Human BCR/ABL1 induces chronic myeloid leukemia-like disease in zebrafish

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Supplemental methods

Human *BCR/ABL1* (h*BCR/ABL1*) transient overexpression in zebrafish

h*BCR/ABL1* mRNA was synthesized by *in vitro* transcription reaction using the mMESSAGE mMACHINE® SP6 Transcription Kit (Invitrogen) according to the manufacturer' s instructions. For transient overexpression of h*BCR/ABL1*, one-cell-stage wild-type (WT) embryos were injected with 8 ng h*BCR/ABL1* mRNA. Embryos injected with diethylpyrocarbonate (DEPC)-treated Water (DNase/RNase free) were used as negative controls.

Cell transfection

h*BCR/ABL1*(b3a2) cDNA fragment isolated from the pToL *hsp70*:p210$^{BCR/ABL1}$ construct and cloned into the expression vector pCS2 under the control of *cmv* promoter to form the pCS2 *cmv*:p210$^{BCR/ABL1}$ construct. Then transfected into 293T cells using the PEI-Transferrinfection Kit (Invitrogen) according to the manufacturer' s instructions.

Heat-shock treatment

Tg(hsp70:p210$^{BCR/ABL1}$) embryos were heat-shock treated (HS) at 38.5°C for 2 hours twice per day from 70%-epiboly to 96 hours post fertilization (hpf), and then once per day after 96 hpf. Recovered at 28.5°C for 1-2 hours before fixed for WISH. *Tg(hsp70:p210$^{BCR/ABL1}$)* adults were heat-shocked at 38.5°C for 2 hours once per day. Performed as described previously[1].

Genotyping

Tg(hsp70:p210$^{BCR/ABL1}$) transgenic zebrafish were identified by PCR using h*BCR/ABL1* transgene-specific primers 5′-GGATTTAAGCAGAGTTCAAAAGCC-3′ and 5′-GTTGATCCTGTAATGGTACACCCT-3′, amplified a 466 bp fragment within the h*BCR/ABL1* fusion section. DNA polymerase (Transgene) was used with amplification conditions of 20 cycles at 94°C for 30 seconds, 65°C-55°C gradient annealing (-1°C per 2 cycles) for 30 seconds, and 72°C for 30 seconds.

In vitro synthesis of antisense RNA probe and whole-mount _in situ_ hybridization (WISH)

Digoxigenin-labeled antisense *cmyb*, *lcp1*, *lyz*, *mpx*, *mfap4*, *rag1*, *βe1*, and *BCR/ABL1* RNA probes were synthesized by *in vitro* transcription reaction according to standard protocols[2]. Then WISH was performed as described[3].

Quantitative RT-PCR

Total RNA from sorted cells and embryos was extracted using the TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. For cDNA preparation from the total RNA of embryos, reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) according to the manufacturer' s instructions. For specific detection of h*BCR/ABL1* transcript, primer sets were 5′-GGCTCTATGGGTTTCTGAATGTC-3′ and 5′-TTTCCTTGGAGTTCCAACGAG-3′. The relative quantity of gene expression was calculated by the 2(-ΔΔCt) method with normalization to the level of *Danio rerio* elongation factor 1α (*ef1α*), primer sets were 5′-TACTTCTCAGGCTGACTGTG-3′ and 5′-ATCTTCTTGATGTATGCGCT-

3′. Primers were designed using the PerlPrimer v1.1.12 software.

Cytological analysis

All experiments were performed under anesthesia, and all efforts were made to minimize suffering. For euthanasia, fish were immersed in an ice water bath (5 parts ice/1 part water at ⩽4°C) for ⩾5 min. Blood cells from the peripheral blood (PB) and kidney marrow (KM) were re-suspended in ice-cold phosphate-buffered saline with 5% fetal bovine serum, followed by cytocentrifuged at 400 rpm for 3 min. The cells were then stained with May-Grunwald's eosin methylene blue (Merck) and Giemsa (Merck) according to the manufacturer' s instructions. Blood cells of KM and PB were calculated manually based on their morphologies[4, 5].

