Supplemental Information

Supplemental Materials and Methods

Probes utilized in the study

Digoxigenin labeled antisense *pu.1* (zebrafish information network), *cebp1* (zebrafish information network), *lyz* (zebrafish information network), *mpx* (zebrafish information network), *runx1* (zebrafish information network), *irf8* (zebrafish information network), *csf1ra* (zebrafish information network), *apoeb* (zebrafish information network); fluorescein (flu) labeled antisense *cepb1* and *lyz* probes.

Antibodies utilized in the study

Primary antibody: goat anti-GFP (1:200, Abcam), rabbit anti-dsred (1:200, Clontech), mouse anti-Brdu (1:50, Sigma), mouse anti-Myc (1:50, Santa Cruz) and rabbit anti-Pu.1 (1:50, made by injection of rabbit with GST-Pu.1 fusion protein). Secondary antibody: donkey anti-goat IgG alexa488 (1:400, Invitrogen), donkey anti-rabbit IgG alexa555 (1:400, Invitrogen), donkey anti-rabbit IgG alexa488 (1:400, Invitrogen), donkey anti-mouse IgG alexa555 (1:400, Invitrogen), donkey anti-mouse IgG alexa488 (1:400, Invitrogen)

Whole mount Brdu incorporation assay

Dechorionated embryos from Tg(*mpx*:GFP)i114$^{+/-}$*runx1*$^{w84x/+}$ intercrosses at desired stages were soaked with 10 mM Brdu in egg water for 1 hour-2 hours and fixed at 36 hpf for staining. Fixed embryos were washed with Phosphate buffered saline with 0.1% Tween20 (PBST) for 2 X 5mins and stored in -20ºC methanol for more than 1 hour (hr). Embryos were then sequentially rehydrated with 75% MeOH/PBST (5mins), 50% MeOH/PBST (5mins), 25% MeOH/PBST (5mins) and PBST (2 X 5mins). Afterwards, embryos were permeabilized with 10 µg/ml

Proteinase K in PBST for 5mins and refixed with 4% PFA for 20mins. Refixed embryos were treated with 2N HCl for 1hr at room temperature. After rinse several times with PBST, embryos were blocked with 1% bovine serum albumin in PBST. Subsequently, embryos were incubated with anti-Brdu antibody (1:50) and anti-GFP antibody (1:200) followed by anti-mouse IgG Alexa 555 (1:400) and anti-goat Alexa 488 (1:400).

Fluorescence activated cell sorting (FACS) and cell cycle analysis with Hoechst staining

FACS isolation and Hoechst staining of *mpx:*GFP^{+} cells were performed as described[1].

Construction

To generate pTolhsp70:6Xmyc-*runx1* or pTolhsp70:6Xmyc-*pu.1*, full-length *runx1* or *pu.1* was first PCR amplified and engineered into pCS2^{+}6Xmyc to generate pCS2^{+}6Xmyc-*runx1* or pCS2^{+}6Xmyc-*pu.1*. Afterwards 6Xmyc-*runx1* or 6Xmyc-*pu.1* was subcloned into pTolhsp70 to produce pTolhsp70:6Xmyc-*runx1* or pTolhsp70:6Xmyc-*pu.1*, respectively.

Establishment of Tg(*hsp70*:myc-*runx1*) and Tg(*hsp70*:myc-*pu.1*) lines and heat shock experiment

A mixture containing pTolhsp70:6Xmyc-*runx1* or pTolhsp70:6Xmyc-*pu.1* construct (25 ng/μl) and capped transposase mRNA (25 ng/μl) was injected into one-cell stage AB embryo at the volume of approximate 2 nl to yield F0 founders. The resulting F0 fish were raised to adulthood and mated pairwisely to identify founders that transmitted transgenes to their offsprings. Genomic DNA was extracted from pools of 50-100 24 hpf embryos from pairwise mating and subject to PCR. PCR positivity was verified using anti-Myc staining with 24 hpf embryos that have been heat shocked (see below). Three founders Tg(*hsp70*:myc-*runx1*^{r1}), Tg(*hsp70*:myc-

runx1^{r2}) and Tg(*hsp70*:myc- *pu.1*), were identified. These lines were propagated by crossing the identified founder with germ-line transmission with AB.

For heat induction of myc-*runx1* or myc-*pu.1* expression, embryos resulting from crossing between heterozygous Tg(*hsp70*:myc-*runx1*^{r1}) or Tg(*hsp70*:myc-*pu.1*) and AB were soaked with pre-heated egg water at 11 hpf and heat shocked at 39.5ºC for 1hr or 38.5ºC for 40mins, respectively. After heat shocking, embryos were grown in 28.5ºC until desired stages for histological examination.

Quantification of Pu.1 protein expression

Embryos stained with anti-Pu.1 antibody were imaged with 20XObjectives in Zeiss LSM510 confocal. Individual positive cells in the image were outlined and calculated for fluorescent signal intensity by data analysis function in the Photoshop.

Functional assays for phagocytes

Escherichia coli expressing Dsred^2 were cultured overnight and concentrated 3X before injection. 3 nl concentrated bacterial culture was delivered into circulatory system via injection into the joint of anterior cardinal vein (ACV) and common cardinal vein (CCV). Phagocytic foci representing functional macrophages were scored 15 minutes (mins) post injection.

Quantitative and semi-quantitative RT-PCR

Total RNA was extracted from embryos using the Rneasy Mini Kit (Qiagen) and was converted to cDNA using Omniscript RT Kit (Qiagen). Quantitative PCR (qPCR) was performed with SYBR Green Supermix on 7500 Fast Real-time PCR system. Primers used in qPCR: eGFP: 5'-accatcttcttcaaggacga-3'/5'-ggctgttgtagttgtactcc-3'; *pu.1*: 5'-atgctgcatccgtacagaatgg-3'/5'-

gtggtcgatagatctctgtttc-3'; *elf1a*: 5'-cttctcaggctgactgtgc-3'/5'-ccgctagcattaccctcc-3'. Semi-qPCR was performed with homemade Taq system.

Pu.1 promoter reporter assay

To test the *runx1* responsiveness of -9.0*pu.1*:eGFP and -5.3*pu.1*:eGFP, wild-type sibling and *runx1*^{w84x} mutant embryos injected with -9.0*pu.1*:eGFP construct (30 pg/embryo) or with a Tg(-5.3*pu.1*:eGFP) transgene were quantified for GFP expression at 17.5 hpf using Quantitative RT-PCR (qRT-PCR). *Runx1* motifs were predicted using online software PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promo.cgi?dirDB=TF_8.3&calledBy=alggen). The two adjoining *Runx1* motifs in the -9.0 kb to -5.3 kb promoter region were deleted in -9.0*pu.1*:eGFP construct by overlapping PCR to form -9.0*pu.1*ΔR:eGFP construct. To assay the reporter activity, -9.0*pu.1*:eGFP or -9.0*pu.1*ΔR:eGFP construct was injected into 1-cell stage AB embryos at the dose of 30pg/embryo. Activity of GFP reporter was quantified by qRT-PCR at 17.5 hpf and normalized with the amount of DNA injected. The relative quantity of DNA injected was determined by sampling 30 injected embryos at 3 hpf for quantitative PCR.

Microscopy and imaging

Video enhanced (VE) DIC microscopy was done with a 60X/1.00 NA water-immersion objective mounted on Nikon 80i microscope according to previously described.[10] VE-DIC image was captured with a HV-D30 Hitachi color camera and recorded using Sony GV-HD700E videocassette recorder. Whole mount or magnified bright field image was taken using spot flex camera mounted on Nikon AZ100 microscope or Nikon 80i microscope, respectively. Fluorescent image was captured with Zeiss LSM510 confocal microscopy.

References

1. Liu Y, Du L, Osato M, Teo EH, Qian F, Jin H, Zhen F, Xu J, Guo L, Huang H, Chen J, Geisler R, Jiang YJ, Peng J, Wen Z. The zebrafish udu gene encodes a novel nuclear factor and is essential for primitive erythroid cell development. *Blood* 2007;**110**(1):99-106.

2. van der Sar AM, Musters RJ, van Eeden FJ, Appelmelk BJ, Vandenbroucke-Grauls CM, Bitter W. Zebrafish embryos as a model host for the real time analysis of Salmonella typhimurium infections. *Cell Microbiol* 2003;**5**(9):601-611.

Supplementary Figure Legends

Figure S1. Lineage tracing experiment shows that RBI gives rise to both embryonic macrophages and neutrophils.

(A) Schematic diagram of lineage tracing experiment. DMNB-caged flu was injected into one-cell stage AB wild-type embryos and photoactivated in RBI myeloid progenitors at 10-14s stage. Uncaged embryos were grown to 36-48 hpf and the identities of cells with activated flu were determined using video-enhanced DIC microscopy. (B) Representative flu^+ cells (green) from 3 wt embryos. Red and yellow arrows indicate flu^+ neutrophils containing abundant granule (red arrowheads) and flu^+ macrophages loaded with engulfed material (yellow arrowheads), respectively.

Figure S2. *lyz* expression preferentially labels neutrophil and *cebp1* is a marker of early embryonic neutrophil progenitor.

