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SHORT COMMUNICATION

Two alloalleles of *Xenopus laevis hairy2* gene—evolution of duplicated gene function from a developmental perspective

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Abstract Gene duplication is a fundamental source of a new gene in the process of evolution. A duplicated gene is able to accept many kinds of mutations that could lead to loss of function or novel phenotypic diversity. Alternatively, the duplicated genes complementarily lose part of their functions to play original roles as a set of genes, a process called subfunctionalization. Pseudotetraploid frog *Xenopus laevis* has four sets of genes, and it is generally thought that the alloalleles in *X. laevis* have mutually indistinguishable functions. In this paper, we report differences and similarities between *Xhairy2a* and *Xhairy2b* in the neural crest, floor plate, and prechordal plate. Knockdown studies showed that *Xhairy2a* seems not to function in the neural crest, although both of them are required in the floor plate and the prechordal plate. Temporal expression pattern analysis revealed that *Xhairy2a* is a maternal factor having lower zygotic expression than *Xhairy2b*, while *Xhairy2b* is not loaded in the egg but has high zygotic expression. Spatial expression pattern analysis demonstrated that future floor plate expression is shared by both alloalleles, but *Xhairy2b* expression in the neural crest is much higher than *Xhairy2a* expression, consistent with the results of individual knockdown experiments. Therefore, our data suggest that subfunctionalization occurs in *Xhairy2*.

Keywords Gene duplication · Subfunctionalization · *Xhairy2* · Pseudotetraploid · *Xenopus laevis*

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Introduction

Gene duplication is an important evolutionary strategy by which organisms acquire phenotypic diversity (Ohno 1970). In this widely cited classic model, gene duplication creates a redundant locus that allows one of the duplicates to accumulate different kinds of mutations on the condition that the other, the original copy, still plays most of the ancestral roles. This mechanism is thought to produce two results. One is nonfunctionalization, in which the duplicate eventually loses its function because of accumulated degenerative mutations and is extinguished from the genome or becomes a pseudogene. The other is neofunctionalization, by which a hitherto nonexisting beneficial function is acquired. This promotes fixation of the duplicate in the genome.

However, neofunctionalization is generally thought to be an event of extremely rare occurrence and is contradictory to the fact that many duplicated genes exist in the extant genome (e.g., Prince and Pickett 2002). Genetic studies of the teleost zebrafish, whose genome is thought to have undergone whole-genome duplication three times, have

produced several lines of evidence providing another explanation for the existence of many duplicated genes in the extant genome, which is subfunctionalization supported by the duplication–degeneration–complementation (DDC) model (Force et al. 1999). Subfunctionalization is the accumulation of degenerative mutations eventually leading to complementary loss of part of subfunctions in the duplicate of the pair. Because the pair complements each other to maintain ancestral roles, this trajectory of gene duplication seems to be preserved in the genome. To illustrate, one study on zebrafish *hoxb1* genes shows the complementary expression and functions of *hoxb1a* and *hoxb1b* in rhombomeres, supporting the DDC model (McClintock et al. 2002).

Xenopus laevis, a popular model of vertebrate development, is a pseudotetraploid species of the *Xenopus* genus (Graf and Kobel 1991). Similar to zebrafish, *X. laevis* has four sets of genes, although there is no evidence supporting zebrafish tetrasomy. Therefore, the duplicated genes of zebrafish and *X. laevis* might mutually differ in the way their genomes are preserved, and the widely accepted DDC model might not always be applicable to *X. laevis*. However, unlike zebrafish, the differences between alloalleles of a gene in *X. laevis* have not been intensively investigated so far. We report herein differences and similarities between *Xhairy2a* (Davis et al. 2001) and *Xhairy2b* (Tsuji et al. 2003) in the neural crest, floor plate, and prechordal plate. Part of our results support the DDC model, suggesting that subfunctionalization might have occurred after ancestral *Xhairy2* had been duplicated.

Materials and methods

Embryonic manipulations

X. laevis embryos were in vitro fertilized, dejellied, and cultured as described (Hawley et al. 1995) and staged according to Nieuwkoop and Faber (1967). For whole-mount in situ hybridization (WISH), embryos were fixed in MEMFA at the indicated stages. For microinjection experiments, injected regions were checked by coinjecting fluorescein dextran amine (FLD, Molecular Probes).

Plasmids

Xhairy2a/pBS: Full-length *Xhairy2a* was PCR-amplified based on published sequence with the following primer sets: F (5′-CCCGAATTCTGAGGTGTAGGATCCAGCCT GACGCACAA) and R (5′-CCCGCGGCCGCTTCAA TAAAACTCCTGCATGTTCGG). The fragment was cut with *Eco*R1 and *Not*1 and inserted into *Eco*R1–*Not*1-digested pBSKS+. *Xhairy2b*/pBS was described elsewhere (Murato et al. 2006). *Xhairy2a*/pCS2AT+ and *Xhairy2b*/pCS2AT+ were constructed by amplifying the 5′ untranslated region and the coding sequence of *Xhairy2a*/pBS and *Xhairy2b*/pBS, respectively, and inserted into pCS2AT+. *Xhairy2a-5′UTR*/pCS2AT+ (for *Xhairy2a* specific probe synthesis): *Xhairy2a*/pCS2AT+ was digested with *Nco*1, and the remaining vector backbone was self-ligated. *Xhairy2b-5′UTR*/pCS2AT+ (for *Xhairy2b* specific probe synthesis): *Xhairy2b*/pCS2AT+ was digested with *Pst*1 and *Asc*1, blunted with T4 deoxyribonucleic acid (DNA) polymerase, and self-ligated.

