SUPPLEMENTARY APPENDIX

Humanized zebrafish enhance human hematopoietic stem cell survival and promote acute myeloid leukemia clonal diversity

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Humanized Zebrafish Enhance Human Hematopoietic Stem Cell Survival and Promote AML Clonal Diversity

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Supplementary Materials

1. Supplementary Methods
2. Supplementary Figure 1-5

Supplementary Methods

Animal use and the creation of transgenic fish. All zebrafish studies reported were approved by Dalhousie Use and Care of Laboratory Animals (UCLA) committee, under protocol #17-007 (formerly14-116). The constructs for the making the transgenic fish were made using traditional and Gateway™ cloning. Briefly, tagBFP was amplified from pME-tagBFP and human SDF1a was amplified from pBabe-SDF1a-Puro (Addgene, #). An overlap extension cloning was performed to make BFP-P2A-hSDF1a with AgeI and NotI-XhoI flanking on 5' and 3' ends respectively and was sub-cloned into pCR2.1 by TOPO cloning. The plasmid containing 4.3kb zebrafish *cxcl12* promoter (pminitol2-zsdf1a-DsRed) was obtained (1). The construct downstream of DsRed that includes the SV40 polyA (pA) site and 3' minitol2 site was amplified with NotI on the 5' end and SacI-XhoI on the 3' end. The created NotI-SV40pA-SacI-XhoI amplicon was inserted into the pCR2.1-BFP-P2A-hSDF1a plasmid creating a pCR2.1- BFP-P2A-hSDF1a-SV40pA-3'minitol2 plasmid. Restriction digestion was performed using AgeI and SacI to excise the DsRed-polyA and 3' minitol2 site from pminitol2-zsdf2a-DsRed and was replaced by BFP-P2A-hSDF1a – polyA-3'minitol2 site that was excised using AgeI and SacI from the pCR2.1 plasmid resulting in pminitol2-zsdf1a-BFP-P2A-hSDF1a plasmid. This plasmid has been deposited in Addgene (#127550).

The human stem cell factor (SCF)/KITLG and human granulocyte macrophage stimulating factor (GM-CSF)/CSF2 were ordered as geneblocks from IDT and cloned into p5E-dA-MCS-biTRE (a kind gift of Dr. Abbie Jensen, University of Massachusetts, Amherst) and pME-MCS (*tol2* kit). The above constructs were assembled together with p3E-poly A (*tol2* kit), using Gateway™ cloning approach such that the SCF and GM-CSF is downstream of the bidirectional tetracycline response element.

The transactivation construct was also cloned using a Gateway™ cloning approach by assembly of p5E-*ubiquitin-C promoter* (*tol2* kit), pL1L2-rtTA-HA (a kind gift of Dr. Abbie Jensen, University of Massachusetts, Amherst) and p3E-poly A (*tol2* kit). Two separate transgenic fish were produced by co-injecting CXCL12 together with *tol2* mRNA and the construct coding for rtTA with the construct coding for SCF/KITLG and GM-CSF/CSF2 together with tol2 mRNA into embryos from *casper* (*nacre*$^{-/-}$; *roy*$^{-/-}$) mutants (2). These fish were further crossed in the F2 generation to produce an F3 multi-transgene expressing GM-CSF/CSF2, SCF/KITLG, and SDF1/CXCL12 zebrafish (referred to as the GSS fish). For all the experiments, we used *casper* larvae as the background control, and for brevity, we refer to it as control throughout the manuscript.

Cell lines and cell culture. The human Jurkat T-ALL cell line was cultured in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Jurkat cells express high levels of CXCR4, a receptor of CXCL12 (CCLE, Broad Institute) and hence was the preferred cell line to carry out migration experiments with the hCXCL12 expressing transgenic fish. The human CMK Down syndrome AML cell line was a kind gift from Dr. Jeffery Taub (Wayne State University, Detroit, MI, USA). The cells were cultured in RPMI1640 (Gibco) with 10% FBS (Wisent). All cell lines used were authenticated for their originality.