(A) In vivo imaging of 2 dpf *Tg(mpx:GFP);Tg(lyz:dsred)* embryos by video-enhanced DIC microscopy (left-most panel: bright field (BF) DIC image; left panel: fluorescent image for GFP; right panel: fluorescent image for dsred; right-most panel: a overlay of BF DIC and fluorescent images). Note that mpx:GFP expression coincides with lyz:dsred expression. Blue arrowheads identify granules contained by neutrophils (white arrows) expressing both transgenes. (B) Triple staining for Sudan Black (SB) (left-most), *lyz:*GFP (left) and *E coli*-dsred (right) with embryos injected with red fluorescent *E coli* at the joint of anterior cardinal vein (acv) and common cardinal vein (ccv) at 2 dpf. An overlay of SB, *lyz:GFP,* and *E coli*-dsred staining is shown in the right-most panel. White arrowheads indicate neutrophils positive for both *lyz:*GFP and SB that are devoid of red-fluoresent *E coli*. White arrows show SB or *lyz:*GFP negative macrophages loaded with red fluorescent *E coli*. (C) Counts of cells expressing *cebp1, mpx,* or *lyz* at 18 or 19.5 hpf. ($cebp1_{18hpf}$ (mean/s.e./n)=2.8/0.6/28, $cebp1_{19.5hpf}$=18.2/2.2/16; mpx_{18hpf}=0.2/0.1/29, $mpx_{19.5hpf}$=4/1.3/11; lyz_{18hpf}=0.1/0.0/29, $lyz_{19.5hpf}$=1.3/0.6/12). (D) Double fluorescence in situ hybridization for *mpx* (left panel) and *cebp1* (middle panel) at 24 hpf. Right panel is an overlay of *mpx*, *cebp1* staining and a corresponding BF image. Arrows indicate cells only expressing *cebp1* and arrowheads indicate co-localizing cells. (E) Double fluorescence in situ hybridization for *csf1ra* (left panel) and *cebp1* (middle panel) at 24 hpf. Right panel is an overlay of *csf1ra*, *cebp1* staining and a corresponding BF image. Arrows and arrowheads indicate cells only expressing *cebp1* and *csf1ra*, respectively.

Figure S3. *apoeb*$^{+}$ microglia significantly diminish in *csf1ra* mutants (*csf1ra*^{j4e1}).

(A-B) WISH of *apoeb* expression in 3 dpf *siblings* (*sib*) (A) and *csf1ra*^{j4e1} mutant (B) embryos.

Figure S4. The zebrafish *pu.1*^{G242D} mutation.

(A-B) *pu.1*^{G242D} mutants harbor a single point mutation (g to a) in the coding region of the *pu.1* gene (A), resulting in a mutated protein in which amino acid G (Gly242) is replaced by D (Asp) in the Ets domain (B).

Figure S5. Characterize Pu.1 expression in *pu.1*^{G242D} mutants.

(A-B) WISH of *pu.1* RNA expression in 14 hpf siblings (*sib*) (A, arrows) and *pu.1*^{G242D} mutants (B, arrows). (C-D) Antibody staining of Pu.1 protein expression in 17.5 hpf *sib* (C, arrows) and *pu.1*^{G242D} mutants (D, arrows). Embryos in A-D are viewed dorsally with the anterior towards the top. (E) Quantification of per-cell fluorescent intensity (FL) of Pu.1 antibody staining in *sib* and *pu.1*^{G242D} mutants shown in C and D. FL_{sib}(mean/s.e/cell number)=1856.1/243.6/33. $FL_{pu.1G242D}$ (mean/s.e/cell number)=515.9/55.5/14. The asterisk indicates statistical significance ($P<0.001$, t-test).

Figure S6. Heat-shock induced expression of Myc-Pu.1 and Myc-Runx1.

(A-B) Whole mount staining of Myc-Pu.1 expression by Myc anti-serum in 20 hpf non-heat-shocked (A, Non-HS) and heat-shocked (B, HS) *Tg(hsp70:myc-pu.1)* embryos. (C-D) Whole mount staining of Myc-Runx1 expression by Myc anti-serum in 20 hpf non-heat-shocked (C, Non-HS) and heat-shocked (D, HS) Tg(*hsp70*:myc-*runx1*) embryos.

Figure S7. Decreased embryonic neutrophils in *runx*^{w84x} mutants.

(A-B) WISH of *lyz* expression in 32 hpf *siblings* (*sib*) (A) and *runx1*^{w84x} mutants (B). Arrows indicate WISH signal of *lyz*. (C-D) SB staining of 36 hpf *sib* (C) and *runx1*^{w84x} (D) embryos. Arrows indicate SB staining signal. (E-F) In vivo image of neutrophils in 2 dpf *sib* (E) and *runx1*^{w84x} (F) embryos in *Tg(mpx:eGFP)* background by video-enhanced DIC microscopy. Left

panels are BF DIC images. Right panels are overlays of BF DIC images with corresponding GFP fluorescent images. Blue arrowheads indicate granules contained by neutrophils (white arrows).

Figure S8. Diminished neutrophil development in *runx1*^{w84x} is not accompanied by a suppressed S phase profile.

Diagram shows the percentage of mpx:GFP^{+} cells with Brdu staining in 36 hpf *siblings* (*sib*) and *runx1*^{w84x} mutants which were pulse labeled with Brdu at 19.5 hpf or 32 hpf for 1 hour and 2 hours, respectively. No statistical difference were found (*t*-test, $sib_{19.5\ hpf}$ (mean/s.e./n)= 59.1%/7.0%/6, $runx1^{w84x}{}_{19.5\ hpf}$ =54.3%/7.5%/10; $sib_{32\ hpf}$ (mean/s.e./n)= 29.5%/4.8%/14, $runx1^{w84x}{}_{32\ hpf}$ =31.0%/10.8%/10).

Figure S9. Dampening Pu.1 activity using *pu.1*^{G242D} mutation corrects neutrophil phenotype in *runx1*^{w84x} mutants.

(A-D) WISH of *cebp1* expression in 23 hpf *runx1*$^{+/+}$*pu.1*$^{+/+}$ (A), *runx1*$^{+/+}$*pu.1*$^{G242D/+}$ (B), *runx1*$^{w84x/w84x}$*pu.1*$^{+/+}$ (C), *runx1*$^{w84x/w84x}$*pu.1*$^{G242D/+}$ (D). (E-H) SB staining of 36 hpf *runx1*$^{+/+}$*pu.1*$^{+/+}$ (E), *runx1*$^{+/+}$*pu.1*$^{G242D/+}$ (F), *runx1*$^{w84x/w84x}$*pu.1*$^{+/+}$ (G), *runx1*$^{w84x/w84x}$*pu.1*$^{G242D/+}$ (H). (I-N) WISH of *lyz* expression in 30 hpf *runx1*$^{+/+}$*pu.1*$^{+/+}$ (I), *runx1*$^{+/+}$*pu.1*$^{G242D/G242D}$ (J), *runx1*$^{+/+}$*pu.1*$^{G242D/+}$ (K), *runx1*$^{w84x/w84x}$*pu.1*$^{+/+}$ (L), *runx1*$^{w84x/w84x}$*pu.1*$^{G242D/G242D}$ (M), *runx1*$^{w84x/w84x}$*pu.1*$^{G242D/+}$ (N). (**O**) Quantification of No. of *cebp1*$^{+}$ cells in 23 hpf *runx1*$^{+/+}$*pu.1*$^{+/+}$, *runx1*$^{+/+}$*pu.1*$^{G242D/+}$, *runx1*$^{w84x/w84x}$*pu.1*$^{+/+}$, *runx1*$^{w84x/w84x}$*pu.1*$^{G242D/+}$ embryos. The asterisk indicates a statistics difference (*t*-test, $cebp1_{runx1+/+\ pu.1+/+}$ (mean/s.e./n)=50.3/3.1/13, $cebp1_{runx1+/+\ pu.1G242D/+}$ =54.2/3.2/23, $cebp1_{runx1w84x/w84x\ \ pu.1+/+}$ =11.0/1.3/10, $cebp1_{runx1w84x/w84x\ \ pu.1G242D/+}$ =24.0/2/20, *: p<0.001). (**P**) Quantification of No. of SB^{+} cells in 36 hpf *runx1*$^{+/+}$*pu.1*$^{+/+}$, *runx1*$^{+/+}$*pu.1*$^{G242D/+}$, *runx1*$^{w84x/w84x}$*pu.1*$^{+/+}$, *runx1*$^{w84x/w84x}$*pu.1*$^{G242D/+}$ embryos. The asterisk

indicates a statistics difference (*t*-test, $SB_{runx1+/+\ pu.1+/+}$ (mean/s.e./n)=105.6/10.7/7, $SB_{runx1\ +/+\ pu.1\ G242D/+}$ =100.8/4.9/18, $SB_{runx1w84x/w84x\ pu.1+/+}$ =28.9/3.9/11, $SB_{runx1w84x/w84x\ pu.1G242D/+}$ =52.3/6.2/15, *: p<0.01). (Q) Quantification of No. of lyz^{+} cells in 30 hpf $runx1^{+/+}pu.1^{+/+}$, $runx1^{+/+}pu.1^{G242D/+}$, $runx1^{+/+}pu.1^{G242D/G242D}$, $runx1^{w84x/w84x}pu.1^{+/+}$, $runx1^{w84x/w84x}pu.1^{G242D/+}$, $runx1^{w84x/w84x}pu.1^{G242D/G242D}$ embryos. The asterisk indicates a statistics difference (*t*-test, $lyz_{runx1+/+\ pu.1+/+}$ (mean/s.e./n)= 79.5/6.0/8, $lyz_{runx1+/+\ pu.1\ G242D/+}$ =63.0/2.9/21, $lyz_{runx1+/+\ pu.1\ G242D/G242D}$ =72.3/12.8/6, $lyz_{runx1w84x/w84x\ pu.1+/+}$ =23.0/2.0/13, $lyz_{runx1w84x/w84x\ pu.1G242D/+}$ =32.7/3.5/18, $lyz_{runx1w84x/w84x\ pu.1G242D/G242D}$ =34.0/3.9/9 *: p<0.05).

Figure S10. Introduction of *pu.1*^{G242D} mutation in the *runx1*$^{W84x/w84x}$ mutants blocks macrophage development.

