The role of transposable elements in hematopoietic stem and progenitor cell development.

A thesis

Submitted to

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by

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CERTIFICATE

This is to certify that this dissertation entitled '**The role of transposable elements in hematopoietic stem and progenitor cell development**' towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Gunwant Patil** at Institute for Research on Cancer and Aging Nice, France under the supervision of **Dr. Eirini Trompouki**, Molecular Hematology- Blood Signals lab, during the academic year 2022-2023.

Dr. Eirini Trompouki

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Name of your TAC: Prof. Dr. Sanjeev Galande

This thesis is dedicated to my supervisors.

(Dr. Eirini Trompouki, and Dr. Sanjeev Galande)

DECLARATION

I hereby declare that the matter embodied in the report entitled '**The role of transposable elements in hematopoietic stem and progenitor cell development'** is the result of the work carried out by me at the Department of Biology, IISER Pune, and the Institute for Research on Cancer and Aging, Nice, France under the supervision of **Dr. Eirini Trompouki** and the same has not been submitted elsewhere for any other degree.

Gunwant Patil

Date: 1st April 2023

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ABSTRACT

Hematopoietic stem cell (HSC) formation is the major developmental process leading to the establishment of the hematopoietic system. HSCs are the foundation of the hematopoietic system and give rise to almost all differentiated hematopoietic cells. HSCs are of high clinical importance as they are used for patients needing a blood transfusion, like in some cases of leukemia. However, as the mechanisms behind the formation of the HSCs are still unexplored, it is hard to find therapeutics against these diseases. Trompouki's lab recently identified that the formation of HSCs during development is mediated by the interplay between RNA sensors involved in innate immune signaling and transposable elements (TEs). The transcripts of transposable elements trigger innate immune receptors like RIG-I and lead to enhanced formation of HSCs. To investigate the role of specific TEs in hematopoiesis, we aim to clone the CRISPR activating and inactivating constructs under a tissue-specific promoter and inject them in zebrafish to generate stable lines. We plan to explore whether overexpression of specific TEs in HSCs or other cell types affects HSC development. We will use whole-mount in situ hybridization, injections, microscopy, and other techniques to dissect the role of specific TEs in developmental hematopoiesis.

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CONTRIBUTIONS

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This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

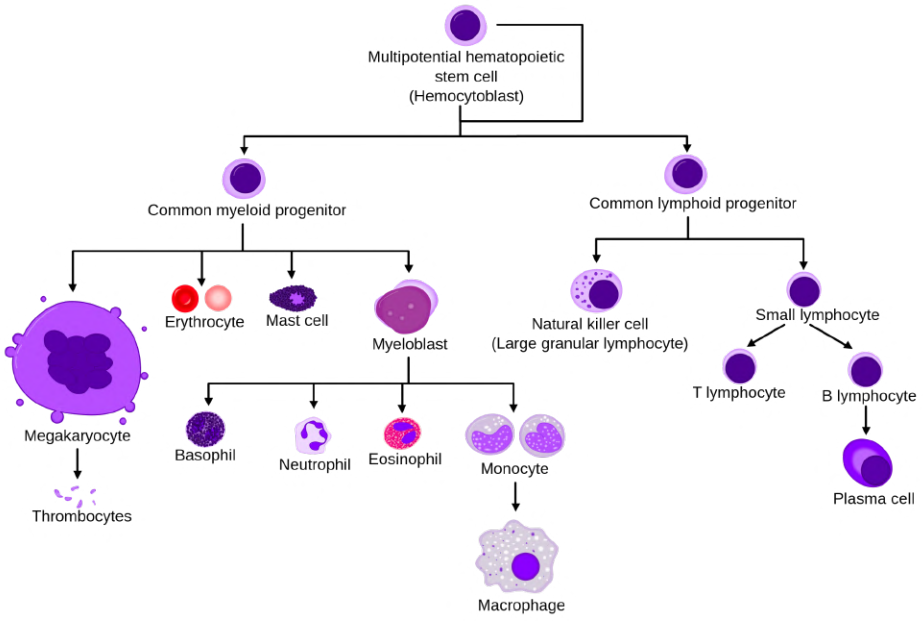
LIST OF ABBREVIATIONS

TEs	Transposable elements
HPF	Hours post fertilization
HSCs	Haematopoietic stem cells
LTR	long terminal repeat
LINE	Long interspersed nuclear elements
SINE	Short interspersed nuclear elements
CRISPR	Clustered regularly interspersed short palindromic sequences
RBC	Red blood cells
AGM	Aorta-gonad-mesonephros
ALM	Anterior lateral mesoderm
PLM	Posterior lateral mesoderm
HDR	Homology-mediated repair
TMEJ	Theta-mediated end joining
NHEJ	Non-homologous end joining
WISH	Whole mount in situ hybridization
ATAC-seq	Assay for transposase-accessible chromatin sequencing
ChIP	Chromatin Immunoprecipation
gRNA	Guide RNA
FACS	Fluorescence-activated cell sorting
CIP	Calf intestinal alkaline phosphatase

Chapter 1
Introduction

1.1 Hematopoiesis

Hematopoiesis is the formation of a blood pool during embryonic development and adulthood. All the cells in this cellular blood component arise from the parent Hematopoietic Stem Cell (HSC)(Monga et al., 2022) In healthy adult persons, these HSCs give rise to 10^{11}–10^{12} new blood cells in the trabecular region of the bone marrow from the limited HSCs daily to keep steady-state levels in the peripheral circulation (Stites et al., 1997). The development of the hematopoietic system in mammals takes place in three phases, i.e., primitive hematopoiesis, prodefinitive, and definitive hematopoiesis. Primitive hematopoiesis forms enucleated erythrocytes and myeloid lineages. Prodefinitive hematopoiesis gives rise to similar cells as primitive hematopoiesis. In contrast, definitive hematopoiesis produces hematopoietic stem cells, from which all other mature blood and immune cells are derived. The earliest definitive HSCs in vertebrates emerge from the endothelium wall of the embryonic aorta-gonad-mesonephros area. (AGM). This is referred to as the endothelial-to-hematopoietic transition. (EHT) (Ottersbach, 2019).



Overview of Normal Hematopoietic System. The diagram shows all the lineages in the hematopoietic system arising from multipotent HSC. Alternative names of the cells are also mentioned in parentheses. HSCs give rise to myeloid and lymphoid lineages. Myeloid lineages give rise to RBCs, Mast cells, Megakaryocytes, Basophil, Neutrophil Eosinophils, and Monocyte,

While lymphoid progenitors differentiate into NKC, T, and B lymphocytes and Plasma cells. The morphological characteristics of cells are represented as seen under Wright's stain, May-Giemsa stain (“Haematopoiesis,” 2023)

The differentiation of the HSCs leads to different blood cell lineages, such as myeloid and lymphoid. These cell lineages are involved in the development of dendritic cells. . Myeloid lineage includes monocytes, macrophages, basophils, neutrophils, eosinophils, erythrocytes, and platelets. Lymphoid progenitors give rise to T, B, Natural killers, and innate lymphoid cells. The hematopoietic tissues can give rise to cells capable of long-term and short-term regeneration as well as committed multipotent or unipotent progenitors.