Flow cytometry

Embryo dissociation and fluorescence-activated cell sorter (FACS) were performed as described previously[6]. *Tg(coro1a:GFP)* specifically labels the leukocytes, including lymphoid cells and myeloid cells, in zebrafish embryos.[7] *Tg(coro1a:GFP)* transgenic zebrafish adults were outcrossed with WT and *Tg(hsp70:p210$^{BCR/ABL1}$)* adults, and then collected the GFP^{+} embryos at 4 days post-fertilization (dpf). *coro1a:*GFP^{+} cells of each group were collected from a total of around 1000 embryos using MoFlo XDP (Beckmann) (around 500 embryos once, performed 2 times).

Hematopoietic cells isolated from adult KM in WT or *Tg(hsp70:p210$^{BCR/ABL1}$)* transgenic zebrafish were washed and resuspended in ice-cold phosphate-buffered saline with 5% fetal bovine serum. Hematopoietic progenitors and myelocytes were

sorted using a flow cytometer (BD Biosciences) based on side scatter characteristics, as described previously[5]. Hematopoietic progenitors and myelocytes were cytocentrifuged at 400 rpm for 3 minutes and subjected to May-Grunwald-Giemsa staining.

Histology

Sudan Black B (SB) staining was performed according to previous report[8].

Leukemic *Tg(hsp70:p210$^{BCR/ABL1}$)* adults and age-matched controls were fixed for 24 hours at 4˚C in 4% paraformaldehyde, then dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Tissues were sectioned at 5 μm and stained with hematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich).

Imaging

Tissue sections were imaged using a Zeiss imager.A2 microscope with a Zeiss AxioCam503 color camera. Blood cell counts were captured on an Olympus BX51 microscope with an Olympus DP80 color camera. Whole-mount and magnified images were captured with an Olympus MVX10 microscope with an Olympus DP71 color camera and an Olympus BX51 microscope with an Olympus DP80 color camera. Cell proliferation and TUNEL assay results were captured under Leica SP8 using a Zeiss LSM880 confocal microscope.

Supplemental References:

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Supplemental Tables

Supplemental Table 1. The complete blood counts of peripheral blood of 77 CML-like *Tg(hsp70:p210$^{BCR/ABL1}$)* transgenic zebrafish at 6-12 months

	blast	myeloid precursors	neutrophil	eosinophil	monocyte/ macrophage	erythroblast	erythrocyte	lymphocyte	platelet
WT (n=55)	**0.00 ± 0.00**	**0.04 ± 0.01**	**0.23 ± 0.07**	**0.01 ± 0.00**	**0.02 ± 0.01**	**0.09 ± 0.03**	**97.86 ± 0.27**	**1.80 ± 0.23**	**0.11 ± 0.04**
					***Tg(hsp70:p210$^{BCR/ABL1}$)* transgenic zebrafish**				
C1	0.44*	2.91†	1.02‡	0.00	0.26	0.08	93.25	1.96	0.08
C5	0.02	0.22†	0.44	0.00	0.00	0.00	98.19	1.10	0.02
C6	0.00	0.08	0.18	0.00	0.03	0.03	97.72	1.58	0.39
C8	0.00	0.10†	0.08	0.00	0.00	0.00	99.29	0.36	0.05
C9	0.03	0.08	0.05	0.00	0.00	0.00	99.12	0.67	0.05
C11	0.00	0.00	0.05	0.00	0.00	0.00	98.18	1.44	0.33
D1	0.00	0.21†	0.15	0.00	0.00	0.03	97.88	1.41	0.32
D3	0.00	0.00	0.00	0.00	0.00	0.00	99.85	0.15	0.00
D4	0.00	0.07	0.02	0.00	0.00	0.00	99.53	0.16	0.23
D6	0.00	0.00	0.02	0.00	0.00	0.00	99.47	0.35	0.15
D8	0.00	0.00	0.00	0.00	0.00	0.00	99.39	0.50	0.11
Tg2	0.00	0.09	0.09	0.00	0.02	0.00	97.41	2.26	0.13
Tg3	0.00	0.00	0.00	0.00	0.00	0.00	99.54	0.46	0.00
Tg4	0.00	0.05	0.48	0.00	0.00	0.00	97.34	2.06	0.07
Tg5	0.03	0.03	0.29	0.03	0.00	0.00	98.57	0.90	0.14

