Figure S1

A

Percent Identity Matrix – created by Clusta12.1

zebrafish Spi1a	100.00	51.78	40.13	35.11
zebrafish Pu.1	51.78	100.00	33.52	37.25
zebrafish Spi2	40.13	33.52	100.00	40.23
zebrafish Spic	35.11	37.25	40.23	100.00

B

Spi2 VS ?	ETS domain	N-terminal
Spic	60.67	18.18
Spi1a	60.67	23.19
Pu.1	55.06	24.75

C

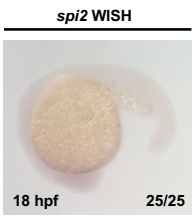


Fig. S1. Protein sequence comparison of zebrafish Spi-family members and *spi2* WISH in zebrafish embryonic hematopoiesis.

(A) Protein sequence comparison of Spi2 with other *spi*-family members in zebrafish. (B) N-terminal and C-terminal (Ets domain) protein sequence comparison. (C) WISH of *spi2* at 18 hpf.

Figure S2

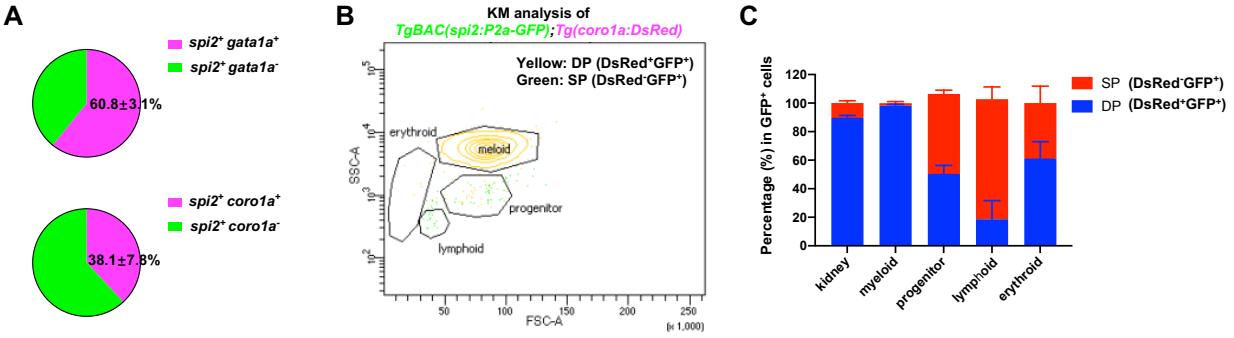


Fig. S2. FACS analysis of whole KM of adult *TgBAC(spi2:P2a-GFP);Tg(coro1a:DsRed)* zebrafish.

(A) Pie charts showing the quantification percentage (%±SD) of *spi2*$^+$ *gata1a*$^+$ cells in *spi2*$^+$ cells (fish n = 5) or *spi2*$^+$*coro1a*$^+$ cells in *spi2*$^+$ cells (fish n = 10) in CHT at 3 dpf. (B) Representative FACS analysis of the whole KM of adult *TgBAC(spi2:P2a-GFP);Tg(coro1a:DsRed)* fish. *spi2* expressing cells are marked by GFP, whereas leukocytes are labeled by DsRed. (C) Quantification of the percentage of GFP$^+$DsRed$^+$ in total GFP$^+$ cells in each gate in B. Data are representative of three independent experiments (4 biological replicates) and represented as mean ± SD.