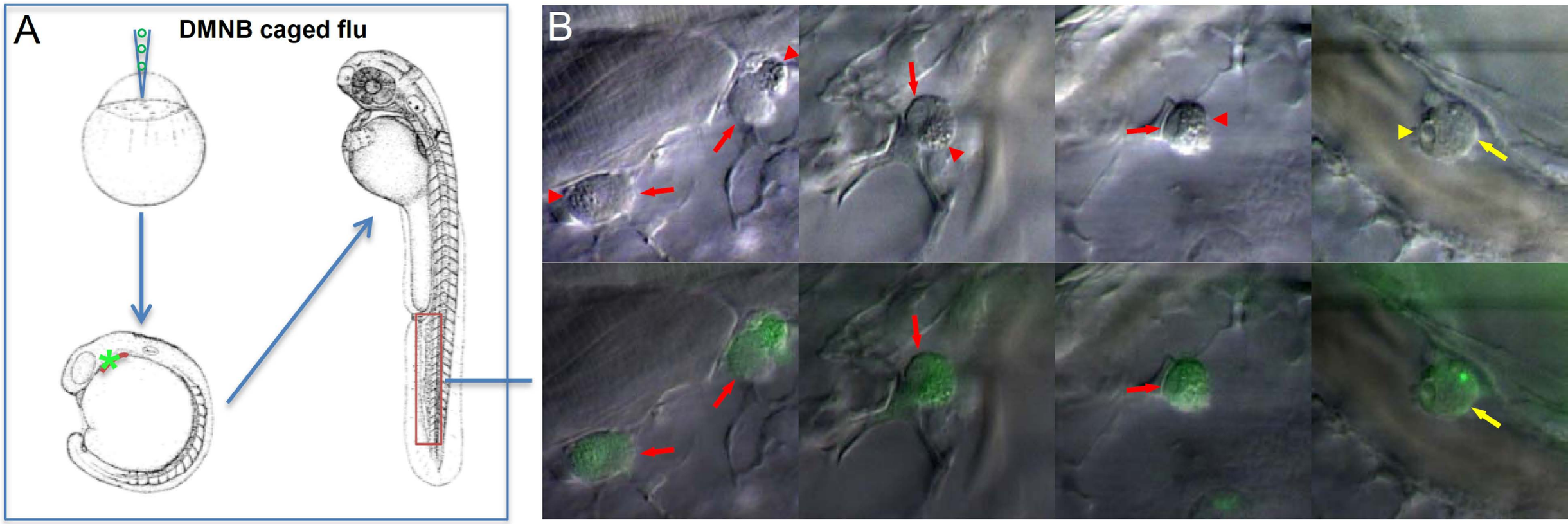
(A-C) WISH of *irf8* expression in 23 hpf $runx1^{w84x/w84x}pu.1^{+/+}$ (A), $runx1^{+/+}pu.1^{G242D/G242D}$ (B), $runx1^{W84x/w84x}pu.1^{G242D/G242D}$ (C). (D-F) WISH of *csf1ra* expression in 30 hpf $runx1^{w84x/w84x}pu.1^{+/+}$ (D), $runx1^{+/+}pu.1^{G242D/G242D}$ (E), $runx1^{W84x/w84x}pu.1^{G242D/G242D}$ (F). (G-I) WISH of *apoeb* expression in 72 hpf $runx1^{w84x/w84x}pu.1^{+/+}$ (G), $runx1^{+/+}pu.1^{G242D/G242D}$ (H), $runx1^{W84x/w84x}pu.1^{G242D/G242D}$ (I). Black arrowheads in D-F indicate *csf1ra* expressed in neural crest cells.

Figure S11. The 9.0kb but not 5.3kb *pu.1* upstream regulatory region contains *runx1* repressible cis-elements.

(A) Quantitative RT-PCR for GFP expression in 17.5 hpf $runx1^{w84x}$/*Tg(-5.3pu.1:eGFP)* mutants and siblings. Units on y-axis represent the relative fold change of GFP expression in siblings and $runx1^{w84x}$ mutant embryos. Expression level was normalized with *elf1α* expression. Error bars, s.e. (B) Quantitative RT-PCR for GFP expression in 17.5 hpf $runx1^{w84x}$ mutants and siblings

injected with Tg(-9.0*pu.1*:eGFP) construct. Units on y-axis represent the relative fold change of GFP expression in siblings and *runx1*^{w84x} mutant embryos. Expression level was normalized with *elf1α* expression. Error bars, s.e.

supplemental Figure 1



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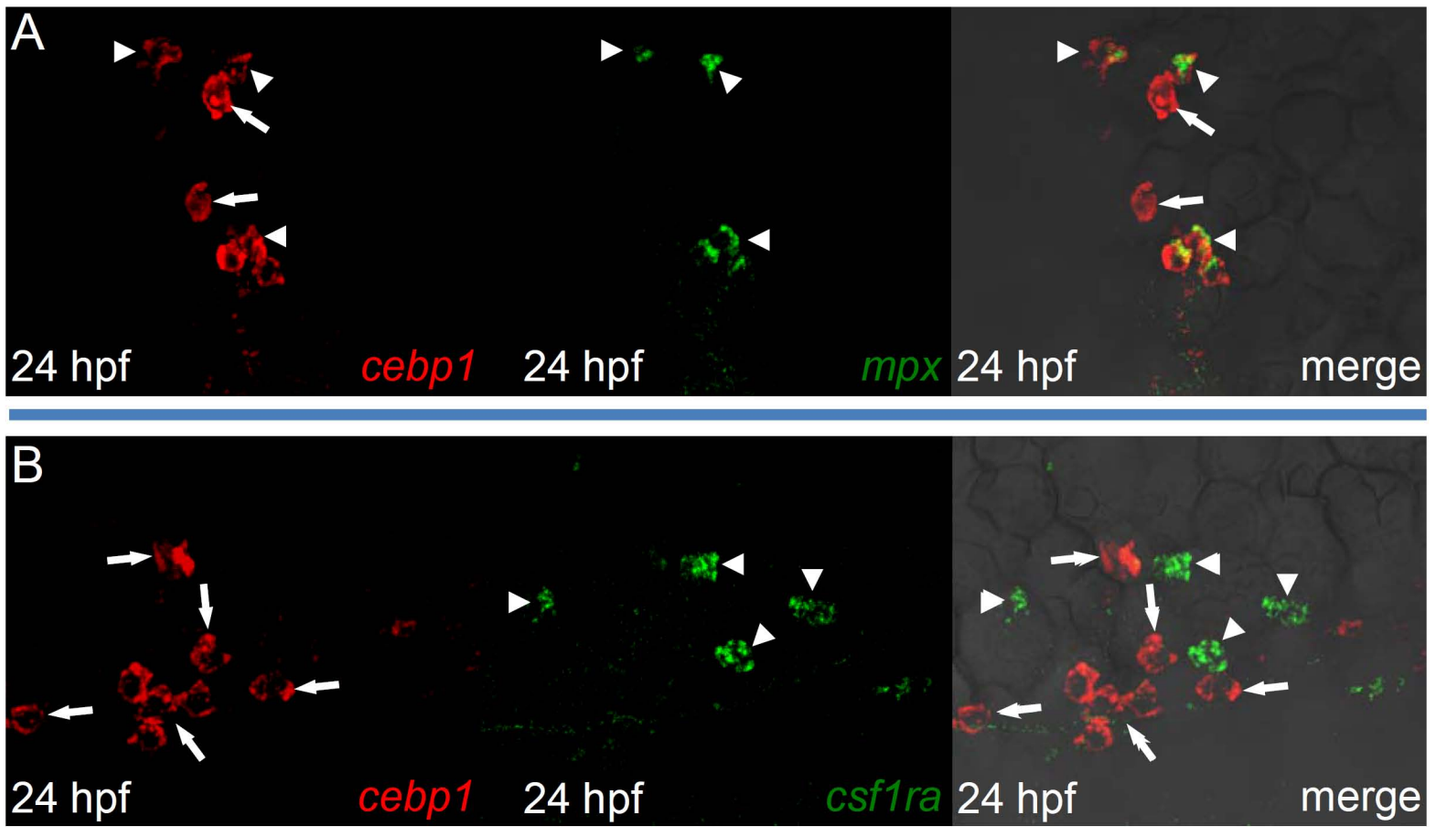

