The Rag-Ragulator Complex Regulates Lysosome Function and Phagocytic Flux in Microglia

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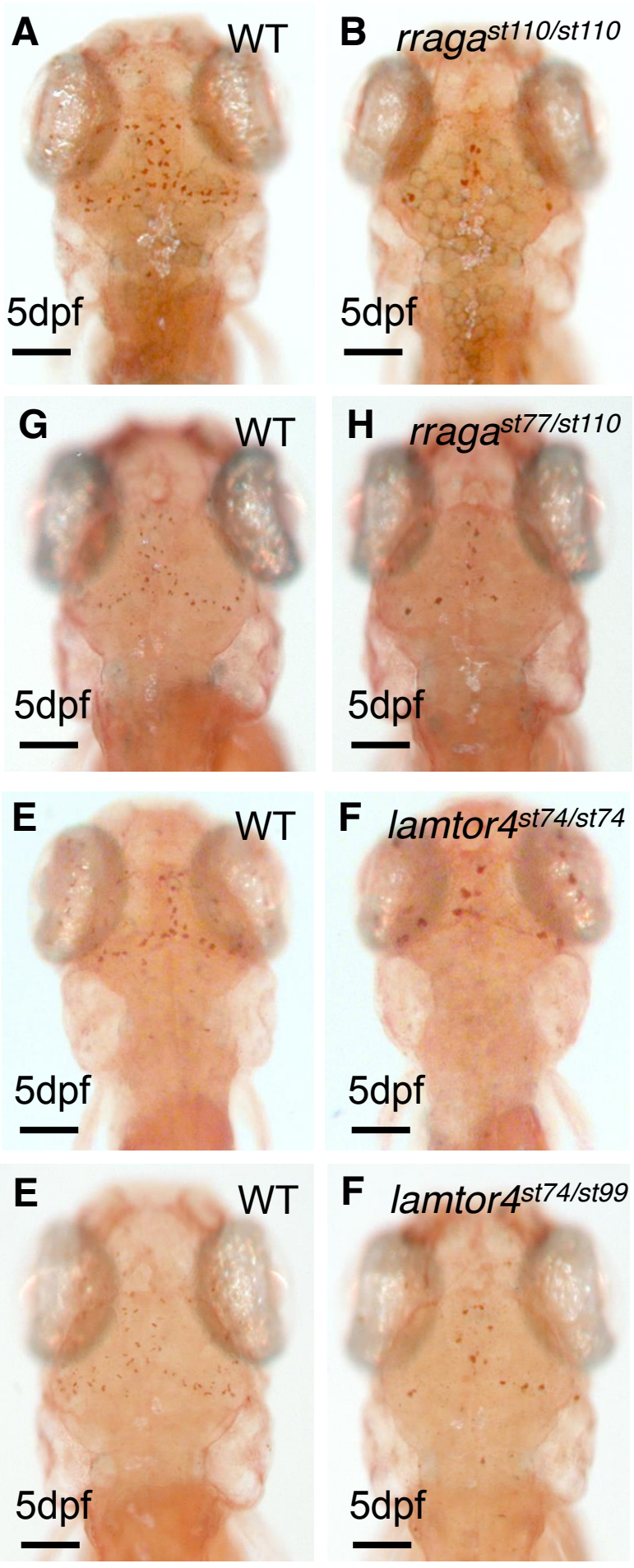
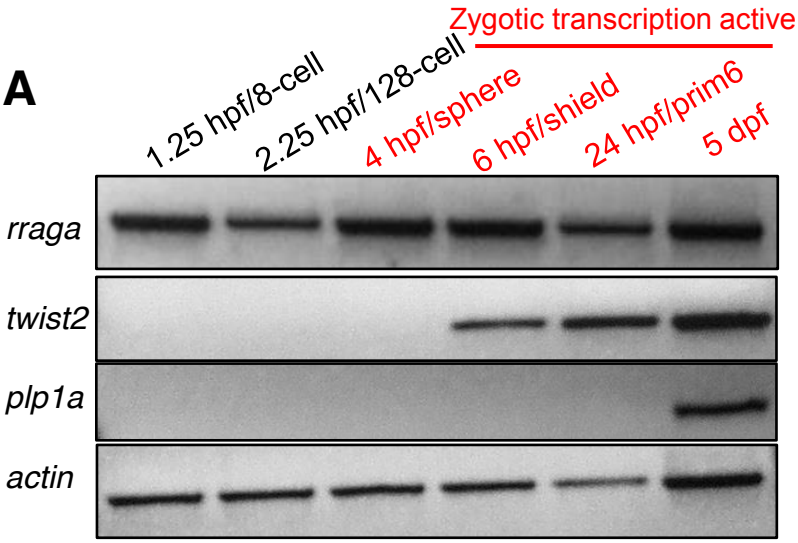
Supplemental Fig. 1