Figure S3

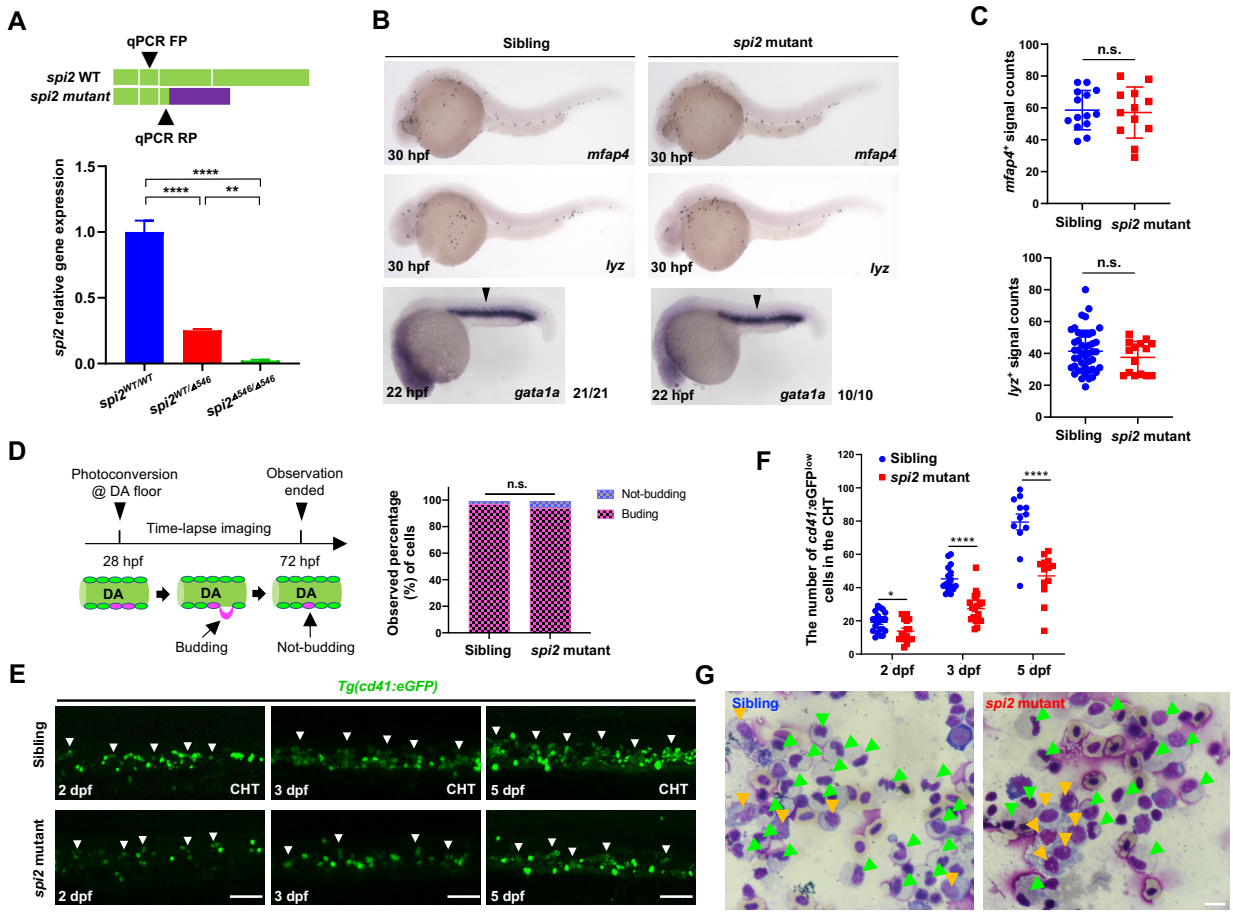


Fig. S3. Primitive hematopoiesis and EHT analysis in *spi2* mutants.

(A) qPCR analysis of *spi2* N-terminal transcripts in WT, *spi2*$^{+/\Delta 546}$ heterozygous, and *spi2*$^{\Delta 546/\Delta 546}$ homozygous embryos (the number of clutches of embryos, n = 3/4/4 for WT, *spi2*$^{+/\Delta 546}$, and *spi2*$^{\Delta 546/\Delta 546}$ respectively). (B) WISH of *mfap4*, *lyz*, and *gata1a* in *spi2* mutants and siblings at 22 hpf and 30 hpf. (C) Quantification of *mfap4* (siblings, n = 14; *spi2* mutants, n = 12) and *lyz* (siblings, n = 46; *spi2* mutants, n = 15) positive cells in B. (D) Diagram showing photoconversion and time-lapse imaging strategy to track EHT process and the percentage quantification of the observed cell behaviors in *spi2* mutants (embryo n = 5; photoconverted ECs n = 38; budding ECs n = 36) and siblings (embryo n = 6; photoconverted ECs n = 41; budding ECs n = 40). (E) Representative images of *Tg(cd41:eGFP)* in the CHT at 2 dpf and 3 dpf in *spi2* mutants and siblings. (F) Quantification of the *cd41:*eGFP^{low} cells in the CHT at 2 dpf and 3 dpf in *spi2* mutants and siblings in E. 2/3/5 dpf siblings, n = 21/16/12; 2/3/5 dpf *spi2* mutants, n = 15/21/13. (G) Representative MGG staining image in *spi2* mutants and siblings. Green triangles indicate myeloid cells. Orange triangles indicate myeloblasts. Scale bar, 20 μm. n/N reports the number of embryos with staining pattern in image/total embryos. Data are represented as mean ± SD, *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001, n.s. not significant (p > 0.05). Student's t test used in A, C, and F. Fisher test used in D.

