A zebrafish model for HAX1-associated congenital neutropenia

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Supplementary Data:

Supplementary Material and Methods

Quantitative RT-PCR

Total RNA was extracted from pools of 8 zebrafish embryos at 48 hpf using TRIzol (Life Technologies) following the manufacturer's protocol. RNA samples were then treated with RQ1 RNAse-Free DNase (Promega) before first-strand cDNA synthesis with random hexamer primers and Superscript III Reverse Transcriptase (ThermoFischer) following the manufacturer's protocol. Quantitative PCR (qPCR) was carried out using the SYBR Green Kit (Applied Biosystems) on the Light Cycler 480 (ROCHE). The data was analyzed in Microsoft Excel using the ΔCT method with *β-actin* as a reference gene for normalization. Primers are listed in the *Supplementary Table 3*.

Fluorescent *in situ* hybridization (FISH)

High resolution double FISH was performed utilizing standard digoxigenin- and fluorescein-labeled RNA antisense probes along with tyramide signal amplification (TSA) as described previously [1]. Briefly, after hybridization and removal of both probes, samples were incubated with 1:500 diluted Anti-Fluorescein-POD antibody in blocking solution for overnight at 4°C and then stained with the TSA Plus Fluorescein solution for 60 minutes in the dark. Samples were treated with 1% H_2O_2 in Methanol for 30 minutes. After stepwise rehydration and blocking, samples were incubated with 1:1000 diluted Anti-DIG-POD antibody in blocking solution for overnight at 4°C. The TSA Plus Cy3 solution (1:50 dilution) was used for the second staining. Images were taken using LSM 710 microscope.

Whole-mount immunostaining

Immunostaining was performed as described previously [2]. Briefly, zebrafish embryos at 2 dpf were fixed with 4% paraformaldehyde in 2xPBS, 0.1% Tween-20 for at least 24 hours at 4°C. For permeabilization, samples were treated with 100% Acetone at -20°C for 20 minutes. After wash steps, samples were incubated with blocking solution (10% FCS, 0.8% Triton X-100, 1% BSA, 0.1% Tween in 1xPBS) at 4°C for 3 hours with a gentle agitation on a turning wheel. For primary Antibody incubation, rabbit anti-phosphohistone 3 antibody (Ser10, Millipore 06-570, 1:500 dilution) was incubated with the sample in the incubation buffer (1% FCS, 0.8% Triton X-100, 1% BSA, 0.1% Tween in 1xPBS) at 4°C in the dark for 3 days. As secondary antibody, the Cy3-donkey anti-rabbit IgG (Jakson laboratories, 711-165-152, 1:500) was used for the

incubation with samples in the incubation buffer at 4°C in the dark for 2.5 days. To remove residual secondary antibody, samples were washed 3 times for 1 hour with PBS-TS (10% FCS, 1% Triton X-100 in PBS).

TUNEL assay

TUNEL staining in whole-mount embryos was performed using the In situ Cell Death Detection kit (Roche) according to manufacturer's protocol. Briefly, zebrafish embryos were fixed in 4% PFA/2xPBS and 0.1% Tween at 4°C overnight. Embryos were then permeabilized in 10µg/ml Proteinase K in PBS and 0.1% Tween for 20 min without shaking. For positive control, embryos were first incubated with DNase I at 25°C for 30 min. For negative control, embryos were incubated only with the labeling solution.

Acridine Orange staining

Zebrafish embryos at 2 dpf were stained for 30 minutes in a 50µg/ml acridine orange (Enzo, ENZ-52405) solution prepared with E3 media. After incubation in the dark, the embryos were washed 10 times for 5 minutes with E3 and imaged directly afterward.

