RESEARCH PAPER

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Incomplete splicing of neutrophil-specific genes affects neutrophil development in a zebrafish model of poikiloderma with neutropenia

Prakash Patil, Tamayo Uechi, and Naoya Kenmochi*

Frontier Science Research Center; University of Miyazaki; Miyazaki, Japan

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Abbreviations: CHT, caudal haematopoietic tissue; CRISPR, clustered regularly interspaced short palindromic repeats; GFP, green fluorescent protein; hpf, hours post fertilization; MO, morpholino antisense oligo; MPN1, mutated in poikiloderma with neutropenia; PN; poikiloderma with neutropenia; snRNPs; small nuclear ribonucleoproteins; sqRT-PCR; semi-quantitative reverse transcription and polymerase chain reaction; USB1, U Six Biogenesis 1

Poikiloderma with neutropenia (PN) is a rare inherited disorder characterized by poikiloderma, facial dysmorphism, pachyonychia, short stature and neutropenia. The molecular testing of PN patients has identified mutations in the *C16orf57* gene, which encodes a protein referred to as USB1 (U Six Biogenesis 1). In this study, we developed a zebrafish model of PN by the microinjection of morpholino antisense oligos to suppress *usb1* gene function. Severe morphological defects, including a bent tail, thin yolk extension and reduced body length, were predominant in the Usb1-suppressed embryos (morphants). We also observed significantly decreased number of neutrophils in the morphants by Sudan Black staining. Interestingly, the splicing of genes involved in neutrophil differentiation and development, such as *mpx*, *ncf1*, *ela3l* and *npsn,* was aberrant in the morphants. However, the splicing of haematopoietic precursors and erythroid-specific genes was unaltered. Importantly, the neutrophil defects were almost completely rescued by co-injection of *ela3l* mRNA, the most markedly affected gene in the morphants. Our study demonstrated a possible role of USB1 in modulating the tissue-specific gene splicing that eventually leads to the impaired development of neutrophils. This zebrafish model could serve as a valuable tool to investigate the causative role of USB1 in PN pathogenesis.

Introduction

Clericuzio-type poikiloderma with neutropenia (PN) (OMIM# 604173) is a unique genodermatosis that was first observed in the Navajo Indian population.[1] It is characterized by poikiloderma together with an erythematous rash on the limbs and face, nail abnormalities, short stature, palmoplantar hyperkeratosis, permanent neutropenia and skeletal defects.[1,2] PN patients are eventually susceptible to myelodysplastic syndrome and acute myeloid leukemia.[1] A recent report on PN also observed an association with squamous cell carcinoma of the skin.[3] The actual number of reported patients is quite limited, mostly because several PN patients have many similar clinical manifestations with dyskeratosis congenita and Rothmund-Thomson syndrome.[4,5] To date, 40 patients with PN have been reported,[6-16] containing 19 different mutations in the *C16orf57* gene that encodes a 265-amino-acid protein, referred to as USB1 (U Six Biogenesis 1).[17,18] In some studies, this protein has also been referred to as MPN1 (Mutated in Poikiloderma with Neutropenia).[19,20] Several types of mutations, including nonsense, deletion/frame-shift and splice site alterations, have been identified in PN patients (**Fig. S1A**).

A high-resolution (1.1 Å) crystal structure categorizes human USB1 as a member of the LigT-like superfamily of 2H phosphodiesterases, which is found in the bacteria, archaea and eukarya. In addition, it was demonstrated *in vitro* that human USB1 is a novel 3′-5′ exoribonuclease that removes terminal oligo(U) and oligo(A) tails at the 3′ end of U6 snRNA and generates the 3′ terminal phosphate modification.[21] Deep-sequencing analysis of U6 snRNA from the PN patient lymphoblasts revealed the non-templated addition of more than 2 adenosine nucleotides to the 3′ end of U6, although no significant difference in the steady-state levels of U6 snRNA was observed.[21] Interestingly, aberrant 3′ end processing of U6 was observed in the USB1-deficient fission yeasts and the human cells derived from PN patients. In human cells, the steady-state levels of U6 were not affected by the diminished USB1 activity, but in yeasts, the cellular U6 levels were greatly reduced, leading to precursor mRNA (pre-mRNA) splicing defects.[18,19] Furthermore, the deep sequencing of the poly(A)$^+$ transcriptome of the

*Correspondence to: Naoya Kenmochi; Email: kenmochi@med.miyazaki-u.ac.jp
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PN-patient cells revealed that the pre-mRNA splicing was normal.[19,21]

These *in vitro* and *in vivo* studies suggest that PN manifestations are not derived from common pre-mRNA splicing defects. Instead, they might have resulted from incomplete splicing of the genes expressed in the tissues that are highly affected by the disease, such as neutrophil precursors. To investigate this possibility, we need an animal model that enables us to explore new insight into the molecular pathogenesis of PN. In the last decade, zebrafish (*Danio rerio*) has been used extensively as a model organism to study vertebrate hematopoiesis owing to its genetic tractability and the embryo transparency that allows simple live-imaging.[22,23] In this study, we developed a zebrafish model of PN using a morpholino antisense oligo (MO)-based loss-of-function approach. We observed acute morphological abnormalities and neutrophil reduction in the Usb1-deficient embryos (morphants). In addition, we found that the splicing of neutrophil-specific genes was altered by the *usb1* knockdown. Interestingly, the neutrophil defects were almost completely rescued in *ela3l* mRNA coinjected embryos. Taken together, our results suggest the existence of a regulatory mechanism by which USB1 modulates the splicing of tissue-specific genes and ultimately leads to abnormal tissue development. This study provides a prime model for PN to unravel its pathogenic mechanism and the role of USB1 in disease development.