Figure S4

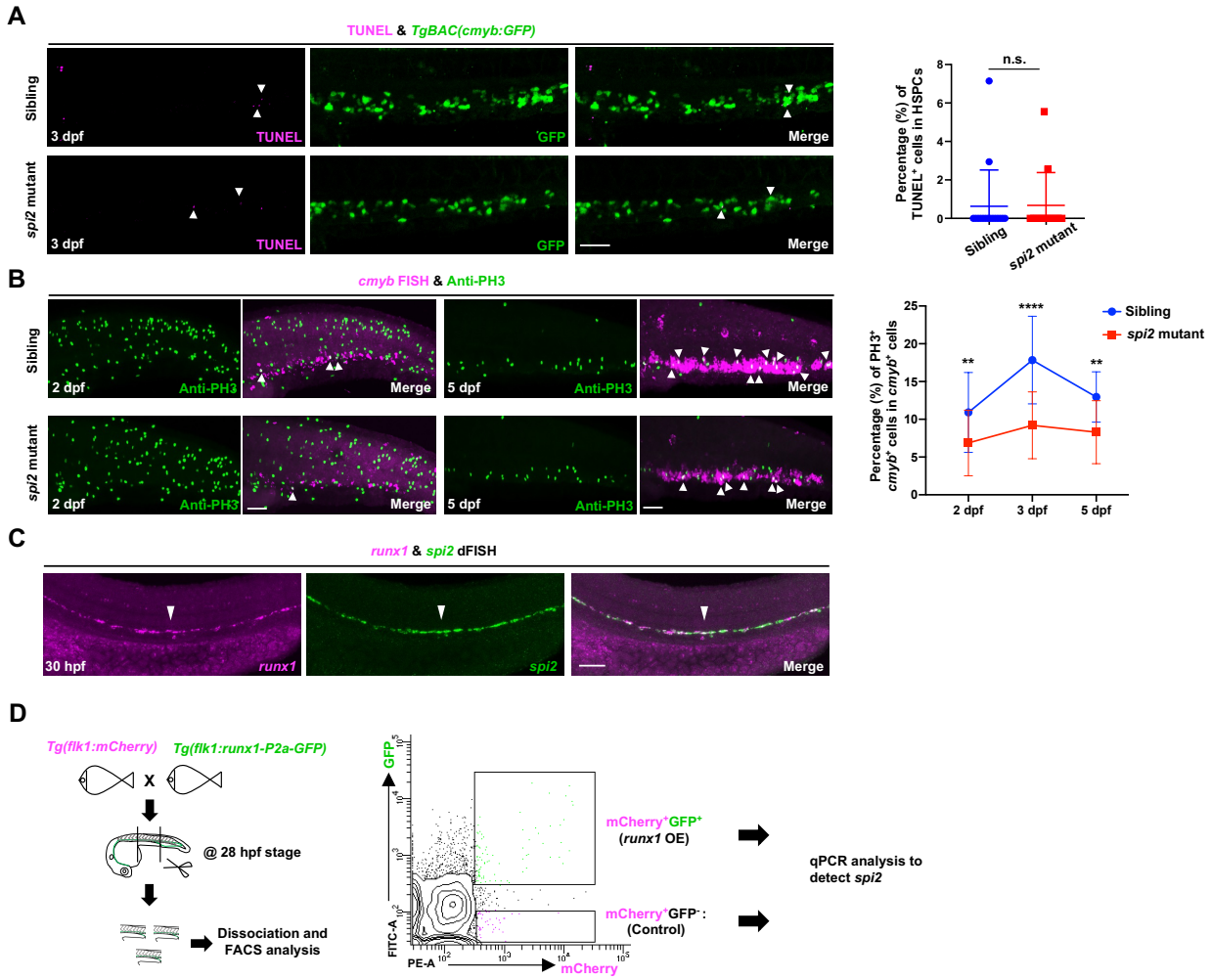
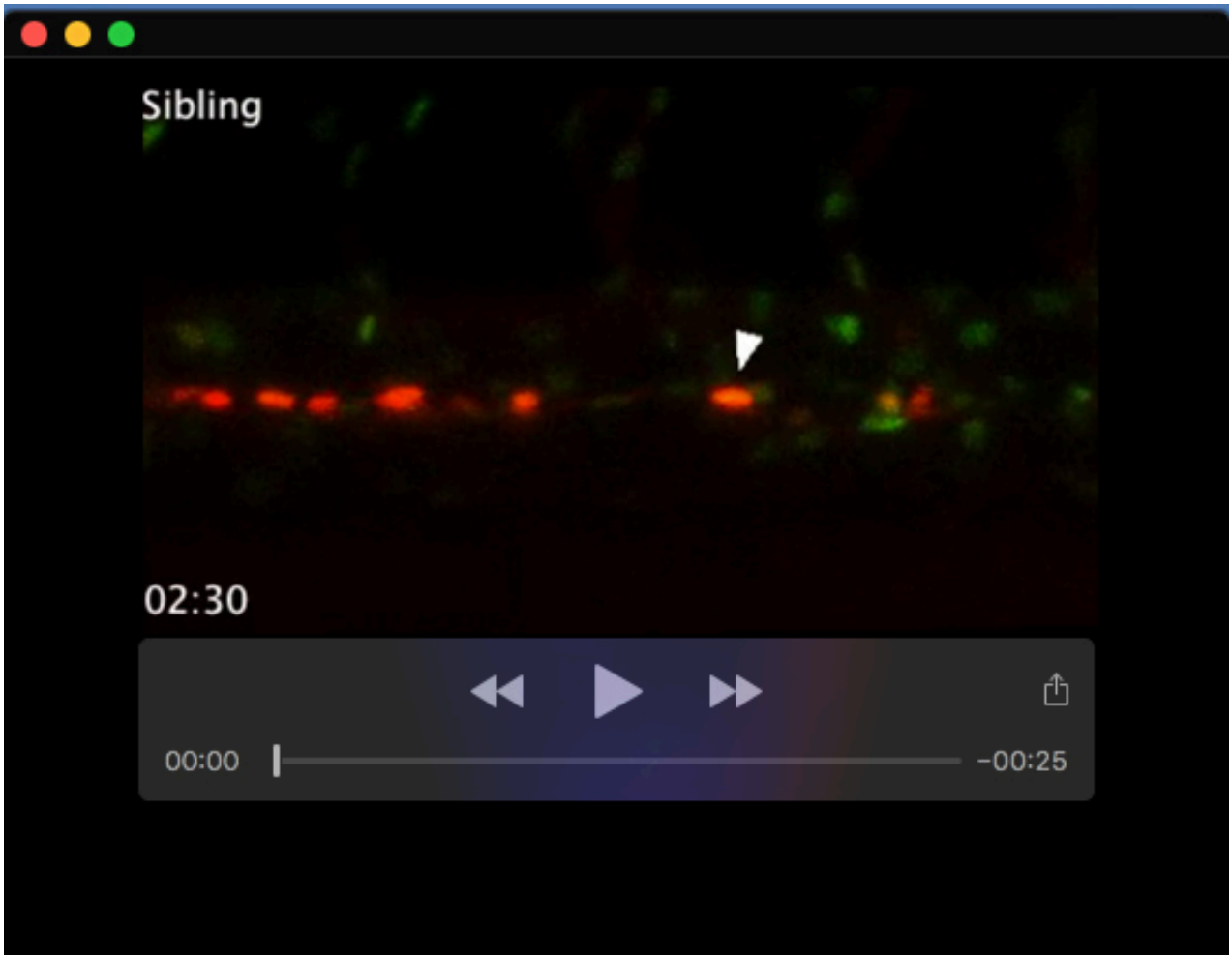