Tg6	0.00	0.00	0.27	0.16§	0.00	0.11	96.47	2.83	0.16
Tg8	0.00	0.02	0.02	0.02	0.00	0.00	98.32	1.45	0.17
Tg18	0.00	0.05	0.49	0.02	0.00	0.00	97.66	1.78	0.00
Tg19	0.00	0.07	2.09‡	0.02	0.00	0.00	95.93	1.88	0.00
Tg20	0.00	0.02	0.00	0.00	0.00	0.00	99.08	0.90	0.00
Tg21	0.00	0.02	0.02	0.00	0.00	0.00	97.97	1.98	0.00
Tg22	0.00	0.02	0.12	0.00	0.00	0.00	97.84	2.02	0.00
Tg23	0.00	0.00	0.02	0.02	0.00	0.07	99.12	0.76	0.00
Tg24	0.00	0.02	0.94‡	0.07	0.00	0.02	96.56	2.29	0.10
Tg25	0.00	0.05	0.23	0.00	0.05	0.00	98.14	1.53	0.00
Tg28	0.00	0.05	0.16	0.02	0.02	0.00	98.19	1.55	0.00
Tg29	0.00	0.00	0.02	0.00	0.00	0.00	99.06	0.92	0.00
Tg30	0.00	0.00	0.20	0.00	0.00	0.00	98.82	0.98	0.00
Tg31	0.00	0.00	0.05	0.00	0.00	0.00	98.85	1.05	0.05
Tg32	0.00	0.00	0.07	0.00	0.00	0.00	99.01	0.91	0.00
Tg111	0.00	0.00	0.00	0.00	0.00	0.02	99.63	0.34	0.00
Tg114	0.00	0.00	0.02	0.00	0.00	0.00	99.30	0.65	0.02
Tg115	0.00	0.05	0.00	0.00	0.05	0.00	98.88	1.02	0.00
Tg116	0.00	0.00	0.00	0.00	0.00	0.00	99.41	0.59	0.00
Tg118	0.00	0.02	0.05	0.00	0.00	0.00	98.67	1.26	0.00
Tg119	0.00	0.00	0.02	0.00	0.00	0.00	99.66	0.31	0.00
Tg120	0.00	0.00	0.02	0.00	0.00	0.00	99.88	0.10	0.00
Tg121	0.00	0.00	0.02	0.00	0.00	0.00	99.25	0.72	0.00
Tg123	0.00	0.00	0.02	0.00	0.00	0.00	98.42	1.56	0.00
Tg124	0.00	0.00	0.05	0.00	0.00	0.00	99.53	0.42	0.00