Morpholino antisense oligos

Xhairy2 morpholino antisense oligos (mo's) were obtained from Gene Tools. The sequences were as follows: control mo (co mo) 5′-GCATTTTCATTTGCTTGTAAAGTAA-3′ and *Xhairy2a* mo (h2A mo) 5′-GCATGTTCAGTTGCTGG TACAGTCA-3′. *Xhairy2b* mo (h2B mo) was described previously (Yamaguti et al. 2005).

In situ hybridization

WISH was performed as described (Harland 1991) with minor modifications. The labeled probe of *Xhairy2* (previously described as *Xhairy2b*) was synthesized as described previously (Yamaguti et al. 2005). The labeled probes of *foxd3* (Sasai et al. 2001), *shh* (Ekker et al. 1995), and *chd* (Yamaguti et al. 2005) were synthesized as described previously. For the synthesis of *Xhairy2a*- or *Xhairy2b*-specific probes, *Xhairy2a-5′UTR/ pCS2AT+* and *Xhairy2b-5′UTR/pCS2AT+* were linearized with *Hin*dIII and transcribed with T7 ribonucleic acid (RNA) polymerase (TOYOBO). To increase the sensitivity of the two specific probes, the concentrations of nucleoside triphosphates and digoxigenin (DIG)-labeled uridine triphosphate (UTP) were modified to increase signal intensity: 10 mM adenosine triphosphate, cytidine triphosphate, or guanosine triphosphate and 5 mM UTP or DIG-UTP. Samples stained with *shh* or *chd* were dehydrated, embedded in paraffin, cut into 10-μm-thick sections, and deparaffinized.

RNA dot blot and RT-PCR analysis

RNA dot blot was performed based on the protocols provided by Amersham Biosciences. *Xhairy2a* and *Xhairy2b* sense RNAs, synthesized with *Xhairy2a/ pBS* and *Xhairy2b/ pBS*, respectively, were spotted on Hybond-N+ (Amersham Biosciences) in a fourfold-decreasing manner (625, 156, 39, 9.8 pg) and fixed with 0.05 N NaOH. DIG-labeled *Xhairy2a*- and *Xhairy2b*-specific probes as well as the fluorescein-labeled *Xhairy2* probe (coding sequence only, synthesized with *Xhairy2b/ pCS2AT+*; Yamaguti et al. 2005) were hybridized at 68°C. After thorough washing, the

membrane was incubated with Anti-Digoxigenin-POD (Roche, 1:2,000) and Anti-Fluorescein-AP (Roche, 1:2,000). For signal detection of the specific probes, electrochemiluminiscence (ECL) Western Blotting Detection Reagents (Amersham Biosciences) were used. After ECL reaction, the membranes were stained with nitroblue tetrazolium (NBT)/bromo-chloro-indolyl phosphate (BCIP) to ensure equal loading of *Xhairy2a* and *Xhairy2b* sense RNAs.

Reverse transcription polymerase chain reaction (RT-PCR) was performed according to the standard method with M-MLV RTase (Gibco). For PCR amplification, the annealing temperature was set at 66°C, and the reaction was run for 25 cycles. The primer set for the detection of *Xhairy2a* and *Xhairy2b* expression was the following: F (5′-AGCGCTGAGTCCGTGTGGAGACCATG) and R (5′-GCAGGGTCCCATAGAACGGAACCAA). To detect *Xhairy2* expression, the following primers were used: F (5′-GATCGTAGCCATGAATTACC) and R (5′-GATAAC AGGTCCGGGGCTGG). For *H4 histone*, the following primers were used: F (5′-ATTTATGAGGAAACTCGTG GGGTCC) and R(5′-TTATCCGCCGAAGCCGTAGAGA GTG). *Xhairy2a/pBS* and *Xhairy2b/pBS* were also used as templates for references in the electrophoresis. The fragment was electrophoresed with Tris–borate–ethylenediamine tetraacetic acid-buffered 8% acrylamide gel.

Results and discussion

Specific knockdown of *Xhairy2* alloalleles *Xhairy2a* and *Xhairy2b* reveals differences between them

We analyzed the functions of the *Xhairy2* gene in the early *Xenopus* embryos. In knockdown experiments, we tried to inhibit completely possible functions by knocking down both A and B alleles with a combination of two specific mo's (Fig. S1a). We designed three morpholinos for functional analyses of the *Xhairy2* gene: co mo (five-base-mismatched control morpholino for *Xhairy2a*), h2A mo (specific for *Xhairy2a*), and h2B mo (specific for *Xhairy2b*). The accuracy and efficiency of these morpholinos were carefully examined by using various Myc-tagged *Xhairy2a* and *Xhairy2b* constructs, and no unwanted cross-reactions were observed (Fig. S1b and its legend).