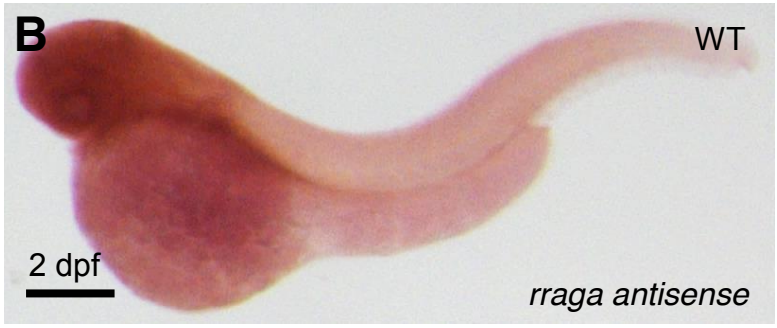


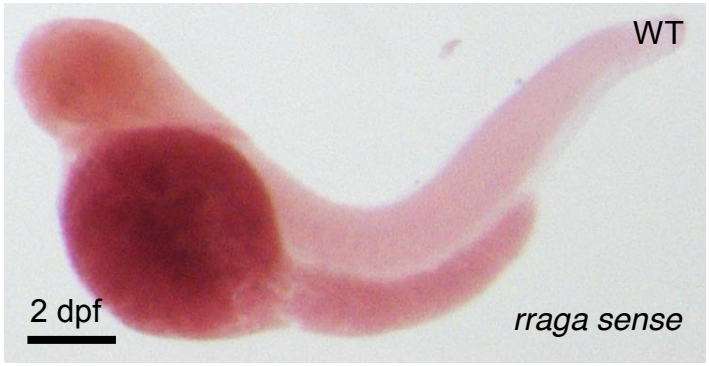
Supplemental Figure 1, related to Figure 1. Neutral red staining of *rraga*$^{st110/st110}$, *rraga*$^{st77/st110}$, *lamtor4*$^{st74/st99}$, *lamtor4*$^{st74/st99}$ mutants.

(A-H) Images of living zebrafish larvae of the indicated genotypes at 5 dpf after neutral red staining. Microglia were reduced to a similar extent in all mutants. Dorsal views, anterior to the top. All scale bars are 50μm. All larvae shown were genotyped by PCR after photography.