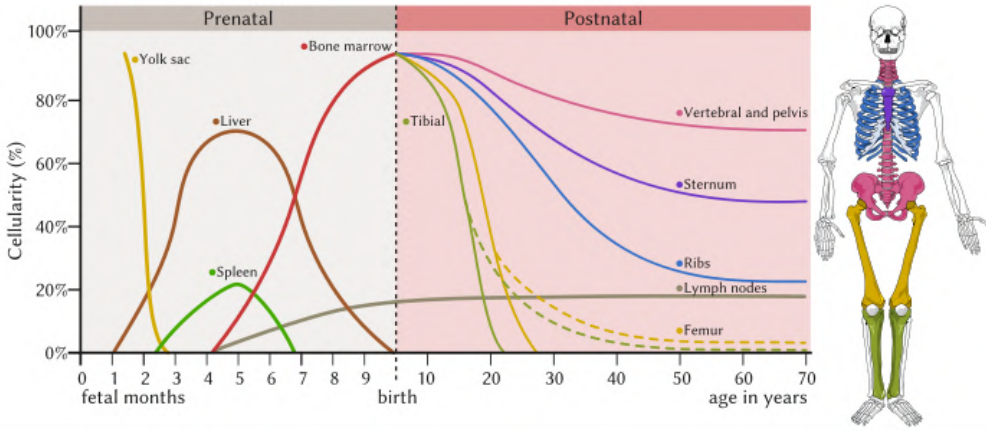


Fig 1.2 Sites of hematopoiesis in pre and postnatal period. The major sites of prenatal hematopoiesis in humans are the yolk sac, liver, and bone marrow. After birth, HSCs arise from the bone marrow of the sternum, ribs, lymph nodes, and femur. (Reproduced from Hematology: Clinical Principles and Applications. 3rd ed. Saunder, 2007).

As previously mentioned, the first few HSCs in humans and mice are found in the endothelial lining of the aorta-gonad-mesonephros and umbilical arteries. In developing embryos, blood formation occurs in so-called blood islands in the yolk sacs, forming the aggregates of blood cells in mammals. Slightly later, HSCs are found in the spleen, lymph nodes, and fetal liver(Dzierzak & Bigas, 2018; Dzierzak & Speck, 2008). Four months into prenatal development, bone marrow takes up the task of blood cell formation for the entire organism (Birbrair & Frenette, 2016). In adults, most of the HSCs are found in the bone marrow of the pelvis, femur, and sternum. However, the

spleen, lymph nodes, thymus, and liver are the major sites for the maturation and activation of the lymphoid cells. [Refer to fig. 1.2]

1.2 Genetic Control of Hematopoiesis

1.2.1 Primitive Hematopoiesis

The major regulators of primitive hematopoiesis are gata1 and pu1 (sfpi1 in mice; spi in zebrafish). *gata1* is the master regulator of erythrocyte development, whereas pu1 is the master regulator of myeloid lineages (Cantor & Orkin, 2002; Scott et al., 1994). *gata1* knockout mice die during gestation due to perturbed pro-erythroblast maturation into erythrocytes. In zebrafish, erythrocyte-specific hemoglobin is visible with benzidine staining in the cells expressing *gata1*, indicating the presence of alpha and beta embryonic globin expression in these cells (Detrich et al., 1995). gata1 knockdown experiments in zebrafish also showed the increased expression of pu1, *mpo* (*myeloperoxidase*, a granulocyte-specific gene), and *l-plastin* (*lcp1*): suggesting the switch in differentiation pattern from erythroid-specific to myeloid cells. In contrast, Pu1 knockdown leads to the increased expression of gata1 and switches to the expression of erythroid fate-specific genes (Rhodes et al., 2005). The studies have shown that gata1 and pu1 interact physically, thus showing the direct cross-inhibitory competition for target genes.

1.2.2 Definitive Hematopoiesis

Runx1, a member of the Runt family of transcription factors, has been shown to play an important role in hematopoiesis (Wang et al., 1996). *Runx1-/-* mice show decreased expression of definitive erythroid, myeloid, and lymphoid lineage cells. In zebrafish, Runx1 is expressed in the posterior lateral mesoderm and neural tissues at five somite stages and in the dorsal aorta at 30 hpf during development. *runx1* is also involved in primitive hematopoiesis in zebrafish but plays a vital role in definitive hematopoiesis. (Burns et al., 2005; Gering & Patient, 2005; Kalev-Zylinska et al., 2002). In zebrafish, *c-myb* is expressed at around the 10–12 somite stage, i.e., during the primitive wave, and at 36 hpf in the ventral aorta, with cells expressing Runx1 in a definitive wave. Also,

studies have shown that *myb* knockout mice die as a result of failure in fetal liver hematopoiesis, thus indicating the role of c-myb in definitive wave of hematopoiesis (Mucenski et al., 1991).

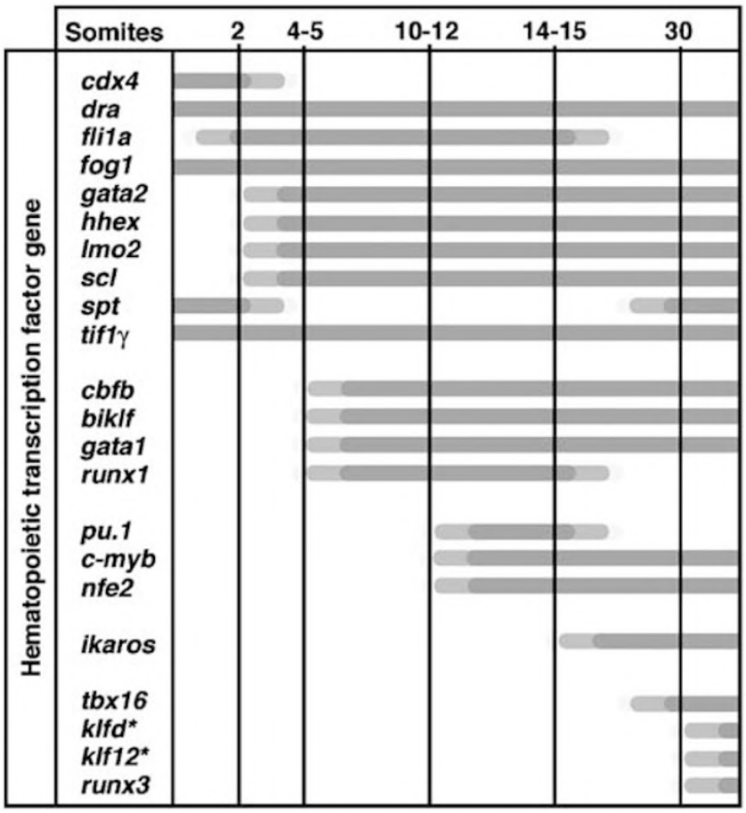


Fig 1.3 Expression patterns of major TFs involved in haematopiesis during zebrafish early developmental stages (1-30 somite stage). (reproduced from Davidson & Zon, 2004).