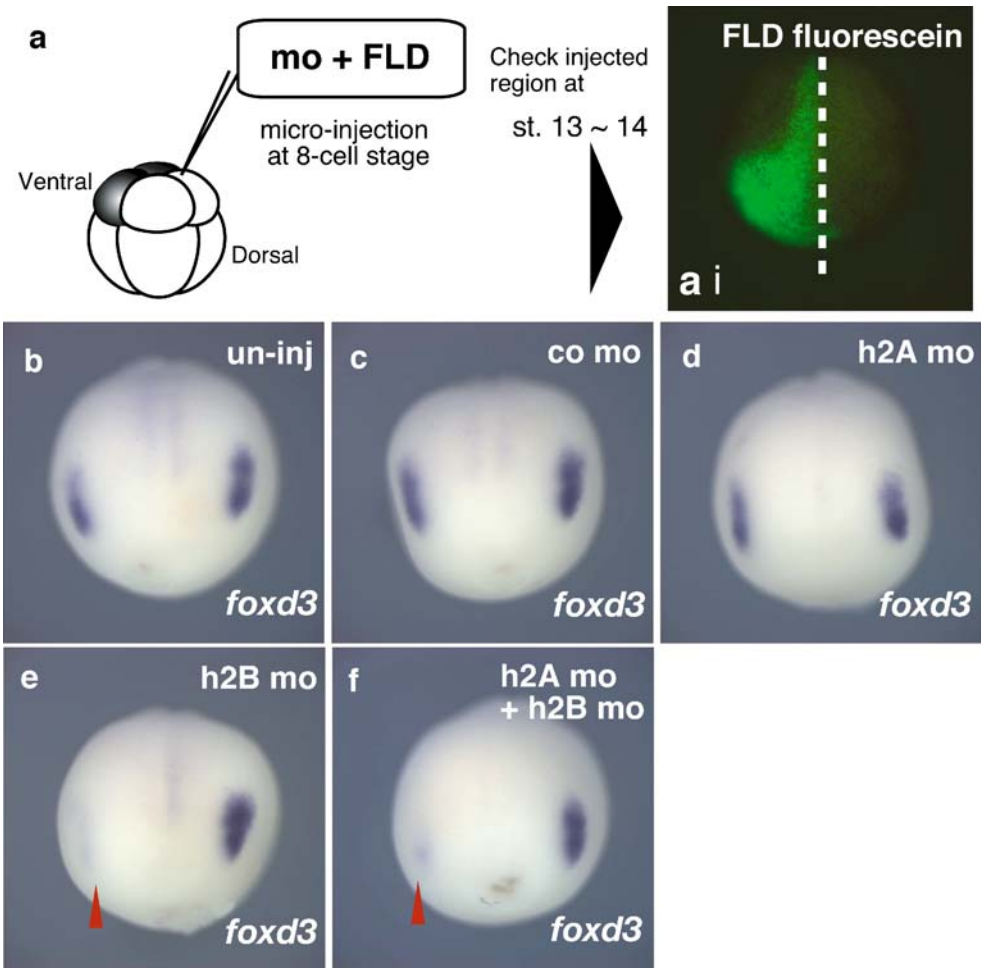

Fig. 1 Functional analysis of *Xhairy2a* and *Xhairy2b* in the neural crest by means of specific morpholinos. **a** Brief summary of experimental scheme. Morpholino and fluorescein dextran amine (FLD) were unilaterally (*right side*) coinjected into one dorsal animal blastomere at the eight-cell stage. Injected regions were checked with FLD fluorescein at stage 13 (early neurula stage), and only correctly injected embryos as shown in **a** *i* were fixed, followed by WISH with *foxd3*. **a** *i* Dorsal view with anterior side down. **b**–**f** WISH results of *foxd3* (anterior–dorsal view). The injected morpholino is indicated at the *upper right corner of each panel* (*un-inj* indicates uninjected control; *h2A mo+ h2B mo* indicates coinjection of h2A mo and h2B mo). The probe is indicated at the *lower right corner of each panel*. *Red arrowheads* indicate repression of *foxd3* expression on the injected side. The dose of morpholinos was as follows: co mo, 3.4 ng; h2A mo, 3.4 ng; h2B mo, 3.5 ng

Specificity of the morpholinos was examined elsewhere with rescue experiments (Nagatomo and Hashimoto 2007).

We previously showed that *Xhairy2b* was strongly expressed in the neural crest (Tsuji et al. 2003). When we knocked down both alleles with a combination of h2A mo and h2B mo in the neural crest (Fig. 1a) and checked the effects on marker gene *foxd3* expression (Fig. 1b; Sasai et al. 2001) by means of WISH, the functional inhibition was found to result in the strong repression of *foxd3* expression at the early neurula stage (in 69%, n=45, Fig. 1f; see also Nagatomo and Hashimoto 2007). It is interesting to note that when we compared the single knockdown of either *Xhairy2a* or *Xhairy2b* to each other, the results were different. Although *Xhairy2b* single knockdown showed repressive effects on *foxd3* expression (in 55%, n=40, Fig. 1e), *Xhairy2a* single knockdown showed virtually no effects on *foxd3* expression (repression in 20%, n=39; Fig. 1d). The control morpholino showed almost no effect, as expected (repression in 14%, n=37, Fig. 1c). These results strongly suggest that *Xhairy2a* and *Xhairy2b* were different in some aspects in the neural crest.

