receptor tyrosine phosphatase ψ is required for Delta/Notch signalling and cyclic gene expression in the presomitic mesoderm

Birgit Aerne and David Ish-Horowicz

Developmental Genetics Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

Accepted 16 April 2004

Development 131, 3391-3399
Published by The Company of Biologists 2004
doi:10.1242/dev.01222

Summary

Segmentation in vertebrate embryos is controlled by a biochemical oscillator ('segmentation clock') intrinsic to the cells in the unsegmented presomitic mesoderm, and is manifested in cyclic transcription of genes involved in establishing somite polarity and boundaries. We show that the *receptor protein tyrosine phosphatase ψ* (*RPTPψ*) gene is essential for normal functioning of the somitogenesis clock in zebrafish. We show that reduction of RPTPψ activity using morpholino antisense oligonucleotides results in severe disruption of the segmental pattern of the embryo, and loss of cyclic gene expression in the presomitic mesoderm. Analysis of cyclic genes in *RPTPψ* morphant embryos indicates an important requirement for *RPTPψ* in the control of the somitogenesis clock upstream of or in parallel with Delta/Notch signalling. Impairing RPTPψ activity also interferes with convergent extension during gastrulation. We discuss this dual requirement for *RPTPψ* in terms of potential functions in Notch and Wnt signalling.

Key words: Receptor tyrosine phosphatase, Somitogenesis clock, Presomitic mesoderm, Notch signalling, Wnt, Convergent extension, Zebrafish

Introduction

The body plan of most higher organisms is made up of serially repeated elements, or segments. In vertebrate embryos, the most obvious metameric structures are the somites. They constitute the basis of the segmental pattern of the body, give rise to the axial skeleton and the muscles and dermis of the trunk, and impose segmentation on the vascular and peripheral nervous system. Somites are formed sequentially from the presomitic mesoderm at a rate that is species-specific (e.g. every 30 minutes in zebrafish and every 90-120 minutes in chick and mouse).

The periodic production of somites along the anteroposterior axis of the vertebrate body involves a molecular oscillator, the 'segmentation clock', which can be visualised through the cyclic activation of a small set of regulatory genes (for a review, see Maroto and Pourquié, 2001). These oscillations result in dynamic wave-like domains that sweep across the presomitic mesoderm (PSM) in a posterior-to-anterior direction, narrowing as they approach its anterior end. The oscillation becomes arrested in each cell as it passes from the presomitic to the somitic region of the mesoderm. One temporal oscillation occurs in the PSM for each somite that is formed, and mutations or treatments that perturb oscillatory gene expression also disrupt segmentation (Evrard et al., 1998; Henry et al., 2002; Holley et al., 2000; Hrabe Angelis et al., 1997; Jiang et al., 2000; Kusumi et al., 1998; Oates and Ho, 2002; Zhang and Gridley, 1998).

In each cycle, these cycling genes are first expressed in the tailbud, and expression is subsequently propagated through the posterior PSM. When it reaches the anterior PSM, it becomes stabilized, and is localized to either the rostral or caudal part of the future somite. Based on these observations and others, the PSM has been subdivided into three different regions in which the oscillator responds to different regulatory cues: the posterior undetermined zone, the anterior committed zone (within which cycling is still seen) and a differentiating anterior most zone, within which somite boundaries and compartments are established (Gajewski et al., 2003; Morales et al., 2002; Saga and Takeda, 2001).

Most oscillatory genes are related to Notch signalling and dependent on Notch signalling for their cyclic expression. In the mouse and chick, these include *lunatic fringe* (*lfng*), which modulates the efficiency of Notch signalling (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998), and various *hairy*-related genes [*hairy1*, *hairy2* and *Hey/Hesr/HRT2* in chick (Jouve et al., 2000; Leimeister et al., 2000; Palmeirim et al., 1997); *Hes1*, *Hes7* and *Hey1* in mouse (Bessho et al., 2001a; Jouve et al., 2000; Nakagawa et al., 1999)] that are transcriptional targets of Notch signalling and encode basic helix-loop-helix (bHLH) repressor proteins. In the zebrafish PSM, three genes have so far been shown to have cyclic expression: the Notch ligand *deltaC* (Jiang et al., 2000) and the *hairy*-related genes, *her1* and *her7* (Henry et al., 2002; Holley et al., 2000; Oates and Ho, 2002). Mutations in these cycling genes and other Delta/Notch components result in defective somite segmentation: intersomitic clefts fail to form or are late and irregular. In zebrafish, *her1* and *her7* appear to cross regulate each other, and it has been proposed that a negative feedback loop involving these genes constitutes the oscillator (Henry et al., 2002; Holley et al., 2002; Lewis, 2003; Oates and Ho, 2002).

In this study we present a novel regulator in the control of the somitogenesis clock, *RPTPψ,* a member of the type IIB family of receptor tyrosine phosphatase. We describe the cloning of zebrafish *RPTPψ* and its expression pattern during

early zebrafish development, and provide evidence that *RPTPψ* is required for normal oscillatory gene expression in the PSM. We show that *RPTPψ* behaves as a positive regulator of *her1* and *her7* expression, acting either upstream of or in parallel with Delta/Notch signalling. We also find that *RPTPψ* is required for convergent extension, a process of cell-rearrangement during gastrulation, raising the possibility that *RPTPψ* functions in Notch and Wnt signalling.

Materials and methods

Fish care and mutant stocks

Zebrafish embryos were obtained by natural spawnings and maintained at 28.5°C in system water. Embryos were fixed in 4% paraformaldehyde using an automated device. *aei*^{tr233} was used to study embryos mutant in Notch signalling (van Eeden et al., 1996; Jiang et al., 1996). Embryos were staged according to Kimmel et al. (Kimmel et al., 1995).

Cloning of zebrafish *RPTPψ* and plasmid construction

Library screening

A chick *RPTPψ* cDNA was used to screen a zebrafish λZapII cDNA library (Haddon et al., 1998) from which several positive cDNA clones were isolated, of which the longest clone (clone 21) spanned sequence nucleotides 1621-4565.

5′-rapid amplification of cDNA ends (RACE)

The missing 5′ sequence was obtained by reverse transcription-PCR from 24 hpf embryo total RNA by using the 5′/3′ RACE kit (Boehringer) according to the manufacturer's protocol. Specific primers used for 5′-RACE were: antisense 5′-RACE-A1, 5′-CCTTCTTGCCCTCGGTGTTGGCGAG-3′ and antisense nested 5′-RACE-A2, 5′-CTCCTCAGTCTGAAACATGACCTCC-3′. The full-length sequence of *RPTPψ* was deposited in the GenBank database under the Accession Number AY555586.

Whole-mount in situ hybridisation and generation of riboprobes

Whole-mount in situ hybridisation was performed essentially as previously described (Haddon et al., 1998). For all experiments using multiple genotypes, hybridisation was carried out in parallel and colour development allowed to run for the same amount of time. The embryos were photographed using a Leica DC500 camera. Digoxigenin-labelled RNA antisense probes were generated with a Stratagene RNA transcription kit. Enzymes for linearization and transcription for probe synthesis were as follows: *RPTPψ, Eco*RI*/T7; deltaC, Xba*I*/T7*; *her1, Xho*I*/T3; her7, Spe*I*/T7; mespa, Eco*RI*/T7; mespb, Hin*dIII*/T3; papC, Apa*I*/T3; fgf8, Eco*RV*/SP6; ntl, Hin*dIII*/T7; spt, Eco*RI*/T7; dlx3, Eco*RI*/T7; hgg1, Xho*I*/T3*.