1.2.3 Genes involved in hemangioblast induction

An array of transcription factors moderates hemangioblast induction during zebrafish development. These hemangioblasts, precursors to the erythroid and endothelial progenitors, are formed within the ALM (ventral lateral mesoderm), the primary site of primitive hematopoiesis during zebrafish early development(Liao et al., 1998; Pham et al., 2007; Sumanas et al., 2005; Thompson et al., 1998). Cells co-expressing Tal1, Gata2, Fli1, Lmo2, and Etsrp, major regulators of hemangioblast development, expressed in both the anterior and posterior lateral mesoderm (Paik & Zon, 2010). These transcription factors are essential for endothelial and hematopoietic differentiation, so these cells are postulated to be hemangioblasts. For example,

Gata2-deficient mice are embryonically lethal and die of severe anemia, proving the importance of Gata2 in HSC maintenance and proliferation (Tsai et al., 1994). Also, Tal1 knockdown shows adverse effects on endothelial differentiation, and the double-negative mutant does not show myelopoiesis or primitive hematopoiesis in zebrafish and mice (Dooley et al., 2005; Patterson et al., 2005; Shivdasani et al., 1995). Furthermore, Lmo2 is expressed with Tal1 and Gata2 and is necessary for erythropoiesis in the yolk sac in mice. In mice, loss of Etsrp (Etv2) results in total depletion of endothelium and blood cells, indicating its role in producing bipotent progenitors. These cells, co-expressing fli1, lmo2, gata2, tal1, and etsrp in the anterior and posterior lateral mesoderm of zebrafish, can differentiate into angioblasts (endothelial progenitors) or HSCs, supporting the hemangioblast theory.

1.3 Transposable elements of the genome

Transposable elements (TEs) are repetitive DNA sequences that move within the organism's genome. They were first discovered by Barbara McClintock in the 1940s, who observed their ability to alter gene expression (McClintock, 1950). TEs are ubiquitous in eukaryotic genomes and can comprise a significant fraction of the genome size, constituting half of the human genome. Previously, TEs were sometimes referred to as "selfish DNA" or "genetic parasites" and considered to have no role in the pathophysiological process. However, the research in recent decades showed a strong correlation between the expression pattern of these elements and essential physiological processes. Transposable elements played a significant role in shaping the evolutionary history of eukaryotic genomes and continue to generate new variants in the population(Feschotte, 2008; Kazazian, 2004).

Transposable elements (TEs) can be divided into two major classes based on their transposition mechanism: Class I and Class II. Class I TEs, also known as retrotransposons, use a two-step "copy and paste" mechanism. An RNA intermediate is first transcribed from the repetitive DNA sequence and reverse-transcribed back into DNA before being inserted at a new genomic location. Retrotransposons can be classified into long terminal repeats (LTR) and non-LTR elementary repeats. LINE (Long

Interspersed Nuclear Elements) are non-LTRs autonomous in their transposase activity, whereas Short Interspersed Nuclear Elements (SINE) are nonautonomous, e.g., Alu, and depend on LINE for retrotransposition.

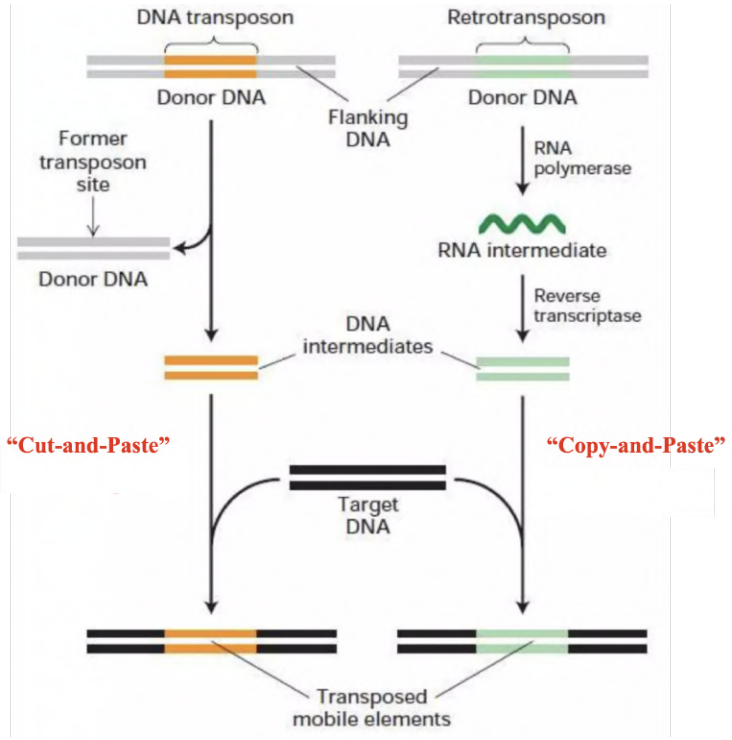


Fig 1.4: Mechanism of action of Class I and Class II TEs. Schematic representation of Class I and Class II TEs showing "Copy Paste" (left) and "Cut-Paste" (right) mechanisms.

Class II TEs, also known as DNA transposons, use a "cut and paste" mechanism in which the TE DNA sequence is directly excised from its original location and inserted at a new site in the genome. Both Class I and Class II TEs can cause mutations and alter the genetic information of an organism by inserting into or near genes or regulatory sequences. However, retrotransposons are more prevalent and have significantly impacted genome evolution due to their ability to amplify and spread through the genome via RNA intermediates.

1.3.1 Transposable elements and hematopoiesis

Although repetitive elements have been recognized for many years, little is known about their role in hematopoietic maintenance and activation. Recent research in the

hematopoietic field has focused on the role of transposable elements in hematopoiesis. Schumann et al. showed that expression levels of transposable elements family LINE1 in the homeostasis of HSCs in humans and mice were not significant, with low levels of transposition(Schumann et al., 2019). Later, it was shown that LINE1 expression and retrotransposition increased with ionizing radiation, leading to the impairment of HSC function. The authors also demonstrated that thrombopoietin induced an interferon response that suppressed TE expression, minimizing retrotransposon-mediated damage to HSCs (Barbieri et al., 2018). Studies have shown that stress signals activate transposable elements (Hummel et al., 2017; Shpyleva et al., 2018; Zovoilis et al., 2016). In HSCs, increased expression of various TEs was observed with physiological stress like aging. Capone et al. and Cecco et al. showed that senescent human HSCs increased transposable elements' activity and activation of inflammatory signaling. (Capone et al., 2018; De Cecco et al., 2019).

