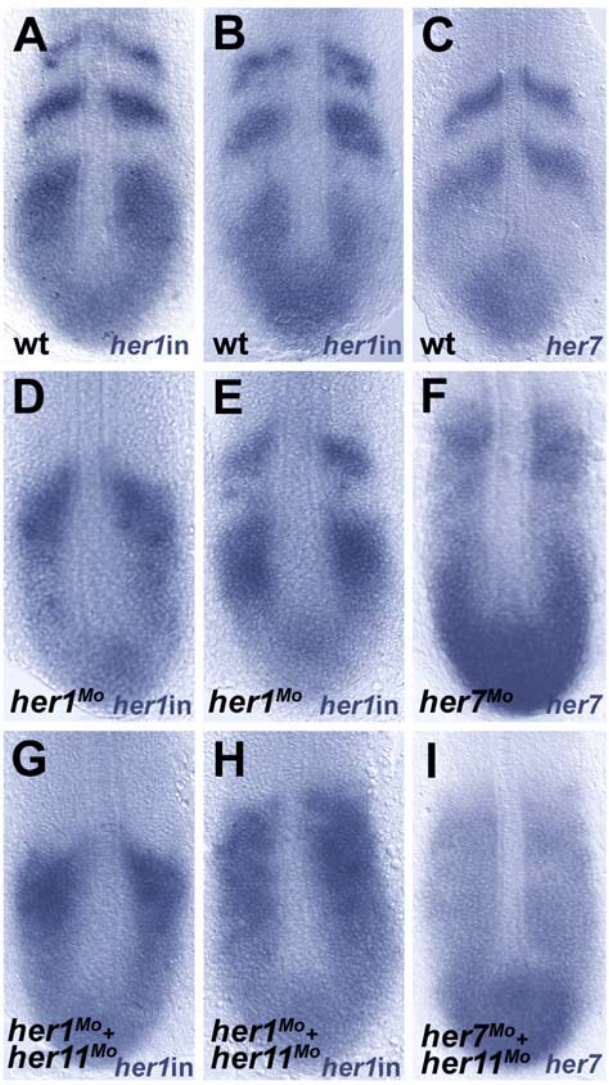

Fig. 9:

Involvement of *her11* in *her1* and *her7* regulation. (A),(B) *her1* intron in situ in wildtype embryos, (C) *her7* expression in wildtype embryos.
(D),(E) altered *her1* expression in *her1* morphants, (G),(H) *her1* expression in *her1/her11* double morphants. (F) *her7* expression after *her7* knockdown, (I) *her7* expression after *her7/her11* double knockdown.

(A)-(I) flat mounted embryos, anterior to the top. All embryos are between the 8-10 somite stage.

4.3 *her12* and its role during zebrafish somitogenesis

Another newly identified *her* gene is *her12*, which belongs together with *her2, her4.1, her4.2* and *her15* to a subclass that is most similar to the cyclically expressed mouse *Hes5* gene (Dunwoodie et al., 2002; Gajewski et al., 2005). *her4.1* has been shown to play a role during late somitogenesis, while the function of *her15* is not yet clear (Pasini et al., 2004; Shankaran 2005). *her2* is not expressed at all in the PSM but in the posterior halves of the 3 anteriormost somites (Weizsäcker 1996; Takke et al., 1999). Here the analysis of zebrafish *her12* is presented, which seems to occur in all fish species analysed, while *her15* seems to be specific for zebrafish (Gajewski et al., 2005).

4.3.1 *her12* is dynamically expressed during zebrafish somitogenesis

her12 expression is first detected at 90% epiboly in a ring like shape around the blastopore. From bud stage to 3s stage *her12* shows a dot like expression in the posterior PSM (Fig.10 A-D). In a batch of embryos of the same age this dot like expression has different diameters in width, indicating an regionally oscillating expression in the posterior PSM. Additionally, *her12* is expressed during bud stage in a thin line at the posterior and lateral border of the embryo with a broader domain in the lateral plate mesoderm (Fig.10 A, B). Transcripts can also be detected in the developing notochord (Fig.10 A, B). These expression compartments fade after the bud stage. The early expression is clearly different compared to the expression of *her1* and *her7*, in that stage, but highly similar to early *her4* and *her15* expression (Gajewski et al., 2003; Holley et al., 2000; Müller et al., 1996, Oates and Ho, 2002, Shankaran 2005, Takke et al., 1999). Starting with the 3s stage also the posterior expression appears in a different shape. Now the expression becomes U-shaped with different arm length or as a dot at the tailbud tip with some residual staining along the arms of the former U (Fig.10 C-F). The variance in the staining pattern suggests that *her12* oscillates and the pattern in the posterior PSM is now highly similar to *her1* and *her7* (Gajewski et al., 2003; Holley et al., 2000; Müller et al., 1996, Oates and Ho, 2002). Cross sections of 8-10s stage embryos reveal that the expression is clearly mesodermal (Fig.10 L). But in contrast to *her1* and *her7*, *her12* does not show any clear stripe like expression up to the 8 somite stage although a weak expression in this area can already be assumed (Fig.10 C-F).

Starting with 8-10 somite stage embryos with none, one or two stripes of *her12* expression in the anterior PSM can be found (Fig.10 G-I). These stripes are again different to the stripe like expression for *her1* and *her7* since the *her12* stripes do not seem to bud off from the posterior U-shaped expression and instead seem to appear at a certain position in the anterior PSM. At around 18 somite stage, *her12* becomes stably expressed as a small U at the posterior end and as one stripe at the anterior end of the PSM (Fig.10K).

Despite the mesodermal expression *her12* transcripts are detected during mid-somitogenesis stages (10s-18s) in the telencephalon, the ear Anlagen, the notochord and the neural tube (Fig.10 J, K).

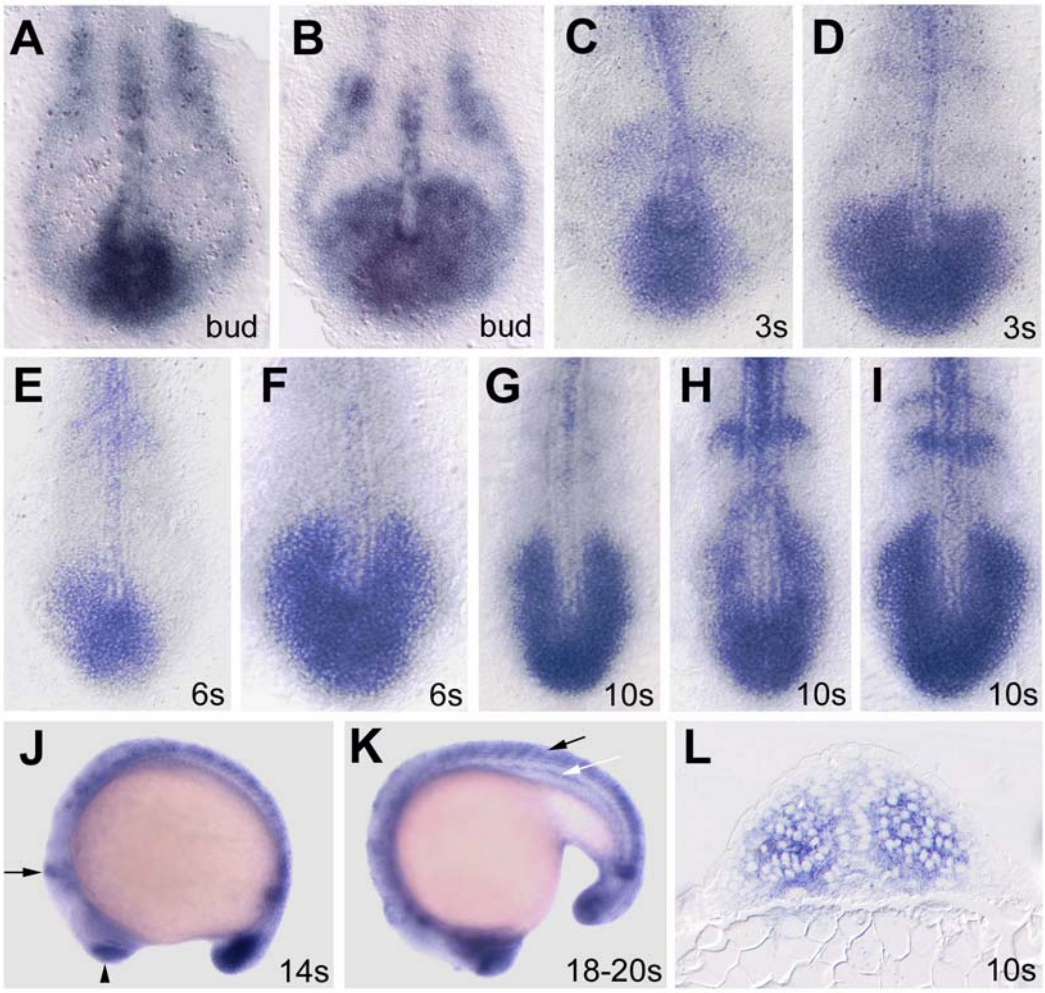

Fig.10 (legend on next page)

Fig.10:

her12 expression during zebrafish somitogenesis. (A), (B) *her12* expression in bud stage, (C), (D) in 3s stage, (E), (F) in 6s stage and (G)-(I) in the 8 to 10s stage. (A)-(I) flat mounted embryos, anterior to the top. (J) *her12* expression in a 12 to 14s stage embryo (arrow marks expression in the ear Anlagen; arrowhead marks expression in the telencephalon), (K) in a 18s stage embryo (white arrow marks expression in the notochord; black arrow marks expression in the neural tube). (J), (K) whole mount embryos, lateral view, anterior to the left. (L) Cross sections through the PSM of a 8-10s stage embryo stained for *her12*, dorsal to the top.

To specify the position of the *her12* stripes in the anterior PSM, double in situ hybridisation using *myoD* as segmental marker were performed. *myoD* is expressed in the posterior part of the already formed somites and shows in addition 2 stripes in the PSM at the position where the next two borders will be formed (Weinberg et al. 1996). Embryos with 2 stripes of *her12* show always a location of these stripes posterior to the last two *myoD* stripes (Fig.11 A). If only one broad *her12* stripe is detected, this stripe is found adjacent to the last *myoD* stripe (Fig.11 B). Furthermore, embryos are found, where a broad *her12* stripe seems to split into two single stripes but this split is not completed yet. Here the signal of the last *myoD* stripe is located in the middle of this splitting *her12* stripe (Fig.11 C). Thus it seems likely that *her12* is first expressed as a broad stripe in the area of the next formed somite, then this stripe is split and the positions of the next two borders are marked (Fig.11 A-C).

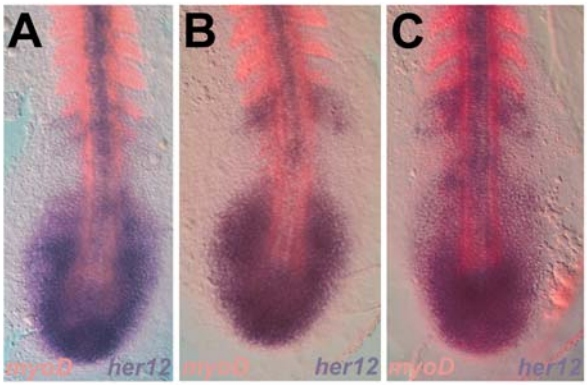

Fig.11:

Double in situ for *myoD* and *her12*. (A), (B), (C), *myoD* expression in red; (A), (B), (C), *her12* expression in blue. (A)-(C) flat mounted embryos (8-10s stage), anterior to the top.

To examine the relation of *her12* expression to *her1* and *her7* expression double fluorescent in situ hybridisation with *her1* was performed. The staining shows that the *her12* stripes appear allways in the interstripe regions of *her1* suggesting that the

expression in the anterior PSM is dynamic (Fig.12 A-F). The posterior domain shows an oscillating expression and is almost completely overlapping to *her1* (Fig.12 G-L).

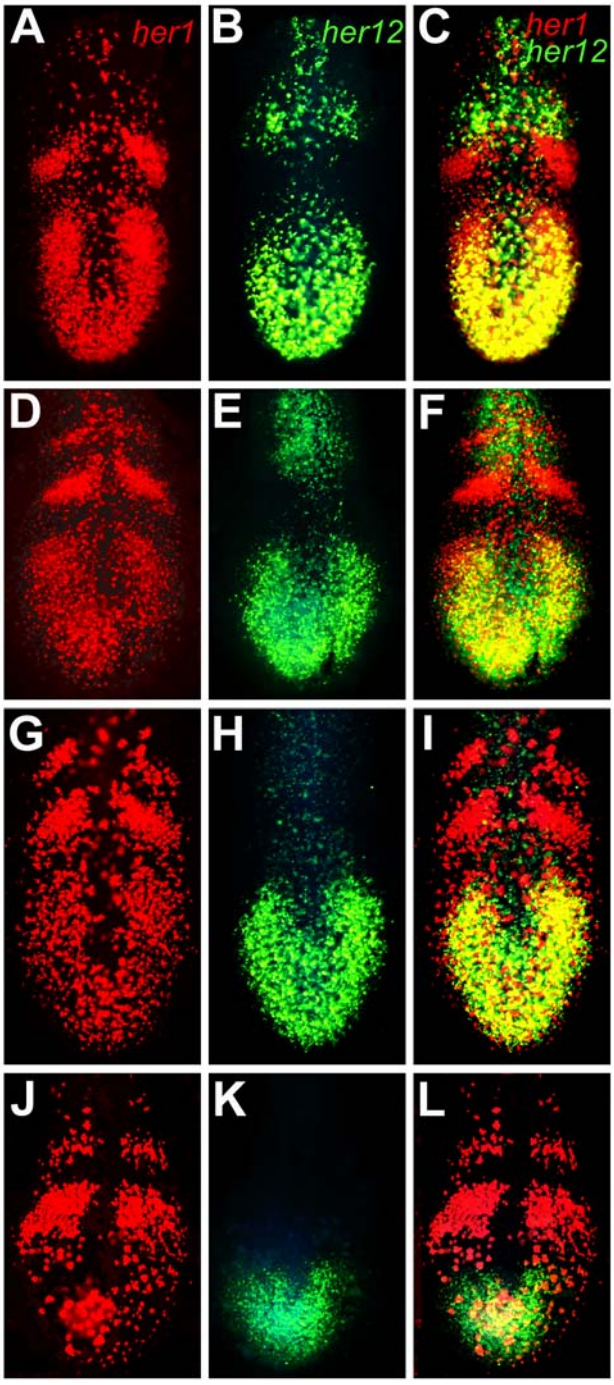

Fig.12 (legend on next page)

Fig.12:

Double fluorescent in situ for *her1* and *her12*. (A), (D), (G), (J) *her1* expression in red; (B), (E), (H), (K) *her12* expression in green. (C), (F), (I), (L) overlay of the respective *her1* and *her12* pictures. (A)-(L) flat mounted embryos (8-10s stage), anterior to the top.