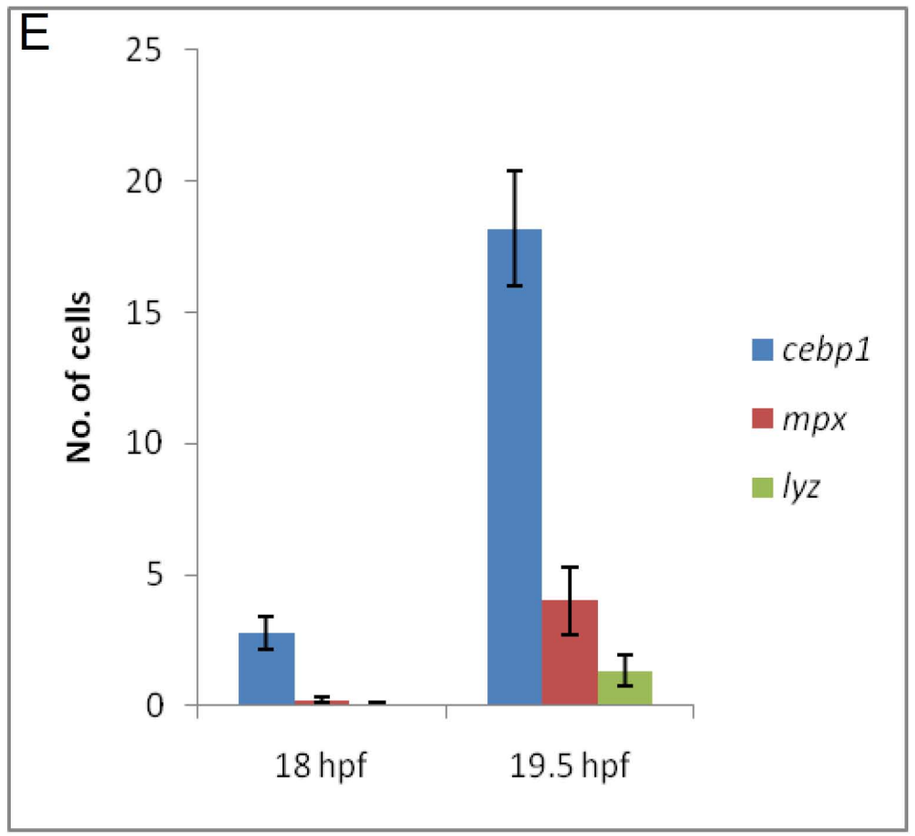

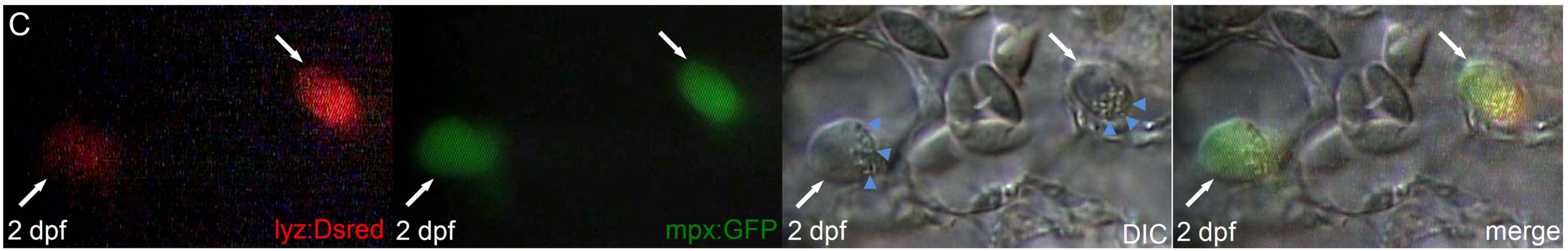

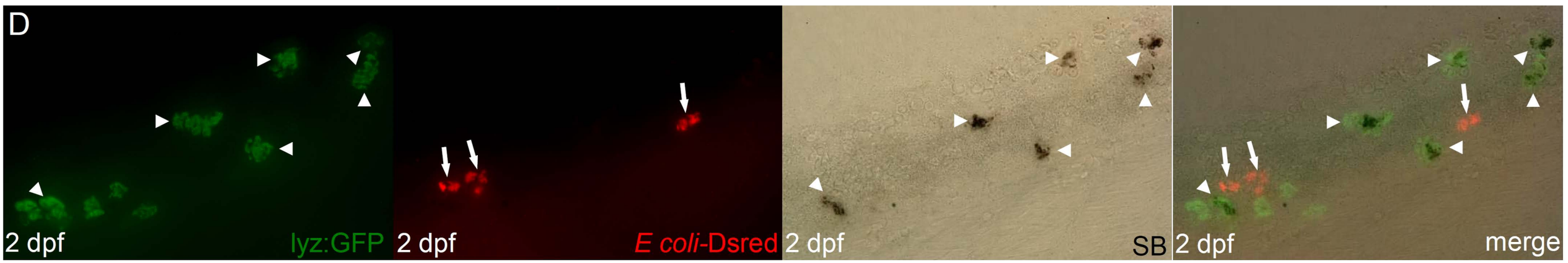

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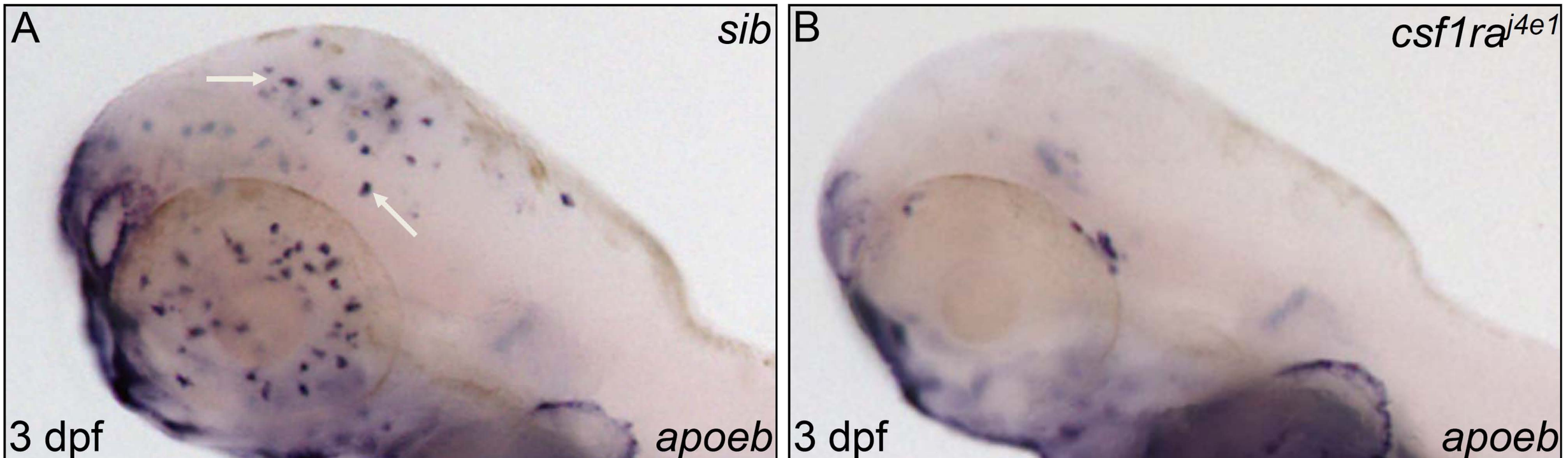

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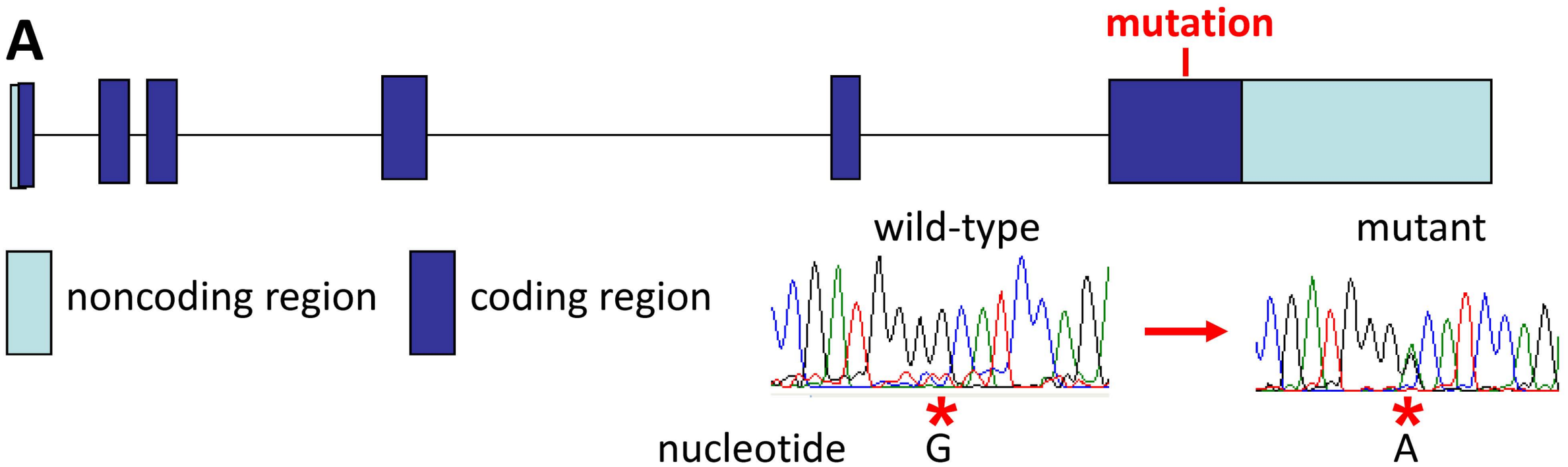