Yolk sac xenograft experiment for evaluation of migration and proliferation. For migration experiments, CXCL12 expressing larvae and WT larvae were gamma irradiated with at a dosage of 15Gy in a 137Cs γ-irradiator at 72 hours post fertilization (hpf). Human Jurkat cells in culture were labelled with a cytoplasmic fluorescent dye (CMTMR Orange, Invitrogen) and resuspended

in flow buffer (1X PBS, 1mM EDTA, 2% FBS). Approximately 50-100 Jurkat cells were injected into the yolk sac of the larvae two hours post-irradiation and observed for migration for 3 days post-injection. The samples were blinded and scored for no migration, local dissemination (cells dispersed throughout the yolk sac) and migration (cells present outside of the yolk sac), and the identity was revealed after scoring.

Cell proliferation was assessed using *casper* larvae and fish expressing SCF/KITLG and GM-CSF/CSF2 (referred to as GS fish). Embryos were maintained in E3 embryo medium (5mM Sodium chloride, 0.17mM Potassium chloride, 0.4mM Calcium chloride and 0.16mM Magnesium sulphate, pH 7.5 supplemented with 0.05% methylene blue [v/v]) and were treated with 10µg /mL of doxycycline hydrochloride daily from 24 hpf to induce hKITLG and hCSF2 expression. CMK cells were labelled with a cytoplasmic fluorescent dye (CellTrackerTM Blue CMAC dye, Gibco) and resuspended in flow buffer. We injected the cells into the yolk sac of 3 dpf larvae and cell proliferation quantified at 1 (baseline), 2 and 3 dpi using a dissociation protocol previously described by our group (3-6); briefly larvae were collected for different conditions and dissociated using collagenase (Sigma Aldrich) at 37C for 20-30 minutes until complete dissociation. The cells were then washed in flow buffer containing EDTA and FBS and resuspended in 10µl of flow buffer per larvae. The dissociation mix was layered as a 10µl dot into a slide and imaged using Zeiss Axio Observer with the wavelength corresponding to the dye used for experiment. The cells were counted using Fiji software.

Human umbilical cord and bone marrow samples. The use of human samples in the study was approved by the IWK Health Centre Research Ethics Board (REB# 1007549). Fresh human umbilical cord blood (UCB) and human leukemia bone marrow (BM) samples were collected from

patients at IWK Health Centre (Halifax, NS, Canada) after formal patient consent. The UCB and BM samples were subjected to density gradient centrifugation using Lymphoprep (StemCell Technologies) and followed by RBC lysis. Further, lineage-depleted (lin-) human hematopoietic stem cell and progenitor cells (HSPCs) were enriched using immunomagnetic separation (Easysep^{TM} human progenitor cell enrichment kit, Stemcell Technologies). Both, the HSPCs isolated from UCB and mononuclear cells isolated from BM samples were stored in liquid nitrogen until required for experimental use.

Orthotropic xenograft experiments with primary samples. Zebrafish larvae (from control and GSS transgenic fish) were collected and grown in E3 Embryo medium. Both the transgenic larvae and control larvae were treated with 10 μg/mL doxycycline hydrochloride (Sigma Aldrich) from 24 hpf to induce the expression of SCF/KITLG and GM-CSF/CSF2 and as a control, respectively. All larvae were irradiated at 72 hpf using a 137Cs γ-irradiator (GammaCell 3000, Theratronics) with a 15Gy central radiation dosage to induce *cxcl12* promoter activity and niche clearance of the organism for transplant. Human patient-derived samples were thawed 2 hours before injection and were revived in MarrowMax (Gibco). Cells were then labelled according to manufacturer's protocol with a cytoplasmic green fluorescent dye (CellTracker™ Green CMFDA Dye, Invitrogen) to facilitate in vivo cell tracking according to manufacturer instructions. The cells were then resuspended in flow buffer (PBS + 2% FBS + 1mM EDTA) and loaded into a pulled-glass capillary tube, and approximately 150-250 cells were injected into the common cardinal vein. The embryos were screened immediately following injection to confirm cells were present in circulation.

Antibody neutralization. Primary T-ALL bone marrow samples were processed as described above. The samples were thawed and seeded in MarrowMax and were treated with either anti-CXCR4 (MAB171, R&D Systems) or IgG2A isotype control (MAB003, R&D Systems). The cells were incubated overnight with 4μg/ml isotype control or CXCR4 targeting antibody before injection. Flow cytometry was used to evaluate successful antibody neutralization. The cells were then orthotropically injected into the circulation of fish as described above.