Trompouki's lab recently identified that the formation of HSCs during development is mediated by the interplay between RNA sensors involved in innate immune signaling and TEs. The transcripts of repetitive elements trigger innate immune receptors like RIG-I and lead to the enhanced formation of HSCs (Pontis et al., 2019). While studying chemotherapy-induced HSC regeneration, our lab has shown that transposable element expression increases within 2 hours of 5-fluorouracil injection. These transposable elements further bind to the innate immune receptor MDA5, which activates the inflammatory signaling necessary for HSCs to exit the quiescent stage (Lefkopoulos et al., 2020).

These studies provide evidence for the involvement of TEs in hematopoiesis and suggest that these elements play a role in the regulation of gene expression and cellular differentiation in the hematopoietic system. Further research is needed to understand the mechanisms underlying this process entirely.

1.4 CRISPR-Cas9: Genome editing technology

CRISPR-Cas9 gene editing is an efficient, specific, and versatile genome editing technology capable of causing irreversible changes in the organism's genome. With

CRISPR, it is now possible to harness, modify, delete, or correct the precise region of the DNA and generate stable transgenics. The discovery of CRISPR led to Jennifer Doudna and Emmanuelle Charpentier winning the Nobel prize in chemistry in 2020. The technique is based on the simplified version of the bacterial antiviral defense system CRISPR-Cas9, which stands for the Clustered Regularly Interspersed Short Palindromic Sequences and consists of two major components, Cas9 nuclease protein complexed with short, unique, and specific guide RNA. On successful delivery in cells, it binds to the desired location, allowing existing genes to be replaced with new ones.

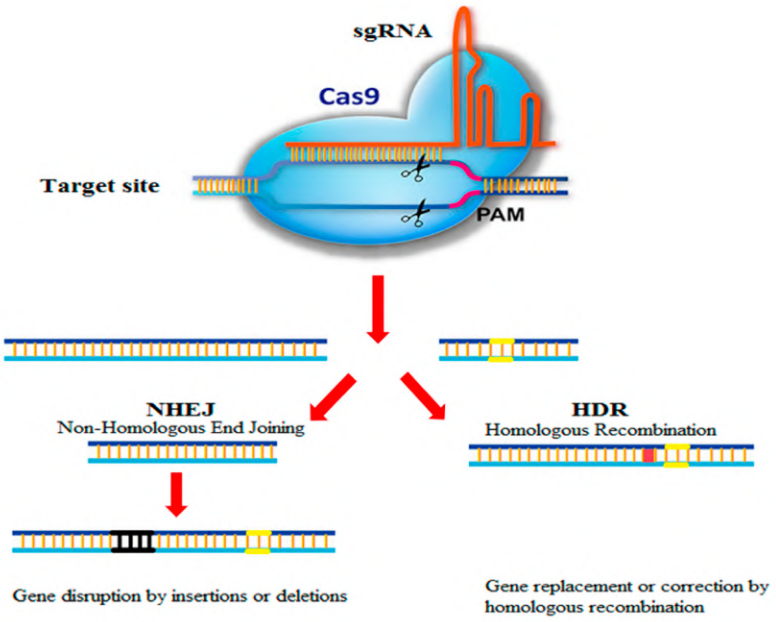


Figure 1.5 Two major pathways mediating CRISPR-Cas9 genome editing system.
(Reproduced from Tavakoli et al., 2021).

Within the cell, the Cas9 nuclease protein act as genetic scissors opening both strands of the targeted DNA sequence to introduce modification by either homology-mediated repair (HDR) or non-homologous end joining or polymerase theta-mediated end joining (TMEJ). HDR is the traditional targeted genome editing pathway to generate knock-in mutations, allowing the introduction of targeted DNA damage and repair. HDR drives the repair of DNA breaks via exogenous DNA as the repair template. Knock-out mutations by CRISPR-Cas9 result from the activity of NHEJ/TMEJ pathways at the double-stranded DNA break sites. The non-homologous end-joining pathways

sometimes result in the random deletion or insertion mutation, which may alter the structural or functional integrity of the gene.(Tavakoli et al., 2021)

1.4.1 CRISPR activation and Inhibition

Recently, CRISPR has been reorchestrated for more sophisticated applications such as CRISPR activation and inhibition. In this technique, catalytically inactive Cas9 protein is fused to the transcriptional activator or repressor domain. This allows the modulation of the expression of genes targeted by sgRNA and manipulates the essential pathways and traits without altering the target sequence. With CRISPRa/i it is now possible to regulate the expression of multiple target genes with the multiplex gRNA array.

CRISPR activation

CRISPRa uses dCas9 fused with the transcriptional activator domains such as VP64, VP160, or VPR to activate gene expression. CRISPRa allows the increased expression of genes in their native context, in contrast to the gain of function plasmids or lentiviral-based ORFs. Transcriptional activators in fusion with dCas9 can be targeted to the promoter or exonic region of the target gene, which assists in the recruitment of transcription machinery, including RNA polymerase II.

CRISPR inhibition

CRISPRi can sterically hinder the transcription of the target gene by blocking transcription initiation or elongation. CRISPRi allows robust modulation of gene expression with up to 99.9 % repression(Larson et al., 2013). The strength of repression can be tuned by the dCas9 complex with KRAB or SALL1-SDS3 domains or by changing the complementarity between the gRNA and template.

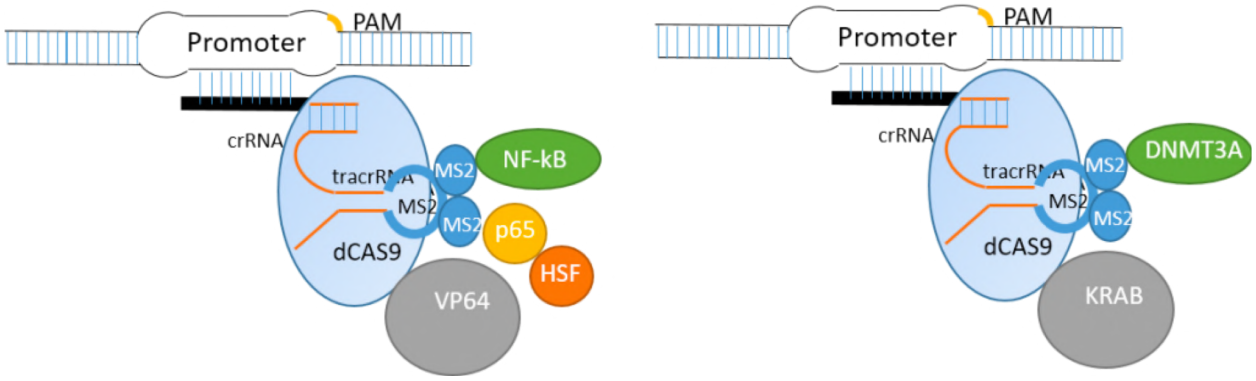


Fig 1.6: Schematic representation of CRISPR activating and inhibiting complex. RNA-programmed activators or repressors can be fused directly to dCas9. Activators form complexes via direct binding of VP64 or indirect via MS2 binding with NF-kb and p65-HSF. In contrast, KRAB transcriptional repressor recruits de-novo methyltransferase 3A and LSD1 directly or indirectly to repress the gene. (Reproduced from Overview of CRISPR/Cas9 Systems | Duke Viral Vector Core).