Morpholino design and injection

Morpholinos (Genetools) were designed with sequences complementary to *RPTPψ* cDNA in a location just upstream or covering the initiating start codon based on the company's recommendations. The morpholino sequences were: *RPTPmo1*, 5′-CGCAGGTATTCATTTTCCGTTGTTA-3′; *RPTPmo2*, 5′-GTTG-GGAAAACAAGTCGAAATCATT-3′; 5-m (5-mispair control oligonucleotide to RPTPmo1), 5′-CGgAGcTATTgATTTTCCcTTc-TTA-3′; *her1mo*, 5′-CGACTTGCCATTTTTGGAGTAACCA-3′. Morpholinos were solubilised and diluted as described by Nasevicius and Ekker (Nasevicius and Ekker, 2000) and injected into one- or two-cell stage embryos at a total amount of 1-8 ng/embryo.

In vitro transcription and translation

To test the specificity and efficiency of the *RPTPψ* morpholinos in knocking down the respective protein, we used in vitro transcription and translation of RPTPψ (TNT Coupled Reticulocyte Lysate System, Promega) performed according to the manufacturer's protocol with the following modifications: in a 25 μl reaction, 0.5 μg of *RPTPψ* cDNA and various amounts of morpholino antisense oligos (25-250 nM) were added to the TNT mix, containing all of the required components for in vitro transcription and translation, and incubated at 30°C for 90 minutes. Five microlitres from the reaction mix were resolved by SDS/PAGE (NuPAGETM, 4-12% Bis-Tris Gel; Invitrogen), and ^{35}S-labeled proteins were visualised by autoradiography.

Results

Cloning and expression of *RPTPψ* during early zebrafish development

We have previously described the cloning and expression of a chick gene encoding receptor protein tyrosine phosphatase ψ (*RPTPψ*) (Aerne et al., 2003). We showed that chick *RPTPψ* is expressed uniformly throughout the PSM and in a dynamic fashion in nascent somites, consistent with a potential role in somitogenesis (Aerne et al., 2003).

To analyse the molecular function of *RPTPψ* during somitogenesis, we used zebrafish, owing to the accessibility of its embryos and the ease of its genetic manipulations. A partial zebrafish cDNA clone was obtained by screening a zebrafish cDNA library with a chick *RPTPψ* probe under low stringency. The missing 5′ end was obtained by 5′RACE (see Materials and methods).

The predicted RPTP protein consists of a 740 amino acid extracellular region, a single transmembrane domain and a 666 amino acid intracellular region. The extracellular sequence contains a MAM (meprin/A5/PTPμ) domain, an immunoglobulin-like domain and four fibronectin type III-like repeats, characteristics of members of the RPTP type IIB family (or MAM domain subfamily) of receptor tyrosine phosphatases (for a review, see Stoker and Dutta, 1998). Comparison of the derived amino acid sequence with other vertebrate receptor tyrosine phosphatases clearly identifies the full-length clone as zebrafish RPTPψ, showing 73-78% homology to human, mouse and chick RPTPψ (Aerne et al., 2003; Wang et al., 1996; Yoneya et al., 1997). Fig. 1A shows a schematic representation of the zebrafish RPTPψ protein domains.

We determined the sites of *RPTPψ* expression during early zebrafish development by in situ hybridisation (Fig. 1B). Low level *RPTPψ* expression is seen throughout the embryo during the first day of development. At later stages (from 10-24 hours), *RPTPψ* is transcribed at slightly increased levels in the somites, and in the pronephric duct, the midbrain hindbrain boundary, the otic vesicle and the retina. Beyond 26 hours post-fertilisation, when somite formation is complete, *RPTPψ* is no longer expressed throughout the embryo but, instead, becomes restricted to the retina, the forebrain-midbrain, the midbrain hindbrain boundary, the otic vesicle and the branchial arches.

RPTP morpholinos inhibit RPTPψ protein synthesis and disrupt segmentation

To examine the effects of reduced RPTPψ activity on segmentation, we used two anti-*RPTPψ* morpholinos *(RPTPmo1* or *RPTPmo2)* targeted to independent regions of the 5′ end of the *RPTPψ* mRNA (Fig. 2A). Antisense morpholino oligos are specific inhibitors of translation that act

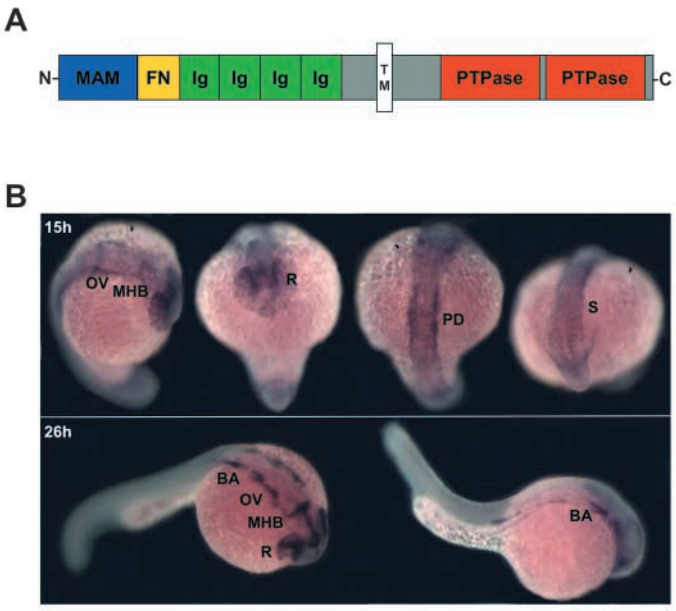

Fig. 1. Domain structure of zebrafish RPTPψ and its expression pattern during early zebrafish development. (A) RPTPψ protein domains. MAM, meprin/A5/PTPμ domain; FN, fibronectin type III-like domain; Ig, immunoglobulin-like domain; PTPase, phosphatase domains. (B) *RPTPψ* expression pattern in 15h and 26h zebrafish embryos. BA, branchial arches; MHB, midbrain hindbrain boundary; OV, otic vesicle; PD, pronephric duct; R, retina; S, somites.

by binding to complementary sequences on mRNA and inhibiting ribosome access (Nasevicius and Ekker, 2000; Summerton and Weller, 1997). In the absence of a specific antibody that recognises the RPTPψ protein, we tested the potency and specificity of *RPTPψ* morpholinos in an in vitro transcription and translation system. Each *RPTPψ* morpholino oligo inhibits protein translation in a dose-dependent manner (Fig. 2B, lanes 5-8). Inhibition by unrelated or mispaired control morpholinos is negligible, even at 250 nM (Fig. 2B, lanes 3,4). These data suggest that morpholino treatment significantly and specifically reduces RPTPψ protein levels. In the experiments described below, the phenotypic effects of *RPTPmo2* were indistinguishable from those of *RPTPmo1*, whereas the mispaired control oligonucleotide did not produce any phenotype.

Injection of *RPTPψ* morpholinos into the one- or two-cell zebrafish embryo results in severe disruption of the segmental pattern of the embryo. The first few somites are relatively normal, but subsequent somite boundaries are indistinct and irregular, like those in embryos mutant for Delta/Notch signalling (Fig. 3; data not shown). The expression pattern of the somite mesodermal marker *myod* in *RPTPmo* embryos reveals a highly penetrant loss of boundary integrity in the disrupted region and a variation in apparent segment size throughout the paraxial mesoderm (Fig. 3B). The number of segments affected and the frequency and severity of boundary defects is dependent on the concentration of injected oligonucleotide. In the extreme, somites are completely lost (Fig. 3B).