RNA isolation and targeted transcriptome analysis. Zebrafish injected with human cells were collected at the identified time points and euthanized. RNA was isolated with Trizol reagent (Invitrogen) using Phase Maker (Invitrogen) tubes according to manufacturer's instruction. RNA was stored at -80ºC until transcriptome sequencing. Targeted transcriptome sequencing was performed using HemeV2 kit (ArcherDx) for the AML samples and using the Human Stem Cell & Differentiation Markers (333002, QIAseq Targeted RNA Panels, Qiagen) for UCB samples.

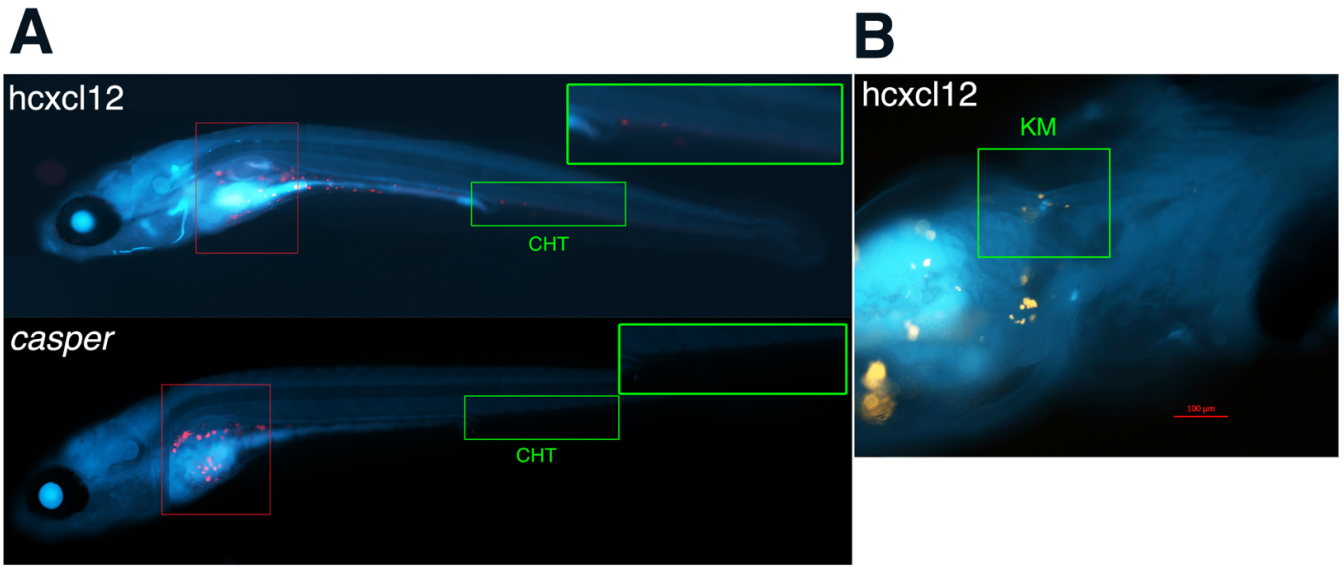
Immunoblotting. The fish samples were homogenized in RIPA with an additional 1% SDS, CompleteMini Protease Inhibitor, 2mM Sodium Orthovanadate, 10mM Sodium Fluoride, 20mM Sodium beta-glycerophosphate and 1mM PMSF. The samples were left to lyse in ice for 40 minutes and the lysate was cleared by centrifugation. The protein was quantified by BCA assay. About 15μg of protein was loaded into each well of a Biorad stain-free Any KD gel. The proteins were transferred to a 0.2-micron PVDF membrane and probed using anti-SCF (C19H6, Cell Signaling; 1:500) and anti-GMCSF (ab9818, Abcam; 1:500) antibodies. The signal was amplified using Western Dura (Life Technologies.)