A previous loss-of-function study of the floor plate expression of *Xhairy2a* (Lopez et al. 2005) showed that *Xhairy2a* positively regulated floor plate development. To further investigate the possible differences between *Xhairy2a* and *Xhairy2b*, we next examined their functions in the floor plate where we reported that *Xhairy2b* was expressed from the mid-gastrula stage (Tsuji et al. 2003). For the analyses of the floor plate, we inhibited functions of either *Xhairy2a* or *Xhairy2b* or both, with specific morpholinos (Fig. 2a) and examined the expression of the marker gene *shh* (Fig. 2b; Ekker et al. 1995) by WISH. Samples were cleared for quantification of data. The results showed that single knockdown of either *Xhairy2a* (repression in 35%, n=20, Fig. 2d) or *Xhairy2b* (repression in 60%, n=20, Fig. 2e) caused remarkable reduction in *shh* expression, whereas the control morpholinos did not show recognizable changes (repression in 5%, n=20, Fig. 2c). As expected, the double knockdown of *Xhairy2a* and *Xhairy2b* showed a stronger penetrance of the phenotype (*shh* repression in 85%, n=20, Fig. 2f; see also Fig. 2g for comparison) than the single knockdown. Because *shh* is expressed in both the notochord and the overlying floor plate in early neurulae, we checked if the morphants lost the expression of *shh* in the floor plate by transverse sectioning. By doing so, we confirmed that the expression of *shh* was indeed repressed in the floor plate but not in the notochord (Figs. 2b′–f′). Taken together, we concluded that *Xhairy2a* and *Xhairy2b* seemed to behave in a similar way in the floor plate expression of *shh*.

We previously showed that *Xhairy2b* was expressed in the anterior part of the prechordal plate and that the inhibition of *Xhairy2b* function in this tissue resulted in anterior expansion of *chd* expression (Yamaguti et al. 2005). Based on this finding, we tried to see if the single knockdown of *Xhairy2a* or the double knockdown of both *Xhairy2a* and *Xhairy2b* caused an anterior expansion of *chd* expression. Embryos that were correctly injected with morpholinos (Fig. 3a) were stained with *chd* and cleared for quantification, and the mid-sagittal sections were compared for confirmation. The results demonstrated that *Xhairy2b* morphants (expanded in 42%, n=19; Fig. 3e) and the double morphants (expanded in 50%, n=14; Fig. 3f) indeed showed anterior expansion of *chd* expression, compared with the uninjected (Fig. 3b) or control-morpholino-injected (expanded in 5%, n=20; Fig. 3c) embryos. In addition, *Xhairy2a* morphants showed expansion of *chd* expression (expanded in 50%, n=20; Fig. 3d) in a similar manner to that of *Xhairy2b* morphants. In this paper, the sectioned embryos shown in Fig. 3 look different with each other in terms of the morphology, possibly because of slight differences in developmental stages or sectioning conditions. However, we double-checked the results by observing the cleared embryos plenty enough to confirm that *chd* expression indeed expanded anteriorly in *Xhairy2* morphants. Therefore, these results suggest that both *Xhairy2a* and *Xhairy2b* were needed to regulate *chd* expression in the anterior part of the prechordal plate.

Differences between *Xhairy2a* and *Xhairy2b*: expression level and spatio-temporal expression pattern

How are *Xhairy2a* and *Xhairy2b* different from each other in the neural crest, and why do they seem to be similar in the floor plate and the prechordal plate? The amino acid sequences of Xhairy2a and Xhairy2b show 92.8% identity with each other (Tsuji et al. 2003). In particular, the sequences of critical domains that serve as a transcriptional repressor, such as the DNA-binding domain and the corepressor-binding motif, are completely identical. For confirmation, Xhairy2a and Xhairy2b, as transcription factors, were compared by means of the luciferase reporter assay. The result clearly showed that Xhairy2a and Xhairy2b were virtually identical in terms of transcriptional regulation via the target sequences (Fig. S2), suggesting that the differences between *Xhairy2a* and *Xhairy2b* seen in the neural crest might be attributed to their expression level and/or expression pattern.

We therefore examined the temporal expression patterns and expression levels of *Xhairy2a* and *Xhairy2b* to explain the differences and similarities observed in the knockdown experiments. We previously showed the temporal expression pattern of *Xhairy2b* by Northern blot analysis (Tsuji et al. 2003). However, we found that the probe cross-hybridized with *Xhairy2a* (Fig. S3). Accordingly, we adopted RT-PCR analysis to distinguish the individual