Supplemental Fig. 2



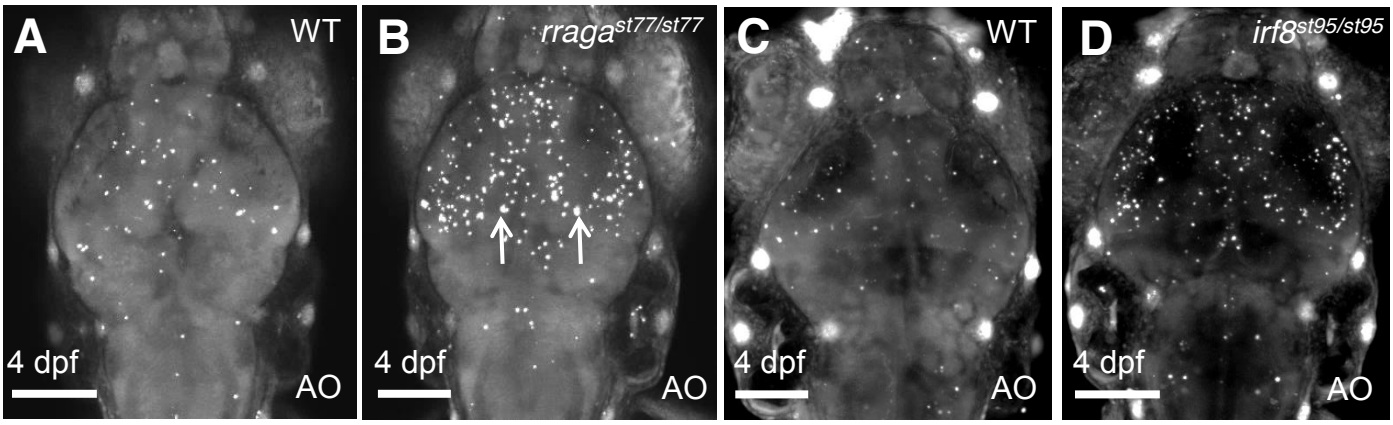




Supplemental Figure 2, related to Figure 2. *rraga* mRNA is maternally expressed and widely distributed in the embryo.

(A) Time course of *rraga* mRNA expression analyzed by RTPCR of whole-animal lysates prepared at the indicated stages before (black) and after (red) the maternal-zygotic transition. RNA for *rraga* is detected before the zygotic transcription is active, in contrast to zygotically expressed control genes *twist2* and *plp1a*. (B) Representative image of a 2 dpf embryo stained by *in situ* hybridization with antisense and sense probes for the *rraga* transcript. The antisense probe detected widespread expression, whereas the control sense probe showed background staining.