Immunofluorescence and imaging. Zebrafish larvae were fixed with 4% PFA overnight and dehydrated in methanol and stored at -20C until further processing. Samples were rehydrated prior to processing. For cryosections the larvae were stored in 30% (w/v) sucrose solution overnight. Larvae were arranged and molded in OCT embedding medium (Fisher HealthCare) and 15-micron sagittal sections were made. Samples were blocked with blocking buffer containing BSA and donkey serum and incubated in primary rabbit anti-human CD33 antibody (ab221558, 1:50) overnight at 4ºC. The secondary antibody incubation with anti-rabbit Alexa Fluor 488 (ab150073, Abcam;1:400) was performed room temperature for an hour for the sections. Nuclear counterstain was performed using DAPI at the concentration of 1μg/ml. Samples were mounted in DAKO fluorescent mounting media and imaged in Zeiss LSM710 (63x, Zoom = 1.5 and NA=1.4; 10x, NA=0.45). All imaging of live zebrafish was performed upon immobilizing the zebrafish in 1% low melting agarose using Zeiss Axio Observer (5x, NA= 0.16; 10x, NA=0.3)

Data Analysis. All data analysis was carried out using R programming language (R Version 3.3.3). For migration experiment Chi-square test of independence was performed to obtain the p-value and for proliferation experiment a Student t-test was performed. Following preparation and sequencing with the QIAseq RNA Panel (Qiagen), reads were demultiplexed via their i7 adapter sequences. Reads sharing the same unique molecular index were aligned to form read families. Error-correction of read families and generation of consensus sequences were performed as described previously (7). Consensus sequences were locally aligned to the human reference genome hg19 using Bowtie2 and processed with Mpileup using parameters -BQ0 -d 10,000,000,000,000 to remove coverage thresholds. Targeted transcriptome analysis from the Heme V2 kit was performed via a custom cloud environment with ArcherDx software (Version

5.1.8). Results from the ArcherDx software was further analyzed using R platform, briefly data cleaning was performed in order to generate high confidence hits by sub-setting the values to Allele Fraction (AF)>=0.002 and Alternate Output (AO, the number of transcripts containing the SNP variant)>4 and data was further subset to only those SNPs that had an existing COSMIC ID or known clinical significance.

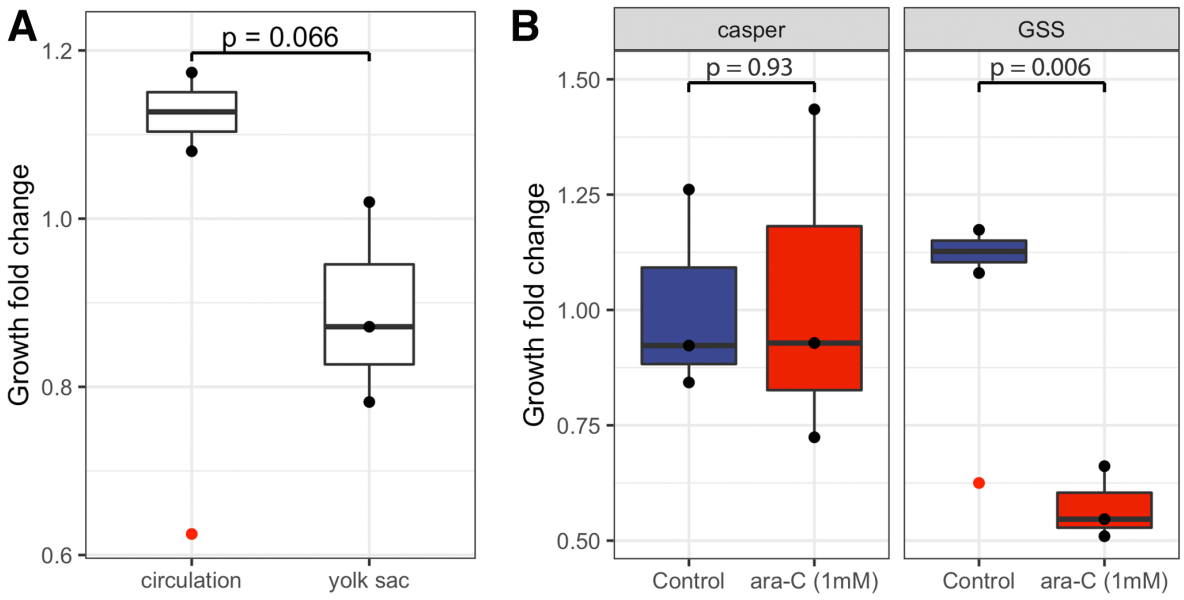
Supplementary Figures



Supplementary Figure 1: Jurkat cells home to the CHT and KM following transplantation into the CXCL12 transgenic fish.