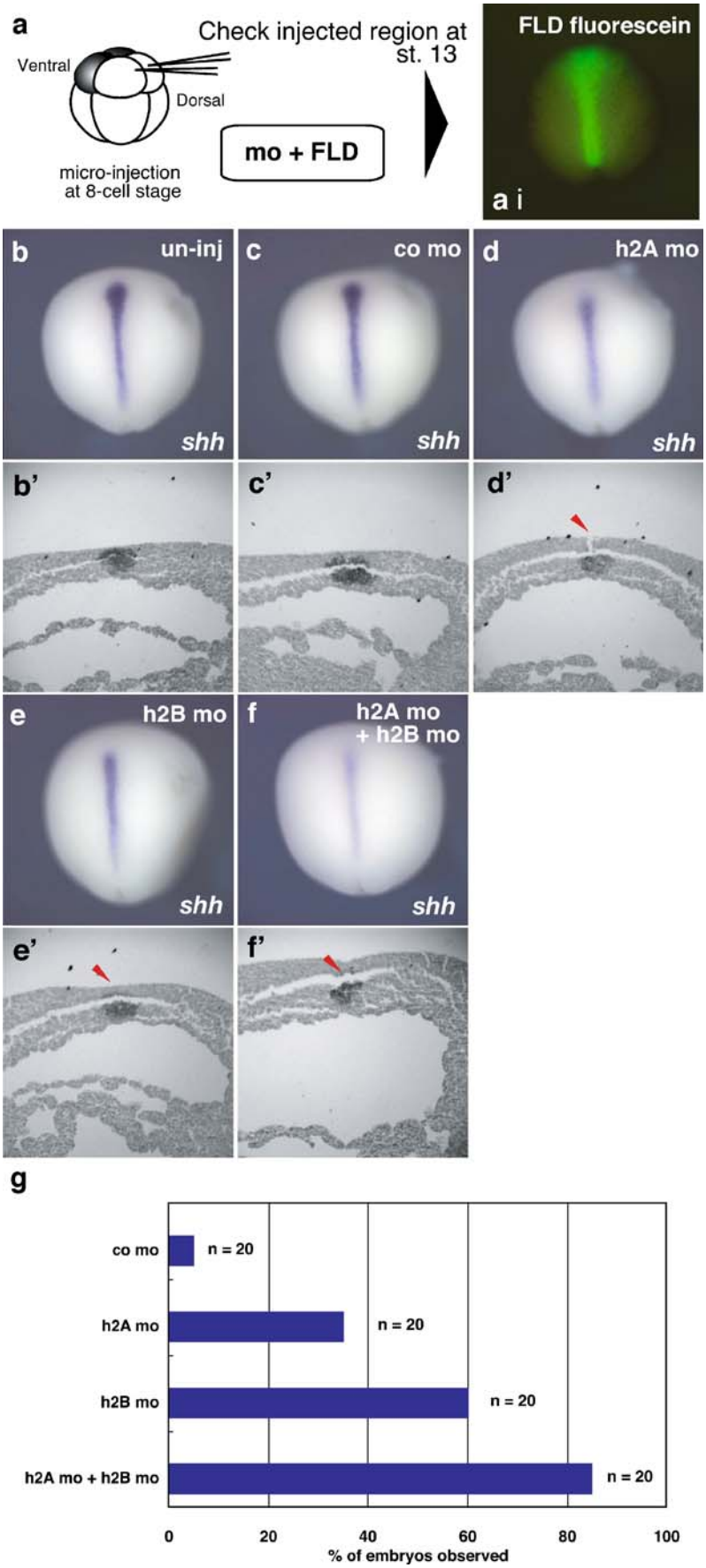

Fig. 2 Functional analysis of *Xhairy2a* and *Xhairy2b* in the floor plate by means of specific morpholinos. **a** Brief summary of experimental scheme. Morpholino and FLD were bilaterally coinjected into the dorsal marginal zone at the eight-cell stage. Injected regions were checked with FLD fluorescein at stage 13 (early neurula stage), and only correctly injected embryos as shown in **a** *i* were fixed, followed by WISH with *shh*. **a** *i* Dorsal view with anterior side up. **b–f** WISH results of *shh* (dorsal view with anterior side up). The injected morpholino is indicated at the *upper right corner of each panel*. The probe is indicated at the *lower right corner of each panel*. **b′–f′** Transverse sections of corresponding samples. In controls **b′** and **c′**, both the floor plate (outside expression) and the notochord (inside expression) were *shh* positive. However, in morphants **d′**, **e′**, and **f′**, the outside floor plate expression was repressed, as indicated by *red arrowheads*. **g** a summary graph showing repression of floor plate *shh* expression in *Xhairy2* morphants. Note that the double knockdown shows higher percentage of *shh* repression in the floor plate than the single knockdown. The dose of morpholinos was as follows: co mo, 6.8 ng; h2A mo, 6.8 ng; h2B mo, 6.9 ng