4.3.2 *her12* is differentially regulated by Delta-Notch signalling, *her7* and *her11*

The Delta-Notch pathway is an essential part of the somitogenesis clock and is known to regulate crucial components of the clock like expression of *her1*, *her7* and *her11* (Holley et al. 2000, 2002; Henry et al 2002; Oates and Ho 2002; Gajewski et al. 2003; results 4.2). To analyse if *her12* is also under control of the Delta-Notch pathway, *her12* expression was monitored in the different *fss*-type mutants and in Su(H) morphants.

her12 expression in the PSM is clearly disrupted in all D-N mutants but slight differences can be detected, indicating a different usage of distinct D-N components for certain expression compartments. The expression in the posterior PSM is absent in *aei/deltaD* but a diffuse expression for *her12* can still be detected in the anterior PSM, indicating a prominent role for *deltaD* in the *her12* activation in the posterior PSM but a minor role in the stripe regulation (Fig.13 B). *deltaC* seems to play an opposite role since in *bea/deltaC* embryos *her12* expression is completely lost in the anterior PSM but a weak residual staining can be detected in the posterior (Fig.13 C). Since in *des/notch1a her12* expression is absent in the whole PSM, the signal seems to be transmitted via *notch1a* to activate *her12* (Fig.13 D). The other neuronal expression compartments of *her12* are not affected in *aei/deltaD, bea/deltaC* and *des/notch1a* embryos but are fully disrupted in *Su(H)* morphants (data not shown). The PSM expression of *her12* is also completely abolished in *Su(H)* morphants, showing only a weak residual staining in the posterior PSM, which might be due to local activators in this morphant situation (Fig.13 E).

fss/tbx24 is responsible for the activation of the anterior most *her1, her7* and *her11* stripe (Holley et al., 2000; Nikaido et al., 2002; van Eeden et al., 1996; results 4.1 and 4.2). *her12* stripe regulation seems to be completely dependent on *tbx24* since *her12* stripes can not be detected in *fss/tbx24* emrbyos (Fig.13 F, G). The posterior PSM expression of *her12* is as expected not altered in the *fss/tbx24* mutant since different forms of the U-shaped expression can be detected in a batch of embryos (Fig.13 F, G).

her1 and *her7* regulate each other and are also involved in *her11* stripe regulation while *her11* interacts with *her1* and *her7* to maintain their cyclic expression (Gajewski et al., 2003; Holley et al., 2002; Oates and Ho 2002; results 4.2). To test whether *her1, her7* and *her11* also influence *her12* expression, the respective morphants were produced and *her12*

expression was monitored. Interestingly, *her1* and *her11* alone seem not to have an impact on *her12* expression (data not shown) while the *her7* knockdown leads to a stripe like expression in the anterior of all embryos (Fig.13 H). This single stripe seems to be fixed at a certain position and a second stripe is never detected. A combined knockdown for *her7* and *her11* leads to an enhancement of this stripe like expression in the anterior, while the combination of *her7* and *her1* Mo results in a decrease of this expression compartment compared to the single *her7* morphant (Fig.13 I, J).

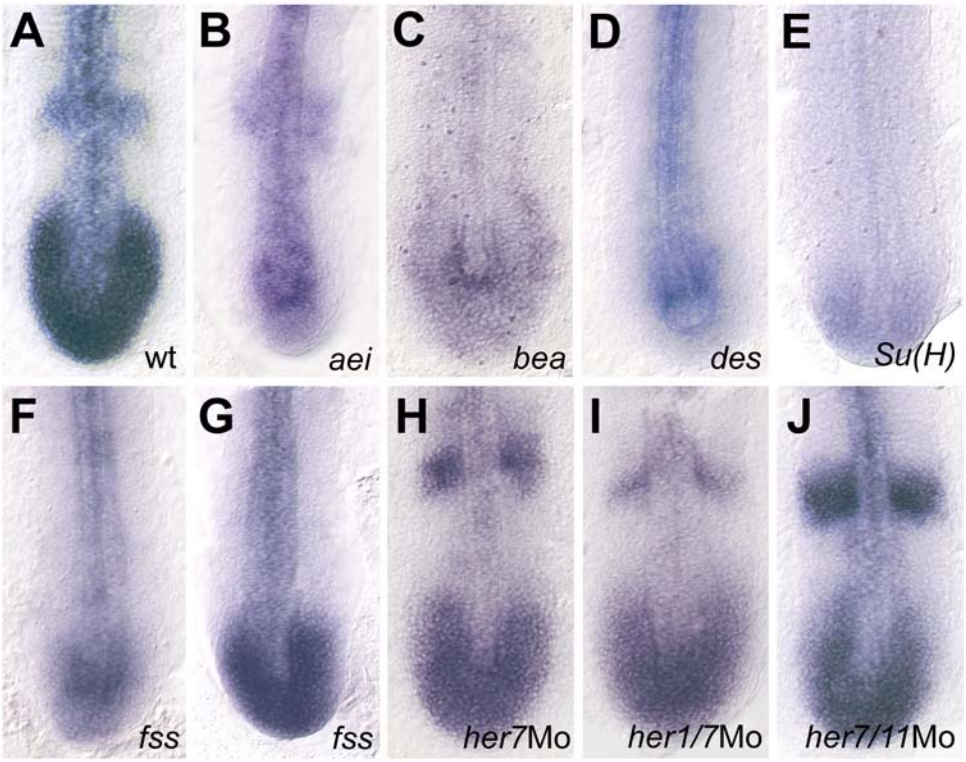

Fig.13:

her12 expression in fss-type mutants and *Su(H)/her* morphants. (A) *her12* expression in the wildtype, (B) in the *aei* mutant, (C) in the *bea* mutant, (D) in the *des* mutant and (E) in *Su(H)* morphants. (F), (G) *her12* expression in the fss mutant, (H) in *her7* morphants, (I) in *her1-her7* double morphants and (J) in *her7-her11* double morphants. (A)-(J) flat mounted embryos (8-10s stage), anterior to the top.

4.3.3 The function of *her12* during somitogenesis

4.3.3.1 *her12* misexpression

To get a first idea of the function of *her12* during somitogenesis, misexpression studies were perfomed. *her12* mRNA was injected in the first cell stage and the embryos were analysed during somitogenesis stages. As control *GFP* RNA and *her11* RNA was injected in the same or even in a higher concentration (for details see figure legend). Control injections showed only in a minority of the embryos (< 5%) unspecific effects, the majority of the embryos (>95%) developed completely normal and did not show any changes in the investigated expression patterns. In contrast, injection of *her12*-RNA caused a disruption of somitic borders along the whole axis of the embryos with high penetrance (Fig.14 D-F). A closer look from dorsal at a higher magnification reveals that most of the somite borders were absent and only a few borders were formed in misplaced position (Fig.14 F). Furthermore embryos misexpressed for *her12* show severe perturbations of the cyclically expressed genes *her1*, *her7* and *deltaC* indicating that the Her12 protein contributes to the clock mechanism (Fig.14 H, J, L).

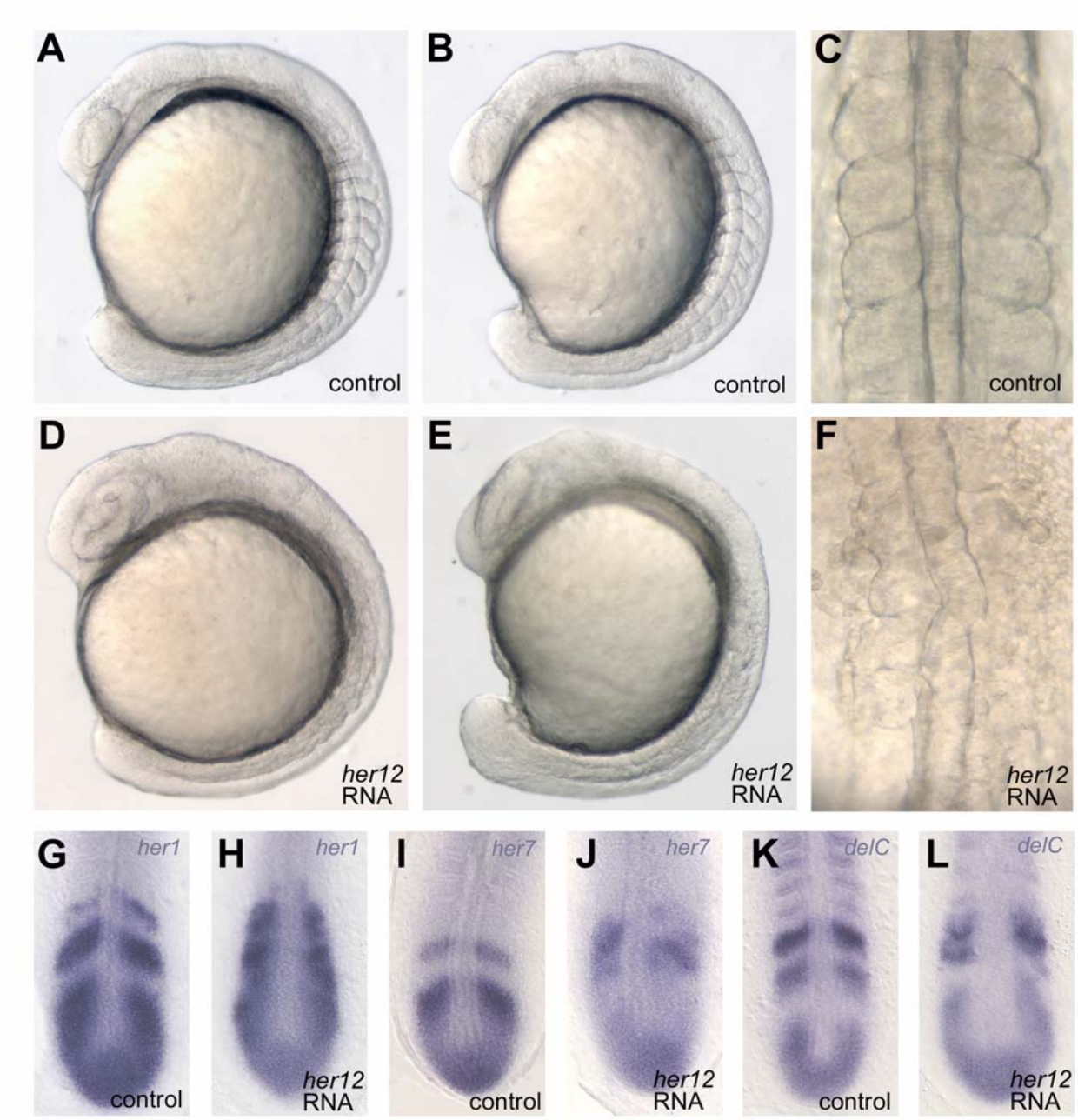

Fig.14:

Effects of *her12* misexpression on somite morphology and cyclically expressed genes. (A), (B), (C) *GFP*- RNA injected embryos (850 ng/µl *GFP*-RNA, 2 experiments, n = 82, 96% show wildtype morphology). (D), (E), (F) *her12* RNA injected embryos (800 ng/µl her12-RNA, 3 experiments, n = 102, 44% affected). (G), (I), (K) *GFP*-RNA injected embryos stained for *her1*, *her7* and *deltaC*, respectively (850 ng/µl *GFP*-RNA, 2 experiments, n = 96, 95% show wildtype expression). (H), (J), (L) *her12*-RNA injected embryos stained for *her1*, *her7* and *deltaC*, respectively (800 ng/µl *her12*-RNA, 2 experiments, n =104, 48% affected). (A), (B), (D), (E) whole mount embryos, lateral view, anterior to the upper left; (C), (F) dorsal view, anterior to the top; (G)-(L) flat mounted embryos, anterior to the top; (A), (C), (D), (F) and (G)-(L) 10s stage embryos; (B), (E) 14s stage embryos.

