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Paf1 complex homologues are required for Notch-regulated transcription during somite segmentation

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Members of the yeast polymerase-associated factor 1 (Paf1) complex, which is composed of at least five components (Paf1, Rtf1, Cdc73, Leo1 and Ctr9), are conserved from yeast to humans. Although these proteins have been implicated in RNA polymerase II-mediated transcription, their roles in vertebrate development have not been explained. Here, we show that a zebrafish mutant with a somite segmentation defect is deficient in *rtf1*. In addition, embryos deficient in *rtf1* or *ctr9* show abnormal development of the heart, ears and neural crest cells. *rtf1* is required for correct RNA levels of the Notch-regulated genes *her1*, *her7* and *deltaC*, and also for Notch-induced *her1* expression in the presomitic mesoderm. Furthermore, the phenotype observed in *rtf1*-deficient mutants is enhanced by an additional deficiency in *mind bomb*, which encodes an effector of Notch signalling. Therefore, zebrafish homologues of the yeast Paf1 complex seem to preferentially affect a subset of genes, including Notch-regulated genes, during embryogenesis.

Keywords: Notch; Paf1 complex; segmentation; somite; transcription

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INTRODUCTION

In addition to transcriptional activators and repressors—which function together with the mediator complex and general transcription factors in transcriptional initiation—factors participating in many of the steps of transcription seem to influence transcription. One such factor is the yeast polymerase-associated factor 1 (Paf1) complex. This complex has been implicated in various processes, such as transcription initiation and elongation, histone modification, phosphorylation of RNA polymerase II (pol II), and RNA processing and export (Sims *et al*, 2004). The components of the Paf1 complex are evolutionarily conserved from yeast to *Drosophila* and humans, although the Rtf1 proteins in human and *Drosophila* are not stably associated with the other members of the Paf1 complex (Zhu *et al*, 2005; Adelman *et al*, 2006).

Although this complex seems to be associated with pol II at all transcriptionally active genes, the loss of each component of the yeast Paf1 complex either reduces or enhances the transcription of a small subset of genes (Penheiter *et al*, 2005), suggesting that the yeast Paf1 complex preferentially regulates a particular subset of genes *in vivo*. Similarly, *Drosophila* homologues of the yeast Paf1 complex seem to have preferential roles during development. For example, knockdown of *Drosophila rtf1* increases the hypomorphic severity of a Notch mutant phenotype during wing development (Tenney *et al*, 2006); however, it is not clear whether Rtf1 preferentially affects transcription of a specific subset of genes involved in Notch signalling.

One aspect of development in which Notch signalling is important is the segmentation of somites (Pourquié, 2003). Periodical segmentation is coupled to an internal oscillator, referred to as the 'segmentation clock', as shown by the cyclic expression of genes in the presomitic mesoderm (PSM) (Palmeirim *et al*, 1997). Interestingly, many genes that show cyclic expression are regulated by Notch signalling in the PSM. Notch signalling is also involved in other aspects of segmentation, synchronization of the oscillating phase between cells in the PSM (Horikawa *et al*, 2006) and establishment of the rostral–caudal compartment inside a somite (Takahashi *et al*, 2000).

In previous studies, we identified numerous ethylnitrosourea (ENU)-mutagenized zebrafish mutants with altered somite morphogenesis (Koshida *et al*, 2005). In contrast to previously identified Notch signalling mutants, in which the striped expression of Notch target genes is perturbed to form salt-and-pepper patterns, one of the mutants obtained in our screens, *kt641*, showed reduced but still striped expression of the Notch target genes in the PSM. In the present study, we found that the gene responsible for this phenotype is a zebrafish homologue of yeast *rtf1*.

RESULTS AND DISCUSSION

In initial experiments, we found that *kt641* homozygous embryos showed a distinct phenotype: in addition to the segmentation