We also see a slight shortening of the body axis and broadening of the notochord, suggestive of a disruption of convergent extension movements during gastrulation (Fig. 3A,B, and see later). *RPTPmo* embryos show neuronal degeneration from the first day of development, with cell death occurring mainly in the brain area (data not shown). *RPTPmo* embryos die 2-3 days post-fertilisation. Presumably, lethality results from requirements in the later expression domains.

Paraxial mesoderm specification and maturation is unaffected in *RPTPmo* embryos

The disruption of somitogenesis observed in the *RPTPmo*-injected embryos could be due to interference with specification and maturation of the PSM. Alternatively, processes during somite patterning, such as the establishment of segment polarity or the timing and maintenance of the somite oscillator, could be defective. To exclude some of these possibilities, we analysed the integrity of the presomitic mesoderm by examining markers for paraxial mesoderm formation (*spadetail*; *spt*) and maturation (*fgf8*).

spt is required for the convergence of mesodermal cells towards the dorsal side during gastrulation, and in the specification of cardiac and presomitic mesoderm (Amacher et al., 2002; Griffin and Kimelman, 2002). Once cells of the paraxial mesoderm are formed, they undergo a maturation

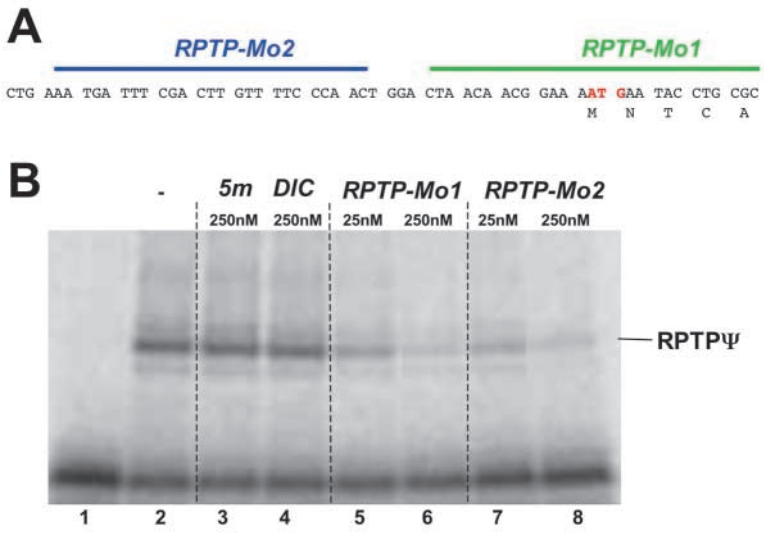

Fig. 2. Morpholinos *RPTPmo1* and *RPTPmo2* specifically block RPTPψ translation. (A) Morpholino target sequences. Part of the 5′ UTR of the *RPTPψ* mRNA is shown illustrating the binding sites of the two morpholinos *RPTPmo1* (spanning the ATG) and *RPTPmo2* (binding 5′ to ATG). (B) In vitro assay showing specific inhibition of RPTPψ translation in the presence of *RPTPmo1* and *RPTPmo2* morpholinos. *RPTPψ* cDNA (0.5 μg) was transcribed and translated in vitro in the presence of ^{35}S-labeled methionine in the absence of morpholino (lane 2) or in the presence of 5-base mismatch control morpholino (5m, lane 3), unrelated morpholinos, e.g. against *deltaC* (*DlC*, lane 4) and various concentrations of *RPTPψ* morpholinos (*RPTPmo1*, lanes 5 and 6; *RPTPmo2*, lanes 7 and 8). Lane 1, in vitro transcription translation in the absence of *RPTPψ* cDNA. ^{35}S-RPTPψ (arrow) was analysed by SDS-PAGE followed by autoradiography.

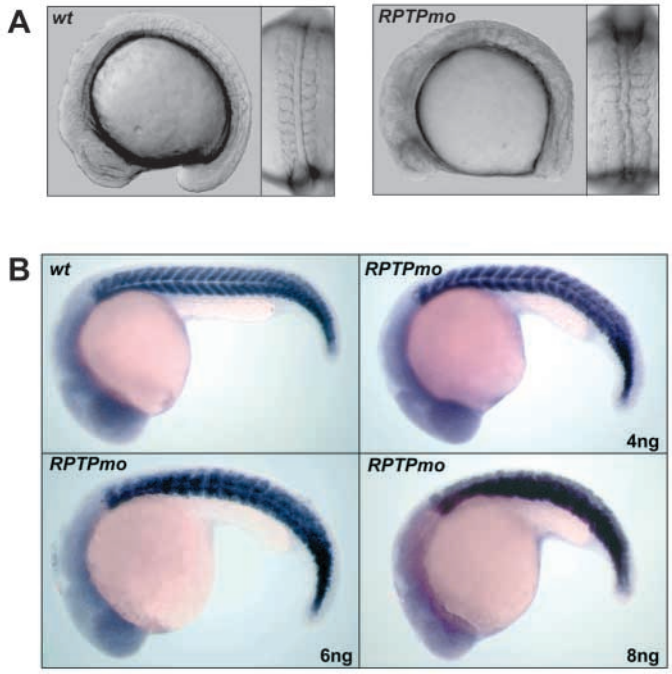

Fig. 3. (A) *RPTPmo* embryos show defects in somitogenesis and a shortening of the body axis. (B) The effect of increasing *RPTPmo* dose on *myod* expression at 26 hpf. Uninjected control, 4 ng, 6 ng and 8 ng *RPTPmo*/embryo.

process, which is determined by a gradient of *fgf8*, with high levels in the posterior and low levels in the anterior presomitic mesoderm. When *fgf8* levels drop below a threshold level, the segmentation clock slows down and somitogenesis is initiated (Dubrulle et al., 2001; Dubrulle and Pourquié, 2004; Sawada et al., 2001). In wild-type embryos, *spt* is expressed strongly in adaxial and tailbud cells, and more weakly in presomitic and lateral mesoderm cells. In *RPTPmo* embryos, the levels and pattern of *spt* expression appear normal (Fig. 4), indicating that *RPTPψ* is not required for specification of presomitic mesoderm tissue. Similarly, the gradient and level of *fgf8* expression is not affected by morpholino treatment (Fig. 4), arguing that the disrupted segmentation seen in *RPTPmo* embryos is not due to impaired mesoderm maturation.

RPTPmo embryos show a defect in segment polarity

Somite boundary formation depends on polarisation of presomites into anterior and posterior compartments. To test if this regionalisation is affected by reduction in *RPTPψ* function, we assayed the expression patterns of markers of rostral and caudal half-segment identity.

Zebrafish *papC* (*pcdh8* – Zebrafish Information Network), a rostral segment polarity marker, is expressed during segmentation in four bilateral pairs of bands in the anterior paraxial mesoderm, and more weakly and uniformly in the rest of the PSM (Yamamoto et al., 1998). The anteriormost bands are located at the anterior borders of the newest somite formed (SI) and the forming somite (S0). Stronger, posterior bands are located in successively less mature somite primordia (S-1, S-2) (Fig. 5A). *papC* expression in *RPTPmo*-injected embryos is very similar to that in somite mutants of the Delta/Notch signalling pathway [e.g. *after eight* (*aei*), a mutation in *deltaD*

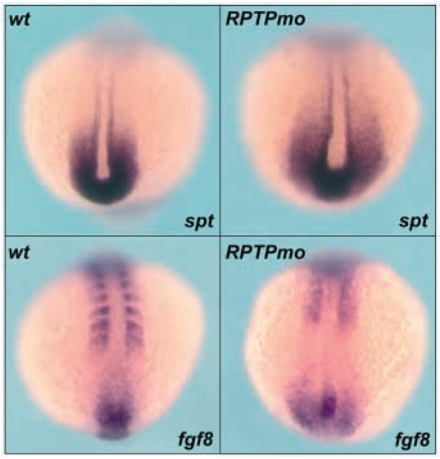

Fig. 4. Paraxial mesoderm specification and maturation is unaffected in *RPTPmo* embryos. In *RPTPmo* embryos, the levels and pattern of expression of markers for paraxial mesoderm formation (*spadetail*; *spt*) and maturation (*fgf8*) appear normal.