Tg126	0.00	0.00	0.10	0.07	0.00	0.00	98.58	1.17	0.07
m42	0.04	0.16†	0.08	0.00	0.00	0.00	97.94	1.79	0.00
m43	0.04	0.80†	0.64	0.04	0.32	0.00	94.42	3.57	0.16
m44	0.00	0.03	0.03	0.03	0.00	0.00	95.10	3.62	1.18¶
m45	0.00	0.31†	0.03	0.00	0.00	0.00	97.33	1.85	0.48
m47	0.00	0.00	0.12	0.00	0.08	0.00	98.44	1.24	0.12
m49	0.03	0.00	0.00	0.00	0.00	0.00	97.05	1.39	1.54¶
m50	0.07*	0.20†	0.10	0.03	0.03	0.03	95.17	2.60	1.76¶
m51	0.00	0.04	0.04	0.00	0.00	0.00	99.49	0.40	0.04
m1	0.00	0.14†	0.05	0.00	0.00	0.23	96.46	3.12	0.00
m2	0.00	0.16†	0.11	0.03	0.03	0.11	96.56	2.77	0.24
m3	0.08*	0.11†	0.00	0.00	0.00	0.11	97.71	1.91	0.08
m5	0.26*	2.95†	8.47‡	7.70§	0.77	0.26	57.51	21.57‖	0.51¶
m7	0.02	0.29†	0.11	0.00	0.00	0.11	97.03	1.71	0.73¶
m8	0.05*	0.32†	0.05	0.00	0.00	1.08	94.19	3.24	1.08¶
m12	0.10*	0.27†	1.04‡	0.00	0.00	1.10	95.27	2.04	0.17
m13	0.00	0.08	0.37	0.16§	0.00	2.10	92.48	3.78	1.03¶
m14	0.00	0.05	0.16	0.00	0.00	0.38	93.30	4.56	1.55¶
m15	0.00	0.00	0.00	0.00	0.00	1.84	96.97	9.16‖	0.31
m17	0.00	0.20†	1.19‡	0.07	0.03	0.24	95.41	2.79	0.10
m18	0.00	0.14†	0.47	0.05	0.00	0.00	95.28	3.98	0.08
m19	0.03	0.69†	5.53‡	0.15§	0.13	0.00	91.63	1.79	0.05
m20	0.10*	1.52†	0.93‡	0.38§	0.21	0.00	90.52	5.21‖	1.14¶
m21	0.12*	1.17†	0.31	0.00	0.19	0.06	94.69	3.15	0.31
m24	0.15*	2.47†	1.31‡	0.30§	0.10	0.71	84.27	10.44‖	0.25

m25	0.04	0.51†	0.18	0.00	0.04	0.04	91.99	6.64‖	0.58¶
m27	0.05*	1.34†	0.21	0.00	0.00	0.05	73.00	24.47‖	0.88¶
m28	0.29*	0.29†	0.08	0.00	0.00	0.00	94.11	5.06‖	0.17
m29	0.00	0.08	0.08	0.00	0.00	0.00	91.68	7.45‖	0.71¶
tg1	0.00	0.00	0.02	0.00	0.02	0.00	99.76	0.19	0.00
tg3	0.00	0.00	0.00	0.00	0.00	0.02	99.85	0.12	0.00
tg5	0.00	0.00	0.02	0.00	0.00	0.00	96.43	3.48	0.00
tg6	0.00	0.11†	0.07	0.00	0.00	0.00	98.30	1.13	0.57¶
tg7	0.00	0.00	0.22	0.00	0.02	0.00	97.61	2.14	0.00
tg8	0.00	0.02	0.15	0.00	0.00	0.00	97.95	1.87	0.00
tg10	0.00	0.00	0.12	0.00	0.00	0.00	98.93	0.95	0.00
F1	74.10*	0.20†	0.10	0.00	0.00	2.11	23.24	0.25	0.00

Cell counts were obtained by identifying at least 1500 cells per peripheral blood (PB) preparation. The percentages were indicated by mean ± SEM. * Indicates blasts in PB increased, > 0.05%. † Indicates myeloid precursors in PB increased, > 0.10%. ‡ Indicates neutrophils in PB increased, > 1.00%. § Indicates eosinophils in PB increased, > 0.10%. ‖ Indicates lymphocytes in PB increased, > 5.00%. ¶ Indicates platelets in PB increased, > 0.50%.