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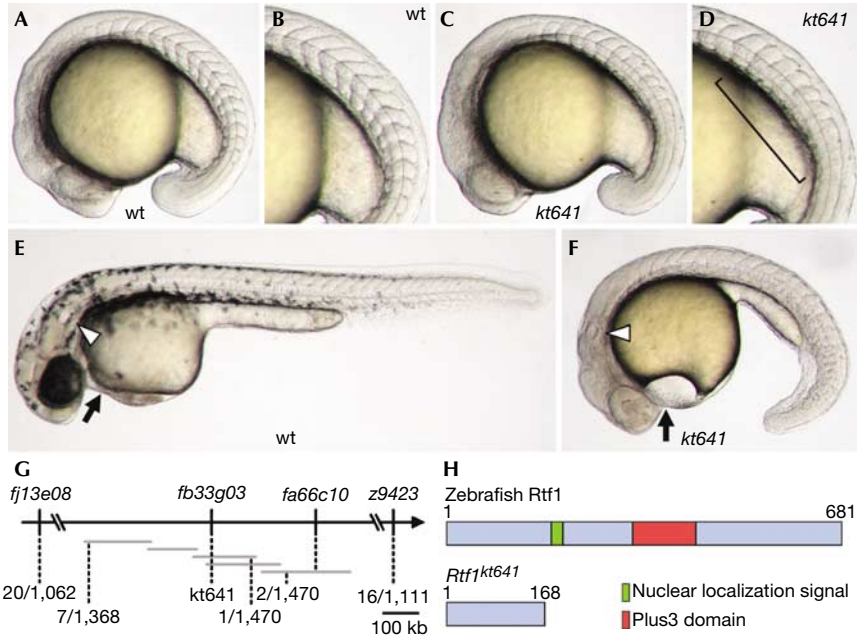

Fig 1 | Isolation and molecular characterization of a zebrafish *rtf1*^{kt641} mutant defective in somite formation. (**A–F**) Lateral views of wild-type (wt) (**A,B,E**) and *kt641* homozygotes (**C,D,F**) at the 17-somite stage (**A–D**) and at 36 h post-fertilization (**E,F**). Panels (**B**) and (**D**) show views of panels (**A**) and (**C**) respectively, at higher magnification. Somite boundaries are disrupted in the posterior trunk of the *kt641* mutant (square bracket in (**D**)). At later stages, the *kt641* mutation causes reduced pigmentation, limited tail growth and abnormal heart (arrow) and ear (arrowhead) development (**F**). (**G**) Meiotic and physical mapping of the *kt641* mutation. Horizontal grey bars represent contiguous sequences deposited in linkage group 13. (**H**) Schematic diagrams of zebrafish Rtf1 proteins encoded by wild-type and *kt641* alleles.

defect (Fig 1A–D), they showed decreased pigmentation of melanophores, abnormal cardiogenesis, small ears, shortened and curled tails in later stages (Fig 1E,F; supplementary Fig S1A–D online), and embryonic lethality. In addition to these defects, *in situ* hybridization analysis showed abnormal neural development (supplementary Fig S1E–H online).

To identify the gene responsible for this phenotype, we mapped its chromosomal position (Fig 1G). The *kt641* mutation was mapped to the region between *fj13e08* (1.9 cM from the mutation) and *fa66c10* (0.14 cM from the mutation) on linkage group (LG) 13, and a polymorphism within a contiguous sequence, BX284673, was found to be near the mutation (1/1,470 recombinants/meioses). Interestingly, a zebrafish homologue of *rtf1* had been located near the suspected mutation. Sequence analysis of *rtf1* in the *kt641* mutant showed a point mutation—C to T—at codon 169, which co-segregated with the phenotype. This mutation produces a termination codon—CAG to TAG—which yielded a truncated version of the Rtf1 protein (Fig 1H). Injection of two individual antisense morpholino oligonucleotides (MOs) specific for *rtf1* also resulted in morphological defects similar to those of the *kt641* phenotype (supplementary Table S1 online). Injection of *rtf1* MO1 into *kt641* mutants did not enhance the phenotype, indicating that this is a null mutation (data not shown). Furthermore, injection of wild-type *rtf1* messenger RNA at the one-cell stage rescued the phenotype, whereas injection of *kt641* mutant mRNA did not (supplementary Table S2 online). Therefore, we conclude that the gene responsible for the *kt641* phenotype is *rtf1*. *In situ* hybridization analysis indicated that the *rtf1* transcript was present in all blastomeres at cleavage stages and in the entire embryo during early developmental stages (data not shown). Maternal expression of the transcript was also detected (data not shown).