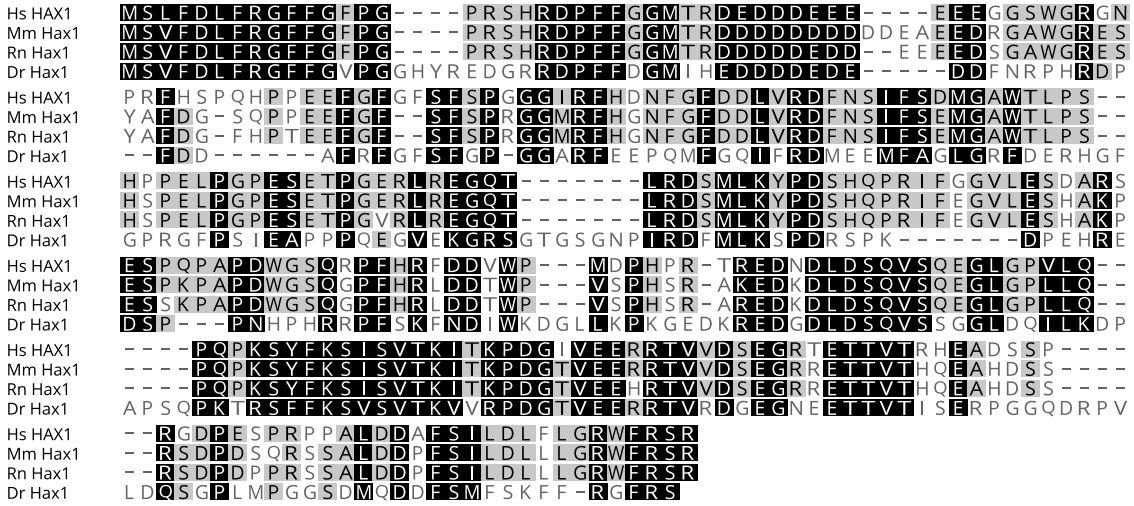
Caspase-3/7 green detection assay

Transgenic tg(*lyz:dsRED*) were stained in 1:100 diluted Caspase-3/7 detection reagent (IncuCyte, 4440) with E3 media. After overnight incubation, embryos were washed five times for 5 minutes with E3 media and imaged directly afterward.