4.3.3.2 *her12* morpholino knockdowns

To further specify the role of *her12* respective morpholino knockdowns were performed. Two specific morpholinos were designed, one against the 5'UTR (5'Mo) and the other against the start AUG of the ORF (ORF-Mo), and a mismatch Mo was applied as control. The control Mo did not have any influence on somite morphology and cyclically expressed genes at all. Injections of the 5'Mo, even in high concentrations (1,2mM), did also not result in altered somite morphology or changed expression patterns (data not shown). In contrast to this, injection of the ORF-Mo led to an obvious disruption of the expression of the cyclic genes *her1, her7* and *deltaC* but no defects in somite border formation could be detected (Fig.15 B, D, F). Since ORF-morphants were often slightly shortened compared to control injections the question arises whether mesoderm specification is affected in these embryos. To answer this, injected embryos were hybridised with *spt*, a marker for mesoderm specification (Amacher et al., 2002; Griffin and Kimmelman 2002). No difference could be detected between control embryos and ORF-Mo injected embryos (Fig.15 G-J), implicating that *her12* is not involved in mesoderm specification but directly in the control of cyclic gene expression in the PSM. The combined knockdown of *her12* and further her genes like *her1*, *her7*, *her11* and *her15* did not have any additional effect when compared to the single knockdowns (not shown).

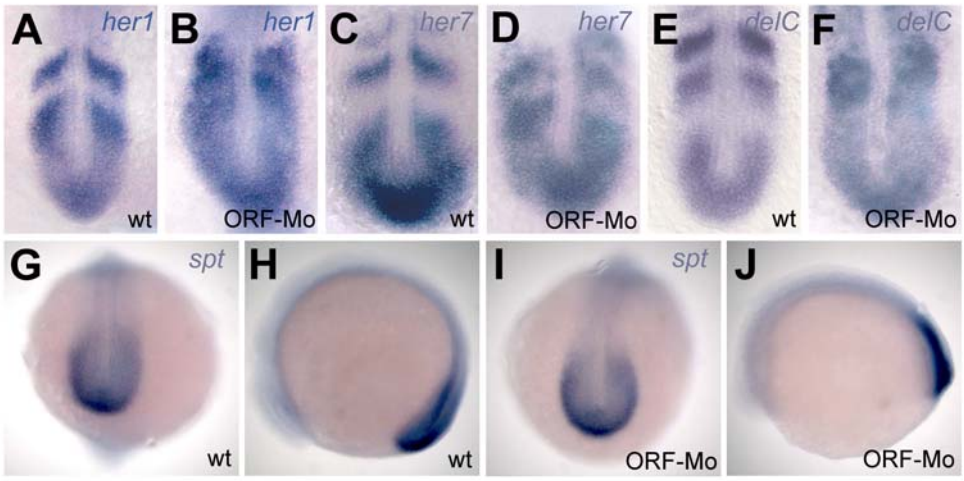

Fig.15:

Influence of the *her12*-ORF-Mo on gene expression in the PSM. (A), (C), (E) wildtype expression of *her1*, *her7* and *deltaC*, respectively. (B), (D), (F) expression of *her1*, *her7* and *deltaC*, respectively, after *her12*-ORF-Mo injection (0,8mM; 3 experiments for *her1*, n=115, 55% affected; 2 experiments for *her7*, n=65, 51% affected; 2 experiments for *deltaC*, n=72, 42% affected). (G), (H) wildtype expression of *spt*, (I), (J) *spt* expression in *her12* ORF-morphants (0,8 mM, 2 experiments, n=68, 96% all embryos show wildtype expression). (A)-(F) flat mounted embryos (8-10s stage), anterior to the top. (G)-(J) whole mount embryos (10s stage); (G), (I) dorsal view, posterior downwards; (H), (J) lateral view, anterior to the left.

However, even the alterations in cyclic gene expression occurred only in 40-60% of the embryos (details see figure legend) implicating that the ORF-Mo does not yield a full *her12* knockdown. To proof the function of the used morpholinos a *her12-GFP* fusion RNA was generated. This RNA consists of the *her12* 5'UTR and the first nucleotides of the ORF fused in frame to the coding sequence of *GFP*, so that both Mo target site are present to perform the knockdown of this fusion RNA (Fig.16).

Fig.16:

Schematic drawing of the *her12*-GFP fusion RNA.

Injection of the *her12-GFP* RNA in the first cell stage resulted in a bright fluorescence in nearly all embryos (Fig.17 A, B; for details see figure legend). As expected, the coinjection of *her12-GFP* RNA and the control Mo had no additional effect and the same strong fluorescence was detected in the majority of the embryos (Fig.17 E, F). In contrast to this, the coinjection of *her12-GFP* RNA and the 5'Mo led to a full knockdown of the transcript since no fluorescence could be detected in the injected embryos (Fig.17 C, D). This could not be observed when injecting *her12-GFP* RNA together with the ORF-Mo. In this combination only a decrease in fluorescence could be detected in the majority of embryos and just a few embryos did not show fluorescence (Fig.17 G, H). Taken together the control experiments imply that the 5'Mo performs a more efficient knockdown compared to the ORF-Mo. This is, however, in contrast to the previous experiments, where the 5'Mo did not have any detectable effect on somite morphology and cyclic gene expression.

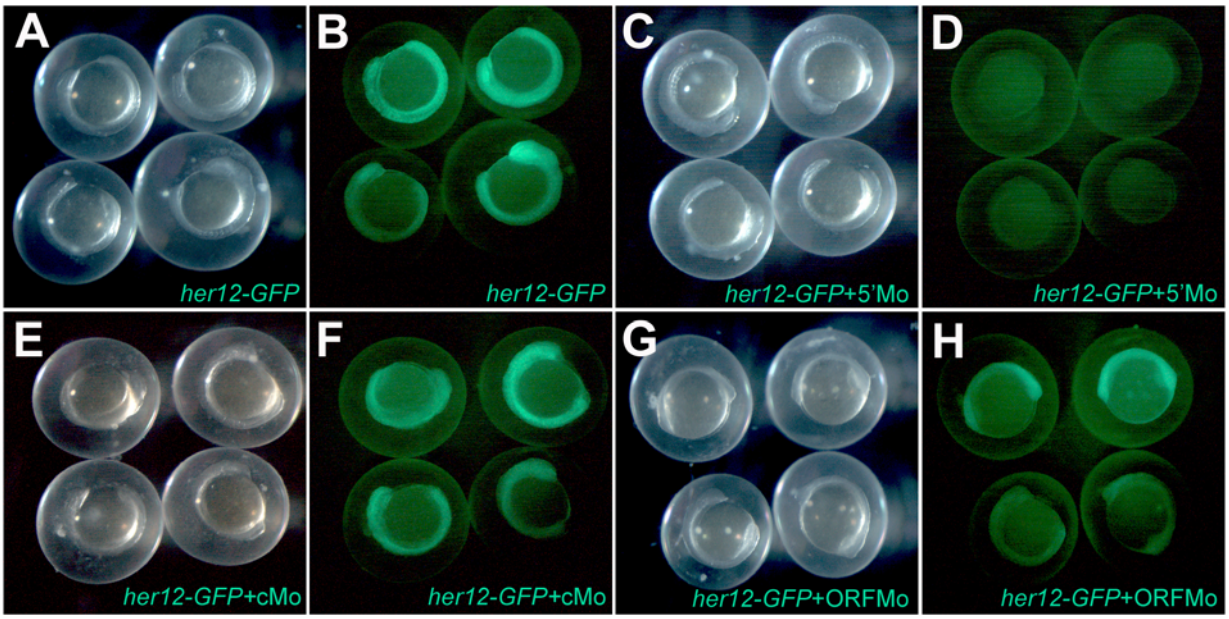

Fig.17:

her12-GFP RNA control injections. (A), (B) *her12-GFP* RNA injected embryos (300ng/µl, 2 experiments, n=76, 97% show fluorescence); (C), (D) *her12-GFP* RNA + 5'Mo injected embryos (300ng/µl RNA + 0,8mM 5'Mo, 2 experiments, n=82, 0% show fluorescence); (E), (F) *her12-GFP* RNA + cMo injected embryos (300ng/µl + 0,8 mM c-Mo, 2 experiments, n=58, 97% show fluorescence); (G), (H) *her12-GFP* RNA + ORF-Mo injected embryos (300ng/µl + 0,8mM ORF-Mo, 2 experiments, n=85, 76% show decreased fluorescence); (A)-(H) whole mount embryos (8-10s stage), lateral view.

4.4 *her1* and *her13.2* play a combinatorial role in anterior somite formation in zebrafish

Previous studies in zebrafish and other vertebrates imply genetic differences in the formation of anterior and posterior somites (reviewed by Rida et al., 2004; Saga and Takeda 2001). For *her1* a mild influence on anterior somites could be detected (Henry et al., 2002) and it has been shown by in vitro studies that *her1* and *her13.2* interact (Kawamura et al., 2005). Now the question arises whether there is a combinatorial role for *her1* and *her13.2* in vivo. Following this idea, knockdowns for the clock dependent gene *her1* and the wavefront (FGF) dependent gene *her13.2* were performed as single as well as double knockdowns and the influence on somite border morphology and cyclic gene expression was analysed.

4.4.1 Anterior somites require *her1* and *her13.2* function

her13.2 shows a gradient like expression in the posterior PSM and in addition a stripe like expression in the anterior part of the youngest 3 somites (Fig.18 A), which was not described by Kawamura et al. (2005). A complete knockdown of the Delta-Notch pathway via *Su(H)*-Mo has no influence on the PSM expression. Only the stripy *her13.2* expression seems to be weaker and more diffuse compared to the wildtype (Fig.18 A, B). This might rather be a secondary effect due to loss of somite borders in this morphant than a direct influence of the Delta-Notch pathway on the somite expression domain.

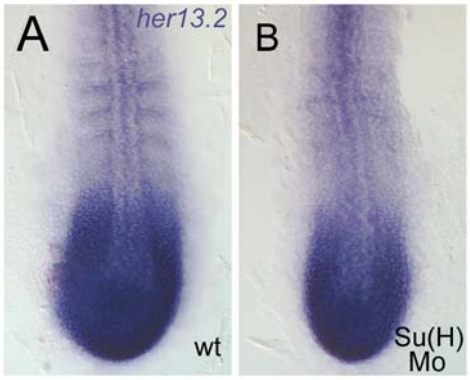

Fig.18:

(A) wt expression of *her13.2* and (B) in *Su(H)* morphants (0,6 mM; 2 experiments, n = 65, 96,92 % affected); Note the reduction of stripes might be rather a secondary effect due to loss of somite borders in this morphant. Embryos are between the 8 and 10 somite stage, dorsal view, flat mounted embryos, anterior to the top.

To examine the process for which the *her1-her13.2* interaction is required in vivo, double knockdowns for *her1* and *her13.2* and as a control the respective single knockdowns were performed. The *her1* morphant embryos show as expected a clear influence on the expression of cyclic genes but still form somites (Fig.19 B, G, M, N). To knock down *her13.2* a different Mo compared to Kawamura et al. (2005) was used, which overlaps in 17 positions with Kawamura-Mo1 but is prolonged towards the start-codon of the gene (see Material and Methods). Using this Mo one finds in ~10-20% of the embryos the described full disturbance on cyclic gene expression (Fig.19 D, I, Kawamura et al., 2005). The remaining ~80-90% of the embryos do also not show the wildtype pattern for *her1* and *her7* but show always an extraordinary long u-shaped expression and only one stripe in the anterior PSM (Fig.19 C, H), which in summary seems to reflect a hypomorphic situation. Injection of this Mo, even in high concentrations, has no effect on somite borders at all (Fig.19 O, P). Since this Mo does obviously not show the full penetrance for *her13.2*, it is suitable to reduce Her13.2 protein amount in other morphant backgrounds to sensitively examine the patterning system. Following this idea the double knockdown for *her1* and *her13.2* was generated. The double morphants show a clear disruption of anterior and posterior borders, indicating that there is indeed an important interaction of *her1* and *her13.2* in formation of anterior somitic borders (Fig.19 Q, R). These morphant embryos scarcely show some rudiments of borders in few positions but fail to generate complete borders (Fig.19 Q, R). The disruption of anterior borders is unique for this combination and does not occur in the *her7/her13.2* morphant situation (Fig.19 S, T). Furthermore there is additional influence on the cyclic *her1* and *her7* expression in the *her1/her13.2* double morphant when compared to the controls. In this case all injected embryos show only a u-shaped expression in the posterior half of the PSM without any dynamics (Fig.19 E, J). The posterior oscillation, which is still there in the *her1* morphant and the two phases, which are seen in the *her13.2* morphant are lost in this combination. *her13.2* expression itself is not affected in the *her1/her13.*2-Mo injected embryos (Fig.19 U, V), indicating that *her13.2* is only controlled via FGF signalling and no *her1/her13.2* driven feed back loops are involved.