So far, posterior segmentation defects in zebrafish similar to those observed in the *rtf1* mutant have been reported to be caused by abnormalities in one of two distinct signalling pathways: Notch or fibroblast growth factor (FGF). Several mutants in Notch signalling components—including *deltaC*, *deltaD* and *notch1a*, and also *mib* in E3 ubiquitin ligase for Notch ligands—lack somite boundaries in the posterior region (Holley & Takeda, 2002; Itoh *et al*, 2003; Jülich *et al*, 2005). Zebrafish embryos defective in *her13.2*, which is a downstream target of FGF signalling, also show a similar phenotype, probably resulting from abnormal regulation of *her1*, a prominent target of Notch signalling (Kawamura *et al*, 2005). To examine whether *rtf1* is involved in either of these signalling pathways, we analysed the expression of genes involved in Notch or FGF signalling in somite segmentation at early somite stages, when tail elongation is not yet defected in *rtf1* homozygous embryos. Striped expression of *her1*, *her7* and *deltaC*, all of which are regulated by Notch signalling (Holley *et al*, 2000; Jiang *et al*, 2000; Oates & Ho, 2002), was strongly reduced in *rtf1* homozygous embryos at early somite stages (Fig 2A–E,L) and at later stages (supplementary Fig S2A and B online). The most anterior expression of these genes also shifted in the posterior direction, although the reason for this shift was uncertain (Fig 2A,D,E, arrowheads). By contrast, the expression of *fgf8* and *her13.2* in the PSM was normal in *rtf1* homozygous embryos (Fig 2F–I,L), suggesting that Rtf1 is preferentially required

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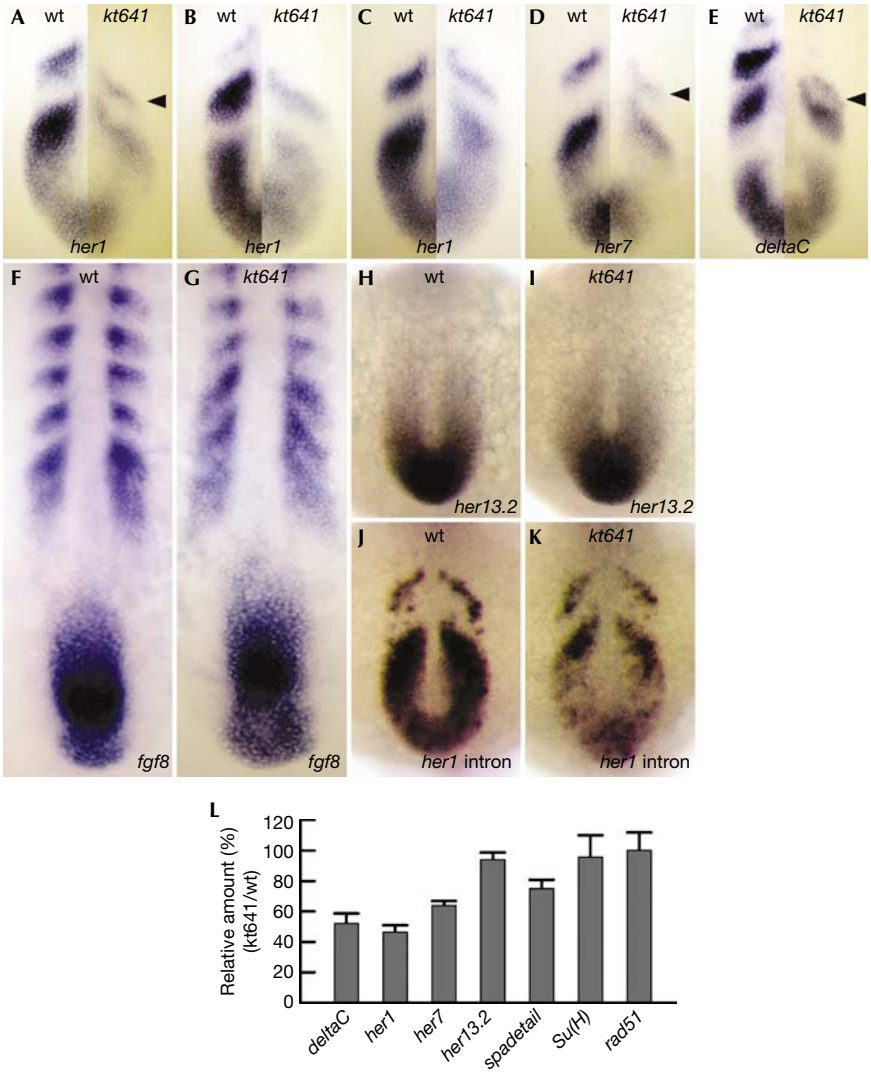