Results

Zebrafish *usb1* gene

USB1, a mutated gene in PN, is highly conserved during evolution. A BLAST search of the zebrafish genome with the human *USB1* coding sequence identified a single homologous gene encoding a 276-amino-acid protein (**Fig. 1A**). The coding region of this gene shares 57% nucleotide and 46% amino acid identity with its human ortholog. Despite the low amino acid identity, the alignment of coding nucleotides revealed that 15 of the 19 mutated nucleotides that have been identified in PN patients are conserved between human *USB1* and zebrafish *usb1* (**Fig. S1A**). The comparisons of the exon lengths and the exon-intron structure of zebrafish *usb1* with those of human *USB1* indicated a high degree of cross-species conservation. The zebrafish *usb1* gene possesses 7 exons and is located on chromosome 25 in a region of shared synteny with the human *USB1* gene that is located on chromosome 16 (**Fig. 1A**, **S1B**).

To determine *usb1* expression during zebrafish development, we collected array data from the Gene Expression Omnibus derived from whole embryos, retina and trunk and tail muscle at various stages of the embryonic development (1.5–120 hpf, hours post fertilization). We calculated the fold changes in *usb1* expression over β-*actin* and found a markedly low level of its expression in the early embryonic stage, peaking at 24 hpf followed by a gradual decrease after 52 hpf (**Fig. S2A**). To verify this expression pattern, we examined the total RNA from wild type embryos at various stages (3–120 hpf) using semi-quantitative reverse transcription and polymerase chain reaction (sqRT-PCR) and confirmed the similar trend of *usb1* expression during zebrafish development (**Fig. S2B**).

Severe developmental defects in Usb1-deficient zebrafish

To investigate the role of Usb1 function in zebrafish development, we knocked down *usb1* gene using MOs and analyzed the morphological status of the morphants at various stages of development (**Fig. 1A**). Injection of the MO^{sp} that targets the 3′-splice site of the second intron altered the splicing and resulted in the exclusion of exon 3 from the mature transcript. In addition, a 50–60% reduction in the expression of *usb1* mature transcript was observed in Usb1-suppressed embryos (**Fig. 1B**). The excision of exon 3 in this aberrant transcript was confirmed by DNA sequencing. These results indicate that the injection of MO^{sp} effectively impeded *usb1* expression in zebrafish.

We compared the morphological features of the MO^{sp} and MO^{aug}-injected embryos with wild-type embryos at 25 hpf, a stage when many organs are almost recognizable, and found that the Usb1-deficient embryos displayed abnormal phenotypes, including a thin yolk extension, a bent tail and reduced body length. We also observed the development of other anomalies at 50 hpf in morphants, such as a significantly smaller head and eyes and a defective heart with pericardial edema (**Fig. 2**). In addition, we found the morphological abnormalities of MO^{sp}-injected embryos were highly similar to those observed in MO^{aug}-injected embryos. The severities of morphological abnormalities were proportional to the MO concentrations (**Fig. S3**). All the morphants died within a week. Co-injection of MO-resistant *usb1* mRNA rescued these developmental defects in the morphants, indicating that the altered phenotypes in the morphants were specifically due to a loss of Usb1 (**Fig. 2**).

Impaired neutrophil development in Usb1-deficient zebrafish

PN is generally associated with characteristic features of poikiloderma and constant neutropenia in all patients.[3] To determine the effects of *usb1* knockdown on neutrophil development, we injected the MOs into one-cell-stage Tg (*mpx*:GFP)^{uwm1} zebrafish embryos in which neutrophils are labeled by a green fluorescent protein (GFP) and analyzed the phenotypes. At 50 hpf, we observed a marked reduction of the green-fluoresce neutrophils in the MO-injected embryos, especially in the caudal haematopoietic tissue (CHT) that is formed around the transient caudal vein plexus where larval neutrophil development takes place (**Fig. 3A**, illustration). We also performed whole-mount Sudan Black (SB) staining that delineates the neutrophils throughout the embryo and counted the neutrophils in the CHT region (**Fig. 3A**). At 50 hpf, we found a substantial decrease in the population of neutrophils, especially in the CHT region of Usb1-deficient embryos (**Fig. 3**). The neutrophil number returned to near normal in the MO-resistant *usb1* mRNA-coinjected embryos (**Fig. 3B**). These results clearly indicate that the development of neutrophils was severely affected by the *usb1* knockdown in zebrafish.