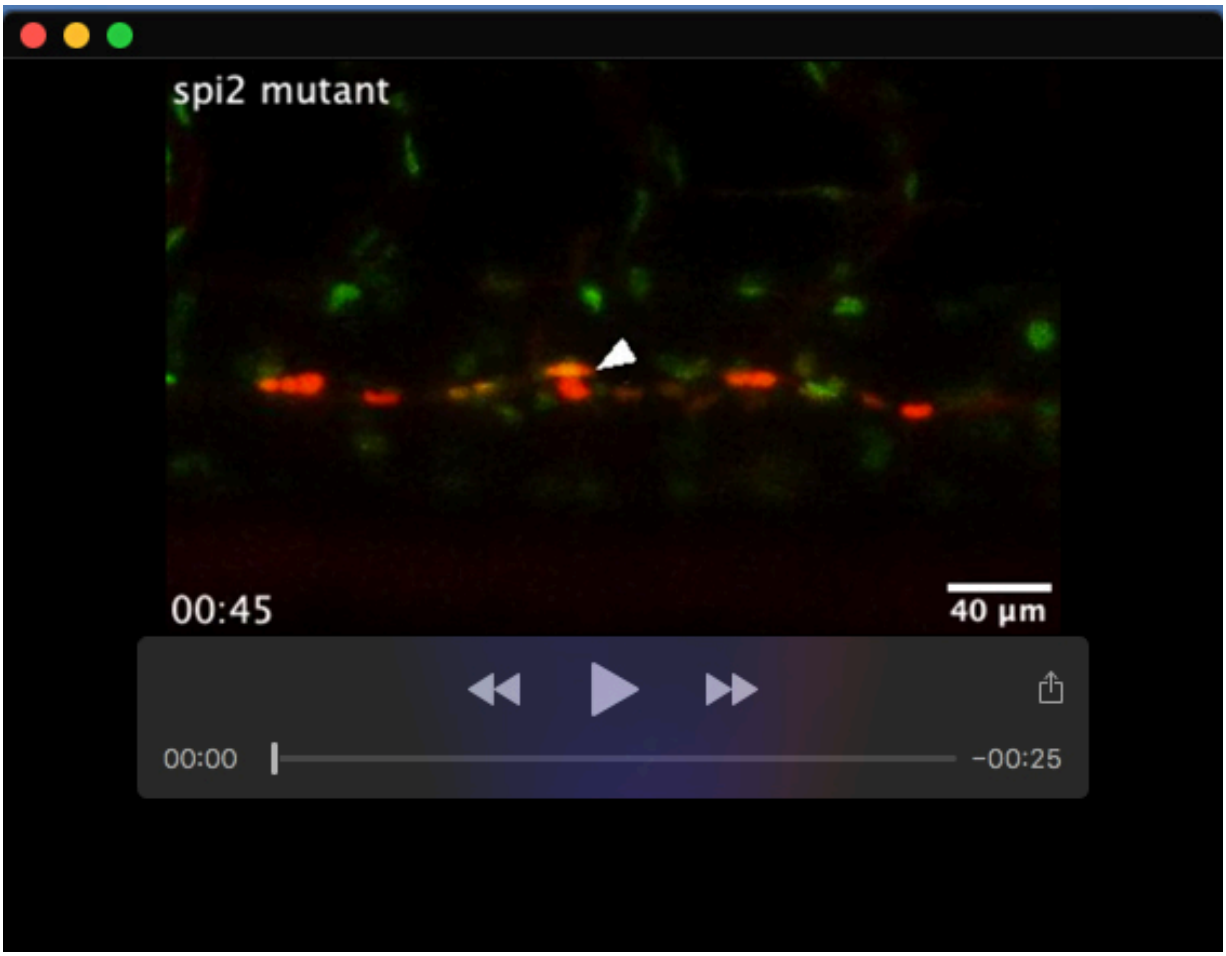
Fig. S4. TUNEL staining of HSPCs and colocalization of *spi2* and *runx1* in the HECs.