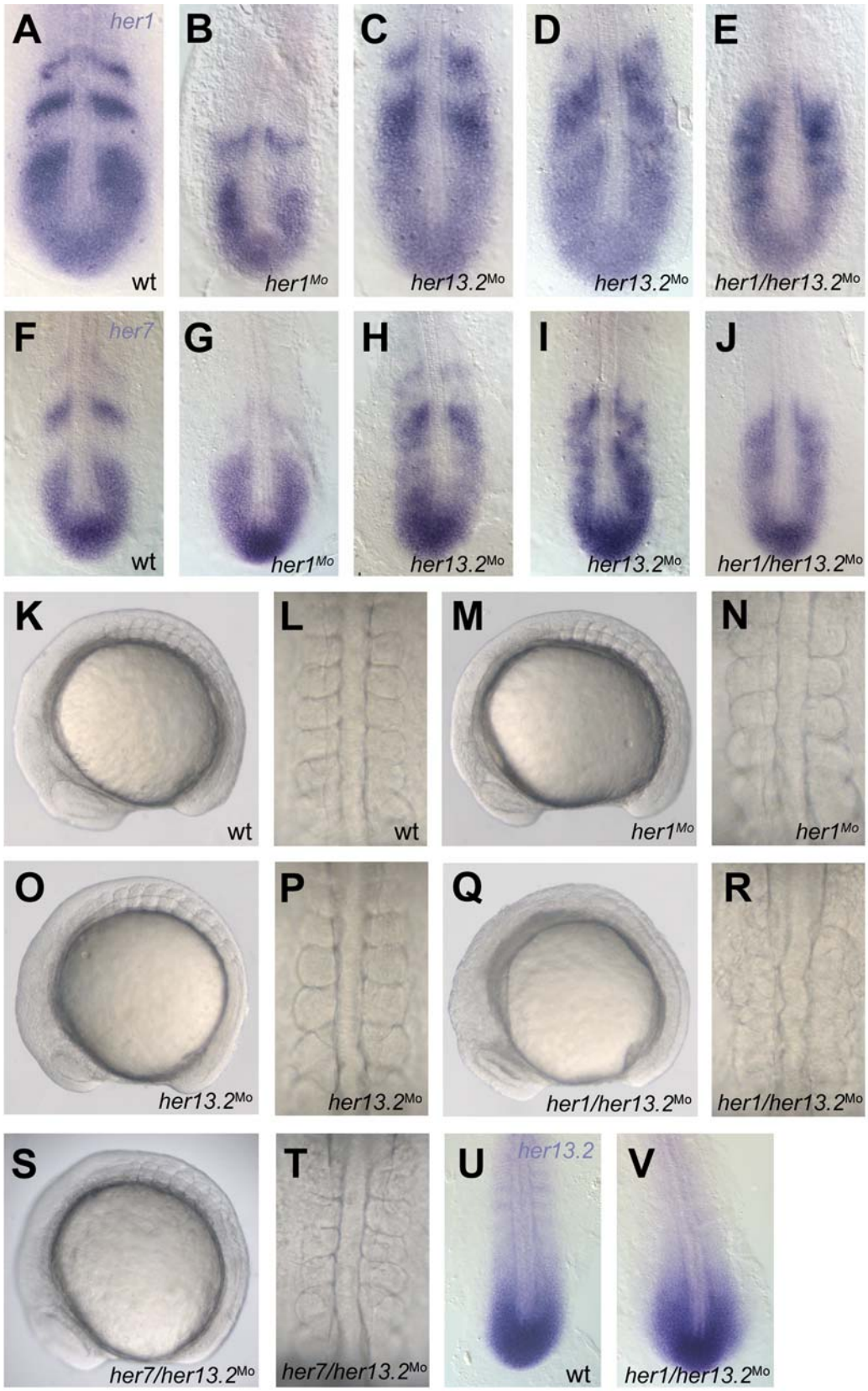

Fig.19 (legend on next page)

Fig.19:

Effects of *her1/her13.2* injections on somite borders and expression patterns. All embryos are between the 8 and 10 somite stage. (A), (F) wildtype expression of *her1* and *her7*, respectively. (B), (G) *her1* and *her7* expression, respectively, after *her1* knockdown (0,6 mM; 2 experiments, n = 76 for each probe, 92,11% affected for *her1*, 94,74% affected for *her7*). *her13.2*-Mo injection (0.6 mM; 2 experiments; n = 53 for *her1*, n = 43 for *her7*) leads in 73,58% of the embryos to a slightly altered *her1* expression (C) and in 81,4% of the embryos to the same alteration in *her7* expression (H). 9,43% of *her1* stained embryos and 18,6% of *her7* stained embryos show a full disruption of the *her1* (D) and the *her7* (I) expression, respectively. A full disruption of *her1* expression (E) and *her7* expression (J) is observed in *her1/her13.2* double morphants (0,6 mM *her1*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 85, 94,11% affected for *her1*, 95,29% affected for *her7*). (A)-(J) dorsal view, flat mounted embryos, anterior to the top.
(K), (L) somite morphology in wildtype embryos, (M), (N) in *her1* morphants (0.6 mM; 2 experiments, n = 56, all embryos show wildtype morphology), (O), (P) in *her13.2* morphants (0.6 mM; 2 experiments, n = 52, all embryos show wildtype morphology), (Q), (R) in *her1/her13.2* double morphants (0,6 mM *her1*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 92, 97,83% affected) and (S), (T) in *her7/her13.2* morphants (0,6 mM *her7*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 46, all embryos show wildtype morphology). (K), (M), (O), (Q) and (S) lateral view, anterior to the left. (L), (N), (P), (R) and (T) dorsal view, anterior to the top. (U) wildtype expression of *her13.2* and (V) in *her1/her13.2* morphants (0,6 mM *her1*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 42, 92,86% affected (loss of stripes, which might rather be a secondary effect due to loss of somite borders)). (U) and (V) flat mounted embryos, anterior to the top.

To confirm the strong effect of the *her1/her13.2*-Mo double injection on somite borders also on the molecular level, in situ hybridisations for *myoD* were performed. While *myoD* expression is almost normal in the *her1* and *her13.2* single morphant, there is a clear disruption of *myoD* expression in the double morphant, showing a diffuse *myoD* expression throughout the whole somitic region (Fig.20 A, B, C, D).

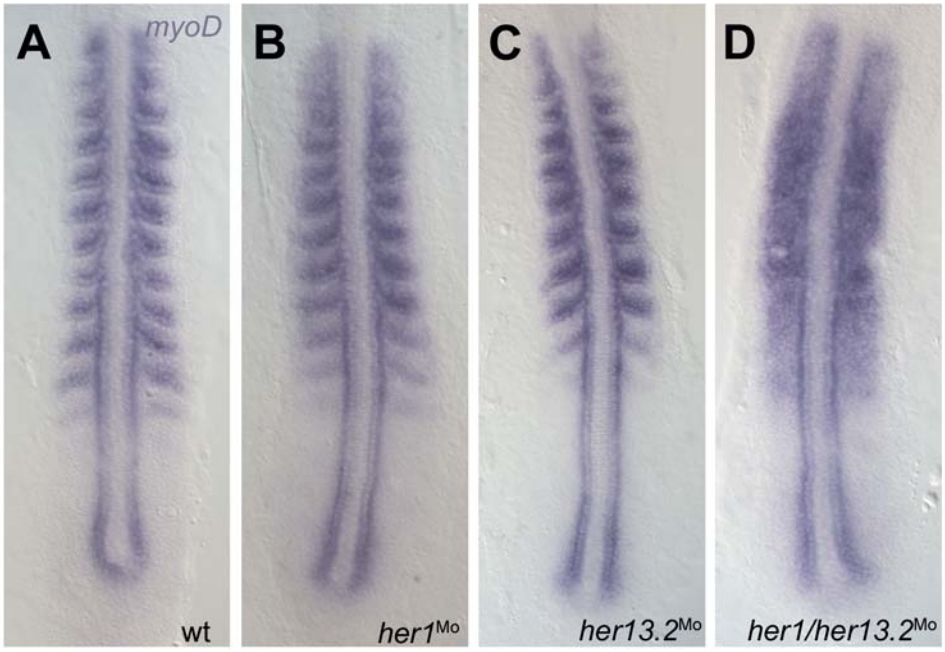

Fig.20:

Expression of *myoD* in the different morphants. (A) wildtype expression of *myoD*, (B) in *her1* morphants (0.6 mM; 2 experiments, n = 40, 95 % show segmental *myoD* expression, which is slightly more diffuse in anterior somites), (C) in *her13.2* morphants (0.6 mM; 2 experiments, n = 38, all embryos show segmental *myoD* expression) and (D) a full disruption of the *myoD* pattern in *her1/her13.2* morphants (0,6 mM *her1*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 57, 96,49 % affected). (A)-(D) 8-10 somite stage embryos, flat mounted embryos, anterior to the top.

4.4.2 Anterior somites and the breakdown of the oscillator

The careful analysis of the D-N mutants and morphants has shown that in these mutants the oscillator is intact in early somitogenesis stages and starts to breakdown between the 4th somite stage and the 8th somite stage, dependent on the mutated gene (Jiang et al., 2000; Oates and Ho et al., 2002; van Eeden et al., 1998; results 4.1). This indicates that a functioning oscillator in early stages might be sufficient to generate the first somite borders. On the other hand the anterior somite borders could be completely independent of the oscillator. Since anterior somite borders are clearly affected in the *her1/her13.2* double morphant it might be feasible that *her1* and *her13.2* act in combination for the early oscillator control. To test this hypothesis, *her1* and *her13.2* single morphants as well as *her1/her13.2* double morphants were generated and the expression of the cyclic genes *her1*, *her7* and *deltaC* was analysed prior to any visible somite border in the bud stage.

The single knockdown of *her1* and *her13.2* has no effect on the early expression of *her1, her7* and *deltaC* since in a batch of embryos all cyclic phases can be detected (Fig.21 A-C, E-G, I-K). In contrast to this, a very early breakdown of cyclic gene expression is found in

the *her1/her13.2* double morphant. These embryos show for *her1* and *her7* only a diffuse u-shaped expression in the posterior PSM without any signs of oscillation (Fig.21 D, H). The cyclic *deltaC* expression is also disrupted in this double morphant but shows a different pattern compared to *her1* and *her7*. All embryos stained for *deltaC* show only a weak signal in the posterior and one stripe in the anterior PSM, which gives the impression that the oscillations are "frozen" at a particular phase of the cyclic expression pattern (Fig.21 L). This clearly suggests that early oscillations are controlled through a combination of D-N signalling, via *her1*, and FGF signalling, via *her13.2*, and these early oscillations seem to be required for the first somite borders.

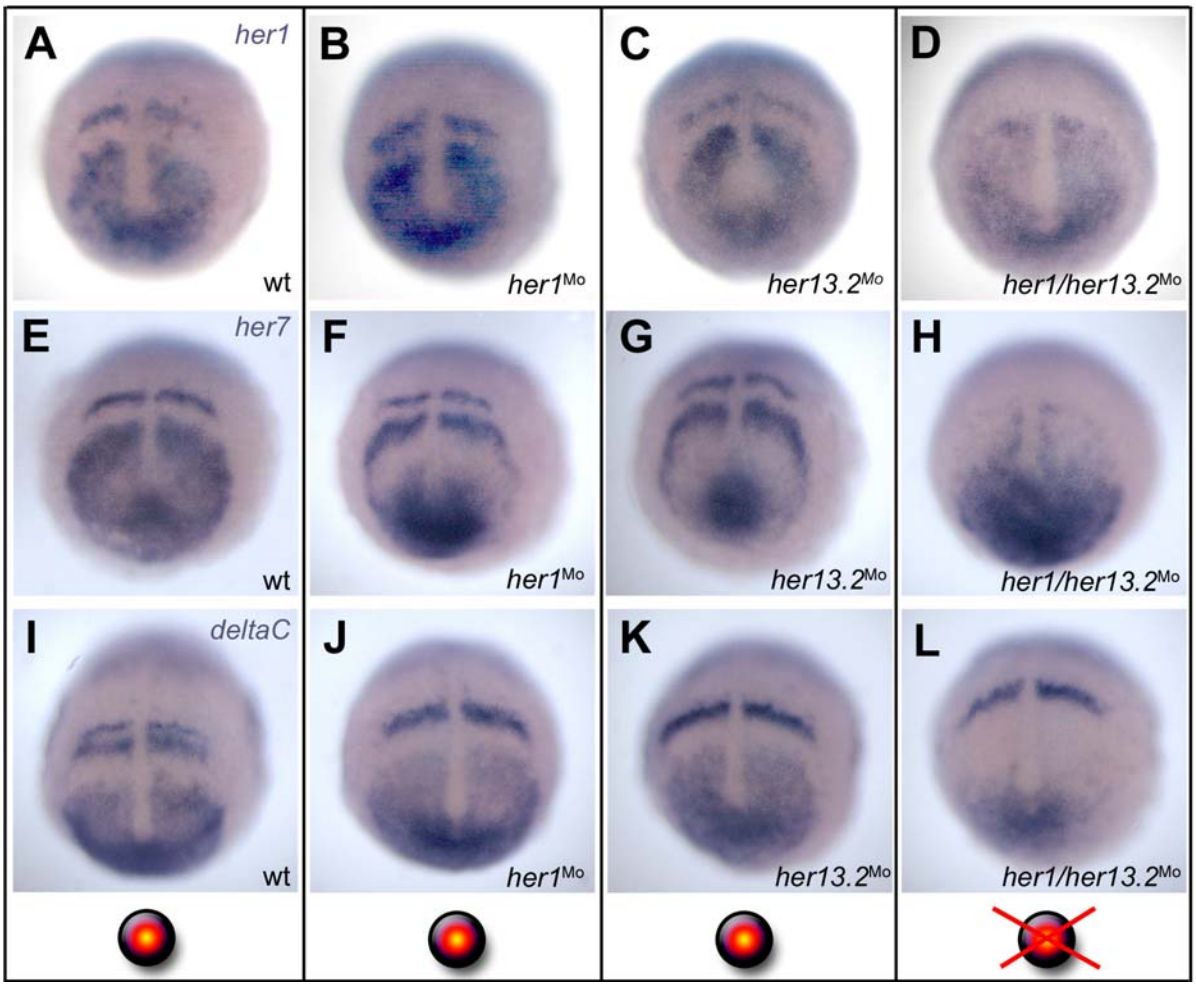

Fig.21:

Disruption of early oscillations in *her1/her13.2* morphants. (A), (E) and (I) wildtype expression of *her1*, *her7* and *deltaC*, respectively. Dynamic gene expression can be found for *her1* (B), *her7* (F) and *deltaC* (J) in *her1* morphants (0.6 mM; 2 experiments, n = 40 for each probe, all embryos show wildtype dynamics) since in a batch of embryos the whole variety of the pattern can be found (only one type is shown here). The same dynamic is seen in *her13.2* morphants for *her1* (C), *her7* (G) and *deltaC* (K) (0.6 mM; 2 experiments, n = 45 for each probe, all embryos show wildtype dynamics). In *her1/her13.2* morphants a full disruption of cyclic *her1* (D), *her7* (H) and *deltaC* (L) expression is detected since the whole batch of embryos shows only one pattern (0,6 mM *her1*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 52 for each probe, 96,15% affected for *her1*, 94,23% affected for *her7* and 92,3% affected for deltaC). (A)-(L) bud stage embryos, whole mounts, dorsal view, posterior downwards; indicates cyclic expression in the respective embryos, while indicates a disruption of cyclic expression in the respective embryos.