(Jiang et al., 2000); Fig. 5D,G]. Expression is strong but non-metameric in the region corresponding to newly formed and nascent somites, with marked random variability of intensity from cell to cell. Expression in the rest of the PSM is normal and diffuse.

papC transcription is dependent on Mesp genes, *mespa* and *mespb*, which code for bHLH transcription factors involved in anteroposterior specification within the presumptive somites (Sawada et al., 2000). In wild-type embryos, *mespa* and *mespb* are segmentally expressed in one to three stripes at the anterior of the PSM, each corresponding to one band of *papC* expression (Fig. 5B,C). *mespb* expression in treated embryos is reduced to a single, broad domain resembling the expression of *mespb* in *aei* mutants (Fig. 5C,F,I). *mespa* expression is completely lost in the morphant embryos, an extreme version of the *aei* phenotype in which expression is very weak but still detectable (Fig. 5B,E,H).

Reduced *RPTPψ* activity also disrupts expression of markers of caudal half-segments, such as *myod* and *deltaC* (Fig. 3B, Fig. 8F). Together, these results show that *RPTPψ* is required for the specification of anteroposterior polarity within somites.

RPTPψ is required for periodic expression of cycling genes in the PSM

Somite compartmentalisation and boundary formation depend on the segmentation oscillator. To analyse whether the somitic defects observed in *RPTPψ* mutants derive from a defective segmentation clock, we analysed oscillator behaviour in *RPTPmo*-injected embryos.

In wild-type embryos, cycling genes show dynamic patterns of expression in the PSM, except at its anterior where somites are formed and expression becomes stable and compartment specific. At high doses of injected morpholino oligonucleotide, cyclic expression in the PSM is lost: expression of *deltaC*, *her1* and *her7* is no longer dynamic, and only one static pattern is observed (Fig. 6). For *deltaC*, expression in these embryos is moderate in the posterior part of the PSM, relatively low in the middle part and high in the anterior part of the PSM. *her1* and *her7*, however, show uniform expression throughout the PSM

(Fig. 6). Thus, dynamic expression of all known cyclic zebrafish genes is disrupted in the treated embryos, indicating that *RPTPψ* is directly involved in the operation of the somitogenesis clock.

The anterior and posterior PSM differ in their threshold requirements for RPTPψ. At lower morpholino doses, cycling continues in the posterior PSM but the normally sharp anterior boundaries of the *deltaC* domains become diffuse. The most anterior stripe becomes weaker and less distinct as interstripe cells begin to express *deltaC* (Fig. 6). A similar dose effect can be seen for *her1* and *her7* expression (Fig. 6). Thus, dynamic expression of cycling genes in the anterior PSM is more sensitive to changes in RPTPψ levels, consistent with domain-specific regulation of cycling genes (Gajewski et al., 2003; Morales et al., 2002; Saga and Takeda, 2001).

RPTPψ acts upstream or in parallel to Delta/Notch signalling and is required for transcriptional activation of both *her1* and *her7*

To consider how *RPTPψ* affects the segmentation clock, we analysed cyclic gene expression in *RPTPmo* embryos in more detail and compared it with that in other known 'clock arrested' embryos, e.g. *aei* mutant and *her1* morphant embryos (Fig. 7A).

We considered, in particular, the posterior PSM, where clock circuitry is not yet affected by differentiation. *her1* and *her7* are downregulated and non-dynamic in *RPTPmo* embryos, as is *deltaC* expression. As Her proteins are repressors, it seems unlikely that their lowered levels are directly responsible for reduced *deltaC* expression. Similar effects are seen in *aei* embryos, suggesting that *RPTPψ* is required to promote Notch signalling (Fig. 7A).

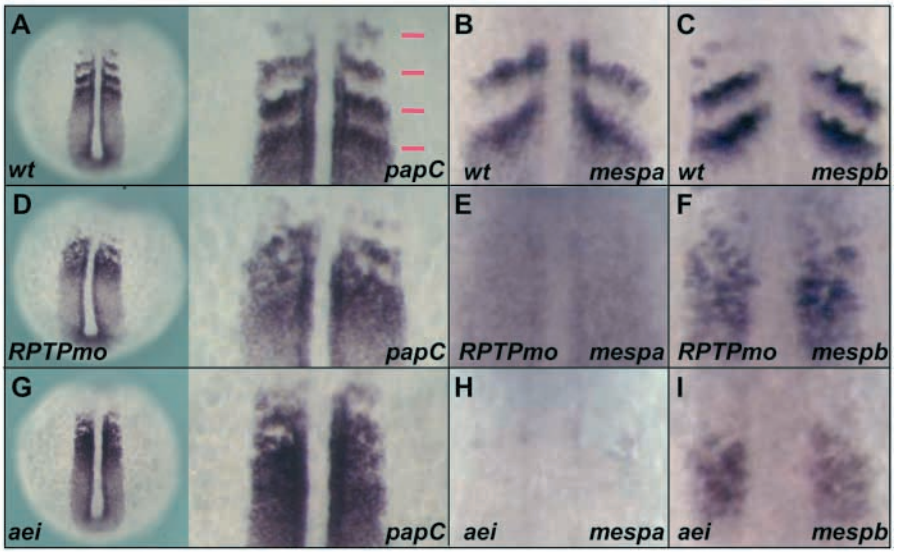

Fig. 5. *RPTPmo* embryos show a defect in segment polarity. The expression patterns of markers of rostral (*papC*, A,D,G; *mespa*, B,E,H; *mespb*, C,F,I) and caudal *(myod, deltaC*; see Fig. 3B and Fig. 8) half-segment identity reveal a requirement for *RPTPψ* for the specification of anteroposterior polarity within somites. (A-C) Wild-type control, (D-F) *RPTPmo* embryos, (E-I) *aei* mutant embryos.

