Materials and Methods

Knock down of sema4a in zebrafish

Zebrafish embryos were raised at 28.5°C and staged according to Kimmel et al.\(^1\). All morpholinos (MOs) used in this study were purchased from Gene Tools LLC (Philomath, OR, USA). We designed a MO, which was named as sema4a perfect match (pm) MO, to block splicing of exon 14 of the zebrafish sema4a (Refseq XM_001338590). The sequence of sema4a pm MO was 5’-CAACACTGGAGAAAGACAGACAGTT-3’. We also designed a MO which had 5 bp mismatch (mm) of the sema4a pm MO and named it sema4a mm MO. The sequence was sema4a mm MO 5’-CAACAgTGAGAAAcACAcACAcTT-3’. A mixture of sema4a pm MO (0.3 mM) or sema4a mm MO (0.3 mM) and control MO with lissamine modification on the 3’ ends (Lis-MO, 0.05 mM) was injected into embryos at the 1–4 cell stage\(^2\). The sequence of control MO was 5’-GAGACTTCATCTTACCTCATTT-3’ which theoretically has no targets in the zebrafish genome. At 3 days post fertilization, only zebrafish embryo expressing fluorescence from lissamine were used for further experiments.

The investigation conformed to the ethical guidelines established by the Institutional Animal Care and Use Committee at Mie University, and was in compliance with the ARVO statement for
the use of animals in vision research.

*In vivo* imaging of zebrafish retina

The embryos were allowed to develop in egg water (Instant Ocean was dissolved in distilled water at 60 µg/ml) containing 200 µM 1-phenyl-2-thiourea to block pigmentation. At 3 days post fertilization, the fluorescence of lissamine (Ex/Em 575/593nm) in the sema4a morphant (zebrafish injected with sema4a pm MO and control MO) was assessed using a fluorescence microscope (MZ16FA, Leica, Tokyo, Japan) and embryos showing the fluorescence of lissamine in their whole bodies were used for further experiments. At 5 days post fertilization, *in vivo* imaging of the retinas of sema4a morphant and control zebrafish (without MO injection) was performed as previously described 3 using ZM-B322.

qPCR using zebrafish

Total RNA was extracted from whole bodies of sema4a morphants or control zebrafish at 3 days post fertilization using a RNeasy Plus Micro kit (Qiagen, Valencia, CA, USA), in order to investigate the expression of sema4a and xbp1 spliced form (xbp1s). The total RNA was used to generate cDNAs using an iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). qPCR was performed using an ABI Prism 7300 (Life Technologies Carlsbad, CA, USA) with SYBR Green Realtime PCR Master Mix Plus (Toyobo, Osaka, Japan). The thermal cycling
procedure consisted of an initial step at 95°C for 1 min followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 45 sec. Data were normalized by the quantity of β-actin (actb). This allowed us to account for any variability in the initial template concentration as well as to calculate the conversion efficiency of the reverse transcription reaction. The primers used in this study were 5’-CAGGACGTGGAAAGAGGAAA-3’ and 5’-GGCAGGACCACAATTTCATT-3’ for sema4a, 5’- ACAGGCTGTGCAGACGAAG -3’ and 5’- CCTGCACCTGCTGCGGACTC -3’ for xbp1s, 5’- CAGCCATGGATGAGGAAATC -3’ and 5’- CACAATACCGTGCTCAATGG -3’ for actb, and 5’- CAGCTGAACAAATGGTTAACATGA -3’ and 5’- AATCAAGTGGATGTGAGTTTGG -3’ for ddit3.

Statistical analysis

Data are presented as the means ± S.E.M. Statistical comparisons were made using Student’s t-test and one-way ANOVA followed by Student’s t-test [using STAT VIEW version 5.0 (SAS Institute, Cary, NC, USA)]. $P < 0.05$ was considered to indicate statistical significance.

Supplemental figure 1. Knockdown of sema4a induced disorganization of retinal lamination and increased the expression of xbp1s in zebrafish. Zebrafish larva (A: control, B: sema4a morphant) at 5 days post fertilization were stained with ZM-B322. The retinas were visualized by confocal laser scanning microscopy. The retinal
lamination in the sema4a morphant (B) was disorganized, especially in the IPL and OPL (red arrow and red arrowhead, respectively), whereas an age-matched control (A) showed no abnormal morphology. The IPL of control showed ring-shaped bands with relatively equal width, whereas the IPL of sema4a morphant showed irregularity in the width (red arrow). The OPL of control was symmetrically ring-shaped, whereas the OPL of sema4a morphant showed irregular shape (red arrowhead). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.  

(C, D) Total RNA was extracted from the whole body of control or sema4a morphant zebrafish and qPCR analysis was performed to examine the expression of sema4a (C) and xbp1s (D). The expression of sema4a mRNA containing exon14 (C) and the spliced form of xbp1 (D) were significantly decreased and increased, respectively, in the sema4a morphant compared to control zebrafish. The expression of sema4a mRNA (E) and ddit3 (F) were decreased and increased, respectively, in zebrafish injected with sema4a perfect match (pm) MO compared to that with sema4a mismatch (mm) MO. Data are expressed as the mean ± S.E.M. (n = 5). * p < 0.05 except for F whose p value was p=0.056.