4.4.3 Cyclic *her* gene expression is crucial for anterior somites

The reduction of Her13.2 protein amount in the *her1* morphant background has clearly shown how sensitive the system reacts, leading to an early breakdown of the oscillator and additional disruption of anterior somite borders. Thus, establishing a link between the clock and the FGF gradient for anterior somitogenesis, which is dependent on *her* gene activity. To further examine the robustness of the patterning system *her13.2*-Mo was injected in the *Su(H)* morphant background. Thus instead of knocking down a repressor of the genetic curcuit, the D-N pathway is inhibited in this way. In the *Su(H)* single morphant early cyclic gene expression is quite normal and somitic border disruption starts at somite 3 to 4 (results 4.1).

Su(H)/her13.2-Mo injected embryos were fixed in the bud stage and stained for *her1, her7* and *deltaC* to analyse the influence on cyclic gene expression. Interestingly the reduction of Her13.2 function in this combination is not sufficient to disrupt the oscillator in early stages (Fig.22 E, H, K). All oscillation phases can be detected in a batch of embryos stained either for *her1*, *her7* or *deltaC* (Fig.22 E, H, K) and coherent with this, the injected embryos still form anterior somites and show only posterior defects (Fig.22 B). The observed phenotype is not due to the used hypomorphic *her13*-Mo in this study since the same results were obtained using the *her13* morpholinos previously described by Kawamura et al. (2005) in combination with the *Su(H)*-Mo (data not shown). This implicates that the Her1 protein itself might be crucial for early oscillations. To test this hypothesis *Su(H)/her1* morphants were generated and examined again for early cyclic gene expression. Here one could detect the same early breakdown of cyclic gene expression as before in the *her1/her13* morphant, namely a diffuse u-shaped expression for *her1* and *her7* and a "frozen pattern" for *deltaC* with one stripe in the anterior PSM (compare Fig.21 D, H, L with Fig.22 F, I, L). Concomitant with the loss of early oscillation control, the *Su(H)/her1* morphants are clearly defective in anterior somite border formation (Fig.22 C).

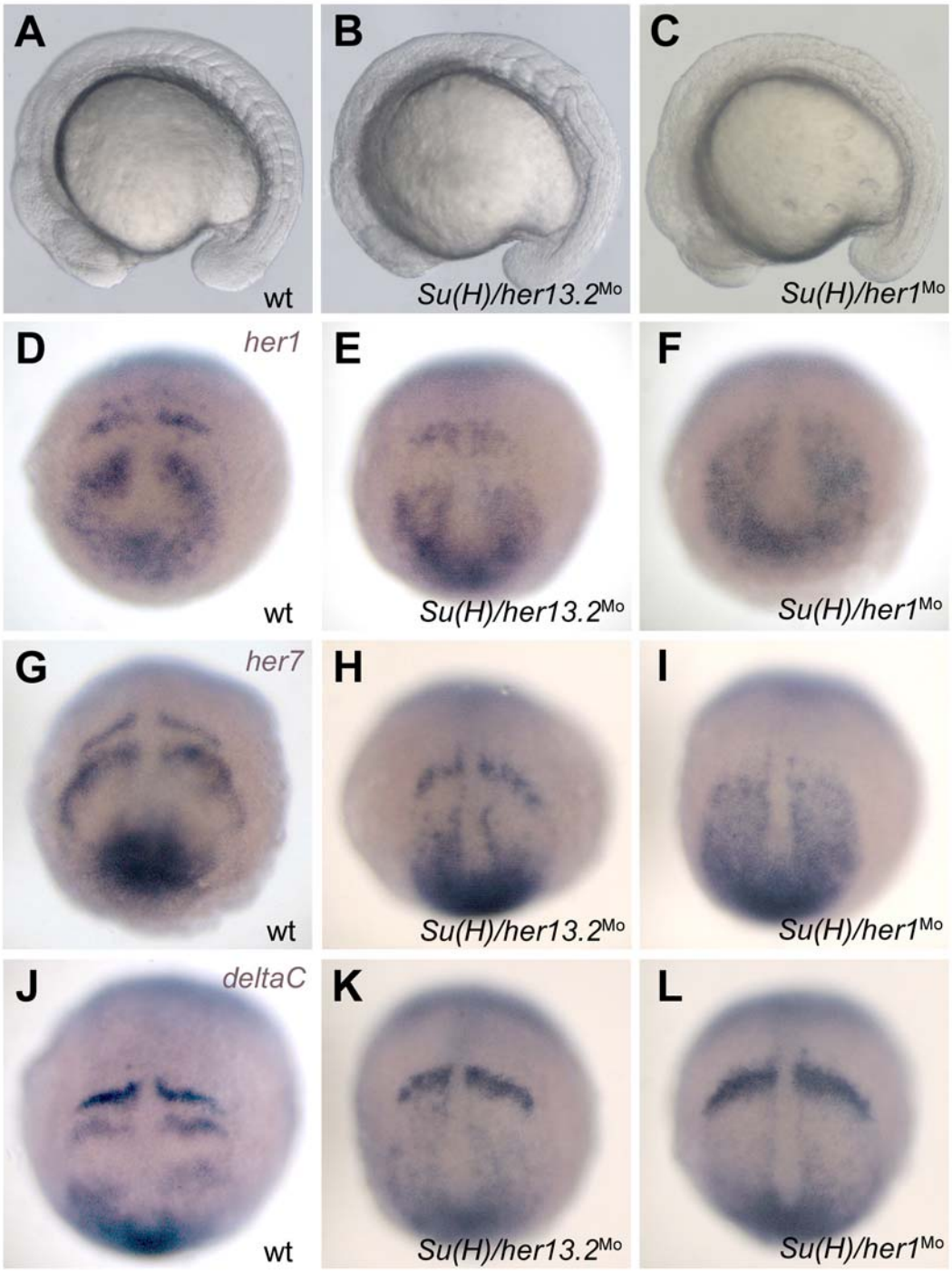

Fig.22:

Somite morphology and early oscillations in *Su(H)/her13.2* and *Su(H)/her1* morphants.
(A) somite morphology in wt embryos, (B) in *Su(H)/her13.2* morphants (0,6 mM *Su(H)*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 47, 95,74% affected) and (C) in *Su(H)/her1* morphants (0,6 mM *Su(H)*-Mo + 0.6 mM *her1*-Mo; 2 experiments, n = 51, 96,07% affected). (A)-(C) 16-18 somite stage embryos, lateral view, anterior to the left. (D), (G) and (J) wildtype expression for *her1*, *her7* and *deltaC*, respectively. Dynamic expression is detected in *Su(H)/her13.2* morphants for *her1* (E), *her7* (H) and *deltaC* (K) (0,6 mM *Su(H)*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 42 for each probe, all embryos show wildtype dynamics). A full disruption of cyclic expression for *her1* (F), *her7* (J) and *deltaC* (L) is found in *Su(H)/her1* morphants (0,6 mM *Su(H)*-Mo + 0.6 mM *her1*-Mo; 2 experiments, n = 46 for each probe, 95,65% affected for *her1*, 93,48% affected for *her7*, and

97,82 affected for *deltaC*), which is comparable to the disruption in *her1/her13.2* morphants (see Fig.21 D, H, L). (D)-(L) bud stage embryos, whole mounts, dorsal view, posterior downwards.

Another difference between *Su(H)/her13.2* morphants and *Su(H)/her1* morphants is observed on cyclic gene expression in later stages. While *Su(H)/her13.2* morphants show, like *Su(H)* single knockdowns (results 4.1), perturbed *her1* and *her7* expression throughout the PSM, a different effect can be detected in the *Su(H)/her1* morphant situation (Fig.23). The expression of *her7* (and *her1*, data not shown) is strongly restricted to the posterior PSM (Fig.23 D) indicating that Delta-Notch signalling is required for the maintenance of oscillation in the intermediate and anterior PSM, while Her1 does not negatively feedback on itself and *her7*.

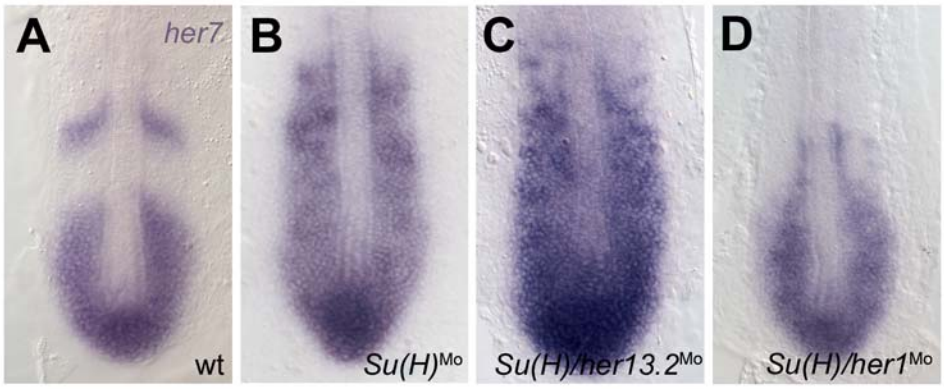

Fig.23:

Additional effects on *her7* expression in *Su(H)/her1* morphants. (A) wildtype expression of *her7*, (B) in *Su(H)* morphants (0,6 mM *Su(H)*-Mo; 1 experiment, n = 25, 92% affected), (C) in *Su(H)/her13.2* morphants (0,6 mM *Su(H)*-Mo + 0.6 mM *her13.2*-Mo; 2 experiments, n = 40, 95% affected). In *Su(H)/her1* morphants (0,6 mM *Su(H)*-Mo + 0.6 mM *her1*-Mo; 2 experiments, n = 55, 94,54% affected) *her7* expression is restricted to the posterior PSM without any dynamics (D). (A)-(D) 8-10 somite stage embryos, flat mounted, anterior to the top.

5. Discussion

The main part of the following discussion is divided into 4 chapters, adequate to the four projects described in the results section. Beyond this, zebrafish somitogenesis as such is compared to somitogenesis in other species in the last part of the discussion (chapter 5.5).

5.1 The role of *Suppressor of Hairless* in *Notch* mediated signalling during zebrafish somitogenesis

5.1.1 *Danio Su(H)* is essential for zebrafish development

The members of the CSL family of transcription factors are highly conserved from human to *Drosophila* (Furukawa et al. 1991; Amakawa et al. 1993). The *Danio Su(H)* homologue analysed here shows a high degree of conservation compared to other vertebrate and invertebrate counterparts, implying a conserved role. But it is not only the coding sequence itself, which is highly conserved. The genomic organisation of this gene, scattered in 10 small exons and an 11th large exon is directly comparable between zebrafish and mouse. The current evidence suggests that there is only one *Su(H)* homologue in zebrafish. First, the cloning approach yielded only one variant (Sieger 2002, diploma thesis). Second, the *Su(H)* homologous sequences that can be found in the new zebrafish whole genome shotgun assembly Zv4 reveal only one complete *Su(H)* gene while the other possible homologous gene fragments appear to be pseudo-genes, similar to the pseudo-genes found in mouse. Third, there is currently no other *Su(H)* homologue in the EST data base. Finally, the ubiquitous expression of the gene suggests that it can potentially be involved in all Notch dependent signalling processes in the zebrafish (Sieger 2002, diploma thesis).

The results of the *Su(H)*-knockdown show that the function of the *Su(H)* gene is essential for zebrafish development and that the lack of function leads to an early death of the embryos. The phenotype of at least the 5' morphants is similar to the *RBP-Jκ* null-mutant in mouse. 5' morphants show more severe defects and die earlier than ORF morphants. The different efficiency between the two Mos concerning the morpholical effects might be due to the known difference in blocking the efficiency of translation depending on the binding position of the Mo. The closer to the 5' end a Mo is targeted, like the 5'Mo, the more efficient is the knockdown (product information Gene Tools). *RBP- Jκ* $^{-/-}$ mutant mice embryos are developmentally retarded, drastically reduced in size, show distinct areas

of cell degeneration in the developing CNS and form only up to 6 somites (Oka et al. 1995). However, the exact reason for the embryonic lethality of the mutants in mouse or morphants in zebrafish is not clear. The lack of proper somitogenesis alone does not lead to embryonic lethality, since the *des/notch1a* and the *aei/deltaD* mutants are homozygous viable (van Eeden et al. 1996). However, it was shown that Notch signalling is also required for proper arterial-venous differentiation (Lawson et al. 2001). Since *Su(H)*-knockdown embryos fail to develop proper blood vessels, disruption of Notch signalling by *Su(H)*-knockdown could cause an insufficient oxygen and nutrient supply of the tissues, which might explain the early embryonic death.