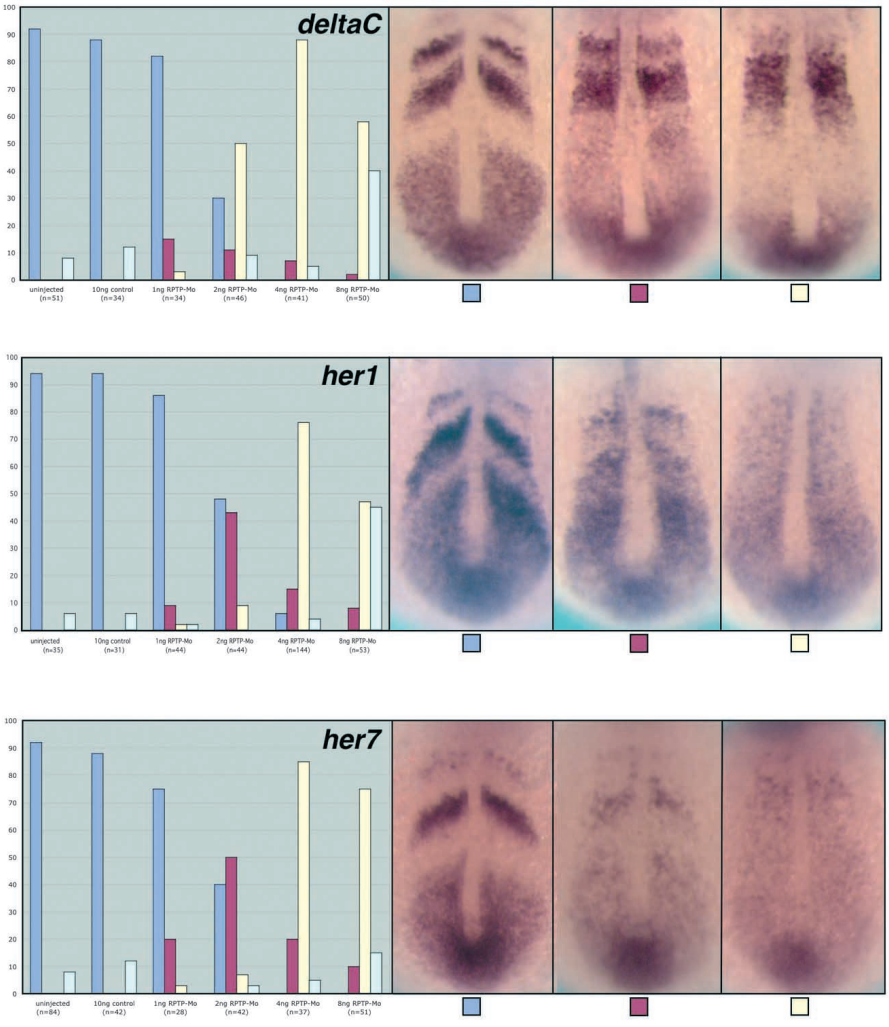

Fig. 6. Cyclic expression of *deltaC, her1* and *her7* in the presomitic mesoderm is disrupted in *RPTPmo*-injected embryos. Histogram shows number of embryos affected dependent on concentration of injected morpholino (uninjected; control, 10 ng/embryo; *RPTPmo*, 1-8 ng/embryo (from right to left)]. Phenotypes are classified into four groups: wild-type expression pattern (blue), partial clock arrest phenotype (red), complete clock arrest phenotype (yellow) and gastrulation defect (sky blue).

Indeed, injecting *RPTPmo* oligonucleotides does not exacerbate the *aei* segmentation phenotype (Fig. 7B), arguing that their major effect is via Notch signalling.

In *her1mo* embryos, levels of *her7* expression are reduced in the cycling PSM, although not as drastically as in *RPTPmo* embryos (Fig. 7A). *her1* levels, on the other hand, are greatly increased (Fig. 7A), but this is probably due to transcript stabilisation by the antisense oligonucleotide (Gajewski et al., 2003; Oates and Ho, 2002). Indeed, this increase is abolished by co-injection of *RPTPmo*, such that the embryos resemble those injected with *RPTPmo* alone (Fig. 7A). These results indicate that RPTPψ activity is needed for efficient transcription of both *her1* and *her7*, and suggest that *RPTPψ* acts upstream or in parallel to Delta/Notch signalling.

The situation in the more anterior PSM, where somite differentiation and boundary formation would normally occur, is rather more complex. There, *RPTPψ* activity is again needed for expression of *her1* and *her7*, but *deltaC* expression is broadened into a single, anterior stripe (Fig. 6, Fig. 7A). Thus, *RPTPψ* is required for final, transient repression of *deltaC* prior to its compartment-specific expression and formation of the somite boundary.

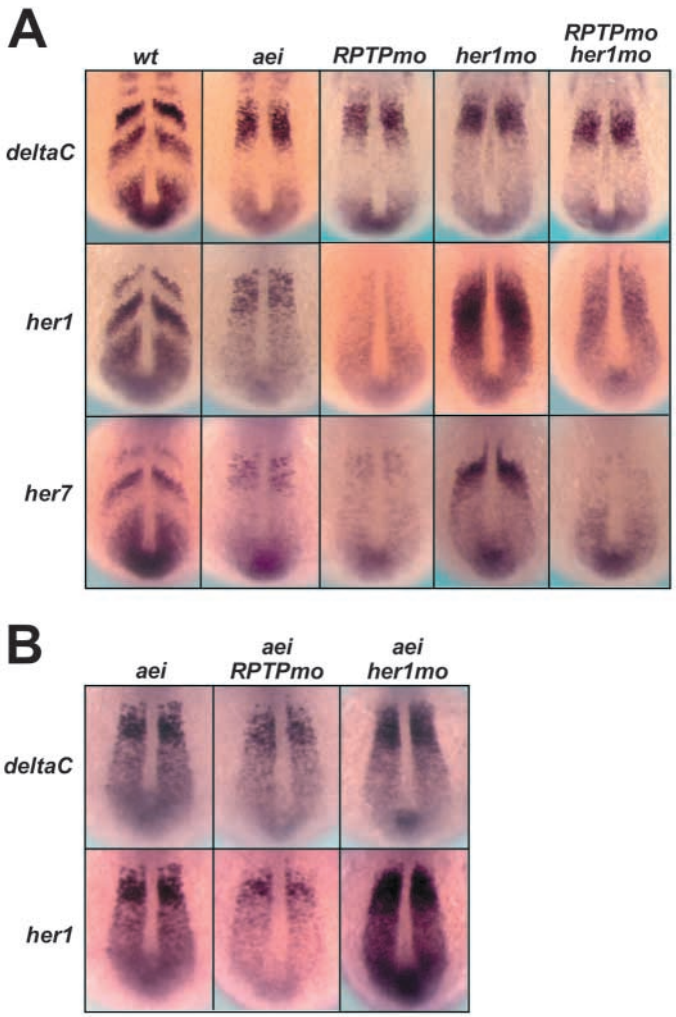

Fig. 7. *RPTPψ* acts upstream or in parallel to Delta/Notch signalling and is required for transcriptional activation of both *her1* and *her7*. (A) Comparison of *deltaC*, *her1* and *her7* expression in *RPTPmo* embryos, *her1* morphants, *aei* mutants and embryos co-injected with *RPTPmo* and *her1mo*. All embryos are stained equivalently. (B) Reduction of neither RPTPψ nor her1 in a *aei* mutant background results in an enhancement of the *aei* mutant phenotype, indicating a role for *RPTPψ* either upstream or in parallel to Delta/Notch signalling.

Reduction of RPTPψ function affects convergent extension

Reduction of RPTPψ activity also results in shortened anteroposterior and broadened mediolateral axes (Fig. 3A,B, Fig. 8). This phenotype is characteristic of a failure of convergent extension, a process of cell polarisation and intercalation that leads to lengthening and narrowing of the embryonic body during gastrulation. An alteration in axial proportions is confirmed by staining for *distal-less3* (*dlx3*) and *no tail* (*ntl*), which mark the boundaries of the neuroectoderm and nascent notochord, respectively (Akimenko et al., 1994; Schulte-Merker et al., 1992). These markers reveal that the neural plate in *RPTPmo* embryos is broader and shorter, and that the notochord is wider and slightly undulated (Fig. 8A,B,D,E).

In addition, staining for a marker for the anteriormost prechordal plate, *hgg1* (*hatching gland gene 1*; *ctlsb* – Zebrafish Information Network) (Vogel and Gerster, 1997) reveals that anterior migration of the prechordal mesoderm is impaired in the treated embryos. *hgg1* expression normally lies rostral to *dlx3*, in the periphery of the neural plate; in *RPTPψ* morphants, *hgg1* is located more caudally, overlapping the edge of the broader neural plate (Fig. 8B,E). Impairment of convergence movements is further indicated by the presence of laterally widened somites as shown by *deltaC* expression in *RPTPmo*-injected embryos compared to the wild-type control (Fig. 8C,F).