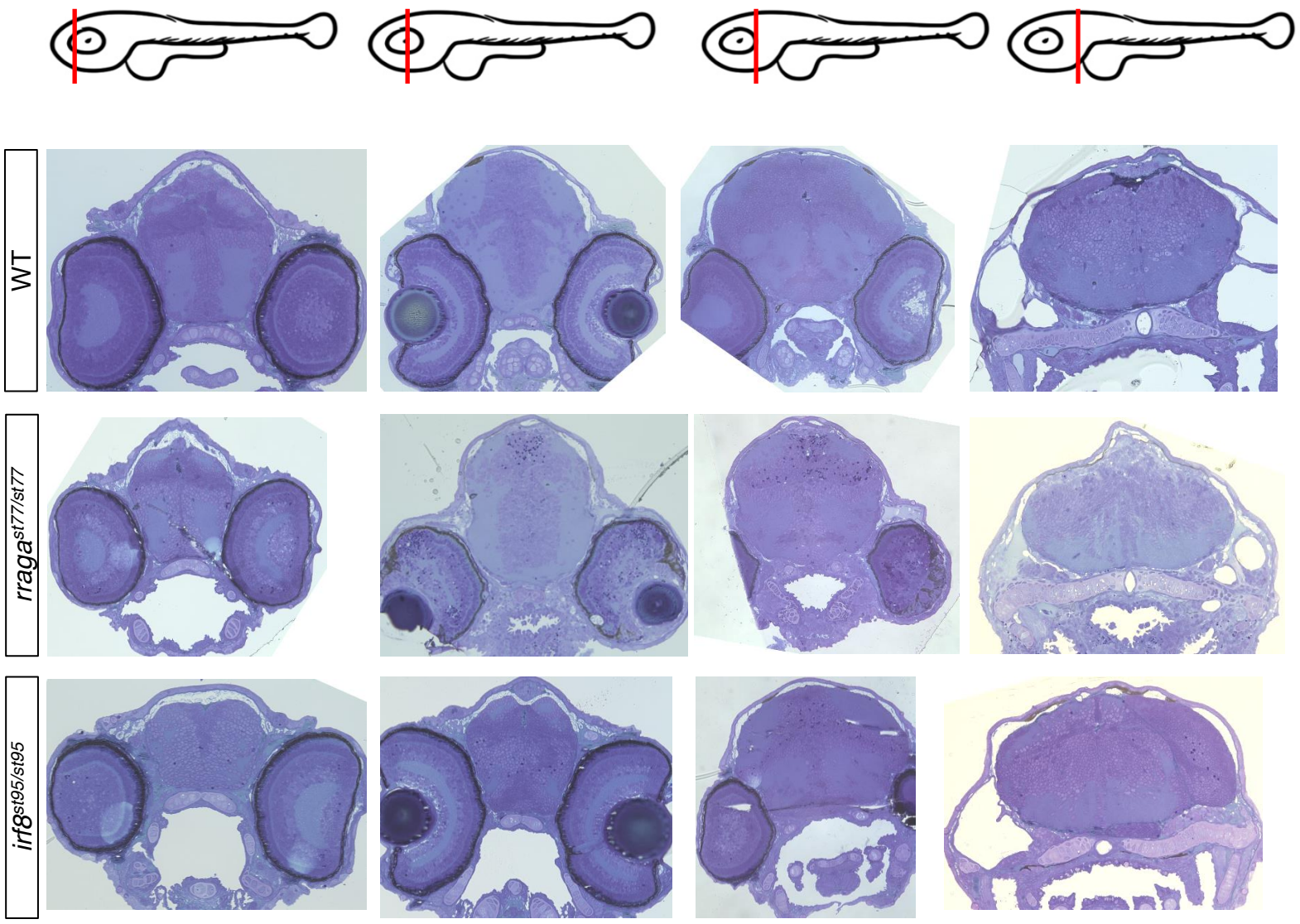
Supplemental Fig. 3



Supplemental Figure 3, related to Figure 3. Acridine orange staining of *rraga*$^{st77/st77}$ and *irf8*$^{st95/st95}$ mutants.

(A-D) Live staining with Acridine Orange at 4 dpf showed increased numbers of apoptotic puncta in *rraga*$^{st77/st77}$ mutants, with some larger clusters (B, arrows), compared to wildtype siblings (A). *irf8*$^{st95/st95}$ mutants also exhibit an increase in the number of acridine orange labeled puncta (D) compared to wildtype siblings (C), but the larger clusters of puncta were not evident. All scale bars are 50μm. All larvae shown were genotyped by PCR after photography.

Supplemental Fig. 4



Supplemental Figure 4, related to Figure 4. Histological sections of *rraga*$^{st77/st77}$ and *irf8*$^{st95/st95}$ mutants.

Toluidine Blue-stained sections of brains from larvae of the indicated genotypes at 4 dpf. The approximate positions of the sections along the anterior-posterior axis are indicated by the red lines in the schematics at the top of each column. Ultrathin sections from these same samples were taken from approximately the second position shown and analyzed by TEM, as shown in Figure 4. Larvae were genotyped by PCR from tail biopsies collected immediately prior to fixation.

Supplemental Information

Supplemental Experimental Procedures

RNA sequencing

Wildtype and *rraga* mutant larvae at 5 dpf were distinguished by neutral red staining. We and isolated RNA from four pools of siblings, each of which contained 10 wildtype or 10 mutant larvae (WT: n=2 pools, mutant: n=2 pools). RNA samples were provided to the Stanford Functional Genomics Facility, which prepared libraries and sequenced them with the Illumina Hi-Seq 2000 to obtain 50-nt paired ends reads. The numbers of reads obtained were: wildtype1-23078044, mutant1-28491733, wildtype2-26485359, mutant2-20836883. Sequencing data was analyzed using the Tuxedo Suite pipeline (Bowtie, Tophat, Cufflinks) as described (Trapnell et al., 2012), to obtain FPKM values. We used a cut-off of 1.5-fold difference of FPKM values.

TALEN-targeting to create *st110* and *st99* mutations

The TAL Effector-Nucleotide Targeter 2.0 (Doyle et al., 2012, Cermak et al., 2011) webtool was used to design a pair of transcription activator-like effector nucleases to target the exon1-intron boundary of the *rraga* gene, and exonic regions of *lamtor4*. The Golden Gate cloning protocol for creating the TALEN plasmids was used (Sanjana et al., 2012). Plasmids were then transcribed using Sp6 mMessage mMachine Kit by Ambion. 400ng of mRNA were injected into 1-cell stage wildtype TL embryos, which were raised to adulthood. To identify founders carrying a null mutation in the germline, we cross injected fish to TLs and genotyped a subset of the progeny at 2-3 dpf. To genotype *st110*, genomic DNA was amplified by PCR (primers: forward 5'- gcagccgcacaaactttgatatt-3' and reverse 5'-tgcagtatggcttccagacacaa-3') and incubated with BspH1, which cleaves the wildtype but not the mutant allele. Based on the disruption of the BspH1 restriction site, we identified founders and raised the remaining F1 progeny to adulthood. Sequencing identified an F1 heterozygous for 16-bp deletion, which was crossed to TL to

establish a stock. Similar protocols were used to generate *st99*; the mutation was identified by PCR of genomic DNA (primers: forward 5'-TTTATCTGCACTGTCTAAAATGGT-3', reverse 5'-CGCGAGTACTCCGTCTTCACTA-3'), followed by digestion with DpnII.