A. Fluorescence microscopyshows Jurkat cell migration towards the zebrafish caudal hematopoietic tissue (CHT), the zebrafish equivalent of the human fetal liver at 6 days post-fertilization (dpf).

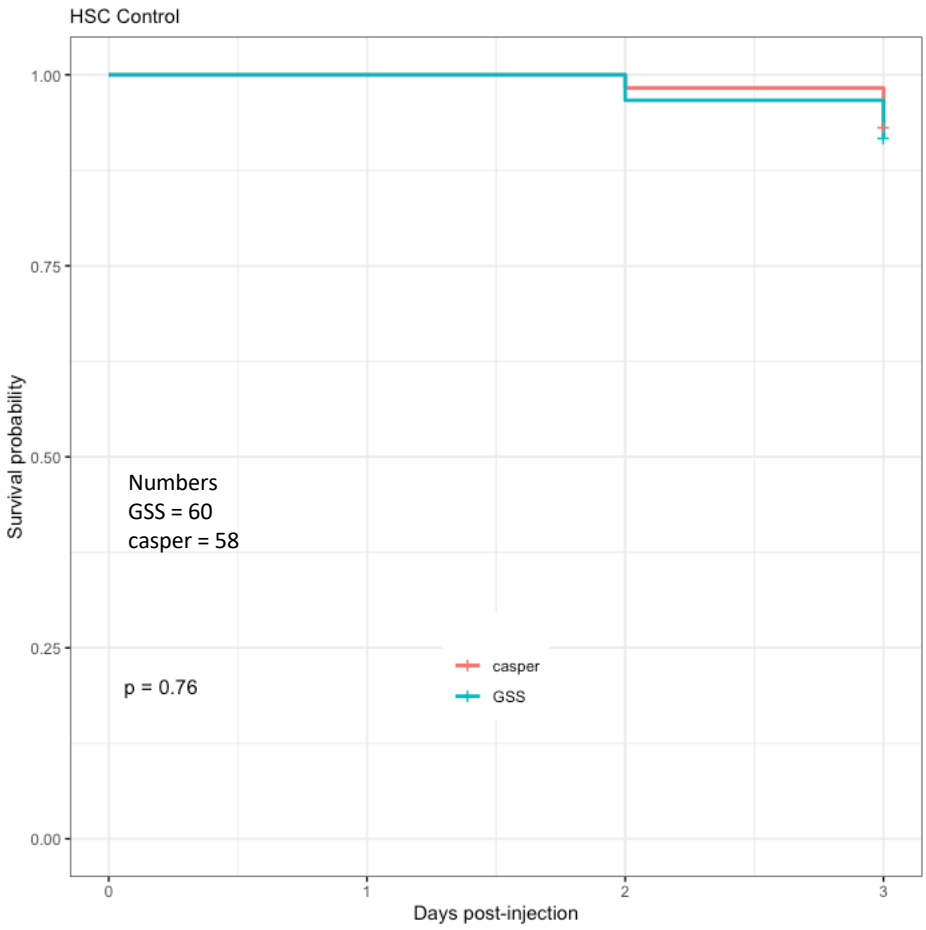
B. Ventral view of zebrafish with cell migration towards the kidney marrow (zebrafish equivalent of bone marrow) at 9 dpf (N=5).



Supplementary Figure 2: GSS larvae show greater sensitivity to chemotherapy compared to control