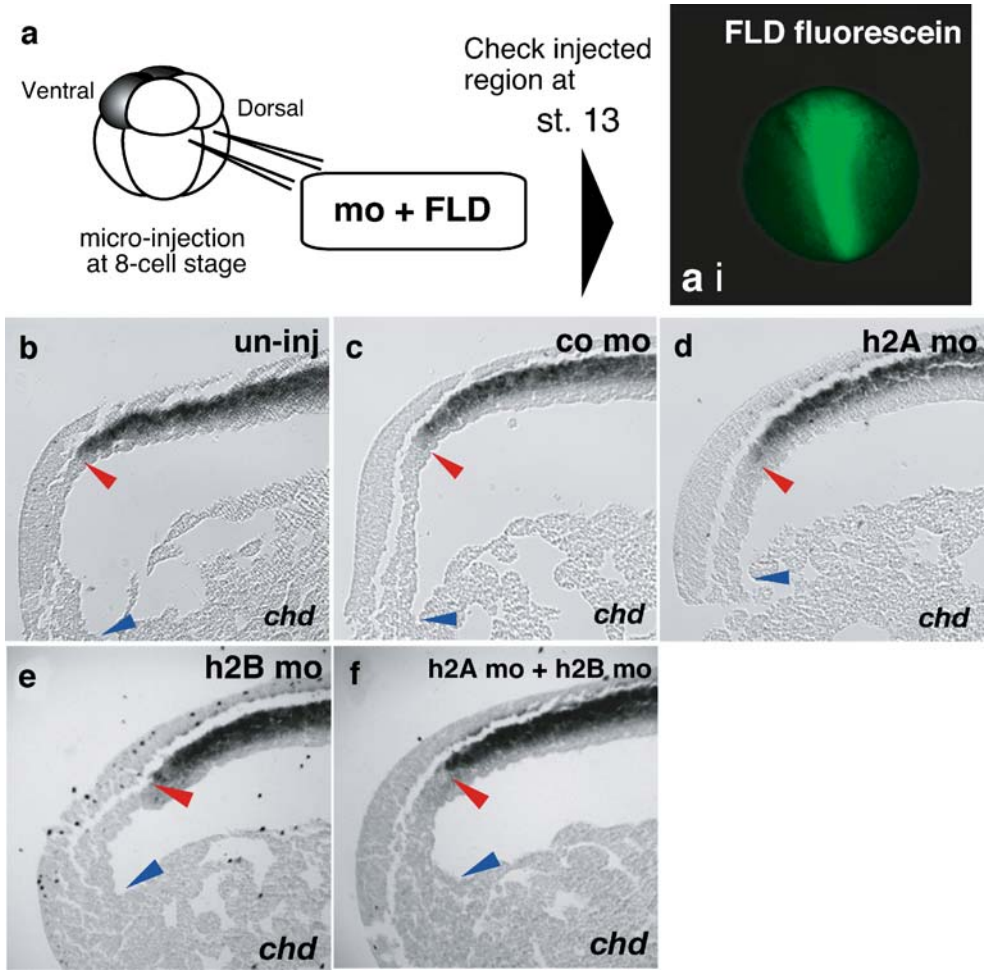

Fig. 3 Functional analysis of *Xhairy2a* and *Xhairy2b* in the prechordal plate by means of specific morpholinos. **a** Brief summary of experimental scheme. Morpholino and FLD were bilaterally coinjected into the deep dorsal marginal zone at the eight-cell stage. Injected regions were checked with FLD fluorescein at stage 13 (early neurula stage), and only correctly injected embryos as shown in **a** *i* were fixed, followed by WISH with *chd*. **a** *i* Dorsal view with anterior side up. **b**–**f** Midsagittal sections of samples stained with *chd* (dorsal side up with anterior side to the left). Because it was virtually impossible to detect the anterior expansion of *chd* expression by whole-mount view, samples were sagittally sectioned after WISH to facilitate analyses. The injected morpholino is indicated at the *lower left corner of each panel*. The probe is indicated at the *lower right corner of each panel*. *Red arrowheads* indicate the anterior limit of *chd* expression, while *blue arrowheads* indicate the anterior edge of archenteron. The distance between them in morphants **d**, **e**, and **f** was shorter than that of controls **b** and **c**, indicating anteriorward expansion of *chd* expression in morphants. The dose of morpholinos was as follows: co mo, 6.8 ng; h2A mo, 6.8 ng; h2B mo, 6.9 ng

expressions of *Xhairy2a* and *Xhairy2b* (Fig. 4a and its legend). The results obtained using a primer set that hybridizes with both *Xhairy2a* and *Xhairy2b* indicated that this method can be used in place of Northern blot analysis (Fig. 4b). Briefly, *Xhairy2* transcripts were maternally loaded and the expression after midblastula transition (MBT) was increased in a stepwise manner at the mid-gastrula stage. The overall expression level was high from neurula stage onward. It is interesting to note that we found that *Xhairy2a* was solely responsible for the maternal loading of *Xhairy2* transcripts and the expression level of *Xhairy2a* after MBT was lower than that of *Xhairy2b* (Fig. 4b and its legend). In contrast, the expression of *Xhairy2b* started after MBT and was maintained at a high level throughout the stages investigated (Fig. 4b). The difference in the zygotic expression level between *Xhairy2a* and *Xhairy2b* is consistent with the results of knockdown experiments in the neural crest (Fig. 1) in which the inhibition of *Xhairy2b* but not *Xhairy2a* affected marker gene expression.

However, the RT-PCR results do not explain the similarity between *Xhairy2a* and *Xhairy2b* in regulating *shh* and *chd* expression in the floor plate (Fig. 2) and the prechordal plate (Fig. 3), respectively. This indicates the need to strictly examine the spatial expression patterns of *Xhairy2a* and *Xhairy2b*. To address this issue, we designed two specific probes for part of the 5′ untranslated region where the sequence similarity between *Xhairy2a* and *Xhairy2b*