Supplemental Figure legends

Supplemental Figure 1. *BCR/ABL1* expressed in blood cells of *Tg(hsp70:p210$^{BCR/ABL1}$)* transgenic zebrafish. (A) *BCR/ABL1* transcript levels in *coro1a:*GFP^{+} cells (around 5×10^{4} cells), collected from *Tg(coro1a:GFP)* and *Tg(hsp70:p210$^{BCR/ABL1}$-coro1a:GFP)* embryos with or without heat shock treatment at 4 dpf, were detected by RT-qPCR. (B) *BCR/ABL1* transcript levels in hematopoietic progenitors and myelocytes in KM blood cells (around 5×10^{4} cells) of 1-year old adults with or without heat shock treatment were detected by RT-qPCR. Hematopoietic progenitors and myelocytes were distinguished by morphology by May-Grunwald-Giemsa staining. Original magnification, ×400. "HS" indicates the *Tg(hsp70:p210$^{BCR/ABL1}$)* transgenic zebrafish with heat-shock treatment. "No-HS" indicates the *Tg(hsp70:p210$^{BCR/ABL1}$)* transgenic zebrafish without heat-shock treatment. Student's *t*-tests; mean ± SEM; $^{*}P< 0.05$; $^{**}P< 0.01$.

Supplemental Figure 2. p210$^{BCR/ABL1}$ expressed in cell lines *in vitro*. HKE293T cells in a 6-well plate were transfected for 24 h with plasmids expressing GFP and p210$^{BCR/ABL}$. Expression of p210$^{BCR/ABL1}$ fusion protein in different cell lines *in vitro* assessed by western blot. K562 cells were used as the positive control, HKE293T cells and HKE293T (GFP) cells were used as the negative control. GAPDH was used as the loading control. Molecular markers are shown on the Right. 293T indicates the HKE293T cells. 293T (GFP) indicates the HKE293T cells transfection with pCS2 *cmv*:GFP plasmid. 293T(p210$^{BCR/ABL1}$) indicates the HKE293T cells transfection with pCS2 *cmv*:p210$^{BCR/ABL1}$ plasmid.

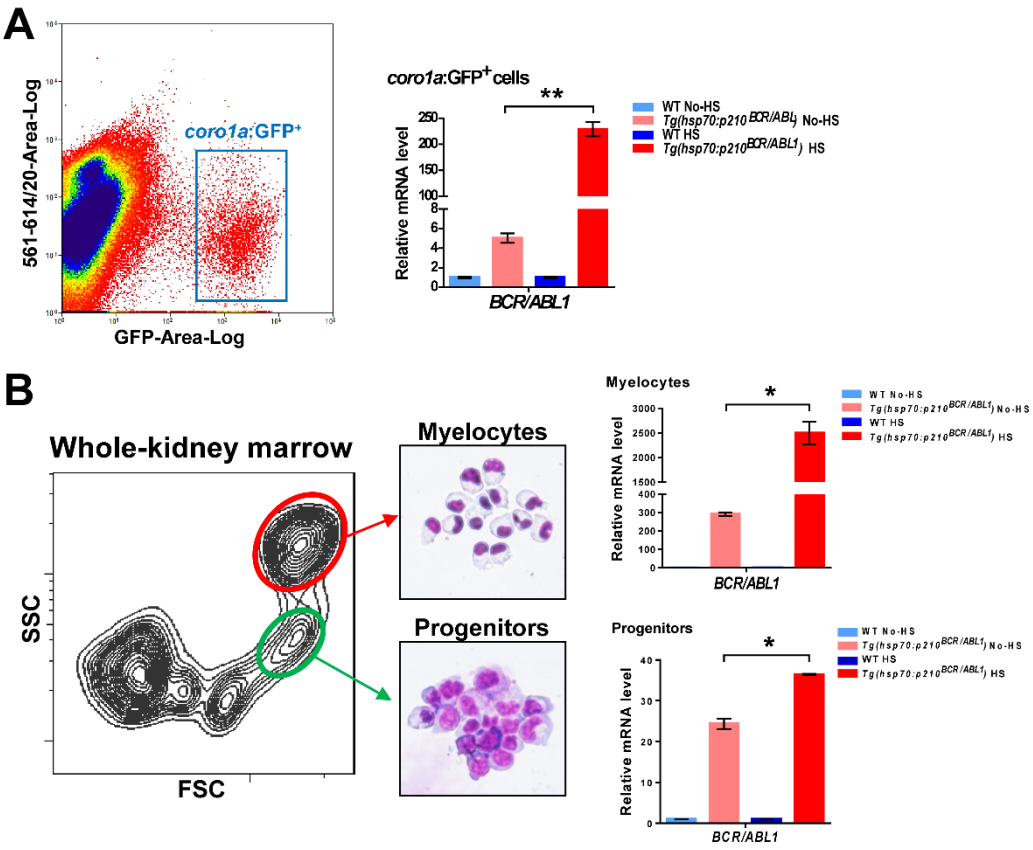
Supplemental Figure 3. Abnormal hematopoiesis in *Tg(hsp70:p210$^{BCR/ABL1}$)* transgenic zebrafish during embryonic hematopoiesis. WISH of *cmyb* (A-B, D-E), *βe1* (G-H), and *rag1* (I-J) expressions in HS *Tg(hsp70:p210$^{BCR/ABL1}$)* embryos and WT controls at 36/60 hpf, 5 dpf, and 5dpf, respectively. n/n, number of zebrafish larvae showing representative phenotype/total number of zebrafish larvae examined. Original magnification, ×40 (A-D), ×32 (E-F), ×50 (G-H). Red rectangles in the panel indicate the *βe1*$^{+}$ erythrocytes in the posterior blood island (PBI) region and the regions were enlarged at the lower right (original magnification ×200). Red oval region indicate the *rag1*$^{+}$ lymphocytes in thymus. (I-J) Statistical analysis. *cmyb*$^{+}$ signals in the whole fish were calculated and compared at 36 and 60 hpf, respectively. Student's *t*-tests; mean ± SEM; *n.s.* indicates no significant difference; $^{**}P< 0.01$.

