Alas1 is essential for neutrophil maturation in zebrafish

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Received: March 27, 2018.

Accepted: June 27, 2018.

Pre-published: June 28, 2018

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Supplementary Methods

Histology

Whole-mount in situ hybridization (WISH) was performed using a standard protocol.[1] Sudan black B (SB) staining was performed as previously described.[2] Diaminobenzidine (DAB) staining was performed using DAB Horseradish Peroxidase Color Development Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. *o*-Dianisidine staining was performed as previously described.[3]

Genotyping

Genomic DNA was extracted as a template from each embryo following the HotSHOT protocol as described by Meeker et al.[4] when the desired assays were done. $alas1^{smu350/smu350}$ and $alas1^{\Delta 2/\Delta 2}$ mutants were genotyped by PCR followed by FaqI (Thermo Scientific, San Jose, CA, USA) and BsrI (New England Biolabs, Ipswich, MA, USA) digestion, respectively. The $alas1^{smu350/smu350}$ PCR products were digested by FaqI into two fragments of 57 bp and 85 bp, the $alas1^{\Delta 2/\Delta 2}$ PCR products were digested by BsrI into two fragments of 43 bp and 97 bp, whereas the WT PCR products were resistant to the digestion. Primers for *alas1* mutants genotyping were as follow: 5'-GGACTCGGTCATGCACAAGAT-3' and

5'-GCTGGGTCACTGAAAACACCA-3'. *gata1a*$^{m651/m651}$ mutants were genotyped by PCR followed by TaqαI (New England Biolabs, Ipswich, MA, USA) digestion. The WT PCR products were digested by TaqαI into two fragments of 121bp and 123bp, whereas the *gata1a*$^{m651/m651}$ PCR products were resistant to the digestion. Primers for *gata1a*$^{m651/m651}$ genotyping were as follow: 5'-GTGAGTATACACAATTACAC-3' and 5'-GGTTCAGAGAATACGCTCCT-3'. The digested products were analyzed by high-resolution melting (HRM) or electrophoresis. Especially, as the swim bladder was absent in *alas1* mutants, *alas1* mutants could be directly distinguished from their siblings under stereomicroscope from 4 dpf onwards. As the *gata1a*$^{m651/m651}$ mutants were lack of erythrocytes, *gata1a*$^{m651/m651}$ mutants could be directly distinguished from their siblings under stereomicroscope from 24 hpf on.

TA cloning

TA cloning was performed using the pMD18-T vector (Takara, Tokyo, Japan) according to the manufacturer's instructions. To obtain the insert fragments, total RNA was extracted from embryos using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was generated using HiScript Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). We used 1 μL of

reverse transcription mixture for PCR reactions with primers specific for *alas1* (5′-CAAGATGATGCCGGGTTGTG-3′ and 5′-GCAGAGACGTGGTGAAGATG-3′).

Generating *alas1* mutants by CRISPR/Cas9

The *alas1* mutants were generated using the CRISPR/Cas9 system by targeting the exon 7 of *alas1* as previously described.[5] The guide RNA (gRNA) targeting sequence was as follow: 5′-GGACATCATCTCGGGCACTCT-3′. The gRNA was synthesized using T7 RNA polymerase (Thermo Scientific, San Jose, CA, USA) and the PCR product as template amplified from the gRNA scaffold vector[4] with the following primers: 5′-TAATACGACTCACTATAGGACATCATCTCGGGCACTCTGTTTT AGAGCTAGAAATAGC-3′ and 5′-AGCACCGACTCGGTGCCACT-3′. The Cas9 mRNA (zebrafish codon optimized) was synthesized using the mMESSAGE mMACHINE T7 Transcription Kit (Life Technologies, Carlsbad, CA, USA) and the XbaI-digested Cas9 expression vector (pGH-T7-zCas9)[6] as a template. Approximately 0.5 nL of Cas9 mRNA (300 ng μl^{-1}) and gRNA (200 ng μl^{-1}) were co-injected into one-cell-stage wildtype embryos using a PLI-100A Pico-Injector (Warner Instruments, Hamden, CT, USA). To determine the mutation efficiency, PCR followed

by HRM analyses were performed as described above. The remainder of the injected embryos were raised to sexual maturity, and founders were mated with wildtypes to obtain the F1 generation. In this study, a mutant with a 2-bp deletion within exon 7 of *alas1* ($alas1^{\Delta 2/\Delta 2}$) was obtained.