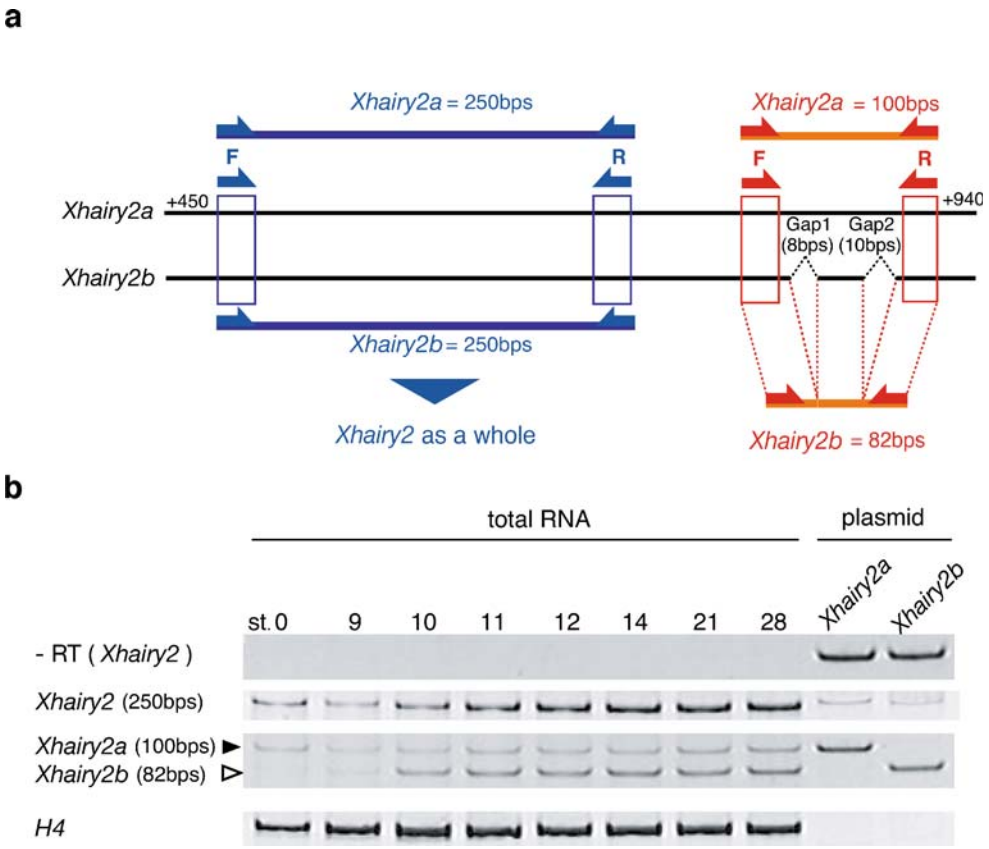

Fig. 4 Temporal expression patterns of *Xhairy2a* and *Xhairy2b* from egg to tail bud stage. **a** Summary of RT-PCR strategy. Use of the same primer set (shown as *red arrows*) resulted in different lengths of fragments (shown as *orange bars*) because of the presence or absence of two gaps (*Xhairy2a*: 100 bp, *Xhairy2b*: 82 bp). To view the temporal expression pattern of the *Xhairy2* gene as a whole, another primer set (shown as *dark blue arrows*) amplifying a 250-bp region (shown as *dark blue bars*) with 100% identity between the alleles without any gaps was used. This enabled us to directly compare the quantitative difference between *Xhairy2a* and *Xhairy2b* expression at each stage. **b** RT-PCR analysis showed that *Xhairy2a* was maternally loaded and was expressed zygotically at quite low levels, while *Xhairy2b* was a bona fide zygotic factor. *H4* served as loading control. Amplification with plasmids containing either *Xhairy2a* or *Xhairy2b* served as reference

was 61% (Fig. S3a). The similarity in sensitivity and specificity of these probes was carefully verified by both in vitro and in vivo experiments (see Fig. S3). As mentioned above, we found that the previously used full-length *Xhairy2b* probe cross-hybridized with *Xhairy2a* (Fig. S3b and d). Therefore, we redefined the two specific probes as *Xhairy2a* and *Xhairy2b* probes and the full-length *Xhairy2b* probe as the *Xhairy2* probe.

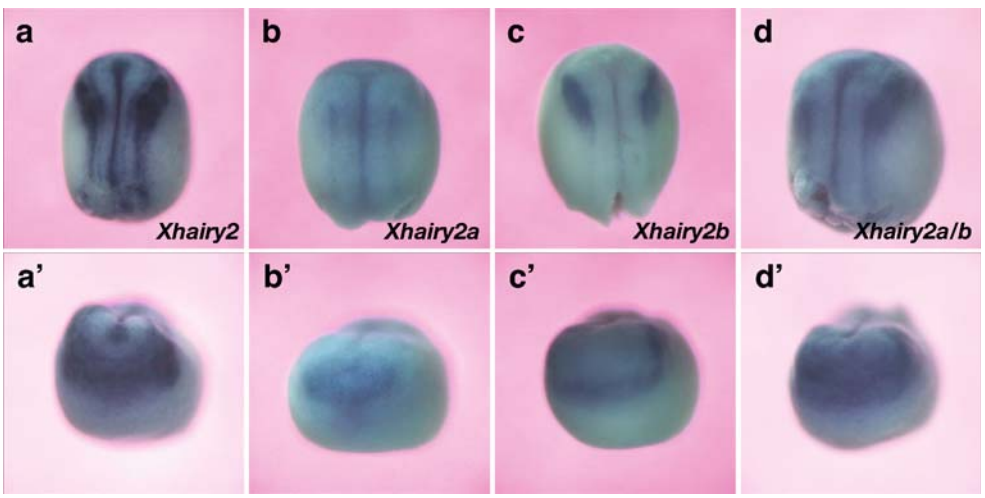

Fig. 5 Spatial expression patterns of *Xhairy2* genes at the mid-neurula stage. **a–d** Stage-15 embryos from the dorsal view with the anterior side up. Note that *Xhairy2b* is expressed at high levels in the neural crest region, while the floor plate expression of *Xhairy2a* and *Xhairy2b* appears to be at a similar level. **a′–d′** The same samples shown in **a**–**d** viewed from the anterior side. The probe is indicated at the *lower right corner of each panel* (*Xhairy2a/b* indicates simultaneous staining with *Xhairy2a* and *Xhairy2b*)