5.1.2 Notch signalling in the PSM

Su(H)-Mo injection leads to a breakdown of cyclic *her1*, *her7* and *deltaC* expression. A weak, diffuse expression of these genes was observed in the whole PSM with a distinct area of stronger expression in the anterior PSM. This is comparable to the *her1*, *her7* and *deltaC* expression in the *des/notch1a*, the *aei/deltaD* and the *bea/deltaC* mutant (van Eeden et al. 1998; Holley et al. 2002; Jülich et al. 2005b). *deltaD* expression, which seems not to be cyclic, shows also a disruption in *Su(H)*-knockdown embryos. As for *deltaC*, the anterior two stripes of expression are lost and instead a broad expression domain appears together with a diffuse expression in the posterior and intermediate PSM. These changes in *deltaC* and *deltaD* expression are also seen after *her1* and *her7* knockdown (Holley et al. 2002; Oates et al. 2002; Gajewski et al. 2003), suggesting that the breakdown of *deltaC* and *deltaD* expression is due to a feedback loop of Her1 and Her7 on *deltaC/deltaD* transcription. The loss of *deltaC* and *deltaD* expression in the later somitic tissue after *Su(H)*-knockdown can not be directly due to this feedback loop since *her1* and *her7* are not expressed in the somites. Thus, *deltaC* and *deltaD* expression in the somites might be controlled by other Notch target genes or directly controlled by Notch via *Su(H)*. Since *deltaD* is upregulated in S*u(H)* morphants, *Su(H)* seems further to be necessary to repress *deltaD* expression in the developing neural tube. The upregulation of a *Delta* homologue from mouse, *Dll1*, has also been observed in the neural tube of *RBP-* $J\kappa^{-/-}$ mice embryos indicating a conservation of Notch signalling for this process between zebrafish and mouse (de la Pompa et al. 1997).

The similarity of *her1* and *her7* expression in *des* and *aei* embryos and in *Su(H)*-knockdown embryos suggests that the Notch signal is mediated via *Su(H)* to control the

expression of *her1* and *her7*. Thus the obtained results support the classical mechanism for Notch signalling during zebrafish somitogenesis. This model assumes that in the absence of Notch signalling *Su(H)* acts as a repressor of its target genes *her1* and *her7* (Fig.24 A). The stronger basal expression of *her1* and *her7* after *Su(H)*-knockdown compared to *her1* and *her7* expression in the *des/notch1a* mutant clearly supports this role as a repressor (Fig.2). When Notch signalling is active *Su(H)* undergoes a switch from a repressor to an activator including the interaction with NIC to promote activation of *her1* and *her7* (Fig.24 B). Because of the recruitment of the *Delta-Notch* pathway in a high number of cell-cell communication processes during development, the use of tissue specific activators is necessary for the proper activation of target genes (Fig.24). The specific activators, which might play a role in the zebrafish PSM are so far not known. Keeping this scenario in mind, the *Su(H)*-knockdown has two effects: on the one hand the derepression of the *her1* and *her7* genes and on the other hand the loss of activation of these genes via Notch signalling (Fig.24 C). Since local activators are still present, this results in a “broadening and weakening” of *her1* and *her7* expression (for review see Barolo and Posakony 2002). According to this model, reduced expression was observed in cells which normally respond to *Notch* signalling and expansion of *her1 and her7* gene expression was found in normally nonresponding cells. But this expansion is limited to cells in which the tissue specific enhancers are sufficient to promote target gene activation.

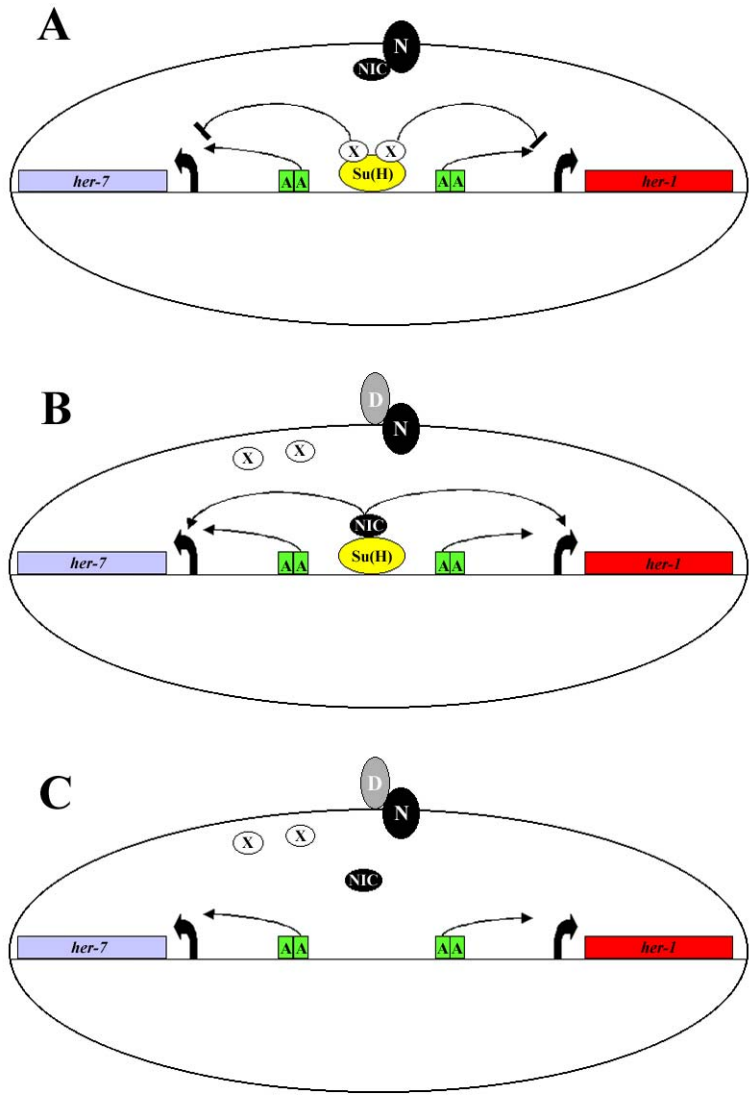

Fig.24:

Schematic drawing of the mediation of Notch signalling via *Su(H)* during zebrafish somitogenesis. (A) Notch signalling is inactive; Su(H) acts as a repressor for *her1* and *her7*. (B) Notch signalling is active; interaction of Su(H) and NIC leads to activation of *her1* and *her7*. (C) knockdown of *Su(H)* has a dual effect: a derepression of *her1 and her7* and a loss of activation via Notch. (A)-(C) potential local activators are represented in green boxes, corepressors for Su(H) are displayed as white circles.

It is known that *Su(H)* recruits different co-repressors for the repression of target genes (for review see Lai 2002). Recent studies in *Drosophila* reveal that the *Hairless* protein acts as an adaptor to recruit the corepressors *groucho* and dCtBP to *Su(H)* (Barolo et al. 2002). Interestingly, *groucho* seems to play a dual role in the Notch pathway. One is the activity as a corepressor for *H/E(spl)-bHLH* proteins while Notch signalling is active and the other is the function as a corepressor for the *Su(H)-H* complex when Notch signalling is absent

(Barolo et al. 2002). The zebrafish *groucho* homologues *gro1* and *gro2* are both expressed in the PSM, within the segmented somites and in the developing nervous system (Wülbeck and Campos-Ortega 1997). This suggests that *gro1* and *gro2* play also a role in the Delta-Notch pathway during zebrafish development.

5.1.3 Cyclic gene expression during early somitogenesis and the formation of the first somites

The first four to seven somites show some peculiarities. They are formed faster than the remaining somites and they show no phenotypic defects in *des/notch1a, bea/deltaC* and the *aei/deltaD* mutants (van Eeden et al. 1998). They are also refractory to the loss of *her7* function (Oates and Ho 2002; Henry et al. 2002), but the formation of their somitic borders is weakly affected by loss of *her1* function (Henry et al. 2002). In *Su(H)* knockdowns they are always only slightly affected with respect to the formation of somitic borders, but not with respect to the formation of somitic tissue. This corresponds also to the results in the mouse (Oka et al. 1995). Given the consistency of this phenotype, it seems very unlikely that it is due to an insufficient reduction of *Su(H)* activity through morpholino inhibition, or a possible partial rescue through maternal RNA in the case of the mouse. According to this phenotype the cyclic expression of *her1* and *her7* is only weakly affected in *Su(H)* morphants during early somitogenesis stages, in the way that the repression in the interstripe regions is lost but a stripe like activation is still observed. This has also been described for *deltaC*, *her1* and *her7* expression during early stages of somitogenesis in the *des/notch1a, bea/deltaC* or *aei/deltaD* mutant background (Oates and Ho 2002; Jiang et al. 2000; van Eeden et al. 1998). A clear breakdown of the oscillation and a so-called salt and pepper like expression in the anterior PSM was only observed from the 4 somite stage on, dependent which mutant was investigated. Based on these results there is an ongoing discussion whether the Notch pathway is only required for the synchronisation of the oscillation or whether Notch signalling is required for both, the progression and the synchronisation of the cyclic expression (Holley et al. 2002; Jiang et al. 2000). The fact that *Su(H)*-knockdown leads to a disruption of cyclic expression in *fss/tbx24* mutant embryos and not only to a desynchronisation, which should cause a salt and pepper like expression, is a clear hint that canonical Notch signalling is involved in the creation of cyclic expression. This observation combined with the fact that disruption of Notch signalling does not lead to a clear breakdown of cyclic expression during early

somitogenesis stages suggests that an additional genetic circuitry might be active in this region. This additional pathway could be necessary for the set-up of cyclic expression and allows the formation and differentiation of the first somites, as well as some limited patterning. The mechanism could be completely independent of Delta-Notch signalling. Meanwhile a mutation in the *integrin5alpha* gene could be identified, which specifically affects only the anterior somites (Jülich et al., 2005a; Koshida et al., 2005). But since this mutation does not affect cyclic gene expression at all, it provides no alternative explanation for the control of early oscillations and the connection to anterior somite formation. A direct correlation between early cyclic gene expression and the formation of the first somites is implied by the recent finding that a double knockdown for *deltaC* and *her7* leads to a breakdown of early oscillations and a loss of anterior and posterior somite borders, although the first border is still formed in this combination (Oates et al., 2005). This suggests that the mechanism for anterior somite formation is more robust and only the removal of two crucial components leads to a disruption. This is further supported by the results of the *her1/her13.2* double Mo injections (results 4.4), which lead to an early disruption of cyclic gene expression and somite borders (discussed in detail in chapter 5.4). However, also in this scenario the expression of cyclic genes is only misregulated and not lost in early stages, leaving the possibility for an alternative pathway, which is responsible for the initial activation of these genes. This initial activation could involve one or two of the Notch genes expressed in this region, but they would then have to act via a *Su(H)* independent pathway. In any case, it is clear that studying the mechanism of the formation of the most anterior somites may yield additional insights in the molecular circuits that are involved in generating the metameric pattern.

5.2 *her11* is involved in the somitogenesis clock in zebrafish

5.2.1 Expression compartments of mouse *Hes7* homologues in zebrafish

her11 belongs together with *her5*, *her1* and *her7* to a subclass that is most similar to the mouse *Hes7* gene (Gajewski et al., 2005). These zebrafish *her* genes are linked in the genome, showing a head to head orientation for *her1* and *her7* as well as for *her5* and *her11* (Gajewski et al., 2003; Gajewski et al., 2005). Comparing the expression of these four genes in zebrafish reveals that there are four separable expression features, namely the midbrain/hindbrain boundary expression (*her5* and *her11*), the somitic expression (*her11*), the anterior PSM (*her11, her1* and *her7*) and the posterior PSM expression (*her1* and *her7*) (note that the latter two were shown to be separable in a reporter gene construct study—Gajewski et al. 2003). Interestingly in medaka (*O.l.*) homologues for *her5* and *her7* can be found but only one homologue for *her1* and *her11* was found, which combines expression features of both zebrafish *her* genes and thus was named *O.l.-her1/11* (Gajewski et al., 2005). *O.l.-her7* shows like its zebrafish homologue cyclic expression in the PSM, while *O.l.-her5* shows cyclic expression in the PSM, which is not there for *D.r.-her5*, and in addition expression in the MHB and the lateral diencephalon is found (Gajewski et al., 2005). *O.l.-her1/11* and *O.l.-her5* are also arranged in a head to head orientation in medaka but for *O.l.-her7* no other gene could be identified in a reasonable distance in head to head orientation (Gajewski et al., 2005). Keeping the scenario in zebrafish and medaka in mind, one might posit according to the duplication-degeneration-complementation model (Force et al. 1999) that a common ancestor gene included all the expression domains and after the duplication events some expression compartments were lost and subfunctionalisation occurred. A more efficient subfunctionalisation might have led to the fixation of more copies in zebrafish (*her1* and *her11*) compared to medaka (*her1/11*). However, if the *Hes7* gene in mouse reflects the ancestral situation one has to consider a different scenario. Since *Hes7* has only a cyclic expression in the PSM (Bessho et al. 2001), which is most similar to the posterior PSM expression in zebrafish and medaka, one would conclude that the other expression features were secondarily added and then lost again differentially for *her5* and *her11*. In any case, both acquisition of new promotor elements and loss of existing elements must have played a role in shaping the current situation.