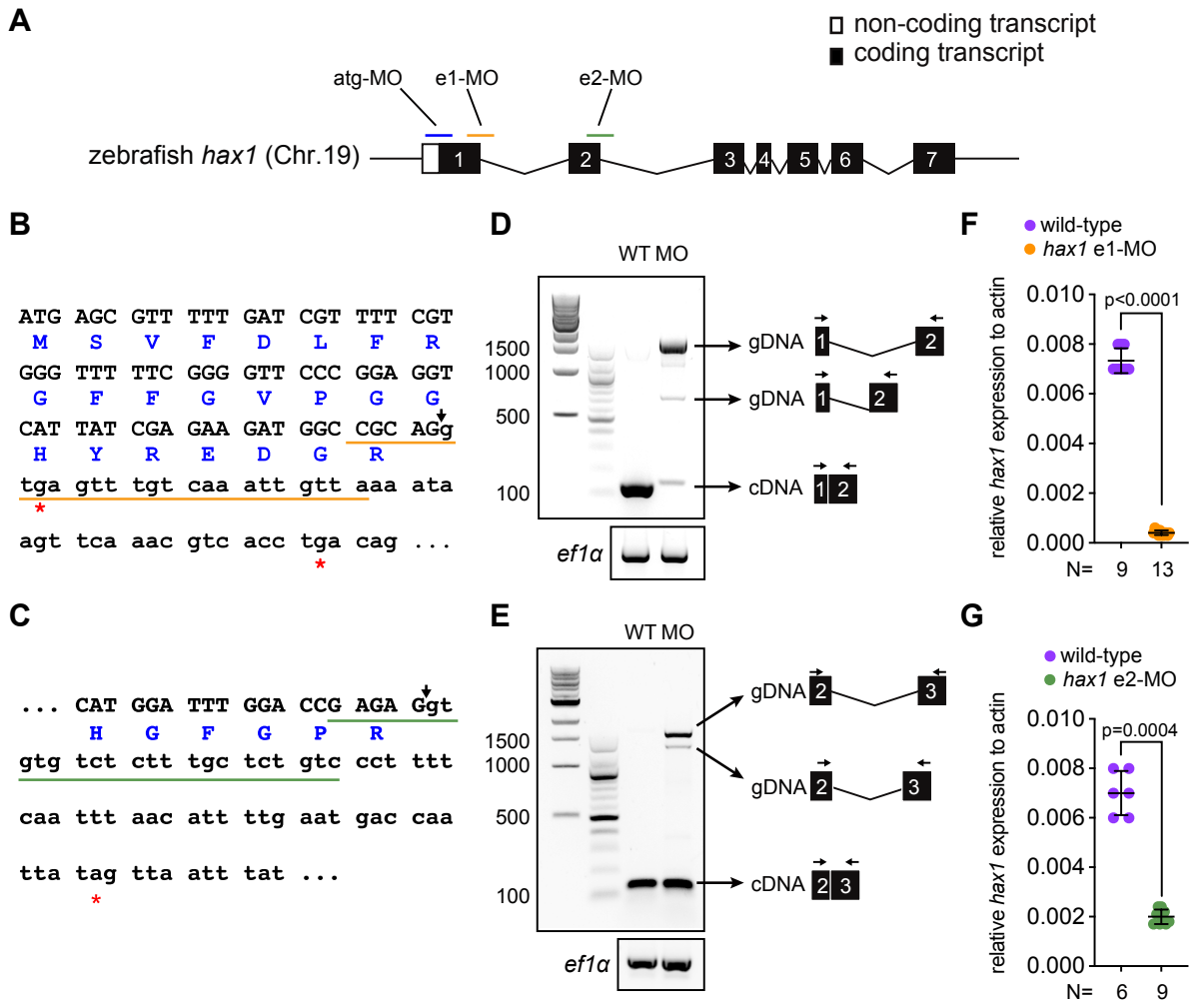
Microscopy and cell enumeration

A Nikon stereo-fluorescent microscope SMZ18 fitted with a Nikon DS-Fi3 camera and NIS-Elements software was used for imaging and manual counting. Confocal images were taken using a Zeiss LSM 710 NLO microscope. Z-stacks of 60-70µm spanning the trunk region (z-space 1µm) were acquired using 10x and 20x objectives. Confocal images were analyzed with Imaris software. The number of GFP-positive cells was automatically quantified using the fast spot tool (diameter: 8 µm, quality >1.25). Single cells detected by Imaris software were then examined manually. In this work, quantification of cell numbers was restricted to the cells that were located in the trunk region caudal to the yolk extension.

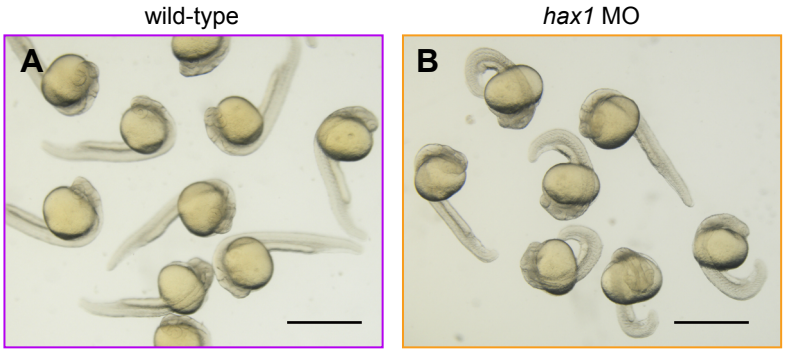
Supplementary Figures



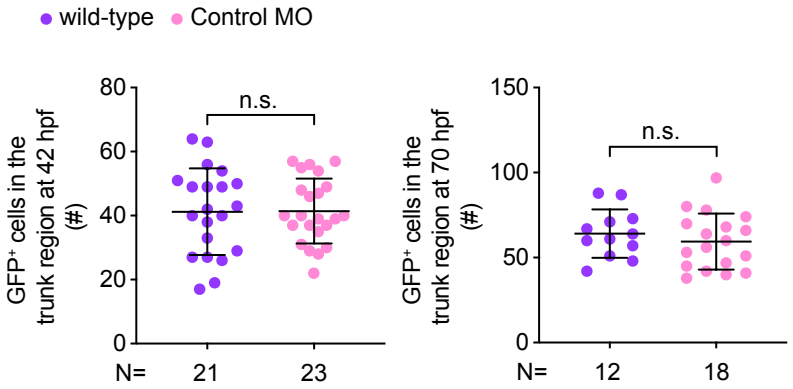
Supplementary Figure 1. Multiple alignment of the Hax1 amino acids sequences. The alignment was produced using Clustal W. Shading indicates conservation, with more strongly conserved positions shaded in darker colors. Hax1 protein from mouse (*Mus musculus*, Mm; Ensebml ID ENSMUSG00000027944), human (*Homo sapiens*, Hs; Accession number NM172219), rat (*Rattus norvegicus*, Rn; Ensebml ID ENSRNOG00000045647), and zebrafish (*Danio rerio*, Dr; ID ENSDARP00000053379), were used for the alignment.