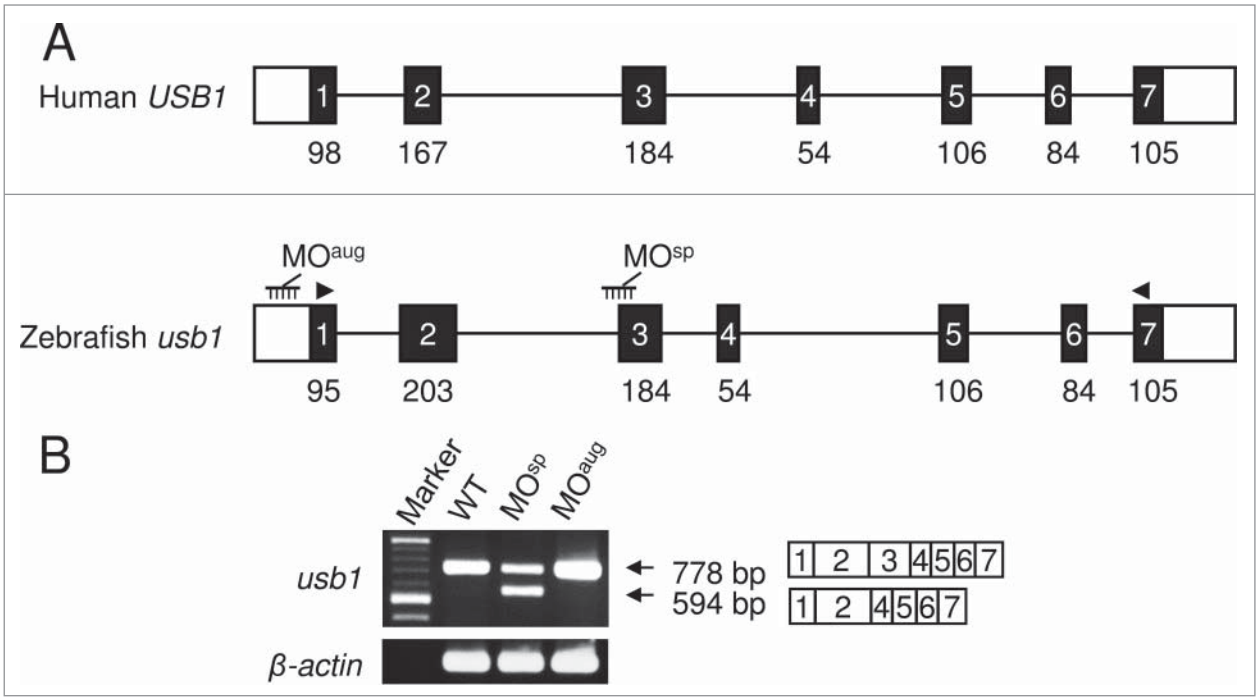

Figure 1. Schematics of human *USB1* and zebrafish *usb1* gene structures and RT-PCR analysis. (**A**) A diagrammatic representation of zebrafish *usb1* and human *USB1* genomic structure. The black and white boxes represent translated and untranslated regions of the exons, respectively. The black lines represent the introns. The numbers below the black bars indicate the exon length (bp). The MOs were designed at the splice site (MO^{sp}) or the translation initiation site (MO^{aug}) to prevent pre-mRNA splicing or to block the translation of *usb1*. The morpholino binding sites are shown in black combs. The arrowheads indicate the primer binding sites for RT–PCR. The human *USB1* and zebrafish *usb1* genomic sequences were obtained from the database under the accession numbers NM_024598.3 and NM_001003460.1, respectively. (**B**) sqRT-PCR analysis of *usb1* and β-*actin* (control) in MO-injected and wild-type embryos. The decreased expression of the full length transcript (778 bp) and the expression of smaller transcript (594 bp) without exon 3 were observed in the MO^{sp} injected embryos. The injection of MO^{aug} had no effect on the splicing. β-*actin* served as a control.

Altered splicing of neutrophil-specific genes in Usb1-deficient zebrafish

It was found recently that USB1 deficiency did not affect either the steady-state levels of U6 snRNA or the general pre-mRNA splicing in PN patients.[21] By contrast, *USB1* deletion in yeast cells showed lower levels of cellular U6, leading to general pre-mRNA splicing defects.[19] To investigate the effects of *usb1* knockdown on the pre-mRNA splicing in zebrafish, we examined the total RNA by sqRT-PCR and analyzed the expression of the genes involving the differentiation and development of neutrophils. We observed aberrant transcripts in the neutrophil-specific genes,[24,25] including *mpx*, *ncf1*, *ela3l* and *npsn*, in the morphants (**Fig. 4A**). The DNA sequencing of these aberrant transcripts confirmed the intron retention in all these genes, suggesting the abnormal regulation of splicing in specific tissues of the morphants.