Supplemental Figure 4. Mortality and abnormality of zebrafish larvae exposed to various concentrations of imatinib, dasatinib, bosutinib during a 120-h test. WT larvae. 6.4‰ DMSO as the placebo group. 30 larvae per concentration group and repeat twice.

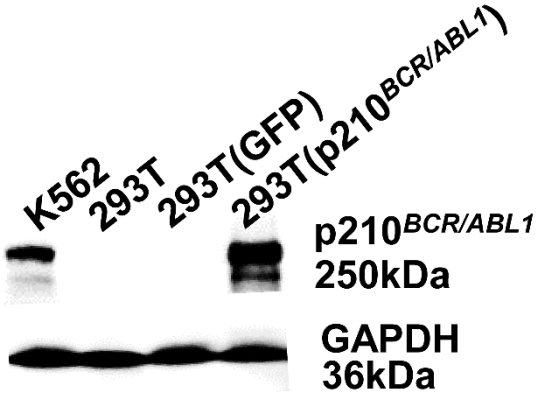
Supplemental Figure 5. High doses of imatinib affect normal myelopoiesis during zebrafish embryonic hematopoiesis. (A) 3 dpf HS *Tg(hsp70:p210$^{BCR/ABL1}$)* (right panels) larvae and WT controls (left panels) treated with DMSO control and Imatinib (20, 40 and 80 μmol/L) for 48 hours and *lcp1* WISH at 5 dpf. n/n, number of zebrafish larvae showing representative phenotype/total number of zebrafish larvae examined. Original magnification, ×200. (B) Statistical analysis. Average numbers of *lcp1*$^{+}$ cells per larva with drug treatment. ANOVA; mean ± SEM; ***P< 0.001; ****P< 0.0001.