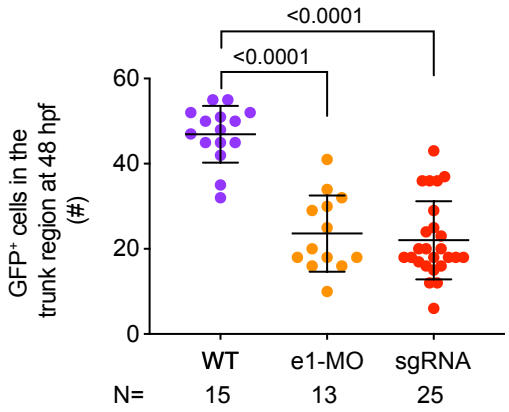
Supplementary Figure 2. Efficiency of *hax1* knockdown by using morpholinos. (**A**) Schematic diagram of zebrafish *hax1* gene organization. The binding sites of three morpholinos (MO) are highlighted by blue, orange and green lines. (**B, C**) The e1-MO and e2-MO target the splice donor site of exon 1-intron 1 (**B**) and exon 2- intron 2 (**C**), respectively. The coding sequences are shown in upper case and intronic sequences in lower cases. The blue letters indicate the amino acid sequences. Arrow indicates the splice donor site. Asterisks mean stop codon. (**D, E**) RT-PCR analysis of *hax1* transcript in wild-type (WT) and *hax1* e1-MO (**D**) or e2-MO (**E**) injected embryos at 48 hpf. (**F**, **G**) Relative expression level of normal *hax1* in WT and e1-MO (**F**) or e2-MO (**G**) injected embryos at 48 hpf. The *β-actin* gene was used as a reference for normalization. N indicates number of biological replicates.



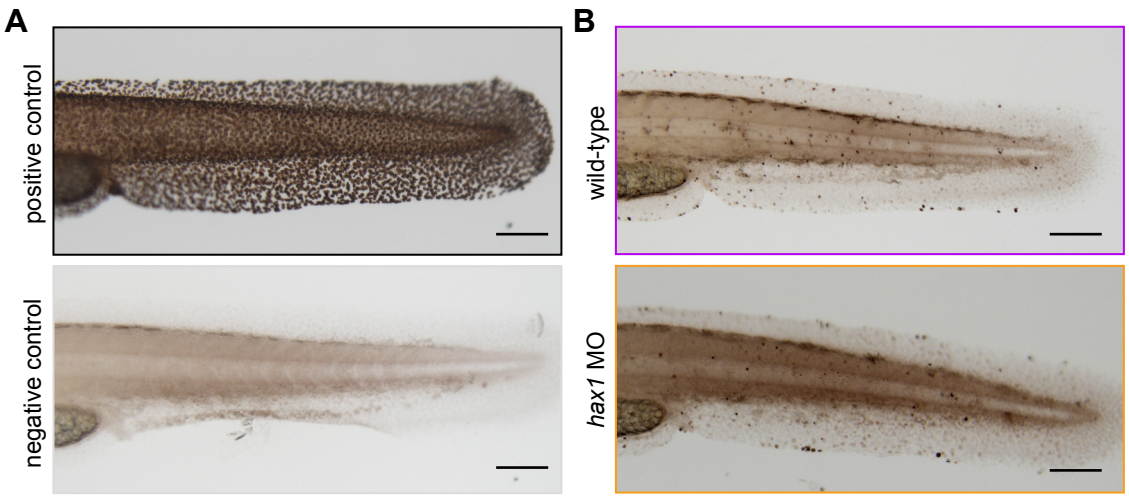
Supplementary Figure 3. No embryonic malformation in the *hax1* morphants. (A) Wild-type embryos at 1 dpf. (B) Embryos injected with e1-MO at 1 dpf. Scale bars indicate 500μm.



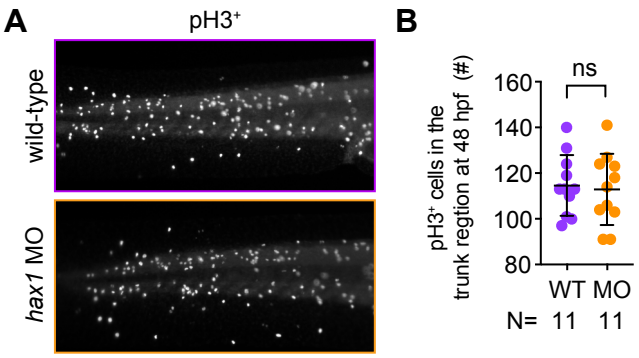
Supplementary Figure 4. Injection of control morpholino does not impair neutrophil development in the tg(*mpo:gfp*) embryos. Numbers of GFP-positive cells in the trunk region in uninjected (WT) and embryos injected with control MO at 42 hpf (left panel) and 70 hpf (right panel). Each dot represents an individual embryo. N indicates number of embryos. Data are means ± SD.