Furthermore, we analyzed the expression of randomly selected genes involving primitive and definitive hematopoiesis (**Fig. S4**) to establish whether the abnormal splicing activity is restricted to specific tissues in the morphants. The expression of genes involving primitive hematopoiesis,[24-26] such as *gata1a*, *pu.1*, *c-myb*, *gata2a*, *nfe2* and *scl*, were normal in the morphants (**Fig. 4B**). Similarly, *usb1* knockdown had no effect on the expression of erythroid- or myeloid-specific genes[24-26] (except neutrophils) including *alas2*, *band-3* and *L-plastin* (**Fig. 4C**). In addition, we examined the total RNA by northern blot analysis and found no difference in the U6 snRNA levels between wild-type and Usb1-deficient embryos (**Fig. S5**). These results suggest that the aberrant pre-mRNA splicing of neutrophil-specific genes induces neutropenia during zebrafish development.

Rescuing neutrophil defects by co-injection of *ela3l* mRNA in Usb1-deficient zebrafish

We observed that the Usb1 deficiency in zebrafish lead to defective neutrophil development and altered splicing of neutrophil-specific genes. To determine whether co-injection of mRNA of neutrophil-specific genes could rescue the neutrophil reduction in Usb1-deficient embryos, we injected *ela3l* mRNA, the most markedly affected neutrophil-specific gene in the morphants, with *usb1* MO into the embryos. We observed a substantial recovery of the neutrophil defects in the *ela3l* mRNA-coinjected embryos, as evidenced by the significant increase in the population of neutrophils in the CHT region (**Fig. 5A**). The recovery was observed in >50% of Usb1-deficient embryos (**Fig. 5B**, **S6**). These results suggest that the decreased neutrophils in the Usb1-deficient embryos were derived from the aberrant splicing of neutrophil-specific genes in zebrafish.

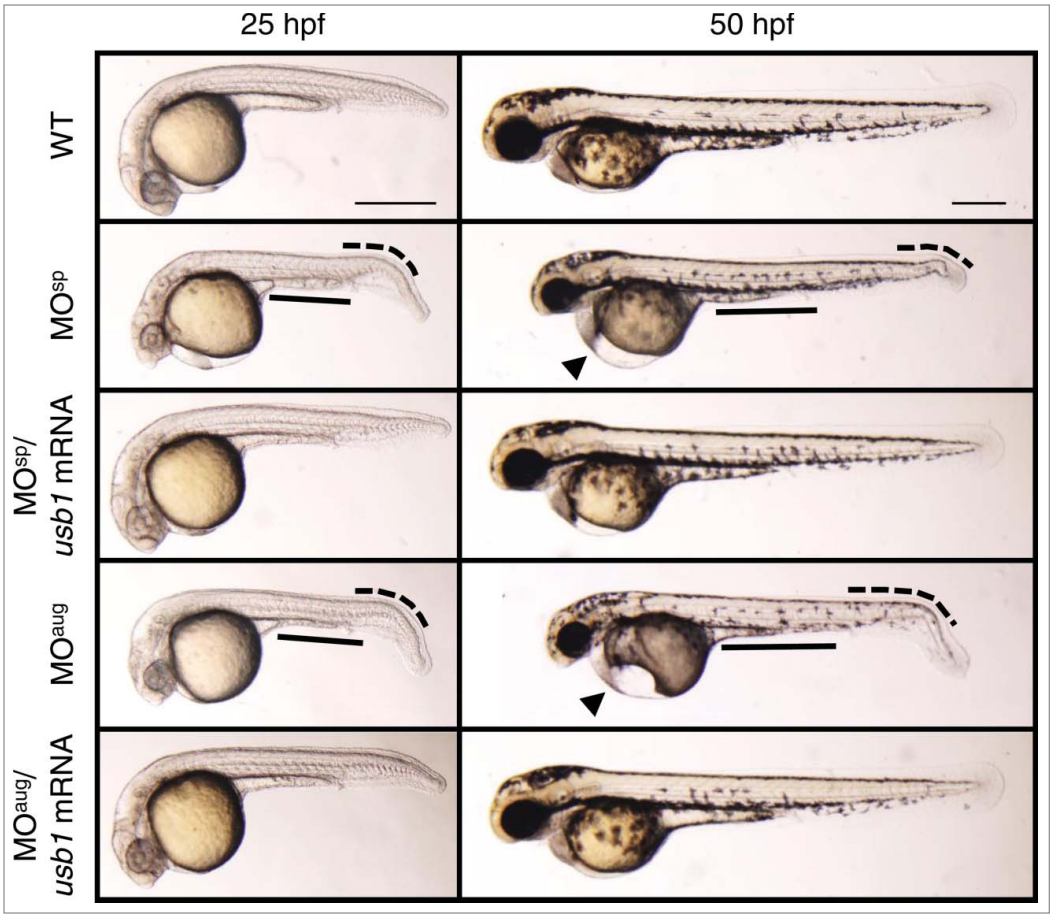

Figure 2. Morphological defects in Usb1-deficient zebrafish. Lateral views of wild-type, MO^{sp} (10 μg/μl) and MO^{aug} (5 μg/μl) injected embryos at 25 and 50 hpf are shown. The morphants displayed a thin yolk extension (black solid line), reduced body length and a bent tail (black dotted curved line) at 25 hpf. The morphants also showed small eyes and pericardial edema (black triangle) at 50 hpf. The co-injection of *usb1* mRNA with MOs nearly completely rescued these morphological abnormalities. Scale bars: 200 μm.