Supplemental Figures

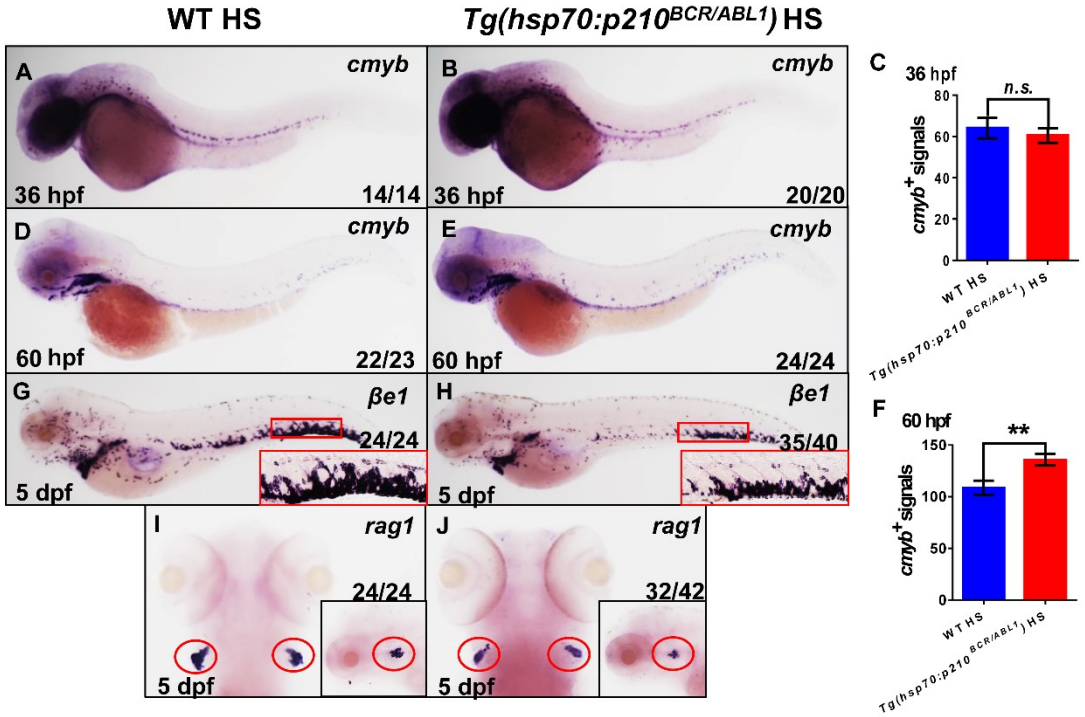
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



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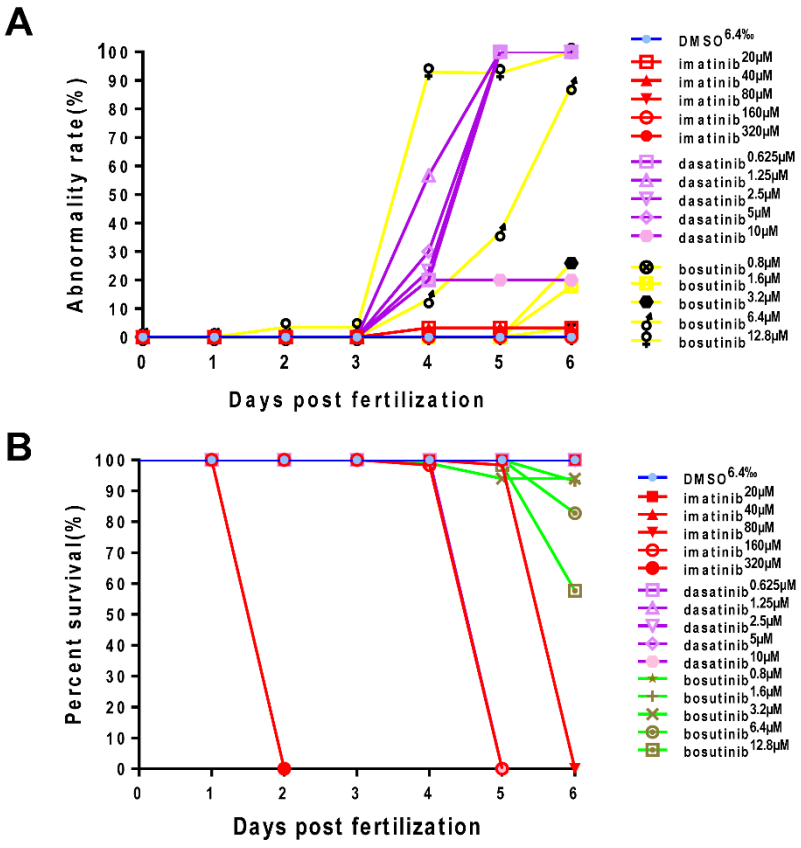
Supplemental Figure 4