Fig 2 | The *rtf1*^{kt641} mutation reduces the expression of genes involved in somite segmentation. (**A–E**) A comparative representation of expression patterns of *her1* (**A–C**), *her7* (**D**) and *deltaC* (**E**) messenger RNAs between wild-type siblings (wt; left halves) and *rtf1*^{kt641} mutants (*kt641*; right halves) indicates a reduction of these genes in *rtf1*^{kt641} mutants. Each half panel represents the cyclic expression patterns of wild-type siblings and *rtf1*^{kt641} mutants at comparative oscillation phases. Three consecutive expression phases of *her1* mRNA are represented (**A–C**). Arrowheads indicate the anterior-most stripes in *rtf1*^{kt641} mutants, which are shifted in the posterior direction. (**F–I**) Expression patterns of *fgf8* (**F,G**) and *her13.2* (**H,I**) mRNA in wild-type siblings (**F,H**) and *rtf1*^{kt641} mutants (**G,I**). (**J,K**) *her1* nascent transcript hybridized with intron probe in wild-type siblings (**J**) and *rtf1*^{kt641} mutants (**K**). Expression of *her1* is reduced at the transcriptional level in *rtf1*^{kt641} mutants. Embryos are at the 10- or 12-somite stage and the anterior is top in (**A–K**). (**L**) Reverse transcription–PCR analysis of transcript abundance for genes involved in somitogenesis. Data are presented as means + s.d. from triplicate assays.

for the correct expression of Notch-regulated genes. As *de novo* mRNA synthesis, as monitored by a *her1* intron probe, was also decreased in *rtf1* mutants (Fig 2J,K), the reduction of Notch-regulated genes apparently occurs at the transcriptional level. By contrast, expression of *Su(H)*, a mediator of Notch signalling, was not significantly altered in the mutants (data not shown; Fig 2L), indicating that the decrease in Notch-regulated genes was not due to a reduction in this Notch signalling mediator. Notably, in contrast to previously identified Notch signalling mutants, expression of the Notch-regulated genes remained periodic in *rtf1* homozygous embryos. Therefore, reduction, but not perturbed patterning, of the expression of these genes might produce defective segmentation of somites in *rtf1* mutants.

It has been proposed that the segmentation process in the posterior PSM is subsequently translated into a rostro–caudal pattern within a segmental unit in the anterior PSM (Pourquié, 2004). In *rtf1* homozygous mutants, the expression patterns of genes that are normally expressed in a segmental fashion in the anterior PSM and/or somites are disrupted. Anterior compartment-specific expression of *papc* and *mesp-b* was broadened and

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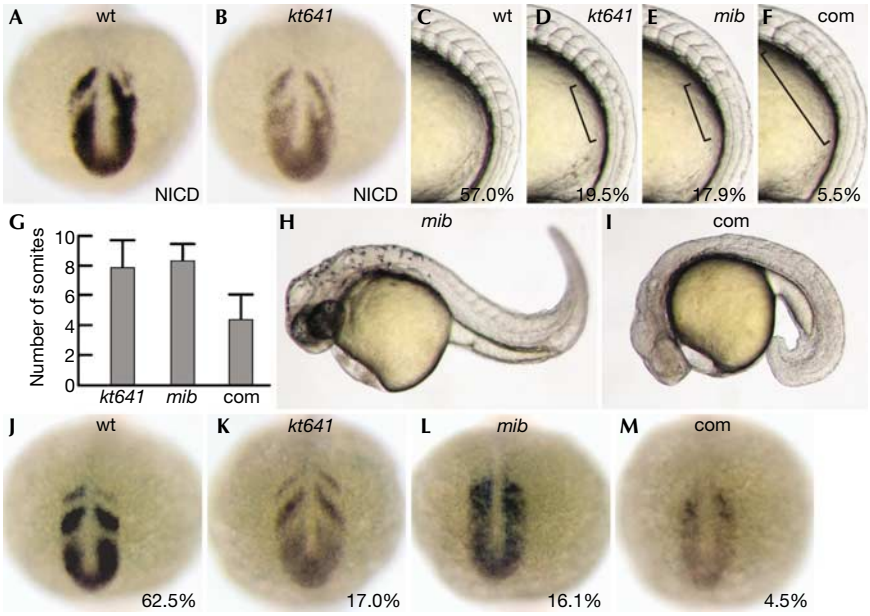