Discussion

In this study, we reported the development of an *in vivo* model of PN by morpholino-mediated *usb1* knockdown. We showed that the loss of *usb1* gene function in zebrafish resulted in a marked reduction in neutrophils and abnormal phenotypes that partially recapitulate the PN defects.[1] Furthermore, we established that the neutrophil defects were derived from the incomplete splicing of neutrophil-specific genes in the morphants. Consistently, the splicing was unaltered in haematopoietic precursors and erythroid-or-myeloid-specific genes (except neutrophils). Moreover, co-injection of *ela3l* mRNA, one of the most aberrantly spliced transcript in Usb1-deficient zebrafish, rescued neutrophil defects. Although we also observed partial rescue of morphological abnormalities, this might be due to the different roles of elastase in other tissues such as exocrine pancreas.[27-29] Overall, our results demonstrated that Usb1 deficiency in zebrafish recapitulates the PN phenotype with constant neutropenia that might have been derived from the similar mechanism of defective splicing of tissue-specific genes, as seen in Usb1-suppressed embryos. This presumption is due to the fact that haematopoietic differentiation and development in zebrafish closely resembles hematopoiesis in humans.[23,30] In addition, zebrafish is a suitable and powerful animal model system for studying various haematopoietic disorders,[31,32] primarily because the embryos can survive for several days, even in the absence of blood cells.[33] Although we focused on neutrophil defects in this study, the *usb1* gene is universally expressed in zebrafish at different stages of development, and when suppressed, embryos show severe morphological abnormalities. Owing to the several symptoms associated with PN, this zebrafish model is appropriate for studying the molecular mechanisms underlying its pathogenesis.

The regulation of pre-mRNA splicing events is crucial for correct haematopoietic lineage specification.[34,35] Nuclear pre-mRNA splicing is catalyzed by a multi-protein/RNA complex called the major spliceosome that consists of 5 small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5 and U6.[36,37] The spliceosome recognizes intron-exon boundaries and removes intervening introns via 2 transesterification reactions that result in the ligation of 2 adjacent exons.[38,39] It was recently disclosed that USB1 acts as 3′–5′ RNA exonuclease that trims the oligouridine tail of U6 snRNA and generates terminal 2′,3′-cyclic phosphate groups. The USB1 dysfunction resulted in the non-templated addition of 2 or more adenosine nucleotides to the 3′ end of U6 was established by the deep-sequencing analysis of U6 snRNA from PN patients. Despite the loss of USB1 activity, the steady state levels of U6 snRNA were unaffected in PN patients.[21] This might be attributed to the presence of multiple and dispersed U6 snRNA genes that have varied transcriptional efficiencies in humans.[40] Although we have not determined the 3′ end sequence of U6 snRNA in this study, instead we observed that the expression of U6 snRNA was not altered by the *usb1* knockdown in zebrafish. As zebrafish has multiple copies of U6

snRNA genes, we presume that zebrafish might also possess a similar mechanism of differential transcriptional efficiency that is present in humans. Although USB1 functions as a U6 biogenesis factor, how it controls target-specific pre-mRNA splicing in Usb1-deficient zebrafish remains to be fully explained.

Previous studies in zebrafish have shown that dysfunction of the splicing apparatus or the deficiency of splicing factors lead to tissue-specific alternative splicing that affects gene expression in specific organs during early embryogenesis. Loss of the *usp39* gene, a component of the RNA splicing machinery, leads to *rb1* mRNA splicing defects and pituitary lineage expansion in zebrafish.[41] Deficiency of the zebrafish *rbfox* genes disrupts splicing regulatory proteins that regulate muscle-specific alternative splicing, which is essential for proper differentiation and function of vertebrate muscle.[42,43] In addition, tri-snRNP dysfunction, by silencing the systemic splicing factors *prpf31* and *prpf4*, led to specific defects in retinal gene expression in a zebrafish model of retinitis pigmentosa, a hereditary eye disease that causes blindness due to a progressive loss of photoreceptors in the retina.[44] *In vivo* mutation of pre-mRNA processing factor 8 (Prpf8) in an ENU-induced zebrafish mutant, *Cephalophonus*, led to accumulation of aberrantly spliced transcripts retaining both U2- and U12-type introns that cause impaired myeloid differentiation in zebrafish.[45]

In summary, our results suggest that Usb1 deficiency in zebrafish affects the splicing of specific transcripts that elicit defects in specific tissues, such as neutrophils. However, understanding how the USB1 protein recognizes the tissue-specific transcripts during pre-mRNA splicing, is of special importance in the context of several symptoms associated with PN disease. A genome-wide RNA-Seq analysis in mutant zebrafish revealed a large set of specific target genes that changed their alternative splicing patterns in the absence of the U1C