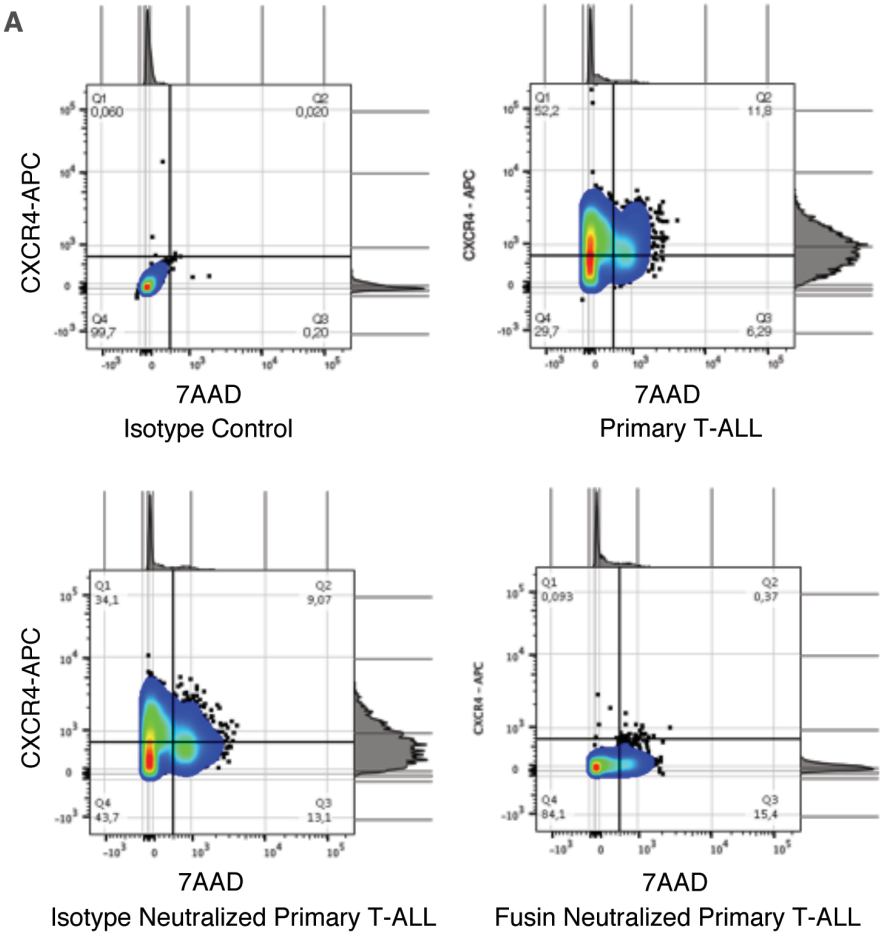
A. Human CMK cells were xenografted into the yolk-sac and orthotopically into the circulation of the GSS larvae. A proliferation assay was performed at 3 dpi and number of cells at 1 dpi was measured as a baseline. Bar plot shows the number of cells normalized to the number of cells at the baseline. A p-value was calculated with a Student-t-test.

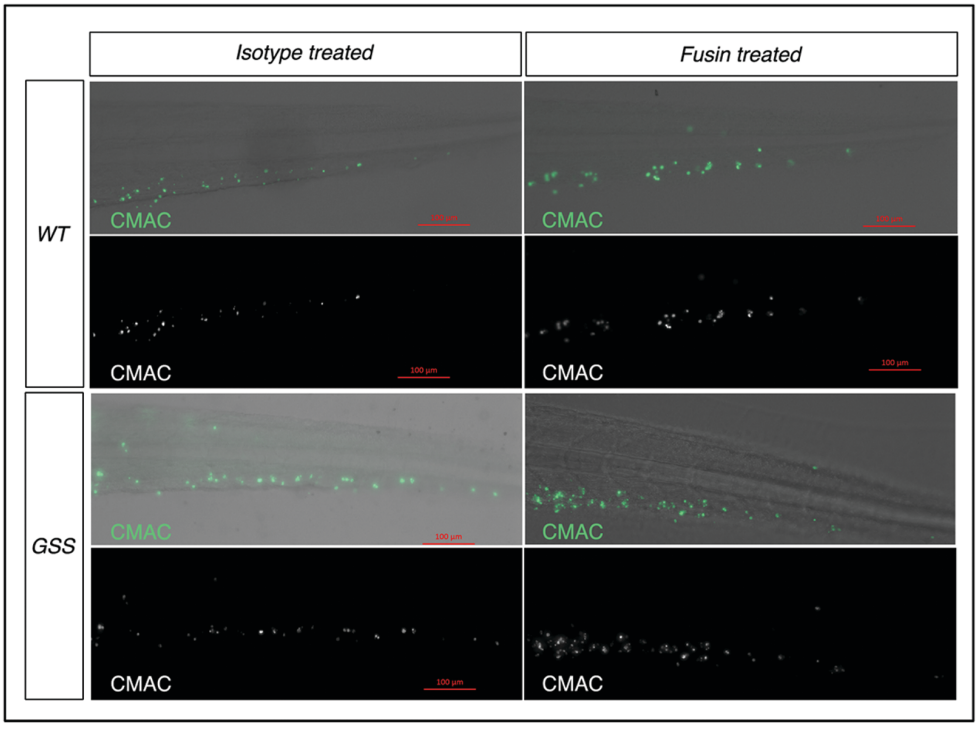
B. Human CMK cells that are responsive to cytarabine (ara-C) were xenografted into control *casper* and GSS larvae. The larvae were further divided into two groups, one of them were administered with 1mM cytarabine, and the other was left untreated. Baseline number of cells in each group was calculated before drug administration (1 dpi) and the proliferation readout was measured 2-days-post-treatment (3 dpi). Bar plot shows the number of cells normalized to the number of cells at the baseline. A p-value was calculated with a Student-t-test on *casper* and GSS larvae separately. Outliers were labelled using red dot and were excluded to determine the mean and standard error.