Fig 3 | Functional cooperation between Rtf1 and Notch signalling. (**A**,**B**) *her1* expression in wild-type (wt; **A**) and *kt641* mutant (**B**) embryos injected with synthetic messenger RNA encoding Notch1a intracellular domain (NICD). In *kt641* mutant embryos, the elevated expression is reduced, although the pattern of *her1* expression is still abnormal. (**C**–**F**) Lateral views of 12-somite-stage embryos from crossing of $rtf1^{kt641/+}$;$mib^{ta52b/+}$ fish. Wild type (**C**), *kt641* mutant (**D**), *mib* mutant (**E**) and *kt641;mib* compound mutant (com; **F**) were generated at a ratio (57.1:18.1:19.3:5.5%; $n = 590$) close to the expected mendelian frequency (56.3:18.8:18.8:6.3%). Square brackets in (**D**–**F**) indicate abnormally segmented somites. The *kt641;mib* double mutation enhances the somite segmentation defect. (**G**) Number of fully segmented somites in *kt641* ($n = 47$), *mib* ($n = 59$) and *kt641;mib* (com; $n = 15$) at 18 h post-fertilization. Data are presented as means + s.d. $P < 0.001$ for differences between single mutants (*kt641* or *mib*) and compound mutants (*kt641;mib*) by Student's *t*-test are represented. (**H**,**I**) In addition to the somite defect, *kt641;mib* mutants (**I**) show more severe defects in the eye, pigmentation and tail elongation than *kt641* (Fig 1F) and *mib* single mutants (**H**) at 36 h post-fertilization. (**J**–**M**) In contrast to wild-type siblings (**J**), *kt641* mutants, which show cyclic but reduced *her1* expression (**K**), and *mib* mutants, which lack the striped expression of *her1* (**L**), *kt641;mib* compound mutants show markedly reduced *her1* expression without stripes (**M**) at the 12-somite stage.

became less localized (supplementary Fig S2C–F online). Furthermore, expression of *mesp-a*, which is specifically expressed in the anterior compartment within a segmental unit of wild-type embryos, was markedly reduced in *rtf1* mutants (supplementary Fig S2G and H online). Caudal-specific expression of *myoD* and rostral-specific expression of *fgf8* in the somite was broadened in *rtf1* embryos (Fig 2F and G; supplementary Fig S2I and J online) probably as a result of abnormal rostro–caudal compartmentation. Thus, *rtf1* is required in the anterior PSM for correct rostro–caudal patterning, and this patterning defect seems to result in morphological defects in somites.

To determine whether Notch-dependent transcription was preferentially affected in the PSM of *rtf1* mutants, we examined *her1* expression in embryos in which Notch signalling was strongly activated by expressing the intracellular domain of Notch1a. Although *her1* expression was elevated in the entire PSM of wild-type embryos (Fig 3A), its expression was reduced in *rtf1* mutant embryos (Fig 3B), indicating that Rtf1 is required for transcriptional activation by Notch signalling. In addition, *rtf1* mutation enhanced the phenotype of *mib*, which encodes the E3 ubiquitin ligase that ubiquitinates Notch ligands. Compared with *rtf1* and *mib* mutants, compound homozygotes for *rtf1* and *mib* showed more severe phenotypes (Fig 3C–M): specifically, a lack of morphological somites (Fig 3C–G) and shortened tails (Figs 1F, 3H,I) and also lower *her1* expression (Figure 3J–M). These results strongly indicate that Rtf1 cooperatively participates in transcription with Notch signalling during somite development. By contrast, overall suppression of pol II-mediated transcription by α-amanitin resulted in abnormal development, but with a phenotype distinct from that of the *rtf1* mutant (supplementary Fig S3 and Table S4 online). Furthermore, in addition to *her13.2*, which is induced by FGF, expression of the Wnt signalling target, *cdx4*, was almost unchanged in the posterior region of *rtf1* homozygous embryos (supplementary Fig S2K and L online). Therefore, we conclude that Rtf1 is preferentially required for Notch-mediated transcription in somite development.