Supplementary Figure 5. Reduced neutrophil numbers in the *hax1* crispants. A sgRNA targeting *hax1* gene with mRNA of Cas9 were co-injected into the tg(*mpo:gfp*) embryos. Number of GFP-positive cells were counted in the trunk region in wild-type (WT) and crispants (sgRNA) at 48 hpf. For comparison, *hax1* e1-MO was also injected. Each dot represents an individual embryo. N indicates number of embryos. Data are means ± SD.



Supplementary Figure 6. TUNEL staining of zebrafish embryos at 2 dpf. (A) Top panel shows a representative image of a TUNEL staining in wild type pretreated with DNase I, as a positive control. Bottom panel shows a representative image of a TUNEL staining in wild type that were incubated with the labeling solution, as a negative control. (B) Representative images of TUNEL-positive cells in wild-type (top panel) and *hax1* morphants (bottom panel) at 2 dpf. Scale bars indicate 100 µm.



Supplementary Figure 7. Knockdown of *hax1* does not impair embryonic proliferation. (A) Representative images of pH3-positive cells in wild-type and *hax1* morphants at 2 dpf. (B) Number of pH3-positive cells counted in the trunk region in wild-type (WT) and morphant (MO) at 48 hpf. Each dot represents an individual embryo. N indicates number of embryos. Data are means ± SD.

Supplementary Table 1: Morpholinos used in this study

Name	Sequence (5‘>3‘)
hax1 atg-MO	AACGCTCATTTATGGACAGAATCCT
hax1 e1-MO	TAACAATTTGACAAACTCACCTGCG
hax1 e2-MO	GACAGAGCAAAGAGACACACCTCTC
Scrambled-MO	CCTCTTACCTCAGTTACAATTTATA

Supplementary Table 2: Gene-specific *in situ* hybridization probes used in this study

Gene	Accession Number/Eensembl ID	Nucleotides X to X
hax1	ENSDARG00000036764	164-755
mpo	BC056287	225-938
l-plastin	NM131320	404-1029
runx1	NM131603	542-1204
cmyb	NM131266	346-966
csf1ra	ENSDARG00000102986	2116-2755
csf3r	NM001113377	124-737
mpeg1.1.	ENSDARG00000055290	859-1417
pu.1 (spi1b)	ENSDARG00000000767	67-614
cebpa	ENSDARG00000036074	323-919
cebpb	ENSDARG00000042725	345-923
hbae1.1	ENSDARG00000088330	45-323

Supplementary Table 3: Primers for quantitative RT-PCR used in this study

Gene	Forward primer (5‘>3‘)	Reverse primer (5‘>3‘)
b-actin	GATCTTCACTCCCCTTGTTCA	GGCAGCGATTTCCTCATC
cebpa	GCTCCATGAAGATTGGCGATCG	CATTTTCGCCTTGTCCCGACTC
cebpb	CTCAAGCGGGAAAGGCAAGAAG	TGCGCATTTTGGCTTTGTCTCT
hcls1	AGAGAACGAGAAGAGGCACGAC	GGGAATTTCCGGCAGTTTCCTG
hax1 [a]	GTTCCCGGAGGTCATTATCGAGAAG	CTGGACCGAAACTGAATCCAAACC
hax1 [b]	CAGATCTTCAGAGACATGGAGG	CTCCTGCCTTTCTCAACTCCTTCC

[a] Primers bind to the exon 1 and 2 of *hax1* gene and were used to validate the efficiency of e1-MO.
[b] Primers bind to the exon 2 and 3 of *hax1* gene and were used to validate the efficiency of e2-MO.

Supplementary References:

1. Brend T, Holley SA. Zebrafish whole mount high-resolution double fluorescent in situ hybridization. J Vis Exp. 2009;25):
2. Bajoghli B, Kuri P, Inoue D, et al. Noninvasive In Toto Imaging of the Thymus Reveals Heterogeneous Migratory Behavior of Developing T Cells. J Immunol. 2015;195(5):2177-2186.