Supplementary Figure 3: GSS larvae no significant change in mortality upon injection of HSPCs

Human HSPCs were xenotransplanted orthotropically into both GSS and casper larvae. The mortality in the larvae were negligible and did not result in any significant change in mortality between the two groups.

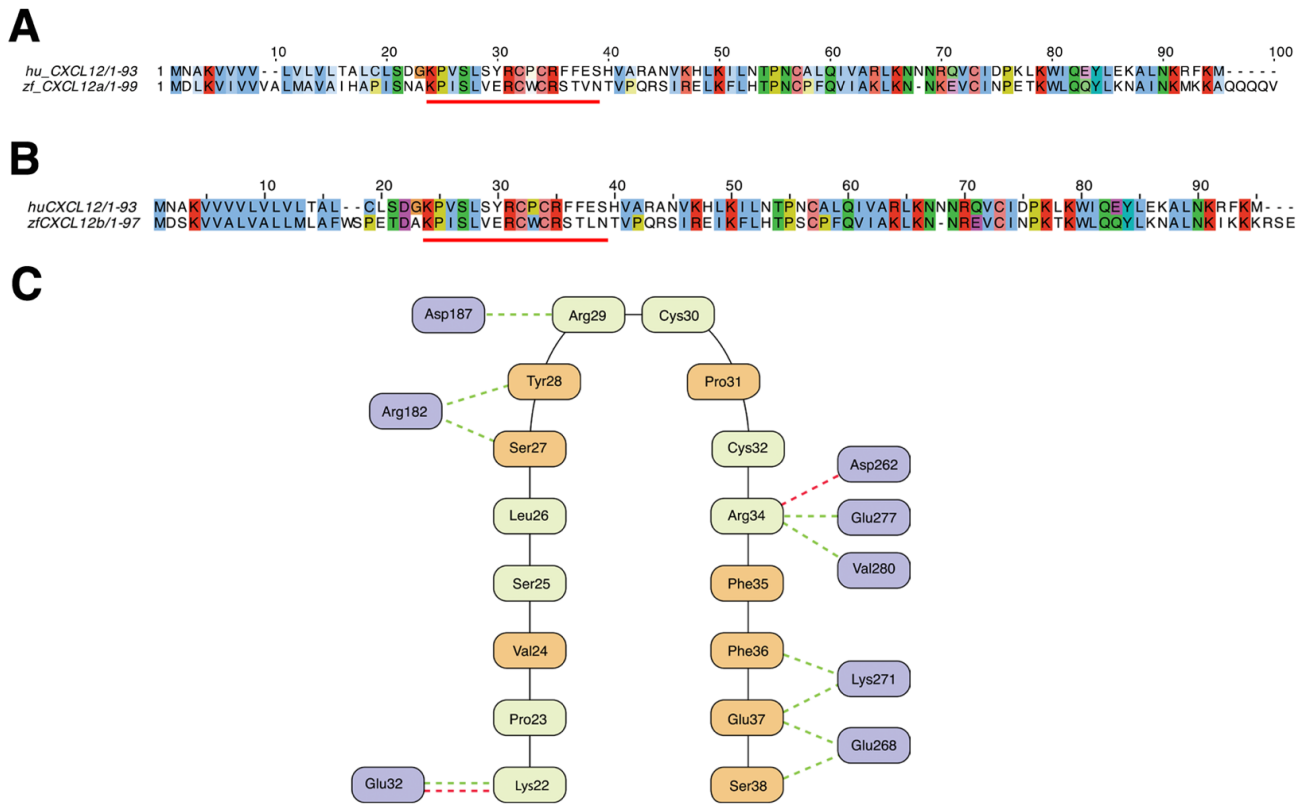

A
CXCR4-APC
Q1 0,060
Q2 0,020
Q4 99,7
Q3 0,20
7AAD
Isotype Control
CXCR4 - APC
Q1 52,2
Q2 11,8
Q4 29,7
Q3 6,29
7AAD
Primary T-ALL
CXCR4-APC
Q1 34,1
Q2 9,07
Q4 43,7
Q3 13,1
7AAD
Isotype Neutralized Primary T-ALL
CXCR4 - APC
Q1 0,093
Q2 0,37
Q4 84,1
Q3 15,4
7AAD
Fusin Neutralized Primary T-ALL


B
Isotype treated
Fusin treated
WT
GSS
CMAC
CMAC
CMAC
CMAC
CMAC
CMAC
CMAC
CMAC

Supplemental Figure 4: CHT migration of human leukemia cells are not dependent on CXCR4-CXCL12 axis.

A. Flow cytometry images demonstrate inhibitionof the CXCR4 receptor using the anti-CXCR4 antibody (fusin).

B. Representative images from *casper* control and GSS larvae transplanted with isotype or fusin treated patient-derived T-ALL sample shows no difference in migration upon neutralization with CXCR4 antibody (N=20/group). (GSS=GM-CSF/CSF, SCF/KITLG, CXCL12/SDF1) expressing transgenic zebrafish)



Supplementary Figure 5: Protein sequence alignment shows low conservation of the CXCR4 locus of CXCL12 between zebrafish and humans.

A&B. Protein sequence alignment between human CXCL12 and zebrafish CXCL12a and CXCL12b respectively. The red line indicates the known CXCR4 binding region, as observed in human.

C. Graphical overview of CXCL12-CXCR4 binding in humans. Amino acids conserved between human and zebrafish are enclosed in green boxes, those lacking conservation are in orange boxes and those from amino acids from CXCR4 are in violet boxes. The green dotted lines represent hydrogen bonds and the red dotted line represents salt bridges. Modified from Xu et al.(8)