(A) TUNEL analysis (magenta) combined with GFP immunofluorescent staining in the CHT in *TgBAC(c-myb:GFP)* of *spi2* mutants and siblings at 3 dpf and the quantification of the percentage of TUNEL$^+$GFP$^+$/GFP$^+$ HSPCs in *spi2* mutants (n = 12) and siblings (n = 16). (B) Representative images of *c-myb* FISH (magenta) and anti-PH3 immunofluorescent staining (green) in the CHT in *spi2* mutants and siblings at 2 dpf and 5 dpf, and the quantification of the percentage of proliferating *c-myb*$^+$PH3$^+$ cells in the CHT in *spi2* mutants (n = 18, 18, 18) and siblings (n = 54, 35, 18) at 2, 3 and 5 dpf. (C) dFISH of *spi2* and *runx1* in the AGM in WT fish at 30 hpf. (D) FACS isolation of HECs and cECs from the trunk region of the progenies (28 hpf stage) of *Tg(flk1:mCherry)* crossing with *Tg(flk1:runx1-P2a-GFP)* zebrafish. *runx1* OE, ECs with *runx1* overexpression; Control, ECs without *runx1* overexpression. Student's t tests used in A and B. Data are represented as mean ± SD, n.s. not significant ($p > 0.05$). Scale bars, 60 μm.



Movie 1. Time-lapse imaging tracks the EHT process in *spi2* siblings.

Photoconverted HECs are labelled in red color. Lines denote DA roof and floor, and triangles denote an example of observed HEC in a *spi2* sibling embryo.



Movie 2.Time-lapse imaging tracks the EHTprocess in *spi2* mutants.

Photoconverted HECs are labelled in red color. Lines denote DA roof and floor, and triangles denote an example of observed HEC in a *spi2* mutant embryo.