1.5 Zebrafish as a model for hematopoiesis

Haematopoiesis is a complex process affected by the multiple signaling pathways regulating each step of the blood formation process, from early precursors to the most differentiated blood cell types. Any perturbations in homeostasis lead to changes in physiologic conditions like oxygen transport and the immune response to pathogens. In recent decades, the zebrafish has emerged as a robust vertebrate model to study various developmental processes, including hematopoiesis. Zebrafish are about 2.5–4 cm long, with a lifespan of approximately three years. However, the major feature includes external fertilization with optical transparency during embryonic development, allowing visual analysis of early development. The use of zebrafish as a model organism increased after the complete genome sequencing project (Howe et al., 2013). Humans and zebrafish share 71% of the same genes, and 82% of diseases associated with human genes are conserved in zebrafish (Howe et al., 2013). Importantly, the hematopoiesis-controlling processes in zebrafish are retained in mammals, including humans. The zebrafish model system is more amenable to large-scale genetic screening than other vertebrate genetic models, such as mice and drosophila, resulting in the characterization of more than 500 mutant phenotypes during early development.

Additionally, the generation of transgenics using CRISPR is very easy in zebrafish, followed by an easy screening of offspring using microscopy.

Chromatin architecture is an essential regulator of gene expression, and any perturbations of this process can lead to hematopoietic malignancies. The zebrafish is a great model organism for studying the mechanisms of chromatin control and epigenetic regulators using chromatin-modifying medicines. The regulatory elements can be identified by performing chromatin immunoprecipitation (ChIP) followed by sequencing. Many conserved antibodies against histone marks from different species have been successfully tested effectively in zebrafish (Bogdanovic et al., 2012; Paik & Zon, 2010; Vastenhouw et al., 2010). Other sequencing techniques, such as ATAC-seq for identifying regulatory elements by detecting chromatin accessible regions requires far less input than ChIP-seq, have been utilized successfully for single cells (Doganli et al., 2017). Additionally, Multi-Omics experiments such as proteomics, Single-cell or bulk RNA sequencing to study the gene expression profiles can be performed in zebrafish.

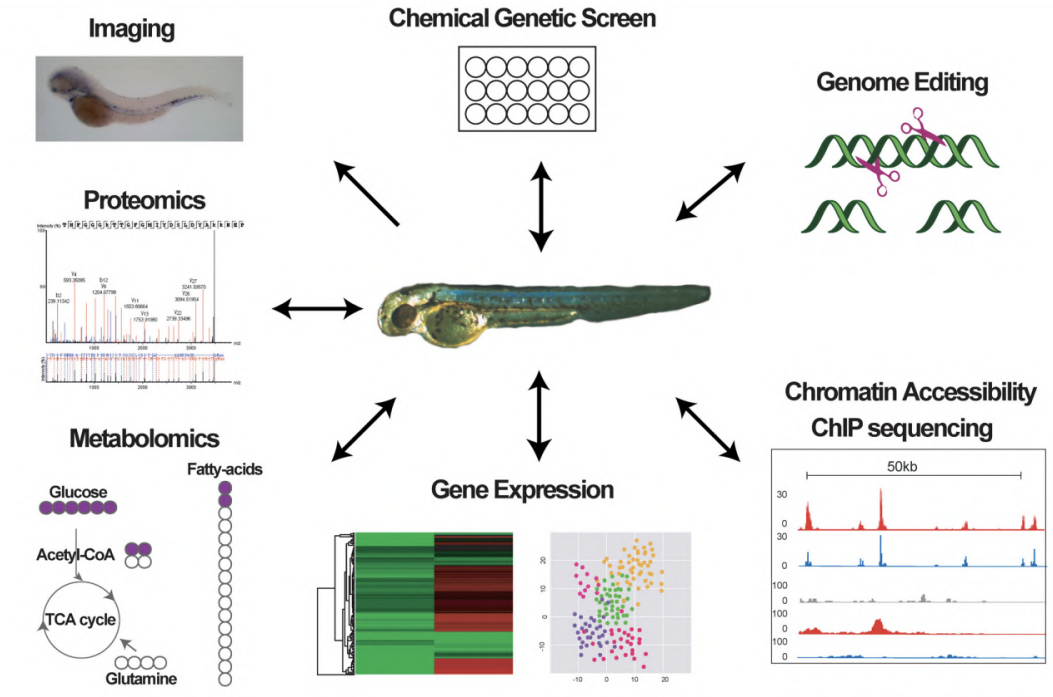


Fig 1.7: The advantages of using Zebrafish as a hematopoietic model organism (de Pater & Trompouki, 2018)

1.5.1 Genetic screens

Forward genetic screens resulted in many blood-related mutants in zebrafish, allowing researchers to dissect genes involved in hematopoietic malignancies (reviewed by Hsia & Zon, 2005). Zebrafish mutants with defects in hematopoiesis mimic human blood disorders, making them significantly important in large-scale drug screens to design therapeutic strategies against diseases. Many human hematopoietic disorders have already been modeled in zebrafish, such as leukemia, lymphoma, anemia, thrombocytopenia, and bone marrow failure syndromes. Such a mutation in the *tbx16* gene in the spadetail mutant showed defective mesodermal tissues, including blood. The genetic features also showed depletion in the expression levels of the hematopoietic markers *tal1, imo2, gata2, fli1*, and *gata1*. The Vladtapes mutant with a nonsense point mutation in gata1 played an essential role in decoding the function of *gata1* in primitive hematopoiesis. The mind-bomb mutant with a defective Notch pathway affected the expression of *runx1* and *cmyb*, critical players in definitive hematopoiesis, confirming the role of Notch signaling in definitive hematopoiesis.