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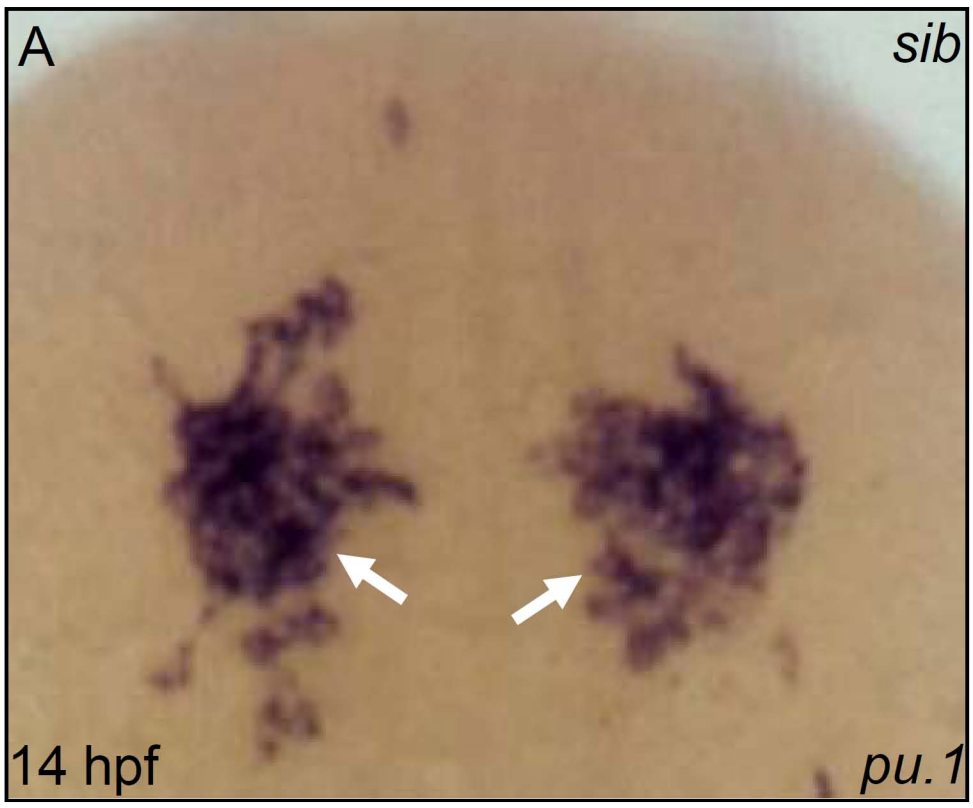
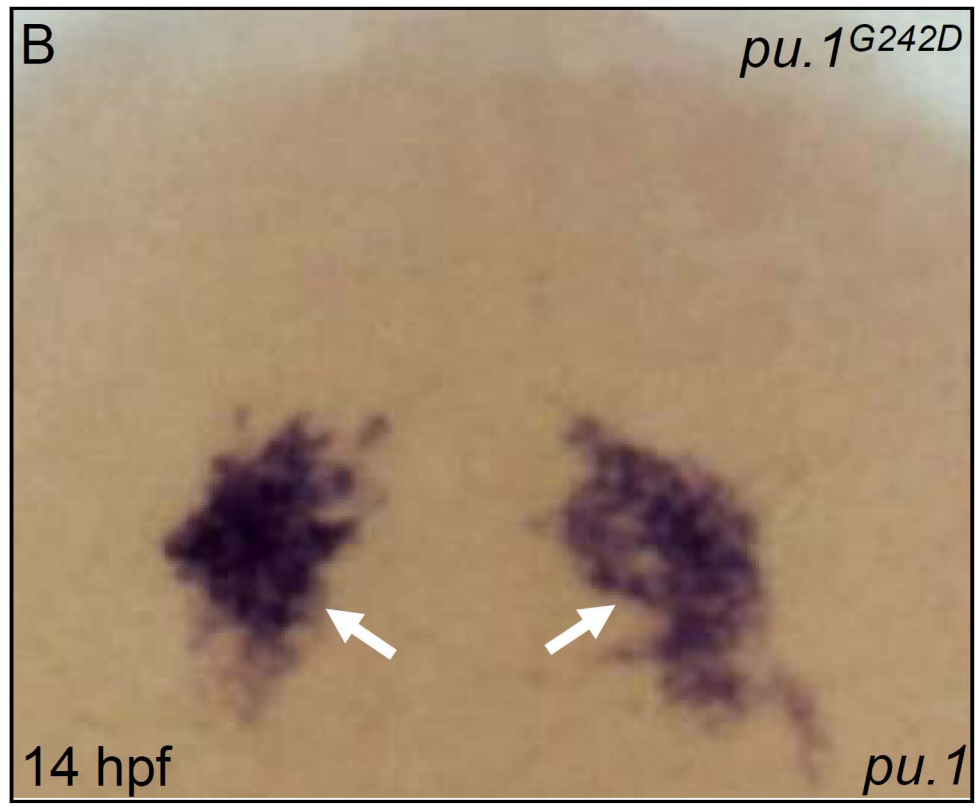
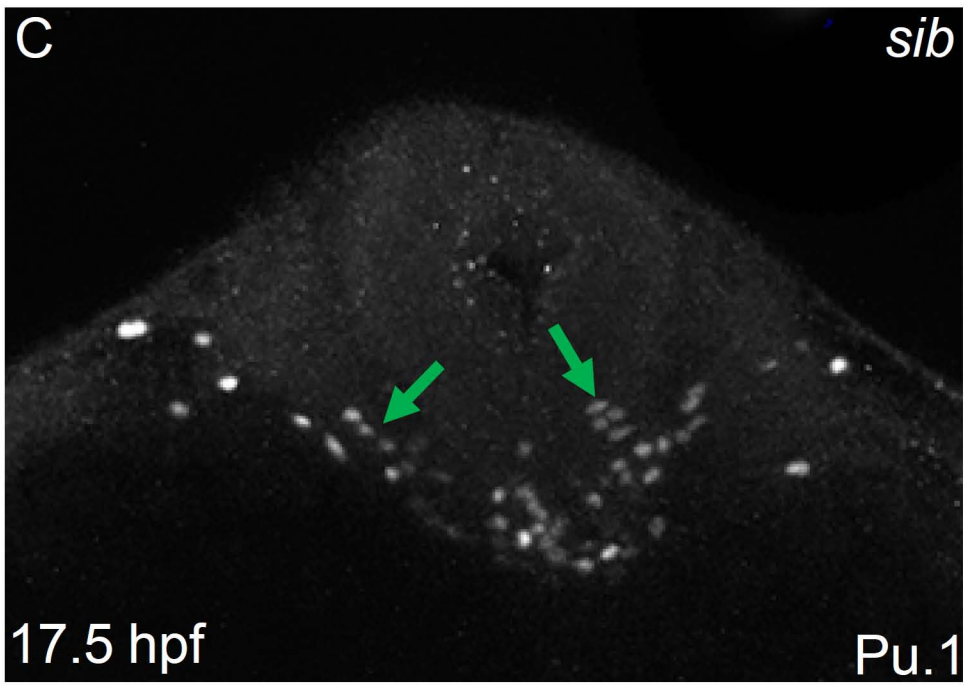
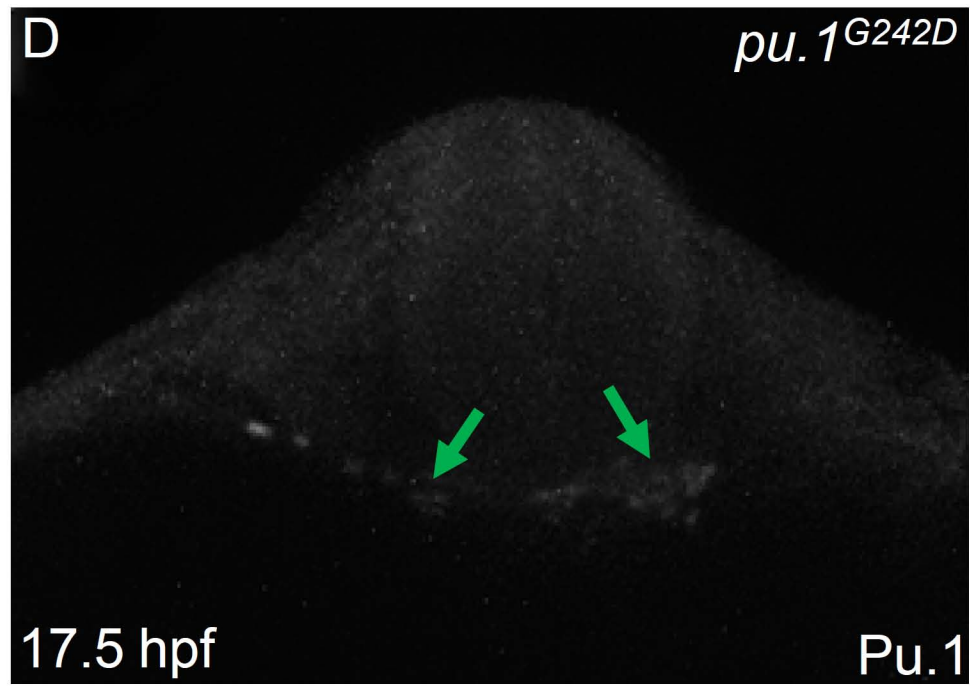
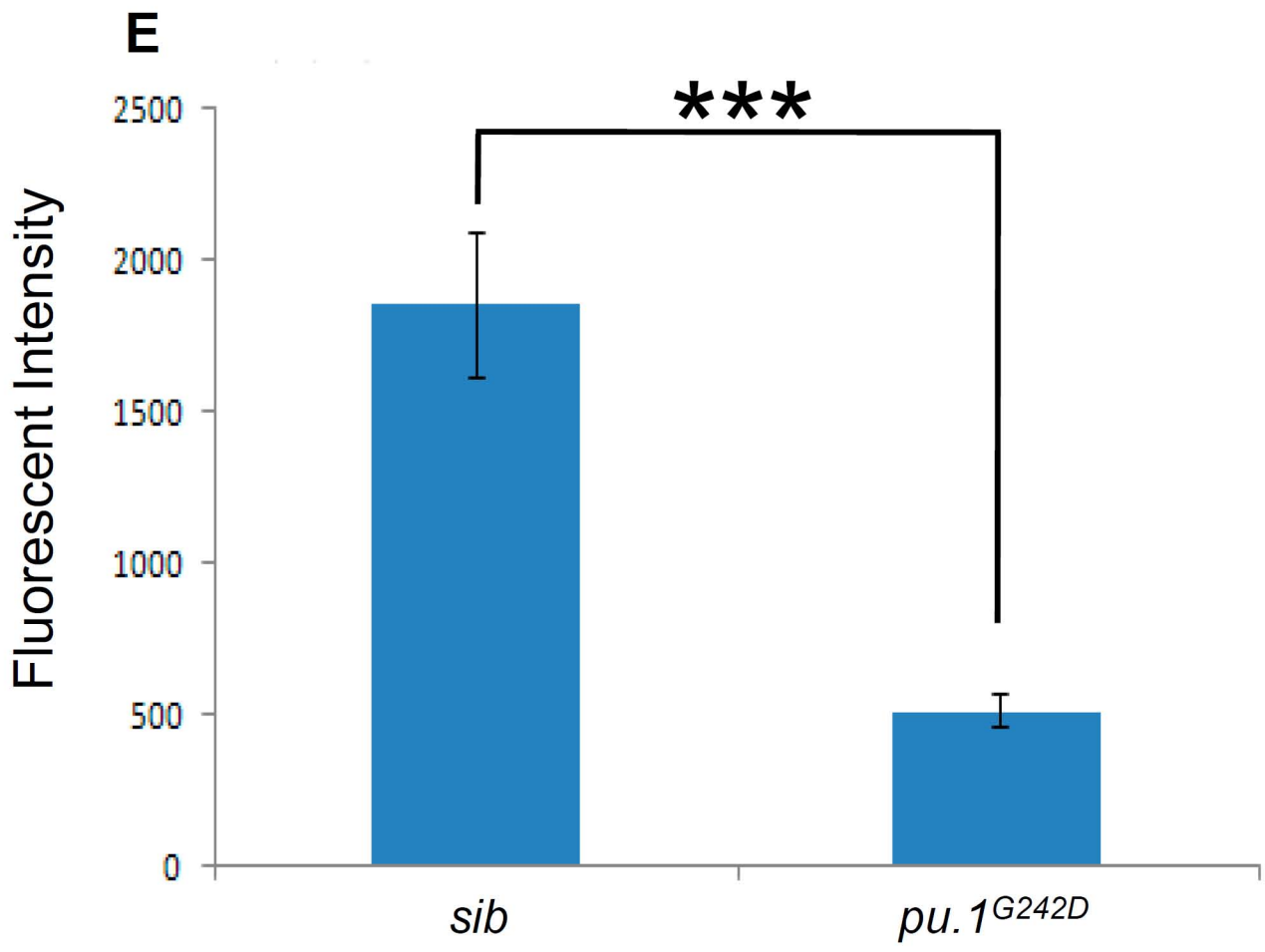