Defects in convergent extension might directly explain the lack of dynamic expression in *RPTPmo*-treated embryos, e.g. if the segmentation clock depends on novel neighbourhood relationships arising during cell intercalation. This explanation seems unlikely because segmentation seems to be more sensitive than convergent extension to reduced RPTPψ activity, judged by the different levels of *RPTPmo* required to observe the described phenotypes (Fig. 6). Nevertheless, we tested this idea by examining cyclic gene expression in embryos mutant for *knypek* (*kny*), which encodes a glypican that promotes non-canonical Wnt signalling during convergent extension (Topczewski et al., 2001). The body axis is shortened in *kny* mutant embryos, but segmentation appears normal, and expression of cyclic genes (e.g. *deltaC*) is still dynamic (Fig. 8G), indicating that non-canonical Wnt signalling is not required for periodic gene expression in the PSM.

Discussion

RPTPs play a significant role in antagonising the activities of protein tyrosine kinases, thereby limiting the amplitude and duration of signalling. Several such phosphatases have been shown to play a role in embryogenesis, in particular in the developing nervous system, but also during gastrulation and hematopoiesis (den Hertog, 1999; Stoker and Dutta, 1998; Van Vactor, 1998). However, so far, little is known about their

biochemical function, ligands or downstream signalling pathways. We have described the zebrafish *RPTPψ* gene, and analysed its expression and activity in early embryos. We show that embryos with reduced *RPTPψ* activity lack oscillatory gene expression in the PSM, and that their neural plates and PSMs are shortened and widened. We discuss these results in terms of requirements for RPTP activity in segmentation and convergent extension.

RPTPψ is a regulator of the somitogenesis clock

Our experiments show that antisense-mediated reduction of RPTPψ activity leads to the loss of oscillatory behaviour of cycling genes and to severe reduction of both *her1* and *her7* transcription. *deltaC* is also downregulated in the posterior PSM of *RPTPmo* embryos. Thus, *RPTPψ* appears to be required for effective Notch signalling in the PSM. All these expression patterns resemble those in zebrafish embryos defective for Delta-Notch signalling, consistent with RPTPψ acting upstream of, or in parallel with, this pathway.

her1 and *her7* code for bHLH transcriptional repressors of the Hairy/E(spl) family, genes encoding which are directly activated by Notch signalling in a variety of developmental contexts, including segmentation (Oates and Ho, 2002; Takke et al., 1999). *Hes1* and *Hes7*, *her1* and *her7* counterparts in mouse, negatively regulate their own expression both in cultured cells and in vivo (Bessho et al., 2003; Hirata et al., 2002). Based on this observation, Lewis showed, using mathematical modelling, that an auto-regulatory feedback loop involving *her1* and *her7* provide a possible molecular basis for an intracellular oscillator (Lewis, 2003).

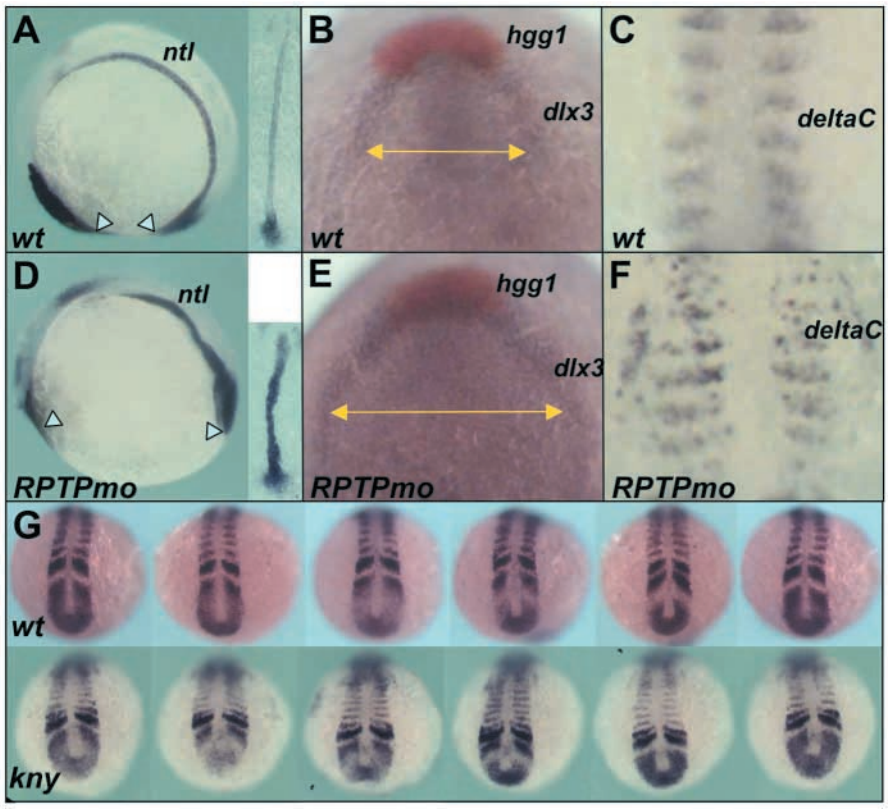

Fig. 8. Reduction of RPTPψ function affects convergent extension. *RPTPmo* embryos show shortening and broadening of the body axis (yellow arrows in B,E), a characteristic of convergent extension mutants. This effect is confirmed by using markers for the nascent notochord (A,D; *ntl*, arrowheads), the boundaries of the neuroectoderm (B,E; *dlx3*), the anteriormost prechordal plate *hgg1* (*hatching gland gene 1*; B,E) and somites (C,F; *deltaC*). **(**A-C) Wild-type control embryos, (D-F) *RPTPmo* embryos. (G**)** Cycling *deltaC* expression in the psm of wild-type and *knypek* (*kny*) mutant embryos.

Reducing RPTPψ levels decreases *her1/7* expression in the cycling PSM, and this reduction is independent of *her1/7* activity (Fig. 6, Fig. 7A). Therefore, *RPTPψ* appears to be required to activate Notch target gene transcription during cycling. This effect appears to be independent of effects on Notch ligand expression because RPTPmo also reduces *her1* expression in *aei* embryos (Fig. 7B). Overall levels of *deltaC*, *her1* and *her7*, and also *mesp* gene expression are much reduced in both the cycling and anterior PSM (Fig. 5, Fig. 6, Fig. 7A,B). However, RPTPψ is also needed for repression of *deltaC* during somite boundary formation – two anterior stripes in wild-type embryos become a single, broad stripe – perhaps because of the failure of anterior *her* expression. This latter, indirect requirement for RPTPψ may reflect differing regulatory circuits operating in different regions of the posterior PSM (Gajewski et al., 2003; Morales et al., 2002; Saga and Takeda, 2001).

Nevertheless, it is still not clear to what extent Delta-Notch signalling is required for the oscillation itself. For example, the first few somites are still formed in zebrafish and mouse embryos defective in Notch signalling. One possibility is that Notch signalling is required only to synchronise neighbouring PSM cells (Jiang et al., 2000), and that an upstream segmentation clock drives cyclic Notch signalling.

One pathway that could account for the latter possibility is that of Wnt signalling. Aulehla et al. (Aulehla et al., 2003) showed recently that *axin2*, which encodes a negative regulator of Wnt signalling, displays oscillating expression in the mouse PSM, alternating with that of *lfng* and *hes7*. They argue that *wnt3a* is necessary for cyclic expression of both *axin2* and the oscillating Notch signalling activity, but that Notch signalling is not required for *axin2* oscillation. This implies that *axin2* oscillations reflect cyclic Wnt signalling that is distinct from, and possibly upstream of, cyclic Notch signalling in the mouse PSM.