1.5.2 CRISPR toolbox in Danio rerio (Zebrafish)

The zebrafish was the first vertebrate model to show that CRISPR/Cas9 can effectively modify the genome in vivo with up to 50% targeting effectiveness (Hwang et al., 2013). Chang et al. further generated biallelic *gata5* and *etsrp* mutants, showing that CRISPR generated mutants phenocopy the genetic mutants (Chang et al., 2013). Jao et al. (2013) demonstrated that codon-optimized Cas9, when injected with sgRNA at the one-cell stage, could efficiently induce biallelic mutation. Up to five genes can be targeted simultaneously (Jao et al., 2013). Multiple kits such as Tol2 and streamlined workflows have been generated to create zebrafish mutants using CRISPR/Cas9 (Gagnon et al., 2014; Varshney et al., 2015). The fusion protein dCas9 with transcriptional activators VP64 or transcriptional repressors KRAB results in CRISPRa/i when targeted to the coding or regulatory region of the gene. The functional validation of CRISPRa and CRISPRi has been shown to work in regulating the expression of the target gene in zebrafish (Long et al., 2015). The authors targeted the fgf8a and foxi1

genes, which are essential for otic placode induction, by sgRNA coinjected with dCas9-KRAB. They observed the reduced expression of both genes at 11 hpf, with smaller otic vesicles at 32 hpf. While in the case of dCas9-VP160, the expression levels of fgf8a and foxi1 were elevated, with relatively bigger otic vesicles at 32 hpf (Long et al., 2015). With CRISPR/i, it is now possible to integrate dCas9 under tissue-specific or conditional promoters for more robust modulation of the expression of target genes.CRISPRa/i has not been used previously to target transposable elements in the zebrafish system.

Aim and Objectives of the study

This project aims to generate the CRISPR-activating and inhibiting zebrafish lines to decipher the role of transposable elements in developmental hematopoiesis.

1 . Generating RNA probes and testing:

- To generate the RNA probes against major hematopoietic TF factors, perform whole mount In-situ hybridization, and optimize the post-fertilization stages with reference to the literature for the best expression profile of these factors.

2. Generation of CRISPR construct using tol2 kit:

- Generating the activating dCas9-VP160, dCas9-VP64, and Inhibiting dCas9-KRAB constructs and cloning them in the middle entry vector for gateway assembly.
- Gateway assembly of these constructs with the 5' tissue-specific or heat shock promoters, middle entry vectors, and 3' PolyA tail using the tol2 kit to generate conditional transgenic lines.

3. Identifying top tags for guide RNA design

- Comparing the expression profiles of transposable elements families of endothelial and hemogenic endothelial cells in zebrafish by transcriptomics analysis.
- To design a target-specific gRNA library for the top hits from TE analysis.

4. Microinjection: Injecting the CRISPR constructs and target specific gRNA at one cell stage in zebrafish.

5. Assays to modulate HSCs number and differentiation:

- Performing in situ hybridization to check the change in expression profile for major hematopoietic factors.
- We will also perform FACS, RNA-Seq, and Microscopy to check for downregulated and upregulated pathways involved in HSCs differentiation.

Chapter 2
Materials and Methods

2.1 Culture and Maintenance of *Danio rerio* (Zebrafish)

Zebrafish were maintained per the standard procedure described previously (Westerfield, 2000). The fish facility was maintained at 28 ℃. Single-pair mating crosses for the experiments were set up the prior evening by separating males and females with a barrier. The barriers were removed the following day, and the sieve was kept at an incline to facilitate breeding. Embryos were harvested by filtering fish water and a quick wash with embryo media. The embryos were incubated at 25-31 ℃. The strains used in this study are AB, Caspr, ikzfp GFP gata1 dsRed.

2.2 Riboprobe synthesis

2.2.1 Whole mount in-situ hybridization

RNA probes were synthesized using the respective template vector for the different probes. 5-10 µg of vector plasmid were linearized using the restriction digestion in a 20 ul reaction incubated at 37 ℃ for 3 hours. The reaction was terminated by adding 0.5M EDTA, 3M NaAc, and 2x(V) 70% ethanol and DNA was allowed to precipitate at -20 for 1 hour. DNA was precipitated in 12 µl of nuclease-free water (NFW) after centrifuge and quantified using nanodrop. The transcription was set up with 300-400 ng of linearized plasmid using the mMESSAGE mMACHINE T7 transcription kit (Invitrogen). The reaction was incubated at 37 ℃ for 2 hours. The template DNA was degraded using the Rnase-free Dnase treatment at 37 ℃ for 30 mins. The synthesized RNA probe was precipitated using LiCl precipitation (30 µl NFW, 30 µl LiCl precipitation solution chilled at -20 for 1 hour). The RNA was precipitated by centrifugation at 13000 RPM, 4 ℃ for 15 mins. The RNA was washed with 70% ethanol, followed by the resuspension in 25 µl of nuclease-free water. The probes used for in situ are gata1, mpo, fli1, flk1, pu1, and runx1.

2.2.2 Transposases RNA synthesis

Transposase RNA was generated using the tol2 vector pCS2FA-transposase(Kwan et al., 2007) plasmid as a template. The plasmid was linearized using the NotI restriction enzyme and purified using a Qiagen PCR purification kit. The mMessage mMachine

SP6 kit (Thermofisher) was used to synthesize capped RNA. RNA was purified using the Licl precipitation, as mentioned above.

2.3 Whole-mount *in situ* hybridization (WISH)

The whole mount In-situ hybridization was performed by the Thisse method (Thisse & Thisse, 2008). Embryos were treated with 1-Phenyl- 2+thiourea (PTU) 12 hpf till the time of fixation to prevent pigmentation. The embryos were dechorionated if necessary and fixed in 4% paraformaldehyde (PFA) overnight at 4 ℃. After fixation, embryos were dehydrated in 100% methanol at -20C overnight. Embryos were rehydrated in various dilutions of Methanol +PBT (75%, 50%, 25%). After a final wash with PBST, the embryos were permeabilized using 0.01mg/ml of Proteinase K in PBST for the following periods in accordance with the stage:

Younger than bud	30 Sec
Early somite stage (until 14)	1 min
Late Somite stages (14–22)	2 min
24 hpf	5 min
36/48 hpf	8-10 min
72 hpf	10 min

After Proteinase K treatment, the embryos were refixed in 4% PFA for 20 minutes at room temperature. After PBST washes, the embryos were pre-hybridized in HM+ for 4 hours at 70℃. The embryos were then incubated in a 200 µl hybridization solution containing 1ng/µl of the desired RNA probe at 70 ℃ overnight. Followed by the Hybe, SSC, and PBT serial washes, embryos were incubated in blocking solution ((2% sheep serum, 2mg/mL BSA) for 30 min at RT. Subsequently, the embryos were treated overnight at 4℃ in a blocking solution containing 0.15U/µl anti-digoxigenin antibody (Roche, 11093274910). Finally, the embryos were washed and incubated in a staining solution at room temperature (for 10mL of staining buffer: 35 µl of 50 mg/mL BCIP, 45µl of 50 mg/mL NBT) till stained.