Next, we examined whether zebrafish homologues of other components of the yeast Paf1 complex are required for expression of Notch-regulated genes and somite segmentation. Injection of two individual MOs specific for zebrafish *ctr9* resulted in morphological defects similar to the *rtf1* mutant phenotype (Fig 4A,B; supplementary Table S1 online). The *ctr9* MO also enhanced somite and neural crest defects, and reduced *her1* expression in *rtf1* mutants (Fig 4C–J; supplementary Fig S5A–D online). These findings indicate that Rtf1 associates with another member of the Paf1 complex in zebrafish embryos. As the yeast Paf1 complex is involved in transcription elongation, we examined the genetic interaction between *rtf1* and genes

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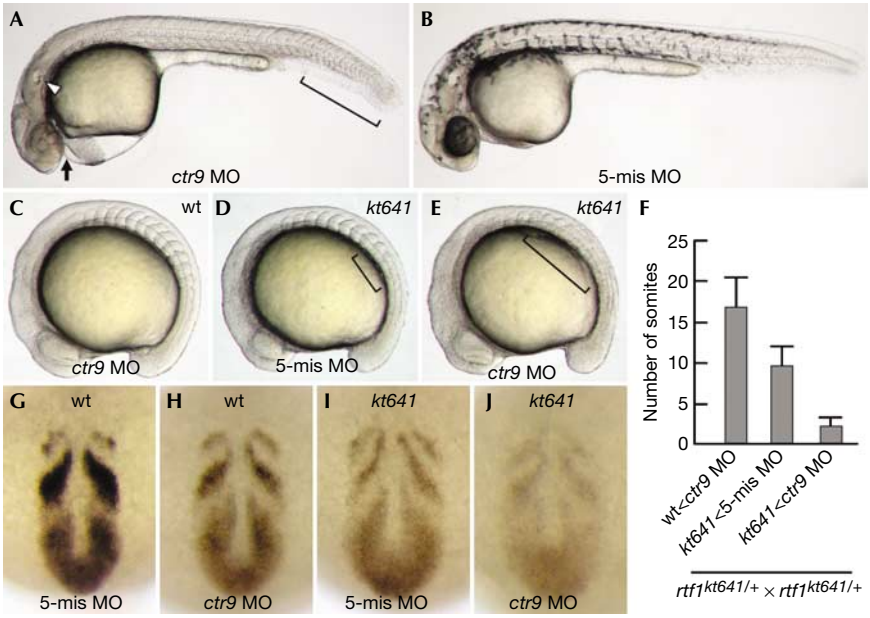

Fig 4 | Rtf1 cooperates with the Paf1 complex during somitogenesis. (**A,B**) Knockdown of *ctr9* results in defects that are similar to but weaker than those of *kt641* mutants. Embryos injected with *ctr9* MO1 (**A**), but not 5-mismatched MO1 (**B**), show reduced pigmentation, abnormal cardiogenesis (arrow), small ears (arrowhead), slightly shortened tails and disorganized somite boundaries in the tail (square bracket) at 36 h post-fertilization. Two independent *ctr9* MOs cause similar morphological defects. (**B**) 5-mispaired *ctr9* MO1 hardly impairs development. (**C–E**) At the 12-somite stage, no marked defects were observed in *ctr9* MO-injected embryos (**C**). 5-mispaired control MO1 had no effect on the *kt641* phenotype (**D**), whereas injection of *ctr9* MO1 into *kt641* mutant embryos resulted in an enhanced segmentation defect with few segmentation boundaries (**E**). The square brackets in (**D**) and (**E**) indicate the posterior somites with obscure boundaries. (**F**) Number of segmented somites in *ctr9* MO1-injected wild-type embryos ($n = 82$), *ctr9* 5-mismatched MO1-injected *kt641* mutants ($n = 18$) and *ctr9* MO1-injected *kt641* mutants ($n = 23$) at 24 h post-fertilization. Error bars represent the s.d. $P < 0.001$ between single mutants (*kt641* < 5-mismatched MO1 or wt < *ctr9* MO1) and double mutants (*kt641* < *ctr9* MO1) are represented. (**G–J**) *her1* expression in 5-mismatched control MO1-injected (**G,I**), *ctr9* MO1-injected (**H,J**) wild-type (**G,H**) or *kt641* mutant (**I,J**) embryos. In comparison with wild-type siblings injected with 5-mismatched control MO1 (**G**), those injected with the *ctr9* MO1 show reduced *her1* expression (**H**). This reduction is enhanced in *kt641* mutant embryos injected with *ctr9* MO1 (**J**). MO, morpholino oligonucleotides.