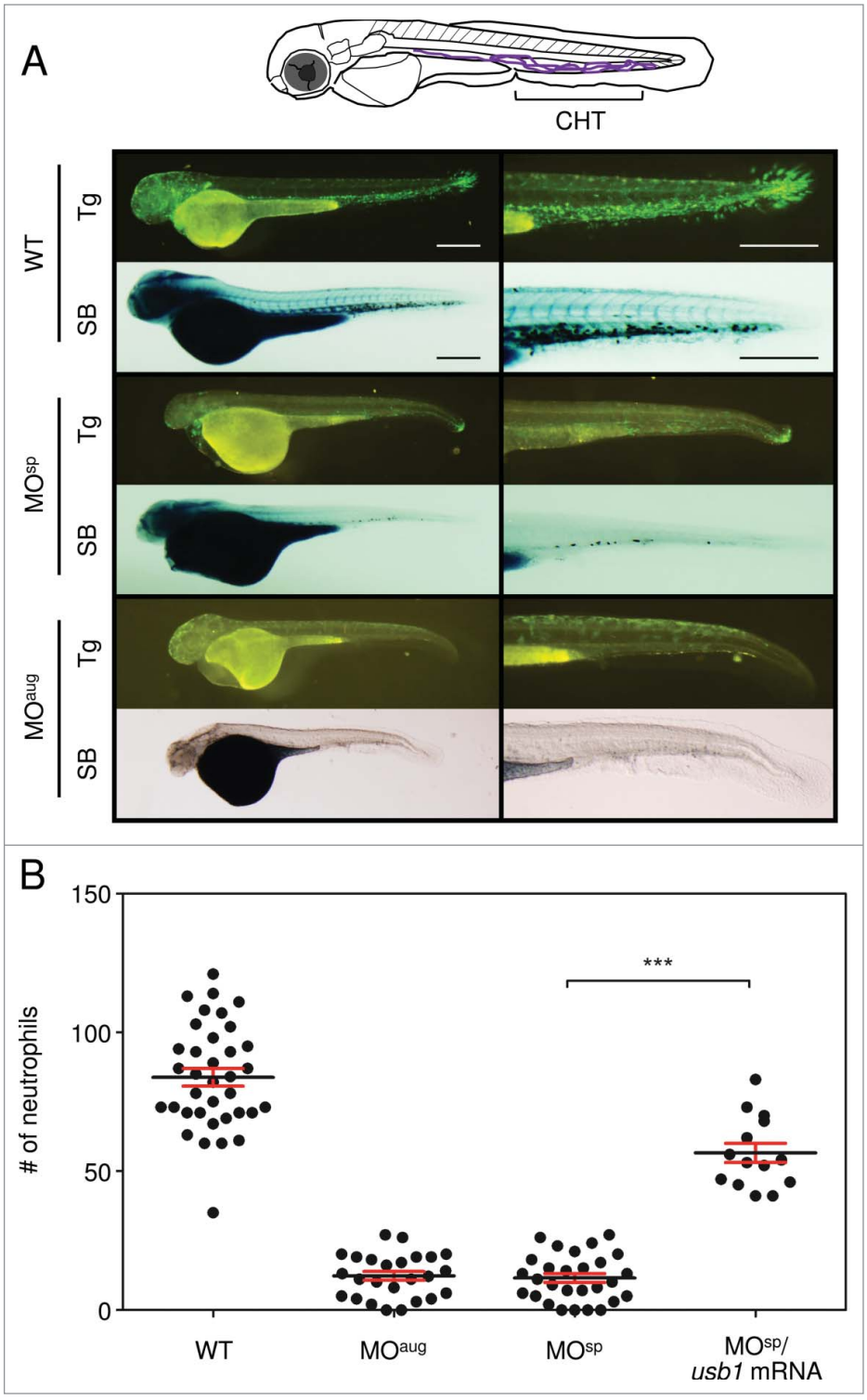

Figure 3. Neutrophil reduction in Usb1-deficient zebrafish. (**A**) Lateral images of the *usb1* MO-injected Tg (*mpx:GFP*) zebrafish and the whole-mount Sudan Black staining at 50 hpf and close-up images of CHT region. Substantial decreases of green-fluoresce and SB-stained neutrophils, especially in the CHT region, were observed in the Usb1-deficient embryos. Scale bars: 200 μm. (**B**) Scatter plot showing the mean number of neutrophils at the CHT region in wild type (WT) and morphants at 50 hpf. The number of neutrophils was significantly reduced in the morphants. Co-injection of *usb1* mRNA with MO^{sp} returned the neutrophils to near normal. ***$P < 0.001$ (one way ANOVA with Dunn's multiple comparison test).