It is not yet clear if cyclic Notch signalling in zebrafish embryos is driven by an upstream Wnt clock. *axin2* expression appears not to cycle in the zebrafish PSM (B.A., unpublished), although Wnt signalling components other than *axin2* could be cycling in zebrafish and thereby generate cyclic Wnt activity. In any case, RPTPψ, like Wnt3a in the mouse, is required for Delta/Notch signalling.

RPTPψ is required for convergent extension during gastrulation

In addition to its role in segmentation, *RPTPψ* seems to be required for convergent extension during gastrulation. *RPTPmo* embryos have a shorter and broader body axis, a phenotype characteristic of convergent extension mutants.

Convergent extension has been shown to depend on the so-called, non-canonical Wnt signalling pathway (for a review, see Tada et al., 2002). Unlike canonical Wnt signalling, which targets the nucleus and directs changes in gene transcription, non-canonical Wnt signalling is independent of β-catenin-mediated transcriptional activity, and directs morphogenetic processes such as changes in cell shape and cell migration. How Wnt signalling is translated into convergent extension movements during gastrulation is poorly understood, but it clearly involves changes in the adhesive properties of cells, e.g. via regulated decreases in the activity of cell adhesion molecules such as cadherins (Kuhl et al., 1996; Marsden and deSimone, 2003). Non-canonical Wnt signalling seems not to be required for the segmentation clock, as we have shown that oscillator behaviour is normal in *kny* mutants (Fig. 8G). Similarly, no role for Notch signalling in convergent extension is known.

How might the dual effect of *RPTPψ* on the somite oscillator and convergent extension be explained? The multiplicity of kinases in the vertebrate genome implies that PTPs have a relatively broad range of substrate specificities. One possibility, therefore, is that RPTPψ affects factors from independent pathways (e.g. Wnt and Notch) that regulate convergent extension and somitogenesis. Alternatively, RPTPψ might affect a single pathway/component that impinges on both convergent extension and somitogenesis. Human and mouse RPTPψ have been shown to associate with β-catenin and to dephosphorylate β-catenin both in vivo and in vitro (Cheng et al., 1997; Wang et al., 1996; Yan et al., 2002). Both these processes could be modulated by RPTPψ, e.g. by acting on tyrosine phosphorylation levels of β-catenin, which is crucial for both instability of the β-catenin/cadherin bond and for enhanced binding to TBP and the Tcf complex (Piedra et al., 2001; Roura et al., 1999). Thus, RPTPψ has the potential to promote adhesion and negatively regulate β-catenin-dependent transcriptional activity (Balsamo et al., 1996; Balsamo et al., 1998). It is therefore possible that changes in RPTPΨ activity impinges both on adhesion and migration processes during convergent extension movements, and on Wnt-directed transcriptional regulation of the somite oscillator.

Clearly, further experiments are needed to pinpoint the targets of RPTPψ activity in both processes. In any case, our study adds an unexpected and novel component to the somitogenesis clock, which, until recently, exclusively implicated members of the Delta/Notch signalling pathway.

We acknowledge members of the Ish-Horowicz and Lewis laboratories for discussion, in particular Francois Giudicelli and Julian Lewis for their comments on the manuscript. We are especially grateful to Phil Taylor for excellent fish-keeping. B.A. was supported by a fellowship from the Swiss National Science Foundation.

References

Aerne, B., Stoker, A. and Ish-Horowicz, D. (2003). Chick receptor tyrosine phosphatase Psi is dynamically expressed during somitogenesis. *Gene Expr. Patt.* **3**, 325-329.

Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to *distal-less*: part of a homeobox gene code for the head. *J. Neurosci.* **16**, 3475-3486.

Amacher, S. L., Draper, B. W., Summers, B. R. and Kimmel, C. B. (2002). The zebrafish T-box genes *no tail* and *spadetail* are required for development of trunk and tail mesoderm and medial floor plate. *Development* **129**, 3311-3323.

Aulehla, A. and Johnson, R. L. (1999). Dynamic expression of *lunatic fringe* suggests a link between Notch signaling and an autonomous cellular oscillator driving somite segmentation. *Dev. Biol.* **207**, 49-61.

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B. and Herrmann, B. G. (2003). *Wnt3a* plays a major role in the segmentation clock controlling somitogenesis. *Dev. Cell* **4**, 395-406.

Balsamo, J., Leung, T., Ernst, H., Zanin, M. K., Hoffman, S. and Lilien, J. (1996). Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. *J. Cell Biol.* **134**, 801-813.

Balsamo, J., Arregui, C., Leung, T. and Lilien, J. (1998). The nonreceptor protein tyrosine phosphatase PTP1B binds to the cytoplasmic domain of N-cadherin and regulates the cadherin-actin linkage. *J. Cell Biol.* **143**, 523-532.

Bessho, Y., Hirata, H., Masamizu, Y. and Kageyama, R. (2003). Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock. *Genes Dev.* **17**, 1451-1456.

Cheng, J., Wu, K., Armanini, M., O'Rourke, N., Dowbenko, D. and Lasky, L. A. (1997). A novel protein-tyrosine phosphatase related to the homotypically adhering kappa and mu receptors. *J. Biol. Chem.* **272**, 7264-7277.

den Hertog, J., Blanchetot, C., Buist, A., Overvoorde, J., van der Sar, A. and Tertoolen, L. G. (1999). Receptor protein-tyrosine phosphatase signalling in development. *Int. J. Dev. Biol.* **43**, 723-733.

Dubrulle, J., McGrew, M. J. and Pourquié, O. (2001). FGF signalling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**, 219-232.

Dubrulle, J. and Pourquié, O. (2004). *fgf8* mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* **427**, 419-422.

Evrard, Y. A., Lun, Y., Aulehla, A., Gan, L. and Johnson, R. L. (1998). *lunatic fringe* is an essential mediator of somite segmentation and patterning. *Nature* **394**, 377-381.

Forsberg, H., Crozet, F. and Brown, N. A. (1998). Waves of mouse *Lunatic fringe* expression, in four-hour cycles at two-hour intervals, precede somite boundary formation. *Curr. Biol.* **8**, 1027-1030.

Gajewski, M., Sieger, D., Alt, B., Leve, C., Hans, S., Wolff, C., Rohr, K. B. and Tautz, D. (2003). Anterior and posterior waves of cyclic *her1* gene expression are differentially regulated in the presomitic mesoderm of zebrafish. *Development* **130**, 4269-4278.

Griffin, K. J. and Kimelman, D. (2002). One-Eyed Pinhead and Spadetail are essential for heart and somite formation. *Nat. Cell. Biol.* **4**, 821-825.

Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D. and Lewis, J. (1998). Multiple *delta* genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125**, 359-370.

Henry, C. A., Urban, M. K., Dill, K. K., Merlie, J. P., Page, M. F., Kimmel, C. B. and Amacher, S. L. (2002). Two linked hairy/Enhancer of split-related zebrafish genes, *her1* and *her7*, function together to refine alternating somite boundaries. *Development* **129**, 3693-3704.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K. and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. *Science* **298**, 840-843.

Holley, S. A., Geisler, R. and Nüsslein-Volhard, C. (2000). Control of her1 expression during zebrafish somitogenesis by a *delta*-dependent oscillator and an independent wave-front activity. *Genes Dev.* **14**, 1678-1690.