Using the specific probes, we investigated the spatial expression patterns of *Xhairy2a* and *Xhairy2b* at the late gastrula stage and early neurula stage. In late gastrulae, we could not detect any signals in both the future floor plate and the anterior prechordal plate (data not shown). In neurulae, the differences in expression between *Xhairy2a* and *Xhairy2b* were obvious (Figs. 5a–d, a′–d′). Ectodermal *Xhairy2* expression in neurulae was dominant in the presumptive neural crest, the anterior neural border, and the future floor plate (Figs. 5a and a′). Clearly, *Xhairy2b* was solely responsible for the expression in the neural crest (Figs. 5c and c′), while *Xhairy2a* and *Xhairy2b* were equally expressed in the floor plate region and the anterior neural border (Figs. 5b and b′). The remarkable difference in the expression level between *Xhairy2a* and *Xhairy2b* in the neural crest (Figs. 5b and c) is again in good agreement with the results of loss-of-function experiments (Fig. 1).

The fact that both *Xhairy2a* and *Xhairy2b* are expressed in the floor plate explained our findings that both *Xhairy2a* and *Xhairy2b* are required in the floor plate when examined for *shh* expression (Fig. 2). Because the overall expression level of *Xhairy2a* is lower than that of *Xhairy2b* at the early neurula stage, the individual WISH as well as the knockdown results might suggest that the floor plate *shh* expression requires Xhairy2 proteins in a dose-dependent manner. In terms of expression in the anterior prechordal plate, again, we could not see any signals when stained with short specific probes (data not shown). To underpin the expression of both *Xhairy2a* and *Xhairy2b* in the anterior prechordal plate as suggested in the knockdown experiments (Fig. 3), technical improvement of signal amplification is required in future studies. As an extra finding, *Xhairy2* expression in the anterior neural border (Fig. 5a′) was subdivided into two domains, each of which was individually marked by *Xhairy2a* (inside expression: Fig. 5b′) and *Xhairy2b* (surrounding expression: Fig. 5c′; see also Fig. 5d′ where the combination of *Xhairy2a* and *Xhairy2b* reconstituted the original *Xhairy2* expression shown in Fig. 5a′). Functions of *Xhairy2* in this region are currently unknown and are under investigation.

Significance of extra copies of a gene in development

Recently, the expression and functions of the *Xfoxi1* gene were investigated, and two copies of this gene were expressed in a mutually indistinguishable manner so that specific knockdown with morpholinos produced similar results (Matsuo-Takasaki et al. 2005). Although not extensively investigated, it is predicted that the two allelic versions of a gene in the pseudotetraploid species *X. laevis* would function in a similar manner. Our results, however, indicate that this is not the case with the *Xhairy2* gene. The *Xhairy2b*allele might compensate for the low expression of the *Xhairy2a* allele after MBT in certain tissues, for instance, in the neural crest, as shown in this study. At the same time, the absence of *Xhairy2b*transcripts in the eggs might be compensated by loading *Xhairy2a*. Therefore, our study could be a clear example of subfunctionalization (e.g., Prince and Pickett 2002; Lynch and Katju 2004) predicted from the DDC model (Force et al. 1999). From this perspective, our finding that both *Xhairy2a* and *Xhairy2b* are required for the floor plate expression of *shh* is interesting because it implies that neither degeneration nor complementation occurred in *shh* regulation. This could be a good subject of studies on gene dosage in pseudotetraploid *X. laevis*.

In support of our findings, a couple of studies revealed differences in temporal expression pattern between two allelic versions of a gene in *X. laevis*. Of the two copies of the *XMyoD* gene, *XMyoDa* is maternally loaded and zygotically expressed from MBT onward, while *XMyoDb* appears to be only maternally loaded (e.g., Harvey 1990). In addition, a recent study on *vg1* whose messenger RNA is maternally localized and critical to mesoderm induction revealed that the knockdown of one allele had no effect at all, although inhibition of the other allele severely disrupted mesoderm induction, suggesting that only one allele of *vg1* is maternally loaded (Birsoy et al. 2006).

Conclusion

This study provides evidence that *Xhairy2a* and *Xhairy2b* are different in terms of expression level and expression pattern. These differences are in good agreement with the results of individual knockdown of either *Xhairy2a* or *Xhairy2b* in the neural crest and the floor plate. This study therefore supports the DDC model in pseudotetraploid *X. laevis*. Together with hitherto unknown functions of *Xhairy2a* and *Xhairy2b*, for instance, how *Xhairy2a* functions as a maternal factor, the comparison of *Xhairy2* functions with those of orthologues in other organisms and the elucidation of their differences in detail will provide further insights into the evolutional and developmental significance of duplicated genes. From this perspective, the differences between *Xhairy2* alloalleles are interesting in that they could relate the process of evolution to the mechanisms of development.

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