5.2.2 Differences in the regulation of *her/hey* genes through the Delta-Notch pathway

her11 expression is severely disturbed in the known Delta-Notch pathway mutants *aei/deltaD*, *bea/deltaC* and *des/notch1a*, although residual oscillation is still evident in each of them (Fig.6). Only the knockdown of *Su(H)* removes this residual cyclic expression, indicating that there are still unknown components, possibly another Notch homologue like *notch5* or *notch6* which are both expressed in the PSM (Westin and Lardelli 1997). A striking difference between the regulation of *her11* and *her1* and *her7* is seen when *Su(H)* is knocked down in the *fss/tbx24* background. Residual *her1* and *her7* expression vanishes in this case in the intermediate PSM (Fig.2), while *her11* shows a broad and uniform expression (Fig.6). This suggests that there is an additional pathway for the transcriptional activation of *her11*, which is not yet known.

The control of the regulation of *hey1* expression is very similar to that of *her11*, with the difference that residual oscillation is only seen in *aei*/*deltaD* mutants, and not in *bea*/*deltaC* and *des/notch1a* mutants. This suggests that *hey1* is more specifically regulated by DeltaC and Notch1 and less specifically by DeltaD. Given that *hey1* is specifically expressed in the posterior half of the somites, one could further speculate that DeltaC and Notch1 are particularly important for the formation of these posterior halves. The observation that *her11* expression is absent in the somitic tissue of *fss*/*tbx24* embryos, whereas *hey1* shows a diffuse expression in this area, gives a further hint that anterior identity of the somites is specified by *fss/tbx24* via *mespb* (Sawada et al. 2000).

5.2.3 The involvement of *her11* in cyclic gene expression

The previous functional analysis of *her1* and *her7* has shown that they act as crucial components of the cyclic somitogenesis oscillator (Gajewski et al. 2003; Holley et al. 2000, 2002; Oates and Ho 2002). Inhibition of either of the two genes results in a disruption of the cyclic expression, although with some differences between the two genes. The analogous analysis for *her11* and *hey1* shown here does not provide such clear evidence for a primary involvement in the cyclic expression. The morpholino-mediated knockdown of either Her11 or Hey1 did not lead to a visible phenotype or changes of expression patterns of other genes. But the RNA of *her11* and *hey1* becomes apparently stabilized by Mo injection against them, as has been observed for *her1* and *her7* (Gajewski et al. 2003; Oates and Ho 2002). This might be taken as evidence that the morpholinos are effective, although one cannot exclude that there is residual translation of the genes, which

would mask the true knockout phenotype. On the other hand, at least for *her11*, knockdown effects are obvious in double injections with either *her1* or *her7*, indicating that at least *her11* is involved in the somitogenesis clock (Fig.9). In each case the residual cyclic expression that is apparent in single injections is lost, indicating that Her11 cooperates with Her1 in *her1* interstripe repression in the budding process and obviously activates (together with Her7) the *her7* transcription in the anterior PSM. Thus, although the protein sequence of *her* genes would classify them as repressors, they appear at least formally also to be involved in activation (Gajewski et al. 2003).

Since for *hey1* no additional effects could be detected after a combined knockdown with *her1* and *her7* one might assume that it reflects an output of the clock rather than being a core component of the mechanism. Furthermore, due to its restricted expression in the posterior half of the somites *hey1* might be involved in the specification of anterior-posterior polarity in the somites.

5.3 *her12* and its role during zebrafish somitogenesis

5.3.1 A complex *her12* expression during somitogenesis and its regulation through Delta-Notch

The expression of the mouse *Hes5* homologue *her12* is highly variable during zebrafish somitogenesis and shares expression domains with other *her* genes in distinct phases of development. During early phases of somitogenesis *her12* expression is highly similar to other *mHes5* homologues like *her4.1* and *her15* (Shankaran 2005; Takke et al., 1999). Bud stage embryos show a thin line of expression at the posterior and lateral border of the embryo with a broader domain in the lateral plate mesoderm and an oscillating expression in the posterior PSM (Fig.10A, B). This early expression of *her12* is completely different to the early expression of *her1*, *her7* and *her11* (Gajewski et al., 2003; Holley et al., 2000; Müller et al., 1999, Oates and Ho 2002; results 4.2). Interestingly the mode of *her12* expression changes with time and from the 6s stage on similarities to *her1* and *her7* expression are observed. The expression in the posterior becomes U-shaped and seems to oscillate almost in phase with the posterior *her1* expression, although a slight shift can be detected (Fig.12). Furthermore a stripe like expression occurs in the anterior PSM starting with the 8-10s stage, which appears in the interstripe regions of *her1* (Fig.12). But the mode of stripe expression seems to be different for *her12* compared to *her1* and *her7*. This is supported by several observations as the nonoverlapping expression of *her1* and *her12* stripes, the lack of transition zones and the variance in stripe number (schematically shown in Fig.25). These findings suggest that *her12* stripes are dynamically switched on and off in the interstripe regions of *her1* when *her1* expression reaches a distinct position in the anterior PSM (Fig.25). This position in the anterior PSM seems further to be related to the area where the next somite border(s) will be formed, as revealed by the double staining with *myoD* (results4.3).

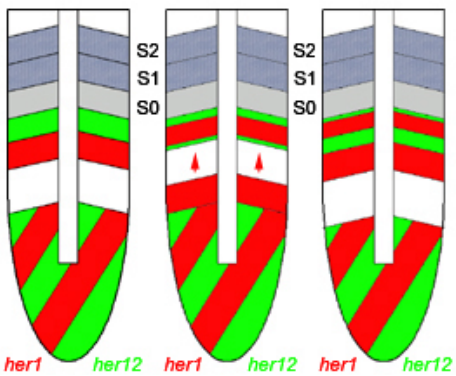

Fig.25 (legend on next page)

Fig.25:

Schematic drawing of *her1* and *her12* expression in the PSM. *her1* expression is marked in red, *her12* in green; S0 (in light grey) shows the forming somite, S1 and S2 (in blue) mark the last two formed somites. Red arrows shall implicate the moving *her1* stripe. *her1* and *her12* expression appears almost in phase in the posterior PSM, while the stripe like expression in the anterior PSM is different. *her12* stripes do not travel from the posterior u-shaped expression to the anterior PSM, like *her1* stripes. Furthermore *her12* expression seems to be switched on in the interstripe regions of *her1* in the anterior PSM.

The stripes and the posterior expression of *her12* appear to be differentially regulated via Delta-Notch signalling and it seems that DeltaD is more important for the posterior regulation while DeltaC plays a more prominent role in stripe regulation (Fig.13). But nevertheless *tbx24* activity is additionally required to activate the stripe like expression (Fig.13), like previously shown for other *her* genes. Furthermore *her7* and *her11* play a combinatorial role in the repression of the stripes, while *her1* seems to have an opposite role (Fig.13). Whether the regulation via other *her* genes is a direct control or whether it is mediated through a feed back loop via Delta-Notch needs to be clarified. The feature that *her12* stripes do not bud off from the U-shaped expression in the posterior but seem to appear at certain position at a distinct time point in the anterior PSM is clearly different to *her1* and *her7* stripes but quite similar to *her4.1* and *her15* stripes (Shankaran et al., 2005, Takke et al., 1999). Since *her4.1*, *her12* and *her15* stripes appear always in the region where somite border formation occurs, one might infer a role for these *her* genes in the positioning of the next border (Shankaran et al., 2005). Furthermore a highly complex crossregulation between these genes might be expected as it has been shown for three *Hes5*-like genes during neurogenesis in chick (Fior and Henrique 2005). Most probably due to redundancy problems their function could not be analysed completely in zebrafish so far (discussed below). The analysis of the mouse *Hes5* homologues in medaka (*Hes5* homologues in zebrafish: *her2, her4.1, her4.2. her12, her15*) revealed only the existence of one copy of *her4* and *her12* (Gajewski et al. 2005). Interestingly both genes show a dynamic expression in the posterior PSM of medaka embryos, which is comparable to zebrafish *her12* but does not exist for zebrafish *her4* (Takke et al., 1999; Shankaran et al., 2005; results 4.3). The stripe like expression, which is found for *her4* and *her12* in the anterior PSM of zebrafish, could not be detected for the homologues in medaka. This is, together with other findings, a further hint that *her* gene regulation in the intermediate to anterior PSM is different in medaka compared to zebrafish (Gajewski et al., 2005; discussed below).

her12 expression in the neuronal compartments of the embryo is not regulated via *deltaC*, *deltaD* and *notch1a* but is definitively controlled via D-N signalling since *Su(H)* morphants show a disruption of all expression compartments (unpublished data). Possible candidates of the D-N pathway, which might be responsible for this regulation, are *deltaA*, *deltaB*, *notch 5* and *notch6* (Westin and Lardelli 1997; Haddon et al., 1998).

Mouse *Hes5*, the homologue of *her2*, *her4.1*, *her4.2*, *her12* and *her15*, shows a dynamic expression in the posterior and anterior PSM as well as a complex expression in different neuronal compartments and is like its zebrafish counterparts completely regulated via Delta-Notch signalling (Dunwoodie et al., 2002; de la Pompa et al., 1997). This strongly supports, at least for this subgroup of *her* genes, that *Hes5* reflects the ancestral situation with all expression features. After the different duplication events some expression features seem to be retained in all zebrafish copies, while others got lost independently.

5.3.2 A role for *her12* in cyclic gene expression and somite border formation?

The functional analyses done in this study reveal that *her12* is involved in both, the regulation of cyclic gene expression and the formation of somite borders (results 4.3). Misexpression of *her12* leads to a clear disruption of cyclic gene expression and somite formation. Since misexpression of other *her* genes like *her11* and *her9* does not affect this process at all, the defects are supposed to be specific for *her12* (results 4.3; Pasini et al., 2004).

However, the results of the morpholino knockdowns are not as clear as expected. Injection of the 5'Mo has no effect at all on somitogenesis, while the ORF-Mo leads in around 50-60% of the embryos to a disruption in cyclic gene expression but does not affect somite border formation (results 4.3). The *GFP* control experiments proof the finding that the ORF-Mo does not perform a full knockdown but show on the other hand that the 5'Mo is able to do so, which is in clear contrast to the outcome that this Mo has no influence on somitogenesis. This might show that although this control is done in vivo, it still reflects an artificial situation. The conditions for the endogenous *her12* RNA could be completely different to the situation for the *her12-5'UTR GFP* RNA when thinking about protein interactions on the RNA to regulate transcription, degradation etc. Thus one has to be careful in interpreting such control experiments. In this case it might therefore be that both Mo's are not capable to reduce the endogenous Her12 protein amount in a manner that strongly affects somitogenesis.

The main explanation for the weak morpholino phenotype, especially on somite border formation, might be redundancy between *her12* and other members of this subgroup like *her4.1* and *her15*. Since all three are expressed in a stripe like fashion in the anterior PSM this is highly possible. Attempts to knock down all three genes did not succeed so far due to toxicity problems (unpublished results). A combined knockdown of two genes of this group did not enhance the phenotype of *her12*-ORF-Mo injections (unpublished results), which might again be a problem of inefficient morpholino binding as discussed before.
Nevertheless, taking the misexpression and morpholino results for *her12* together, one can infer an involvement of *her12* in regulation of cyclic gene expression and somite border formation.

5.4 *her1* and *her13.2* play a combinatorial role in anterior somite formation in zebrafish

5.4.1 Combined "clock and wavefront" signalling for anterior somites?

Single mutations or morpholino knockdowns in *delta-notch* genes, in the *her7* gene and *receptor tyrosine phosphatase ψ* lead only to posterior defects in zebrafish, as discussed earlier, and leave the first 3 to 8 somites unaffected (Aerne and Ish-Horowicz 2004; Holley et al., 2000; Oates et al. 2005; results 4.1; van Eeden et al. 1996). This has also been described for knockdowns of the *her13.2* gene, which is dependent on FGF signalling in the PSM (Kawamura et al., 2005). These results would imply that the oscillator, controlled via D-N signalling as well as the gradient, generated via FGF signalling, are only responsible for posterior somites. The only mutation found so far, which affects specifically anterior somites is a mutation in the *integrin5α* gene giving a phenotype complementary to the posterior defects of Delta-Notch mutants (Jülich et al., 2005a; Koshida et al., 2005). However a direct relation of Delta-Notch to integrin5α remains to be shown although indirect interactions may exist (for review Katsube and Sakamoto, 2005). At least on a certain level a common mechanism in the control of anterior and posterior somites must be present in zebrafish since all somites are defective in the *fss/tbx24* mutant (van Eeden et al., 1996).

The *her1/her13.2* knockdowns show that both, the oscillator and the gradient/wavefront act in combination to generate anterior somites. The cyclic Delta-Notch gene *her1* and the FGF controlled gene *her13.2* seem to be crucial for this role since the double morphant shows disrupted somites over the whole body length, while in the *her7/her13.2* morphant anterior somites are not affected. These results are in line with the recent finding that *her1* and *her13.2* form heterodimers in vitro (Kawamura et al., 2005) although the heterodimerization has not been tested in vivo and only the combinatorial role has been proven here in vivo (results 4.4). The ability of *her1* and *her13.2* to repress the *her1* promotor in vitro (Kawamura et al., 2005) is obviously not possible for Her1 alone in vivo (Gajewski et al., 2003 and this study Fig.19). Anterior *her1* and *her7* expression is clearly missing in the *her1* morphant, while cyclic expression in the posterior is ongoing in the absence of Her1. This is indicative that a Her1 homodimer does not regulate its own expression in the posterior PSM and rather points to an activatory role in the anterior in a so far unknown way. Reducing Her13.2 by injection of a respective Mo leads to disruption of cyclic expression in the whole PSM but only at later stages concomitant with the

posterior border disruption (Kawamura et al., 2005). Reduction of Her13.2 in the *her1* morphant background leads to an additional loss of anterior somite borders and hints for a combinatorial role of both genes, maybe as heterodimers, in this process, which cannot be compensated by other *her* genes. The fact that the *her1/her13.2* morphant does not show any dynamics in *her1* and *her7* expression compared to the *her1* single morphant, which still shows cyclic expression in the posterior PSM, might be a hint that in the absence of Her1 Her13.2 might interact with another *her* gene to keep the oscillation going on in the posterior PSM. A possible interaction partner for this could be Her7. A different explanation would be that in the *her1* morphant still a small amount of Her1 protein is translated, which is enough for the interaction with Her13.2. To exclude this, a *her1* null mutant would be necessary.