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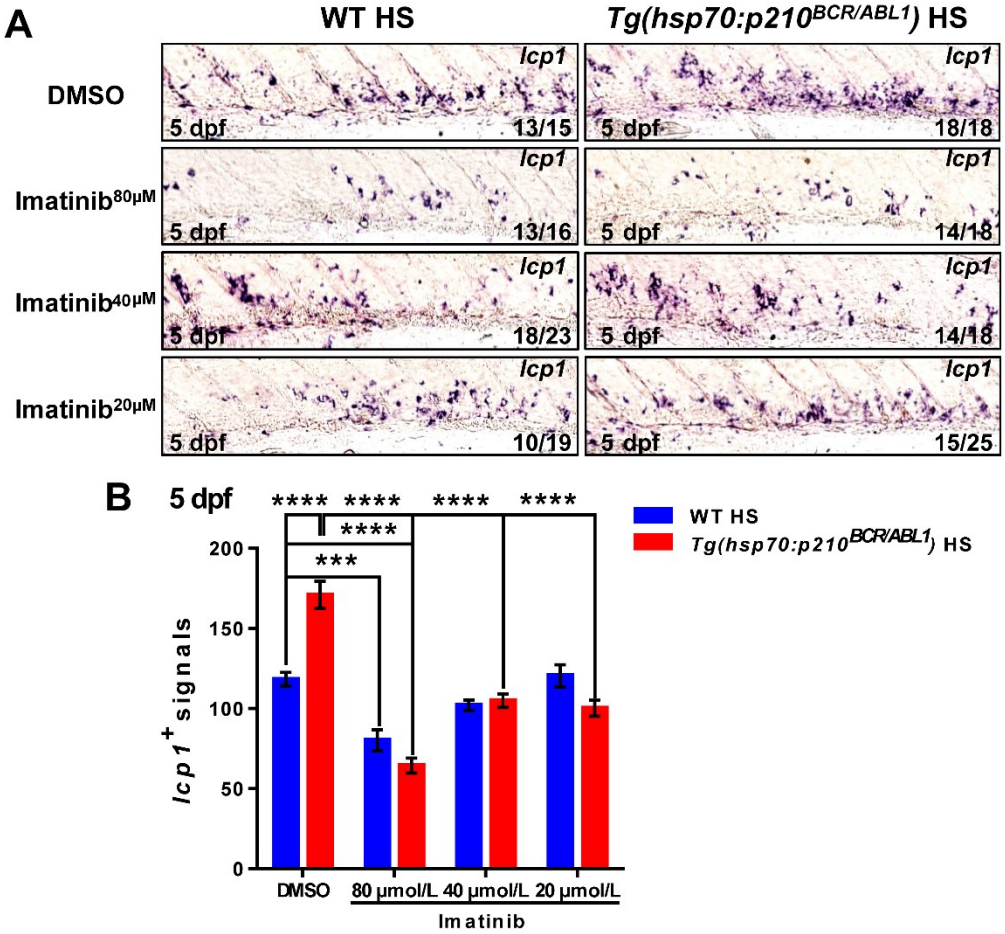
Supplemental Figure 5



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