Holley, S. A., Julich, D., Rauch, G. J., Geisler, R. and Nüsslein-Volhard, C. (2002). *her1* and the notch pathway function within the oscillator mechanism that regulates zebrafish somitogenesis. *Development* **129**, 1175-1183.

Hrabe de Angelis, M., McIntyre, J., 2nd and Gossler, A. (1997). Maintenance of somite borders in mice requires the *Delta* homologue *DII1*. *Nature* **386**, 717-721.

Jiang, Y. J., Brand, M., Heisenberg, C. P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt,

M. et al. (1996). Mutations affecting neurogenesis and brain morphology in the zebrafish, *Danio rerio*. *Development* **123**, 205-216.

Jiang, Y. J., Aerne, B. L., Smithers, L., Haddon, C., Ish-Horowicz, D. and Lewis, J. (2000). Notch signalling and the synchronization of the somite segmentation clock. *Nature* **408**, 475-479.

Jouve, C., Palmeirim, I., Henrique, D., Beckers, J., Gossler, A., Ish-Horowicz, D. and Pourquié, O. (2000). Notch signalling is required for cyclic expression of the *hairy*-like gene *HES1* in the presomitic mesoderm. *Development* **127**, 1421-1429.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Kuhl, M., Finnemann, S., Binder, O. and Wedlich, D. (1996). Dominant negative expression of a cytoplasmically deleted mutant of XB/U-cadherin disturbs mesoderm migration during gastrulation in *Xenopus laevis*. *Mech. Dev.* **54**, 71-82.

Kusumi, K., Sun, E. S., Kerrebrock, A. W., Bronson, R. T., Chi, D. C., Bulotsky, M. S., Spencer, J. B., Birren, B. W., Frankel, W. N. and Lander, E. S. (1998). The mouse *pudgy* mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. *Nat. Genet.* **19**, 274-278.

Leimeister, C., Dale, K., Fischer, A., Klamt, B., Hrabe de Angelis, M., Radtke, F., McGrew, M. J., Pourquié, O. and Gessler, M. (2000). Oscillating expression of *c-Hey2* in the presomitic mesoderm suggests that the segmentation clock may use combinatorial signaling through multiple interacting bHLH factors. *Dev. Biol.* **227**, 91-103.

Lewis, J. (2003). Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator. *Curr. Biol.* **13**, 1398-1408.

Maroto, M. and Pourquié, O. (2001). A molecular clock involved in somite segmentation. *Curr. Top. Dev. Biol.* **51**, 221-248.

Marsden, M. and DeSimone, D. W. (2003). Integrin-ECM interactions regulate cadherin-dependent cell adhesion and are required for convergent extension in Xenopus. *Curr. Biol.* **13**, 1182-1191.

McGrew, M. J., Dale, J. K., Fraboulet, S. and Pourquie, O. (1998). The *lunatic fringe* gene is a target of the molecular clock linked to somite segmentation in avian embryos. *Curr. Biol.* **8**, 979-982.

Morales, A. V., Yasuda, Y. and Ish-Horowicz, D. (2002). Periodic *Lunatic fringe* expression is controlled during segmentation by a cyclic transcriptional enhancer responsive to Notch signaling. *Dev. Cell.* **3**, 63-74.

Nakagawa, O., Nakagawa, M., Richardson, J. A., Olson, E. N. and Srivastava, D. (1999). HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev. Biol.* **216**, 72-84.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat. Genet.* **26**, 216-220.

Oates, A. C. and Ho, R. K. (2002). *hairy*/*E(spl)*-related (*her*) genes are central components of the segmentation oscillator and display redundancy with the Delta/Notch signalling pathway in the formation of anterior segmental boundaries in the zebrafish. *Development* **129**, 2929-2946.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquié, O. (1997). Avian *hairy* gene expression identifies a molecular clock linked to vertebrate segmentation andsomitogenesis. *Cell* **91**, 639-648.

Piedra, J., Martinez, D., Castano, J., Miravet, S., Dunach, M. and de Herreros, A. G. (2001). Regulation of beta-catenin structure and activity by tyrosine phosphorylation. *J Biol. Chem.* **276**, 20436-20443.

Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A. and Dunach, M. (1999). Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. *J. Biol. Chem.* **274**, 36734-36740.

Saga, Y. and Takeda, H. (2001). The making of the somite: molecular events in vertebrate segmentation. *Nat. Rev. Genet.* **2**, 835-845.

Sawada, A., Fritz, A., Jiang, Y. J., Yamamoto, A., Yamasu, K., Kuroiwa, A., Saga, Y. and Takeda, H. (2000). Zebrafish Mesp family genes, *mesp-a* and *mesp-b* are segmentally expressed in the presomitic mesoderm, and Mesp-b confers the anterior identity to the developing somites. *Development* **127**, 1691-1702.

Sawada, A., Shinya, M., Jiang, Y. J., Kawakami, A., Kuroiwa, A. and Takeda, H. (2001). Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. *Development* **128**, 4873-4880.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nüsslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.

Stoker, A. and Dutta, R. (1998). Protein tyrosine phosphatases and neural development. *BioEssays* **20**, 463-472.

Summerton, J. and Weller, D. (1997). Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev.* **7**, 187-195.

Tada, M., Concha, M. L. and Heisenberg, C. P. (2002). Non-canonical Wnt signalling and regulation of gastrulation movements. *Semin. Cell Dev. Biol.* **13**, 251-260.

Takke, C. and Campos-Ortega, J. A. (1999). *her1*, a zebrafish pair-rule like gene, acts downstream of Notch signalling to control somite development. *Development* **126**, 3005-3014.

Topczewski, J., Sepich, D. S., Myers, D. C., Walker, C., Amores, A., Lele, Z., Hammerschmidt, M., Postlethwait, J. and Solnica-Krezel, L. (2001). The zebrafish glypican Knypek controls cell polarity during gastrulation movements of convergent extension. *Dev. Cell* **1**, 251-264.

van Eeden, F. J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A. et al. (1996). Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* **123**, 153-164.

Van Vactor, D. (1998). Protein tyrosine phosphatases in the developing nervous system. *Curr. Opin. Cell Biol.* **10**, 174-181.

Vogel, A. and Gerster, T. (1997). Expression of a zebrafish cathepsin L gene in anterior mesendoderm and hatching gland. *Dev. Genes Evol.* **206**, 477-479.

Wang, H., Lian, Z., Lerch, M. M., Chen, Z., Xie, W. and Ullrich, A. (1996). Characterization of PCP-2, a novel receptor protein tyrosine phosphatase of the MAM domain family. *Oncogene* **12**, 2555-2562.

Yamamoto, A., Amacher, S. L., Kim, S. H., Geissert, D., Kimmel, C. B. and de Robertis, E. M. (1998). Zebrafish paraxial protocadherin is a downstream target of spadetail involved in morphogenesis of gastrula mesoderm. *Development* **125**, 3389-3397.

Yan, H. X., He, Y. Q., Dong, H., Zhang, P., Zeng, J. Z., Cao, H. F., Wu, M. C. and Wang, H. Y. (2002). Physical and functional interaction between receptor-like protein tyrosine phosphatase PCP-2 and beta-catenin. *Biochemistry* **41**, 15854-15860.

Yoneya, T., Yamada, Y., Kakeda, M., Osawa, M., Arai, E., Hayashi, K., Nishi, N., Inoue, H. and Nishikawa, M. (1997). Molecular cloning of a novel receptor-type protein tyrosine phosphatase from murine fetal liver. *Gene* **194**, 241-247.

Zhang, N. and Gridley, T. (1998). Defects in somite formation in *lunatic fringe*-deficient mice. *Nature* **394**, 374-377.