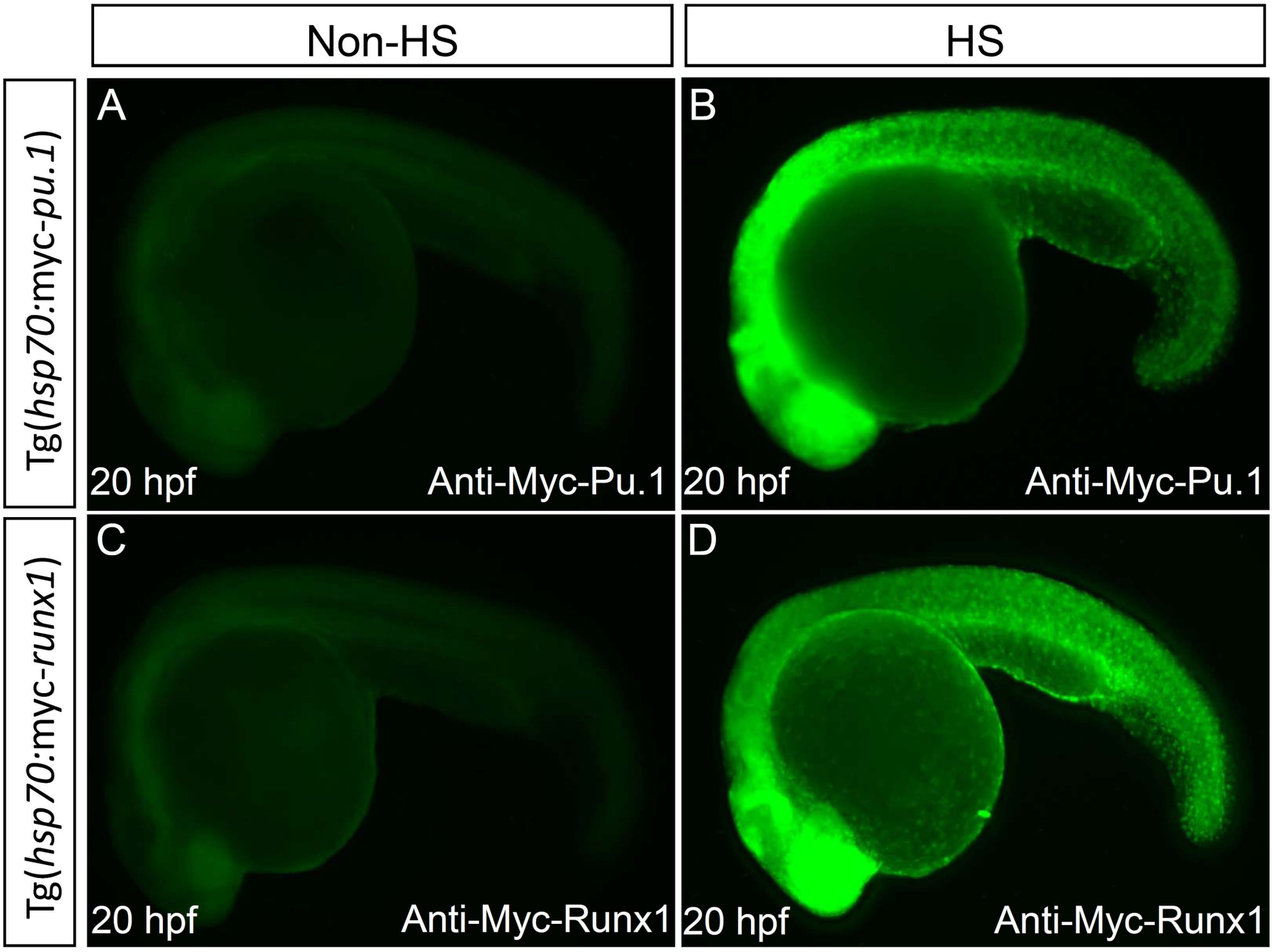








Supplemental Figure 6



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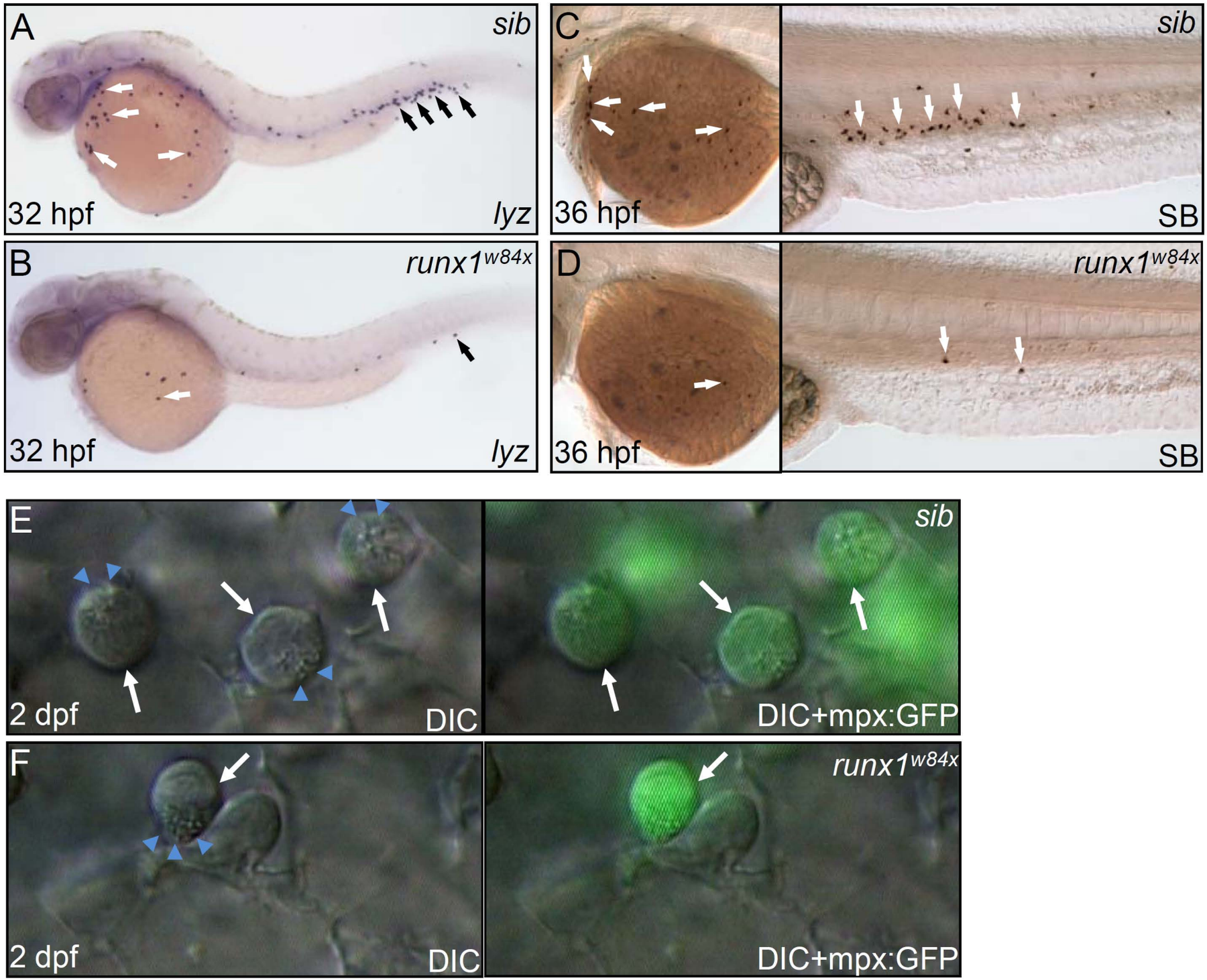

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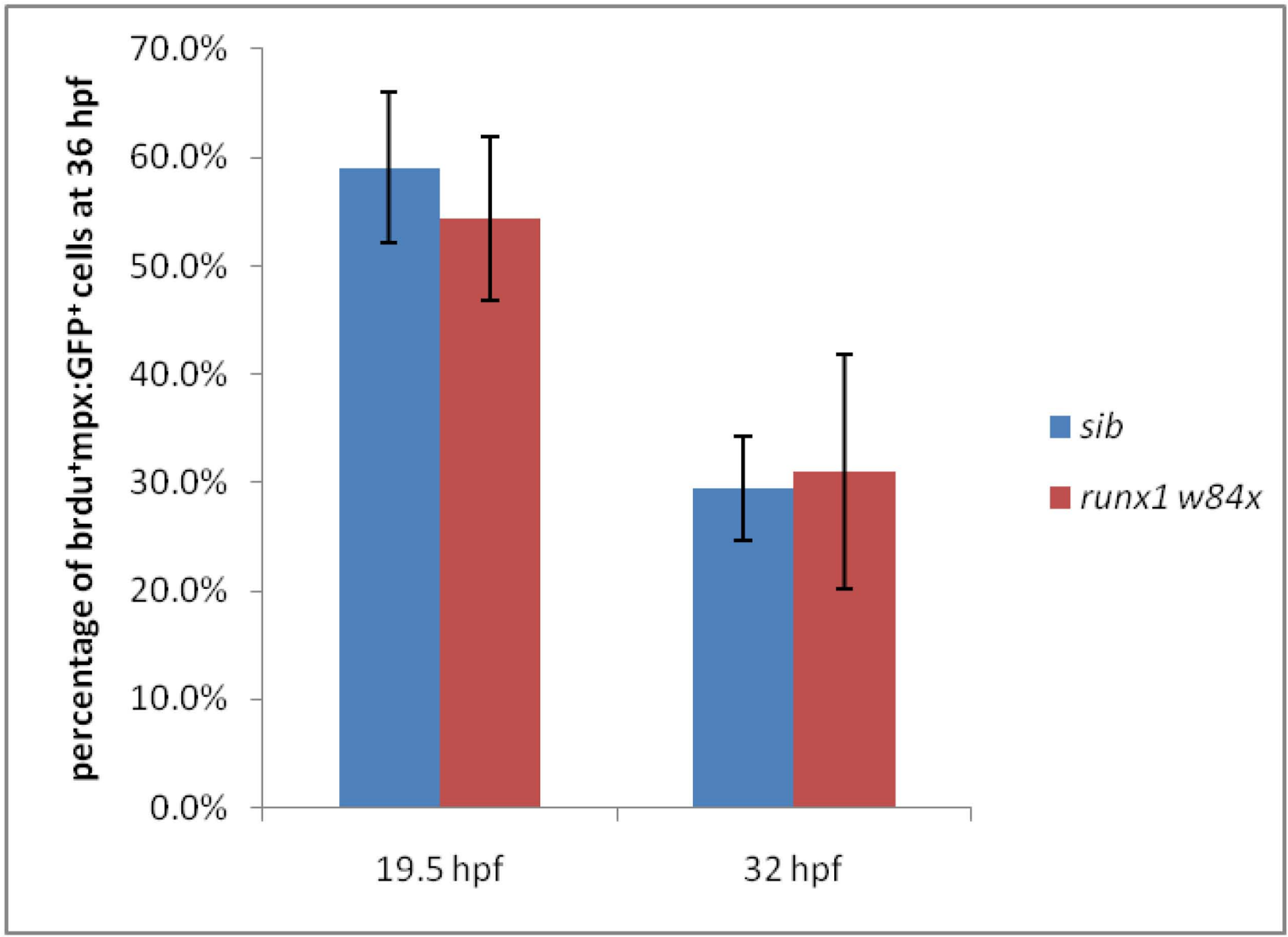