In Situ Hybridization

ISH on embryos and larvae was performed using standard methods (Thisse et al., 2004). Embryos were fixed overnight in 4% paraformaldehyde, dehydrated for at least 2h in 100% methanol, rehydrated in PBS, permeabilized with proteinase K, and incubated overnight with antisense riboprobes at 65ºC. The probe was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase. Images were captured using the Zeiss AxioCam HRc camera with the AxioVision software. Probes for *mfap4* and *apoe* were previously described (Zakrzewska et al., 2010, Shiau et al., 2013). Primers used to generate probes for *rraga* and *slc7a7* are found in Supplemental Experimental Procedures.

Confocal fluorescent imaging

Zebrafish embryos were mounted in 1.5% low melting point agarose in distilled water. Images were captured using a Zeiss LSM 5 Pascal or Zeiss LSM confocal microscope. Objectives used were Plan-Neofluar 10x (numerical aperture 0.30) and 20x (numerical aperture 0.75).

Toluidine blue staining

Thick sections (500-1000µm) for Toluidine blue staining were collected on glass slides, stained at 60ºC for 5 seconds and imaged with the Leica DM 2000 microscope using the Leica DFC290 HD camera and Leica Application Suite software.

Primers used for making *slc7a7* and *rraga* probes for in situ hybridization:

ISH_slc7a7_F	5'-gcgttcaggacttcttcacc-3'

ISH_slc7a7_R	5’- ttcagtggtcgtggtctgtc-3’
ISH_rraga_F	5’-atgtccagcacagccatgaaaa-3’
ISH_rraga_R	5’- ttagcgcatacgcatgtgtagact-3’

Primers used for RT-PCR (Fig. 6K, L):

Hexa_F	5’-aaagttgagccacttgatttcac-3’
Hexa_R	5’-catactctccccaaagacaagc-3’
Asah1b_F	5’-tcagctggtggacaaggac-3’
Asah1b_R	5’-tgttgaagagcaccacctctc-3’
Bloc1s6_F	5’-cagatggattcttgtctcactacc-3’
Bloc1s6_R	5’-gtgacttcctgctccagagtatc-3’
Ctsc_F	5’-gcctccagaattccacgac-3’
Ctsc_R	5’-tccattaacgtttctccaatcc-3’
Mapk1_F	5’-cagccaggctacagaccatag-3’
Mapk1_R	5’-acgataaaggccgaaagagg-3’
Mapk3_F	5’-gcccctgagatcatgcttaac-3’
Mapk3_R	5’-ggaagatgggtctgttggac-3’
Arghgdia_F	5’-cctgcgcaagtacaaggag-3’
Arghgdia_R	5’-ccgcgtcacctgaacatt-3’
Dusp1_F	5’-cgaggccatcgaatttattg-3’
Dusp1_R	5’-cgcatcagataagccagaca-3’
Gabarap_F	5’-ccggacagagtgcctgtt-3’
Gabarap_R	5’-cgaggtatttctttttgtccaga-3’

Supplemental References

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.J., Bogdanove, A.J., and Voytas, D.F. (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. 39:e82.

Doyle, E.L., Booher, N.J., Standage, D.S., Voytas, D.F., Brendel, V.P., VanDyk, J.K., and Bogdanove, A.J. (2012) TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res.

Sanjana, N. E., Cong, L., Zhou, Y., Cunniff, M. M., Feng, G., & Zhang, F. (2012). A transcription activator-like effector toolbox for genome engineering. Nature protocols, 7(1), 171–92.

Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., Kirchner, J., Parkhill, J.P., and Thisse, C. (2004) Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. The Zebrafish: Genetics, Genomics and Informatics, 2nd ed., Methods Cell Biol. 77: 505-519.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., … Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols, 7(3), 562–78.

Zakrzewska, A., Cui, C., Stockhammer, O. W., Benard, E. L., Spaink, H. P., & Meijer, A. H. (2010). Macrophage-specific gene functions in Spi1-directed innate immunity. Blood, 116(3), e1–e11.