2.4 Site-Directed Mutagenesis

The protocol from Phusion Site-directed mutagenesis kit (Thermofisher scientific) was followed for site-directed mutagenesis. The non-overlapping primers to induce point mutation were designed according to guidelines. The oligos were phosphorylated and purified using a spin column kit before the reaction. The middle entry vector pME-Cas9 containing Cas9 codon optimized for the zebrafish was used as a template in standard three-step PCR with the point mutation primers. The PCR product was purified using a Qiagen min-elute PCR purification kit. For Dpn1 digestion of the Dam-methylated parental plasmid DNA, 1 µl of FastDigest Dpn1 was added directly to the mutagenesis reaction and incubated at 37 ℃ for 15 minutes. The ligation reaction was performed with T4 DNA ligase and 5x Rapid ligation buffer at 25 ℃ for up to one hour. The ligation reaction was directly transformed in the DH5 alpha-competent bacteria, plated on the kanamycin plates, and incubated at 37 ℃ for 16 hours.

2.5 Restriction-based Cloning:

We used a restriction-based cloning strategy to insert VP160, VP64, and KRAB into the pME-nls-dCas9-nls vector, a kind gift from the lab of Dr. Robert Wilkinson at the Department of Infection, University of Sheffield, UK.

PCR amplification:

Inserts were amplified using the NEB 2x High fidelity PCR master mix, and the primers were explicitly designed for restriction-based cloning flanking BamH1 sites. All PCR reactions were performed according to the manufacturer's protocol. The PCR products were purified using the Qiagen min-elute PCR purification kit.

Digestion:

The 1.5 µg of vector and inserts were digested using Promega BamH1 restriction enzyme in 20 µl reaction incubated at 37 ℃ for 3-4 hours. The CIP treatment was given by adding 1 µl of CIP to the digestion reaction of the vector. The quick CIP catalyzes the

dephosphorylation of the 5’ and 3’ ends of the DNA strand, thereby preventing self-ligation. The digested product was analyzed by gel electrophoresis and column purified.

Ligation

The digested vectors and inserts were mixed in a 10 µl ligation reaction with a vector-to-insert ratio of 1:10 with a quick ligase enzyme. The reaction was incubated at 25 ℃ for one hour.

vector DNA	100 ng
insert DNA	17ng
Ligase 10X Buffer	1µl
T4 DNA Ligase (Weiss units)	0.1–1µl
Nuclease-Free Water to a final volume of 10 µl	

Transformation and Plasmid isolation

Transform the ligated mixture into DH5α competent cells by heat shock transformation. Transformed colonies were grown in 3 ml of LB media supplemented with suitable antibiotic resistance. The plasmid preps were done using the QiaPrep Spin miniprep and midiprep kits. The 3-5 ml bacterial culture was used for mini preps, while 50 ul of culture was inoculated for midi preps. The plasmids were quantified using nanodrop. Further, the clones were confirmed by PCR validation and Sequencing.

2.6 Gateway assembly

The three-fragment gateway assembly was performed using LR Clonase plus II enzyme mix. The equimolar concentration of the entry clones was used with a 20 fmol destination vector for a 10 ul LR reaction**.**

Entry clones (10 fmoles)	1 µL−7 µL total
Destination vector (20 fmoles)	1 µL
1X TE Buffer, pH 8.0	to 8 µL

2 ul of LR Clonase plus II enzyme mix was added to the multisite gateway reaction Prol LR reaction. The reaction was incubated at 25℃ for 16 hours. The reaction was terminated by adding 1 µl ProK to the reaction mixture and incubating at 37 ℃ for 20 mins. The DNA was precipitated using NaAc and 70% ethanol. The purified plasmid DNA was transformed using 25 µl of K10 electro-competent bacteria. 50 µl of inoculate was spread over ampicillin resistance plates and incubated at 37 ℃ for 16 hours.

2.6 Microinjection

Expression constructs are being tested by injection of plasmid DNA, with or without transposases RNA. Different plasmid DNA concentrations were injected for verification (50 pg, 25 pg). For practice injections and positive control, 50 pg of EGFP vector was injected at the one-cell stage.

2.7 RNA extraction

The RNA from zebrafish embryos was extracted by the trizol method. The larval stages were collected and homogenized in 1 ml of trizol reagent. 200 µl of RNA grade Chloroform was added and mixed well by inverting the tube a few times (until milky appearance). The tube was left at room temperature for 10 minutes. The microcentrifuge tube was centrifuged at 12000 rpm for 15 minutes at 4℃. The upper aqueous layer was transferred into a new microcentrifuge tube. 500 µl of Isopropanol was added to the aqueous layer, and the content was mixed by vortexing gently. The tube was incubated at RT for 15 mins allowing RNA to precipitate. The tube was centrifuged at 12000 rpm for 30 minutes at 4℃. The resultant pellet was washed twice with 75% ethanol. The pellet was allowed to dry at room temperature for 10-15 minutes until residual ethanol evaporated. The pellet was dissolved in 11 µl of nuclease-free-water at 55℃ for 10 minutes. The concentration of RNA was determined by Nanodrop, and the RNA was diluted to the required concentration.

2.8 Transposable elements transcriptomics analysis

The transposable elements transcriptomic analysis was performed on a previously published dataset from Lefkopoulos et al., 2020 to compare Endothelial cells and

hemogenic endothelial cell types. The raw SRA files were downloaded from NCBI. RNA-Seq reads were trimmed with trim-galore. Reads were then aligned to the GRCz11 zebrafish genome version with STAR aligner (Dobin et al., 2013) with the options:--outFilterMultimapNmax 1 --outFilterMismatchNmax 3 --outMultimapperOrder Random --winAnchorMultimapNmax 5000 --alignEndsType EndToEnd --alignIntronMax 1 --alignMatesGapMax 350 --alignTranscriptsPerReadNmax 30000 --alignWindowsPerReadNmax 30000 --alignTranscriptsPerWindowNmax 300 --seedPerReadNmax 3000 -readFilesCommand -outFilterMulti- mapNmax 100 -winAnchorMultimapNmax 100 -outMultimapperOrder Random -outSAMmultNmax 1 -outSAMtype BAM -outFilter- TypeBySJou -alignSJDBoverhangMin 1 -outFilterMismatchNmax. The annotation gtf file was generated by combining the genomic annotation file for transposable elements from RepeatMasker (downloaded from http://www.repeatmasker.org) with the zebrafish genome version GRCz11 gtf file using the cat command. The red count matrix was generated by the Rsubread package feature counts. Differentially expressed TEs were identified using the edgeR package with (FDR < 0.05, FC 1.5). Sample clustering and dispersion were plotted by plotMDS and estimateDisp functions, respectively.