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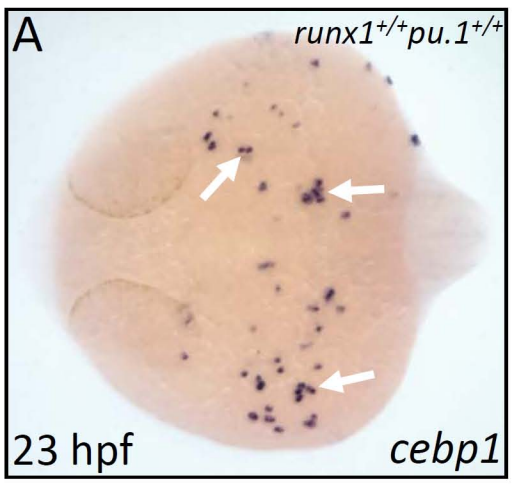
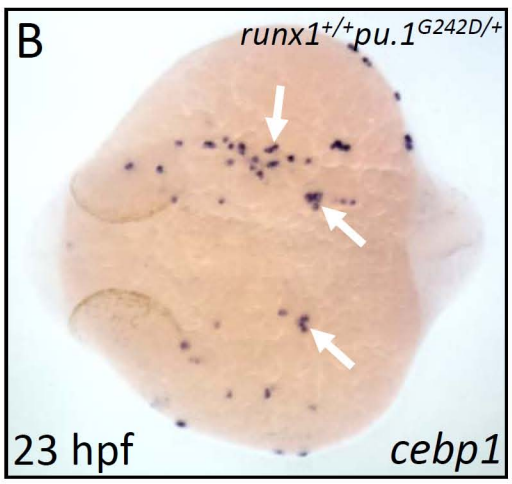
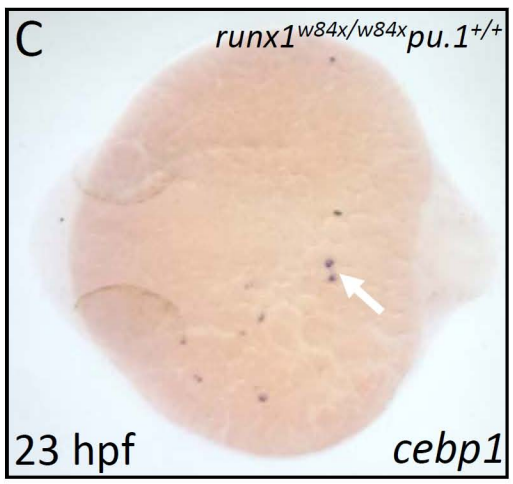
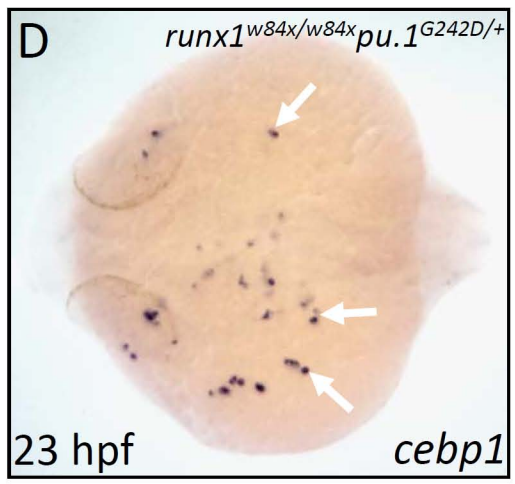
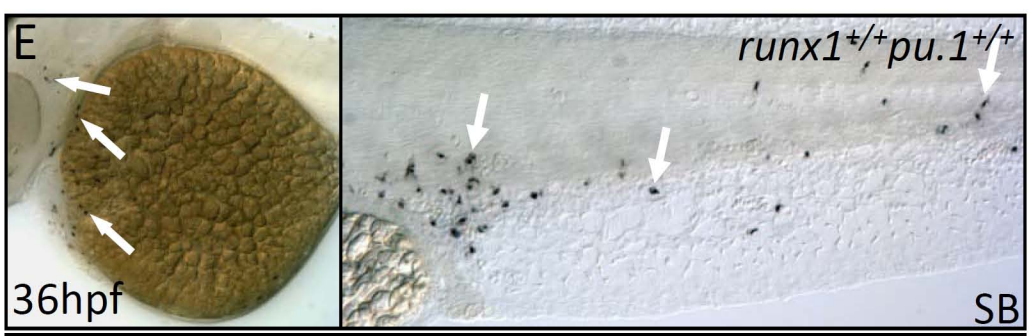
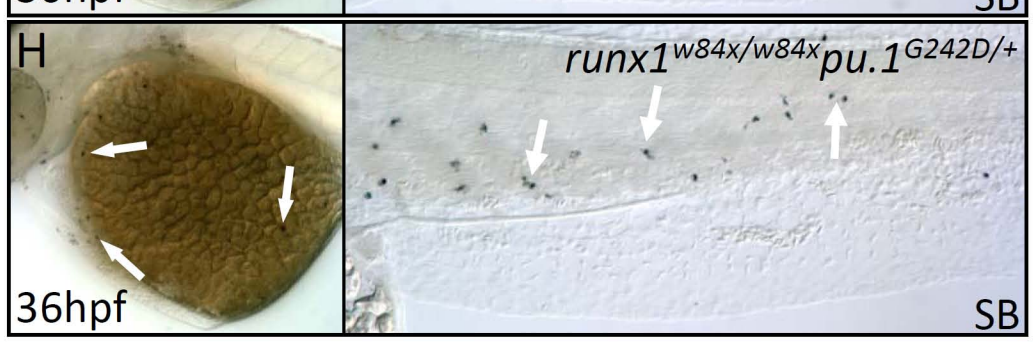