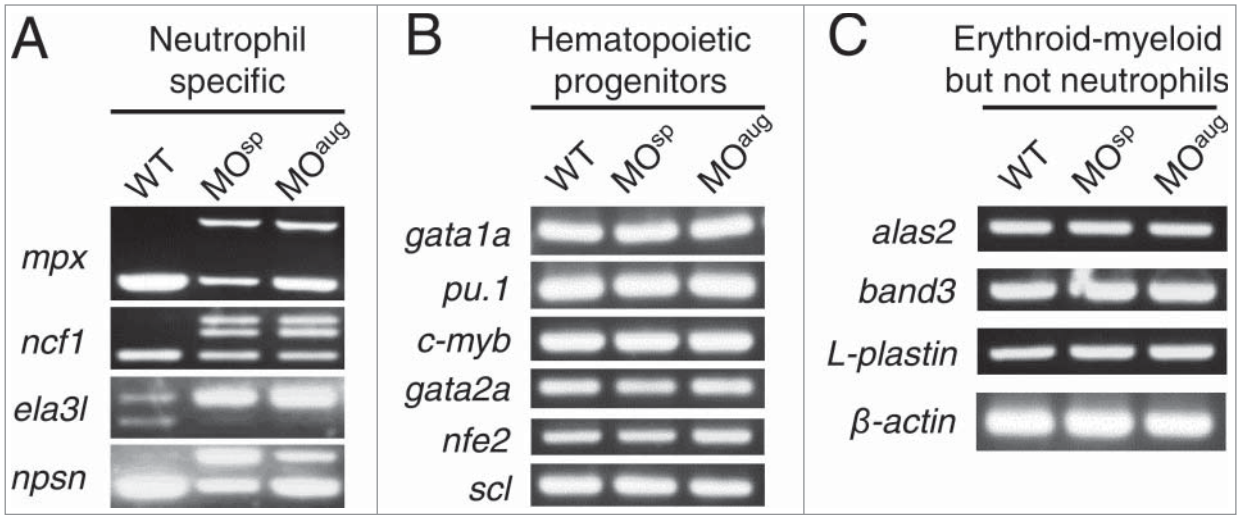

Figure 4. Splicing of haematopoietic genes in Usb1-deficient zebrafish. (**A**) sqRT-PCR analysis of neutrophil-specific genes, including *mpx*, *ncf1*, *ela3l* and *npsn*, in the embryos at 25 hpf. The decreased expression of the normal transcripts and the expression of aberrant transcripts retaining the introns were observed in Usb1-deficient embryos. (**B**–**C**) sqRT-PCR analysis of haematopoietic - but not neutrophil-specific genes in MO-injected and wild-type embryos. The normal transcripts of haematopoietic progenitors (**B**) and erythroid and myeloid-but not neutrophil-specific genes (**C**) were observed in the morphants. β-*actin* served as a control.

protein.[46] Similarly, we expect that the full transcriptome analysis of *usb1*-mutant zebrafish, which we are now generating using the CRISPR (clustered regularly interspaced short palindromic repeats) RNA-guided Cas9 nuclease system,[47] will allow us to resolve this question.

Materials and Methods

Zebrafish maintenance

Zebrafish (wild-type AB line) were raised and maintained according to standard laboratory conditions[48] in the Bio-resource Division at the Frontier Science Research Center, University of Miyazaki, Japan. Current Japanese rules do not require approval for research on zebrafish embryos. The embryos were raised in E3 embryo medium at 28.5°C. The transgenic zebrafish line Tg(*mpx:GFP*) was purchased from the Zebrafish International Resource Center (ZIRC; http://zebrafish.org/zirc/fish/lineAll.php).

Morpholino injections

To knock down *usb1*, we used 2 types of MOs that were obtained from Gene Tools, LLC (USA). The MOs were designed to target either the splice site (MO^{sp}, 5′-AGGATCATCT-GAAATTTAGGCAGGA-3′) in the intron 2/exon 3 boundary region to interrupt *usb1* splicing or the complementary sequence between -60 and -35 nucleotides from the translation start site (MO^{aug}, 5′-TAGAAGAATGTCATCTCAGACACGT-3′) to inhibit Usb1 protein expression. MOs were injected into one-cell-stage embryos at varied concentrations (MO^{sp} at 5, 10 and 20 μg/μl; MO^{aug} at 2.5, 5 and 10 μg/μl), using an IM-30 Electric Microinjector (Narishige, Japan).

Sudan Black staining

Sudan Black staining was performed as previously described.[49] Two-day-old embryos were fixed with 4% paraformaldehyde (Polysciences, Warrington, PA) in phosphate-buffered saline (PBS) for overnight at 4°C and incubated in Sudan Black solution (Sigma-Aldrich, France) for 20 minutes after rinsing thoroughly in PBS. Then, the embryos were washed extensively in 70% ethanol and rehydrated in PBS containing 0.1% Tween 20 (PBS-T). The stained neutrophils in the CHT region were counted under a stereomicroscope (Olympus, SZX12). Statistical analyses were performed using GraphPad Prism (Ver. 5) software.

In vitro mRNA synthesis and rescue experiments

Zebrafish *usb1* and *ela3l* mRNAs were synthesized from their full-length cDNA sequences (GenBank accession numbers NM_001003460 and NM_001024408, respectively) following a previously described protocol.[22] The synthesized mRNAs were injected into the embryos at a concentration of 500 ng/μl.

Semi-quantitative RT-PCR

The total RNA was extracted from 25 hpf morphants and control embryos using TRIZOL reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with random primers. The PCR analysis was performed according to the FastStart Taq DNA polymerase (Roche, Germany) using specific primers (Table S1).