Supplemental References

1. Glass TJ, Lund TC, Patrinostro X, Tolar J, Bowman TV, Zon LI, et al. Stromal cell–derived factor-1 and hematopoietic cell homing in an adult zebrafish model of hematopoietic cell transplantation. Blood. 2011;118(3):766-74.

2. White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, et al. Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. Cell Stem Cell. 2008;2(2):183-9.

3. Bentley VL, Veinotte CJ, Corkery DP, Pinder JB, LeBlanc MA, Bedard K, et al. Focused chemical genomics using zebrafish xenotransplantation as a preclinical therapeutic platform for T-cell acute lymphoblastic leukemia. Haematologica. 2014:haematol. 2014.110742.

4. Melong N, Steele S, MacDonald M, Holly A, Collins CC, Zoubeidi A, et al. Enzalutamide inhibits testosterone-induced growth of human prostate cancer xenografts in zebrafish and can induce bradycardia. Scientific Reports. 2017 2017/10/31;7(1):14698.

5. Rajan V, Dellaire G, Berman JN. Modeling Leukemogenesis in the Zebrafish Using Genetic and Xenograft Models. Zebrafish: Methods and Protocols. 2016:171-89.

6. Corkery DP, Dellaire G, Berman JN. Leukaemia xenotransplantation in zebrafish–chemotherapy response assay in vivo. British journal of haematology. 2011;153(6):786-9.

7. Wong WH, Tong RS, Young AL, Druley TE. Rare Event Detection Using Error-corrected DNA and RNA Sequencing. JoVE. 2018 2018/08/03/(138):e57509.

8. Xu L, Li Y, Sun H, Li D, Hou T. Structural basis of the interactions between CXCR4 and CXCL12/SDF-1 revealed by theoretical approaches. Molecular BioSystems. 2013;9(8):2107-17.