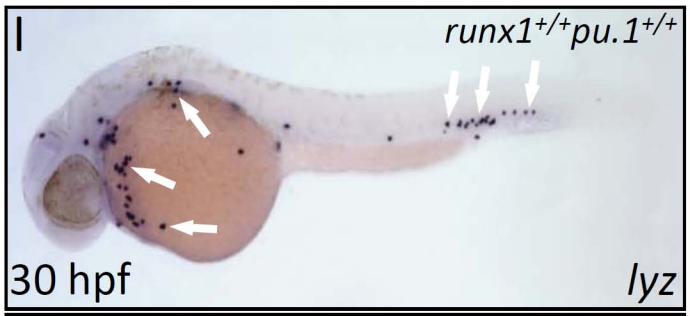


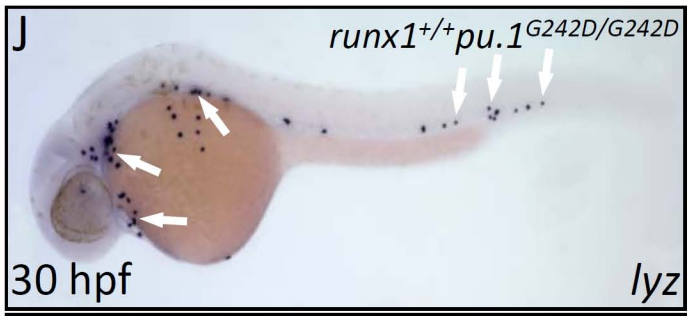


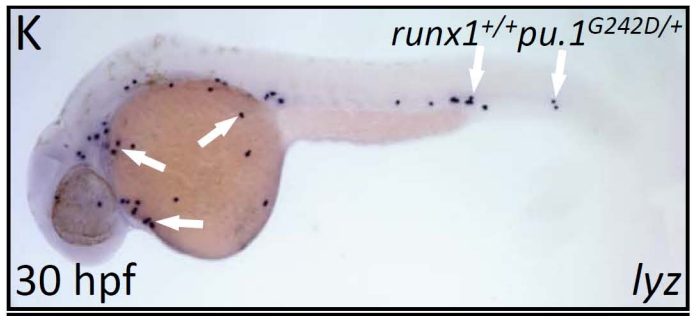


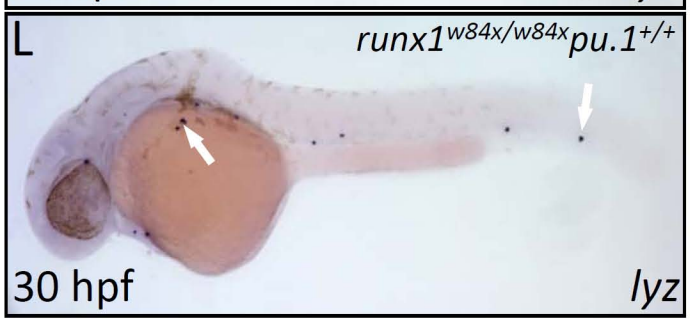


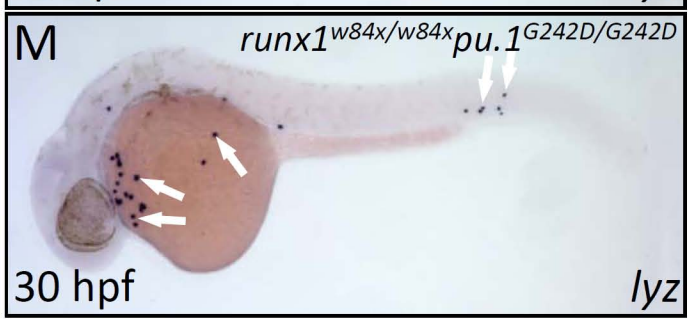


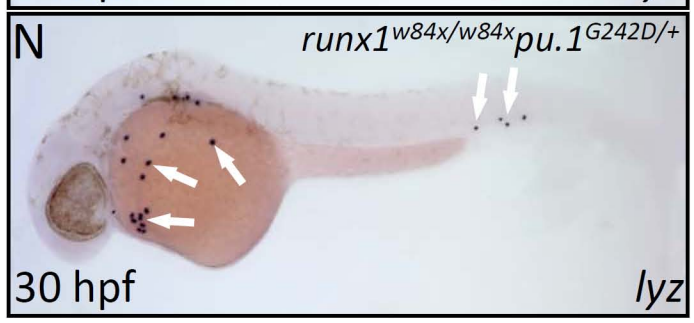


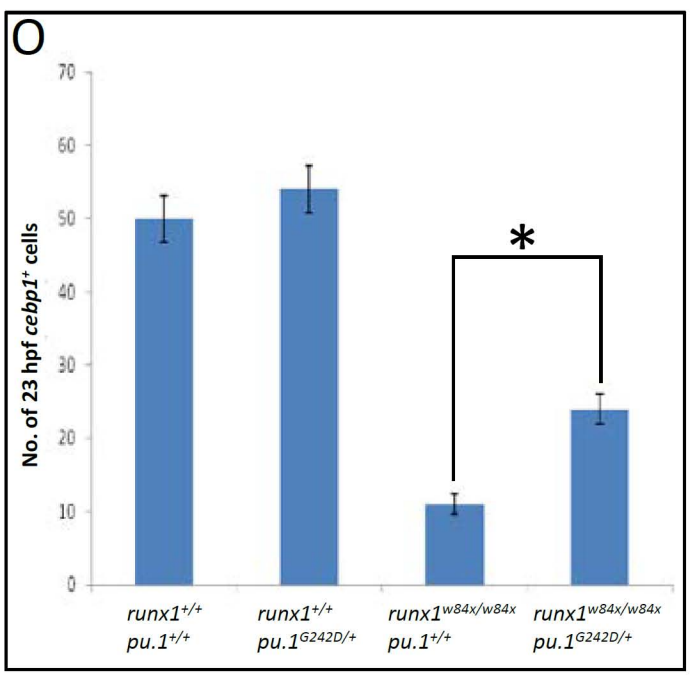


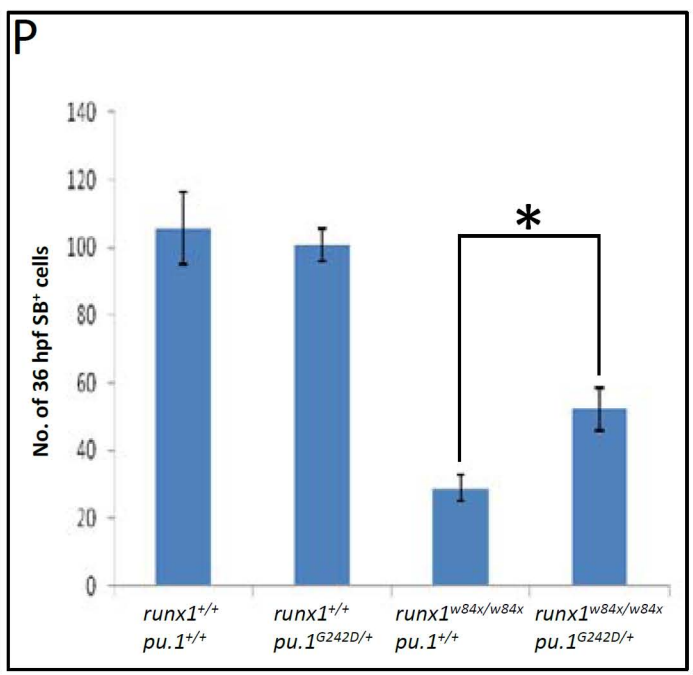


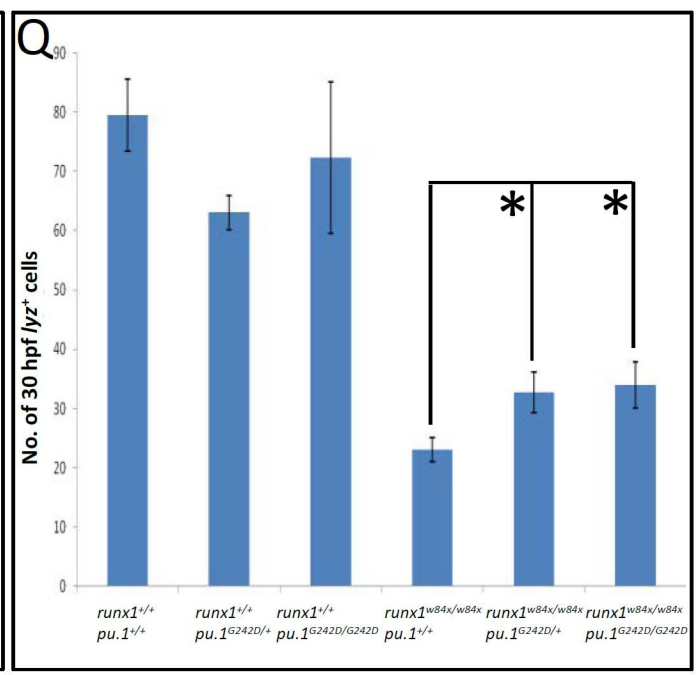




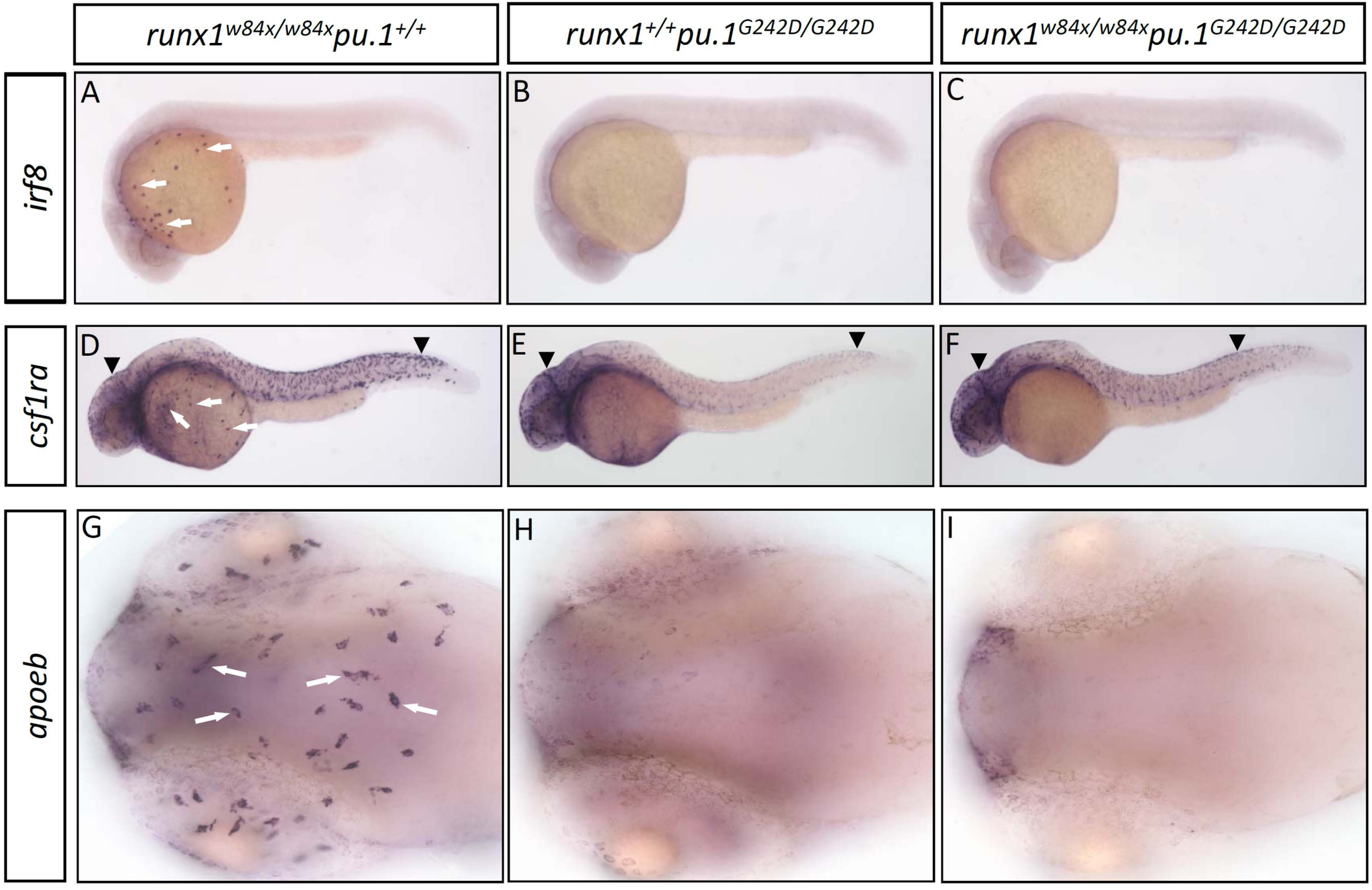








Supplemental Figure 10



supplemental Figure 11