encoding two factors involved in transcriptional elongation, *spt5* and *spt6*. Injection of an *rtf1*-specific antisense MO enhanced the defects in somite segmentation and neural crest formation, and reduced *her1* expression in *foggy/spt5* homozygous embryos (Keegan *et al*, 2002), suggesting a genetic interaction between these genes (supplementary Figs S4 and S5 online). We also observed genetic interaction between *rtf1* and *pandra/spt6* (Keegan *et al*, 2002; data not shown). These results indicate that Rtf1 functions together with Spt5 and Spt6 during the elongation process in the transcription of *her1* in the PSM.

Our results indicate a developmental role of the Paf1 complex homologues in vertebrates. As in yeast (Penheiter *et al*, 2005), zebrafish Rtf1 and Ctr9 function preferentially in the transcription of a specific subset of genes, including Notch-regulated genes. Ligand stimulation of Notch signalling causes proteolytic cleavage of the Notch receptor and translocation of its intracellular fragment ($Notch^{ICD}$) to the nucleus. $Notch^{ICD}$ binds to a transcription factor of the CBF1/Su(H)/Lag-1 family (CBF1 in vertebrates) and functions as its co-activator (Lubman *et al*, 2004). As *rtf1*-defective PSM cells show reduced $Notch^{ICD}$-induced *her1* expression, Rtf1 seems to function downstream of $Notch^{ICD}$. This suggests that Rtf1 functions in Notch-specific gene expression in association with $Notch^{ICD}$ or CBF1.

In contrast to somite development, we have not yet determined whether abnormal development of other tissues in *rtf1* homozygous embryos is due to a decrease in Notch signalling. At present, it is uncertain whether other signals cooperate with Rtf1 in the development of these tissues. The *rtf1* mutation did not affect the expression of genes affected by other signals, including *foxd3*, a Wnt target in neural crest development, or *her9*, *prdm1/blimp1* and *gata5*, the expression patterns of which are affected by bone morphogenetic protein signalling in neural, neural crest and cardiac development (data not shown). Conversely, zebrafish Rtf1 might also function in transcriptional elongation. Our results showed that defective translation of the transcriptional elongation factors *fog/spt5* and *pan/spt6* also enhanced the phenotype of *rtf1* mutants. This result suggests another possibility: that the transcription of the Notch-regulated genes preferentially involves co-operative interaction between Rtf1 and these elongation factors.

Finally, the segmentation phenotype of the *rtf1* mutant showed a unique feature in comparison with previously reported mutants. Although periodic expression of the clock genes *her1, her7* and *deltaC* was maintained in *rtf1* mutants, the expression levels of these genes were markedly decreased. This observation suggests that the periodicity of their transcription is maintained even when their expression levels are reduced, which is consistent with a

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theoretical analysis reported previously by Lewis (2003). However, despite periodic gene expression in the posterior PSM, segmental gene expression in the anterior PSM was disrupted. This might imply that sufficient expression levels of the clock genes in the posterior PSM are required for distinct expression boundaries between genes in the anterior PSM, as well as in somites. Conversely, it also seems possible that the segmentation process in the anterior PSM, which is also regulated by the Notch signalling, is affected by the reduction of *rtf1*.

METHODS

Fish strains and mutant screening. All studies on wild-type zebrafish (*Danio rerio*) were carried out using fish with a TL2 closed-colony background (Kishimoto *et al*, 2004). Embryos obtained from natural crosses were maintained in egg water (0.03% artificial sea salt in water) at 23 °C or 28 °C. ENU-based mutagenesis was carried out as described previously (Kishimoto *et al*, 2004).

Genetic mapping. *kt641* heterozygous fish (TL background) were mated with wild-type Tübingen fish to generate F1 families. Homozygous mutant embryos were obtained from the F1 crosses. We then carried out PCR for specific simple sequence length polymorphism or single-strand conformation polymorphism markers using their genomic DNA. The PCR was carried out for 30–40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s.

Whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization was carried out as described previously (Nikaido *et al*, 1997). In the case of the *her1* intron probe, embryos were hybridized at 55 °C because of its high AT content (Gajewski *et al*, 2003).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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