Fluorescence heme assay

The fluorescence heme assay was performed as previously described.[7] For whole fish heme detection, one to five embryos were solubilized with 5 μL of 0.02 N NaOH at room temperature for 15 min, after which 200 μL of 2 M oxalic acid was added. For sorted cell heme detection, 10^3~10^4 sorted cells for each group were washed with PBS three times and centrifugated at 5,000 × *g* for 5 min. 50 μL of solution was left and 200 μL of 2 M oxalic acid was added. The mixed solution was evenly divided into two tubes: one was heated to 100°C for 30 min in a dry bath, while the other was not heated. Porphyrin fluorescence was then measured using a Tecan infinite M200 multimode microplate reader (Tecan, San Jose, CA, USA) at 408 nm excitation and 655 nm emission. The relative heme level was acquired by subtracting values of unheated from heated samples and then normalized to per-fish level and per-cell level, respectively.

Reverse transcription quantitative real-time polymerase chain

reaction (RT-qPCR)

Total RNA and cDNA were generated as described above. The qPCR was performed in triplicate with the LightCycler 96 system (Roche, Mountain View, CA, USA) and PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. Zebrafish *actb2* was used as a reference gene. The qPCR primers are listed in Table S1. The results are the average of two separate experiments, and p values were calculated using the Student *t*-test.

Western blot

Western blotting was performed as previously described.[8] Specifically, a mouse polyclonal antibody against the zebrafish Alas1 N-terminal region (amino acids 4–148; generated by Institute of Zoology, Chinese Academy of Sciences, Beijing, China) and a rabbit monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #2118; Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies.

Fluorescence-activated cell sorting (FACS)

FACS was performed as described previously.[9] 4-dpf self-progenies of *Tg(lyz:DsRed);alas1*$^{smu350/+}$ and *Tg(gata1:DsRed);alas1*$^{smu350/+}$ in-crosses respectively were obtained. Mutants were distinguished from their

siblings under stereomicroscope as the swim bladder was absent. Cells of each group were collected using a MoFlo™ XDP (Beckman Coulter, Brea, CA, USA).

Flow cytometry

To detect erythrocyte numbers, samples were prepared as described previously.[9] Each sample contained 8 mutants and 8 siblings respectively using 4-dpf self-progeny of *Tg(gata1:DsRed);alas1*$^{smu350/+}$ in-cross. The cell suspensions were constant-volumed to 500 μL and analyzed using a Cytoflex (Beckman Coulter, Brea, CA, USA) (performed in triplicate).

Microscopy and imaging

Video-enhanced differential interference contrast (VE-DIC) imaging was performed using an Olympus BX51 microscope with a 60×/1.00 numerical aperture water-immersion lens as previously described.[10] VE-DIC images were captured by a DP71 Olympus color camera. Histological images were captured using an Olympus MVX10 microscope with a DP80 Olympus camera. Fluorescent images were captured using a Zeiss LSM880 confocal microscope system.

Supplemental References

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Supplementary Tables

Table S1. Primers used for qPCR.

Gene	Primer sequences (5′-3′)
actb2	CCAGACATCAGGGTGTCATGGTTG
	TGGATGGCAACGTACATGGCG
alas1	ATCATTCGCTGCCCATTCCT
	CATCACTGGCTTCATGTCCG
alas2	TGGTCAAGGCCATTGGTGAT
	GACCAGCGCTCCATCTTTCT
hmox1a	CAGCAGTGCAGCCCATTTAC
	GCTCTTGCCAATCTCTCTCAGT
hmox2a	ATGGCGGTCAGTGGAAACACAACC
	GGCAACAGCAGCAACCAATGTGGC
flvcr1a	CCTGTGCCTTTGCTGTGTT
	AGTAGAACGATCCGGTCATG
hpx[11]	CTCATAAAGGCAAACCTGGTG
	TGGACAGCTCAGCCTTGCCA
slc48a1a	ATGGGCGGATTATCAGGAGTC
	AGCGACAGCTAAGAGGGAGA
slc48a1b	GGGTGTCATTTTCTCGGTGC
	GGGATCTGTGAGGGACTGTTG
abcc5	CTCTTCGTCGTGTGGTCTGG
	CACAAACGCTGGGCTACTGA
il1b[12]	GTACTCAAGGAGATCAGCGG
	CTCGGTGTCTTTCCTGTCCA
cxcl8a[12]	TGTTTTCCTGGCATTTCTGACC
	TTTACAGTGTGGGCTTGGAGGG

Movie legends

Movie 1. *In vivo* movie of neutrophils in 2-dpf *Tg(lyz:DsRed);smu350* sibling embryos by VE-DIC microscopy. Neutrophils of siblings had abundant visible and highly mobilized granules.

Movie 2. *In vivo* movie of neutrophils in 2-dpf *Tg(lyz:DsRed);smu350* mutant embryos by VE-DIC microscopy. Neutrophils of *smu350* mutants lacked granules.