Northern blot analysis

The total RNA (10 μg/lane) was separated on a 1.5% denaturing agarose gel and blotted according to standard

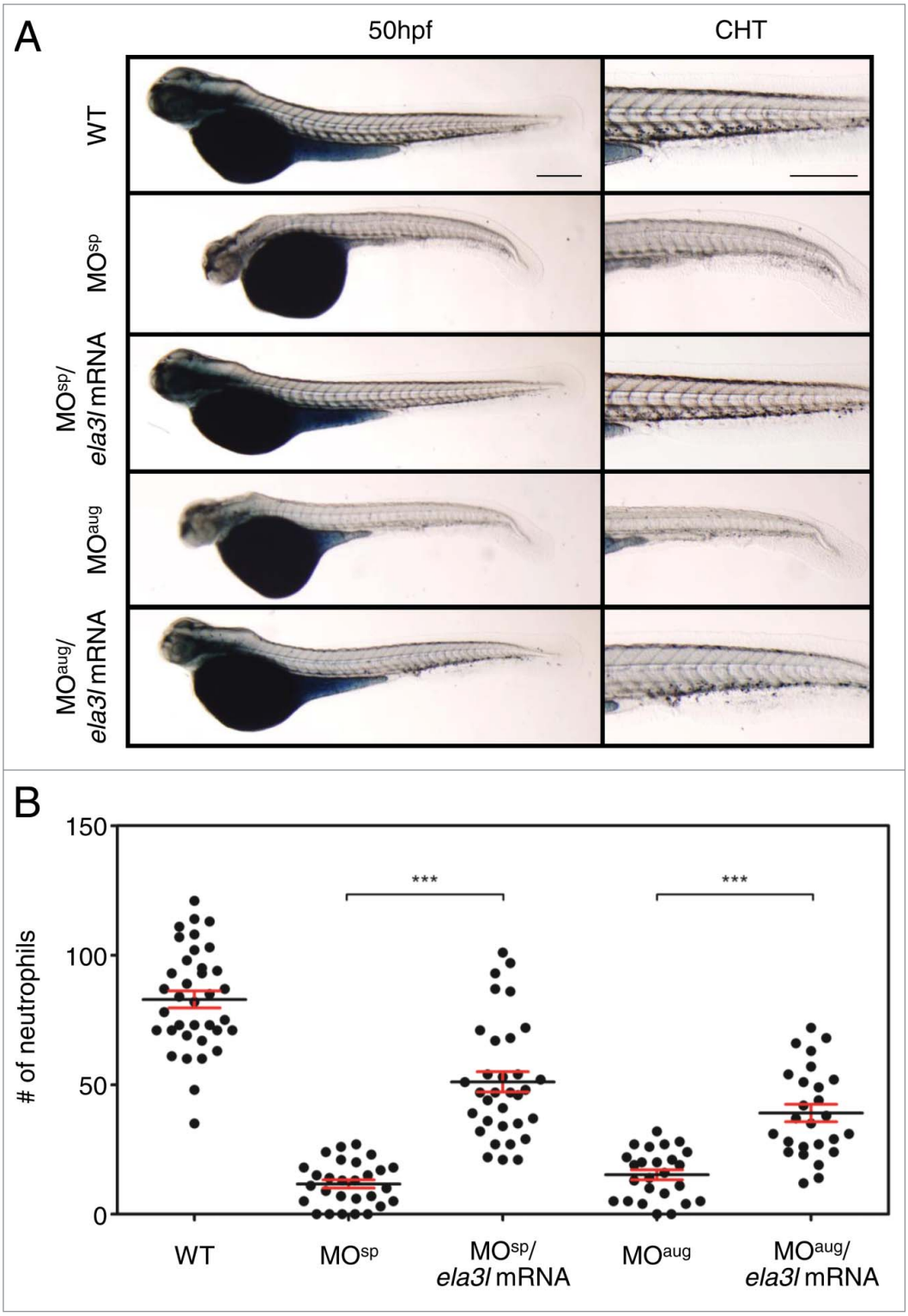

Figure 5. Recovery of neutrophil defects by co-injection of *ela3l* mRNA in Usb1-deficient zebrafish. (**A**) A lateral view of the Sudan Black-stained embryos and the close-up images of the CHT region at 50 hpf. The embryos coinjected with *ela3l* mRNA show the almost complete rescue of the neutrophil defects. Scale bars: 200 μm. (**B**) Scatter plot showing the mean number of neutrophils at the CHT region in wild type (WT) and *ela3l* mRNA-coinjected morphants at 50 hpf. ***$P < 0.001$ (one-way ANOVA with Tukey's multiple comparison test).

procedures.[50] Then, the blots were hybridized overnight at 65°C in modified Church-Gilbert hybridization buffer (0.5 M NaHPO4, 1 mM EDTA, 0.5% BSA and 7% SDS) containing LNA (locked nucleic acid) probes labeled with digoxigenin (DIG) using the DIG oligonucleotide Tailing Kit (Roche, Germany). The sequence of the U6 LNA probe is 5′-aAagAtgGaaCgcTtcAcgAatTtgCgtGt-3′ (uppercase letters indicate the LNAs).

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interests.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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