5.4.2 Early oscillation and anterior borders

The *her1/her13.2* double morphant is unique with respect in combining the D-N pathway and the FGF gradient, but it is not the only morphant, which leads to an early breakdown of cyclic gene expression and a disruption of anterior somites. It has previously been shown that a *her1* and *her7* deletion mutant (b567) as well as the double morphant for *her1* and *her7* shows defective anterior and posterior somites (Henry et al., 2002; Oates and Ho, 2002). Concerning the morphological phenotype it is not clear whether the b567 mutant can be directly compared with the *her1/her13.2* morphant since alternating weak and strong boundaries have been described for the deletion mutant, which can not be seen in *her1/her13.2* morphants (this study; Henry et al., 2002). But nevertheless the *her1/7* morphant shows a breakdown of early oscillations (own unpublished observations), implying that removing two D-N controlled *her* genes is sufficient to disrupt the oscillator right from the start. In this mutant background the Her13.2 protein lacks its binding partners and is not capable to maintain oscillations. This seems to be different in the complementary scenario where *her13.2* is knocked down. Here *her1* and *her7* seem to be able to maintain at least a few cycles and anterior somites are formed (this study and Kawamura et al., 2005). On the one hand this could be interpreted as a more prominent role for the D-N dependent *her* genes in early oscillations but on the other hand it might be a hint that FGF signalling is not only transduced via *her13.2* to the oscillator machinery. Since the phenotypes of embryos, which are inhibited in FGF signalling are quite different

to *her13.2* morphants (Kawamura et al., 2005, Sawada et al., 2001), the latter one might be the more probable explanation.

A further scenario leading to an early breakdown of cyclic gene expression and missing anterior somites is the combined knockdown of *deltaC* and *her7* (Oates et al., 2005). The absence of an important activator (DeltaC) and a crucial repressor (Her7) in this combination leads to a very early disruption of *her1* expression (Oates et al., 2005). Although the interaction between *her1* and *her13.2* is here possible, the heterodimer is not able to rescue the disturbed oscillations, showing that cyclic *her1* expression is a crucial feature for early oscillations. This is also reflected in the *Su(H)/her1* morphant situation. Comparable to the *deltaC/her7* double knockdown, absence of Su(H) mediated activation and absence of Her1 repression leads to a direct early breakdown of the oscillator (this study; Oates et al., 2005).

Taken together all these results show the complexity of anterior somitogenesis. While posterior somitogenesis can be easily disrupted by removing only one component of the oscillator the mechanism for the anterior seems to be more robust. The starting of the oscillator requires a crucial interaction of different components of the Delta-Notch pathway and the FGF pathway. Due to this redundancy it is at least guaranteed that the process of somitogenesis is started even if one component is not functioning properly in the beginning. The evolutionary reason for this redundancy might be an ancestral difference in the regulation of the gastrulation derived somites and the tailbud derived somites, which has evolved to a combinatorial network to control the whole somitogenesis process.

5.4.3 A function for mHes6 homologues during somitogenesis only in lower vertebrates?

Zebrafish Her13.2 and Her13.1 are homologues of the mouse Hes6 protein. Since for *her13.1* no expression could be detected so far, although it was amplified from somitogenesis stage cDNA (personal communication M.Gajewski), the focus here will be on the comparison of *her13.2* with the respective homologues in mouse and Xenopus. A similarity between all these m*Hes6* homologues is the independence on D-N signalling (Kawamura et al., 2005; Koyano-Nakagawa et al., 2000). But there is a striking difference in the location of their expression. While zebrafish *her13.2* and Xenopus *Hes6* are expressed in the posterior PSM and in 2-3 stripes in the youngest somites or anterior PSM,

respectively, the mouse PSM is free of *Hes6* transcripts (Bae et al., 2000; Cossins et al., 2002; Kawamura et al., 2005; Koyano-Nakagawa et al., 2000; this study). In contrast to the situation in zebrafish *mHes6* is mainly expressed in the developing nervous system and later on in the developing myotome (Bae et al., 2000; Koyano-Nakagawa et al., 2000). These expression features are interestingly also seen in Xenopus and medaka (Gajewski et al., 2005), suggesting that this situation might reflect the ancestral role while zebrafish has lost the expression in the nervous system and the PSM expression got lost in mice. Furthermore there seems to be some functional redundancy in mice since a homozygous deletion of *Hes6* results in phenotypically normal mice (Cossins et al., 2002). But there is no obvious redundancy concerning somitogenesis in zebrafish or Xenopus, since knockdown or overexpression of the respective mHes6 homologues leads to a clear segmentation defect (Kawamura et al., 2005; Cossins et al., 2002).

The zebrafish experiments clearly suggest that the FGF gradient interacts with the oscillator via *her13.2*. And this interaction, probably via the Her1/Her13.2 heterodimer is necessary to maintain cyclic gene expression (this study; Kawamura et al., 2005). Since *mHes6* is not expressed in the mouse PSM, the situation appears to be different in mice. Here it is still not clear how the FGF signal is coupled to the oscillator and if the FGF signal is directly coupled to the oscillator at all. May be the additional control of oscillations is done by Wnt signalling via *axin2* in the mouse and not via FGF signalling like in zebrafish (Aulehla et al., 2003). This would be a further detail, which shows that the basic framework of the oscillator is similar in all vertebrates but many different mechanisms are used for the fine-tuning of the system.

5.5 Zebrafish somitogenesis – a rather derived mode?

Due to duplication events the number of *h/E(spl)/hey*-related genes is higher in fish than in mammals (Gajewski and Voolstra 2001; Gajewski et al., 2005). In analogy to this, there are more *h/E(spl)/hey*-related genes involved in fish somitogenesis compared to mouse somitogenesis for example. In mouse *Hes1, Hes5, Hes7* and *Hey2* have been shown to be dynamically expressed during somitogenesis (Jouve et al. 2000; Bessho et al. 2001b+2003; Leimeister et al., 2000; Dunwoodie et al. 2002). While in zebrafish *her1*, *her7, her11*, *her12* and *her15* show a dynamic expression in the PSM, *her4* and *her6* are expressed in stripes in the anterior PSM and additionally *her13.2* is expressed in a gradient in the posterior PSM (Holley et al., 2000; Takke et al., 1999; Pasini et al., 2001; Pasini et al., 2004; Oates and Ho 2002; Gajewski et al., 2003; results 4.2 + 4.3; Shankaran 2005; Kawamura et al., 2005). Although there are differences between the mentioned zebrafish *her* genes in their expression and function during somitogenesis they seem nevertheless all to contribute to this process. In medaka the number of *her* genes is almost similar to zebrafish although zebrafish contains some additional copies involved in somitogenesis like *her1* and *her15* (Gajewski et al., 2005). Interestingly the recruitment of *her* genes is slightly different between zebrafish and medaka, since for example *O.l.-her5* shows a dynamic expression in the PSM while *D.r.-her5* is not expressed at all in the PSM. But the most obvious difference is the appearance of cyclic gene expression in medaka compared to zebrafish. Cyclic gene expression in zebrafish, like for *her1* and *her7*, shows in addition to the U-shaped expression in the posterior PSM two to three stripes of expression which oscillate in the intermediate to anterior PSM (Holley et al., 2000; Oates and Ho 2000; Gajewski et al., 2003). This is different in medaka, since the U-shaped expression in the posterior extends more to the intermediate PSM and only one stripe can be detected in the anterior PSM (Gajewski et al., 2005). This reminds undoubtedly of cyclic gene expression in higher vertebrates, suggesting that intermediate stripe regulation is an additional feature in zebrafish. Alternatively this feature might have been lost in medaka and higher vertebrates.

All investigated *h/E(spl)/hey*-related genes involved in somitogenesis in higher vertebrates and in zebrafish are regulated by Delta-Notch, despite *her13.2*, which is activated via FGF signalling and seems to play only a role in lower vertebrates (discussed before; Kawamura et al., 2005). Delta-Notch signalling and the *h/E(spl)/hey*-related genes built up a genetic circuit, which is the core component of the oscillator (reviewed by Rida et al., 2004).

However even on this level differences can be detected between zebrafish and higher vertebrates. While in higher vertebrates the Notch modulator *Lunatic fringe* (*Lfng*) shows an oscillating expression in the PSM (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998), the homologue in zebrafish is not involved in somitogenesis at all (Leve et al., 2001). In contrast to this, another member of the Delta-Notch pathway, namely *deltaC*, was found to oscillate in the zebrafish PSM in phase with *her1* and *her7* (Jiang et al., 2001; Oates and Ho 2002; Jülich et al., 2005b). Interestingly, recent findings in mice imply that the *delta* homologue *Dll1* is also cyclically expressed during mouse somitogenesis (Maruhashi et al., 2005). This indicates that, dependent on the species, one or more components of the canonical D-N pathway need to have a cyclic expression in the PSM to ensure the oscillator mechanism. The cyclic expression of the *her*- and *Hes* genes seems to be a common feature in all species but also here slight differences can be detected (discussed before).

Recently it could be shown that the *lfng* homologue in medaka shows a comparable expression to its zebrafish homologue (Elmasri et al., 2004). Whether *deltaC* also cycles in medaka and how the genetic circuit is built up has not been investigated so far.

All present evidence suggests that some features of zebrafish somitogenesis appear to be fish specific and are conserved between zebrafish and medaka. But nevertheless there are some details, like intermediate stripe regulation, which are found only in zebrafish and not in medaka. Given that the medaka patterns are more similar to higher vertebrates, one can infer that zebrafish represents a rather derived mode of somitogenesis, which is not fully comparable to other species.

To fully understand zebrafish somitogenesis one first has to specify the various similarities and differences in the expression of the mentioned *her* genes in the zebrafish PSM. Although they are all clearly regulated via Delta-Notch signalling (despite *her13.2*), the differences show how diverse the signals of this pathway are interpreted to regulate gene expression. At least part of this is achieved by a different usage of local activators, which allows an exact regulation for every gene. Most of these local activators in the PSM are so far not known. The only known activating protein that is necessary for the activation of several *her* genes in the anterior PSM is Tbx24 (Nikaido et al., 2002; van Eeden et al., 1998; results 4.1, 4.2 and 4.3; Shankaran 2005). But *tbx24* does not seem to be involved in the specific regulation of certain *her* genes, as it turned out to be a general activator for all investigated *her* genes in the anterior PSM. Furthermore it seems that special activators or

a further pathway is required to activate cyclic gene expression in the posterior PSM. Even the effective combinatorial knockdown of *Su(H)* and *tbx24* function or *Su(H)* and *her1* function results in a still persisting expression in the posterior PSM (results 4.1+4.4), indicating the requirement for a strong activator there.

Beyond the regulation of *her* genes their function has to be analysed individually and in combination as already started here. Since morpholinos are not always effective enough and the combination of several morpholinos causes toxicity problems, mutants will be required for this purpose in the future. Due to the possibility to use TILLING to detect the full spectrum of ENU-induced mutations in the zebrafish genome (Wienholds et al., 2002+2003), the required *her* gene mutants might be available in the near future.

Another important point, which has to be further investigated, is the ability of *her* genes to form hetero- and homodimers. Since in vitro interaction studies represent always an artificial situation, this feature needs to be necessarily analysed in vivo.

Additionally one needs to understand the connection of other signals and pathways to the genetic circuit of Delta-Notch signalling and *her* genes. Recently it could be shown that *gadd45ß1* and *gadd45ß2* as well as *receptor protein tyrosine phosphatase ψ* (*RPTPψ*) influence cyclic gene expression and somite border formation, but how this connection is established is not clear in the moment (Aerne and Ish-Horowicz 2004; Kawahara et al., 2005).

Only when all these aspects are analysed in zebrafish and other vertebrates, it will be possible to judge which features are conserved core components of the somitogenesis mechanism and how derived the zebrafish situation really is.

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Declaration of collaborators contributions

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Martin Gajewski performed the search for *her* genes in the zebrafish genome and thus provided the *her* gene candidates analysed here. Furthermore we contributed equally to the functional analysis of *her11*. Ideas concerning the comparison of somitogenesis in zebrafish, medaka and higher vertebrates were adapted from his initial work in medaka (Gajewski et al., 2005).

Irene Steinfartz prepared the flat mounted embryos for the *Su(H)*- and *her11* project, as well as the sections for the *her12* project.

Eva Schetter contributed some of the flat mount preparations for the *her12* project.

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsquellen 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 angeführten Teilpublikationen- noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahren nicht vornehmen werde. Die Bestimmungen dieser Promotionsordung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. D. Tautz betreut worden.

Teilpublikationen

Auszüge dieser Arbeit wurden bereits, bzw. werden voraussichtlich in Kürze publiziert. Teile der folgenden Publikationen entsprechen den Kapiteln 4.1 bis 4.4 dieser Arbeit.

Sieger, D., Tautz, D. and Gajewski, M. (2003). The role of Suppressor of Hairless in Notch mediated signalling during zebrafish somitogenesis. *Mech Dev* **120**, 1083-94.

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