Table1 :Primer Sequences

pME-Cas9_pointmutation_TGA-GGA_F	TCCTAAGAAGAAGAGAAAGGTGGGACCGCGGAATCTAGAGCGGCC
pME-Cas9_pointmutation_TGA-GGA_R	GATCTCACCGGTGAGTCGCCACCCAGCTGAGACAGGTCAA
pME-Cas9_TGA-deletion_F	CCGCGGAATCTAGAGCGGCCGCCACCGCGGTGGAGCTCC
pME-Cas9_TGA-deletion_R	CACCTTTCTCTTCTTCTTAGGAGATCTCACCGGTGAGTCGCCACCCAGCT
pME-Cas9-D10A Cas9_to_dCas9_F	GTATAGCATCGGCCTGGCTATTGGAACTAACTCCG
pME-Cas9-D10A Cas9_to_dCas9_R	TTCTTATCCATGCTTCCCACCTTTCTCTT
pME-Cas9-H840A Cas9_to_dCas9_F	GATTACGACGTGGATGCTATCGTCCCCCAGAG
pME-Cas9-H840A Cas9_to_dCas9_R	AGACAGCCTGTTAATATCCAGCTCCTGGTC
pMECas9_seqprimer_1	TAAGAAGAAGAGAAAGGTGGGAAG
pMECas9_seqprimer_9	CGACACTACAATCGATAGAAAGAG
(From dCas9-KRAB) KRAB_Forward	GCAGGATCCTCGAGAACACTGGTTACG

(dCas9-KRAB) KRAB_Reverse	GCAGGATCCCTATACCAGCCAAGGTTCTTC
dCas9-VP64_GFP_F	GCAGGATCCGCTGACGCATTGGACGATTTT
dCas9-VP64_GFP_R	GCAGGATCCCTACAGCATGTCCAGGTCGAA
#48221 VP160_BamH1_Forward	TAAGGATCCGCCGGATCCGGGCGCGCC
#48221 VP160_BamH1_Reverse	GCAGGATCCATCGATATACAACATATCCAA
p5E HSP 70 Reverse_1	TGCAATTGTTTCATTATGAAAG
p5E HSP 70 Reverse_2	AAGATTACATATACAAAGTTG
p5E HSP 70 Reverse_3	CAGCGGTCAAAAATACAAAG
p5E_UAS_Forward	CGAGGTCGACGGTATCG
p5E_UAS_Reverse	AAAGGGAACAAAAGCTGG
EGFP Primers_1	GGGGAGGTGTGGGAGGTTTTTT
EGFP Primers_2	AAGTTCACCTTGATGCCGTTC
HSP70_Forward_1	CAGGGGTGTCGCTTGGTTA
HSP70_Forward_2	GACCTCTCAAGTTAGCAAGT
HSP70_Forward_3	TGCAACTGGAAAAACAACACATCA
HSP70_Forward_4	AAATTAGCGTTTTACTTGTAC
dCas9_Forward	TGTCTCAGCTGGGTGGCG
PolyA_Tai_Forwardl	TCCAGACATGATAAGATAC
attB1_fw	CAAGTTTGTACAAAAAAGCAGGCT
attB1_rv	AGCCTGCTTTTTTGTACAAACTTG
attB2_fw	ACCCAGCTTTCTTGTACAAAGTGG
attB2_rv	CCACTTTGTACAAGAAAGCTGGGT
attB3_fw	CAACTTTATTATACAAAGTTG
attB3_rv	CAACTTTGTATAATAAAGTTG
attB4_fw	CAACTTTGTATAGAAAAGTTG
attB4_rv	CAACTTTTCTATACAAAGTTG

Table 2: Plasmids used and generated in study

p5E-hsp70l	1.5 kb hsp70l promoter for heat-shock induction
p5E-UAS	10x UAS element and basal promoter for Gal4
p3E-polyA	SV40 late polyA signal
pME-Cas9	Middle Entry clone (zebrafish codon-optimized Cas9 flanked by 2 NLS)
dCas9-KRAB	Conventional dCas9 repressor-dCas9-KRAB

pAC149-pCR8-dCas9VP160	dCas9VP160 on Gateway donor vector pCR8/GW/TOPO
dCAS9-VP64_GFP	Expresses dCAS9-VP64 activator with 2A GFP
pDestTol2pACryGFP	Gateway destination vector with zebrafish aA-crystallin promoter driving GFP in lens
pME-nls-dCas9-nls	Middle entry clone (Zebrafish codon optimized dCas9 flanked by 2 NLS sequence)
pME-nls-dCas9-KRAB-nls	Middle entry clone (Zebrafish codon optimized dCas9 fused with transcriptional repressor KRAB flanked by 2 NLS sequence)
pME-nls-dCas9-VP160-nls	Middle entry clone (Zebrafish codon optimized dCas9 fused with transcriptional activator VP160 flanked by 2 NLS sequence)
pME-nls-dCas9-VP64-nls	Middle entry clone (Zebrafish codon optimized dCas9 fused with transcriptional activator VP64 flanked by 2 NLS sequence)
pDestTol2pA-UAS-dCas9-VP160-polyA-CryGFP	Gateway destination vector with reporter alpha crystalline GFP in lens (expressing dCas9-VP160 under UAS promoter)
pDestTol2pA-UAS-dCas9-VP64-polyA-CryGFP	Gateway destination vector with reporter alpha crystalline GFP in lens (expressing dCas9-VP64 under UAS promoter)
pDestTol2pA-UAS-dCas9-KRAB-polyA-CryGFP	Gateway destination vector with reporter alpha crystalline GFP in lens (expressing repressor dCas9-KRAB under UAS promoter)
pDestTol2pA-HSP70-dCas9-VP160-polyA-CryGFP	Gateway destination vector with reporter alpha crystalline GFP in lens (expressing dCas9-VP160 under HSP70 promoter)
pDestTol2pA-HSP70-dCas9-VP64-polyA-CryGFP	Gateway destination vector with reporter alpha crystalline GFP in lens (expressing dCas9-VP64 under HSP70 promoter)
pDestTol2pA-HSP70-dCas9-KRAB-polyA-CryGFP	Gateway destination vector with reporter alpha crystalline GFP in lens (expressing dCas9-KRAB under HSP70 promoter)

Chapter 3
Results and Discussion

3.1 The expression pattern of gata1 and pu1 in primitive hematopoiesis

As we want to study the expression profile of various regulators of hematopoiesis with the change in expression of the transposable elements family, we generated the RNA probes against the major regulators of hematopoiesis *gata1, pu1, mpo, fli1,* and *flk1*. We tested these probes and optimized the whole mount in-situ hybridization protocol for the best expression profile during early developmental stages, i.e., 24 hpf, 30 hpf, 36 hpf, and 72 hpf based on the previous research.

In zebrafish, the hematopoietic markers identify erythrocyte precursors in the posterior mesoderm at the 5-somite stage. By this stage, the cell fate of ICM precursors is determined as cells adapt either myeloid cell fate, characterized by *pu1* expression, erythroid (marked by gata1 expression), or vascular (angioblast) fate characterized by *flk1 (kdrl)* expression. At 24 hpf, erythrocyte (ICM) precursor cells are surrounded by an axial trunk vein from the posterior blood island (Fig 3.1). The overlapping expression of gata1 and granulocyte markers mpo (myeloperoxidase) in the posterior lateral mesoderm cells may represent a bipotent myelo-erythroid cell population. In zebrafish, circulation begins around 24 hpf with the development of primitive erythrocytes.

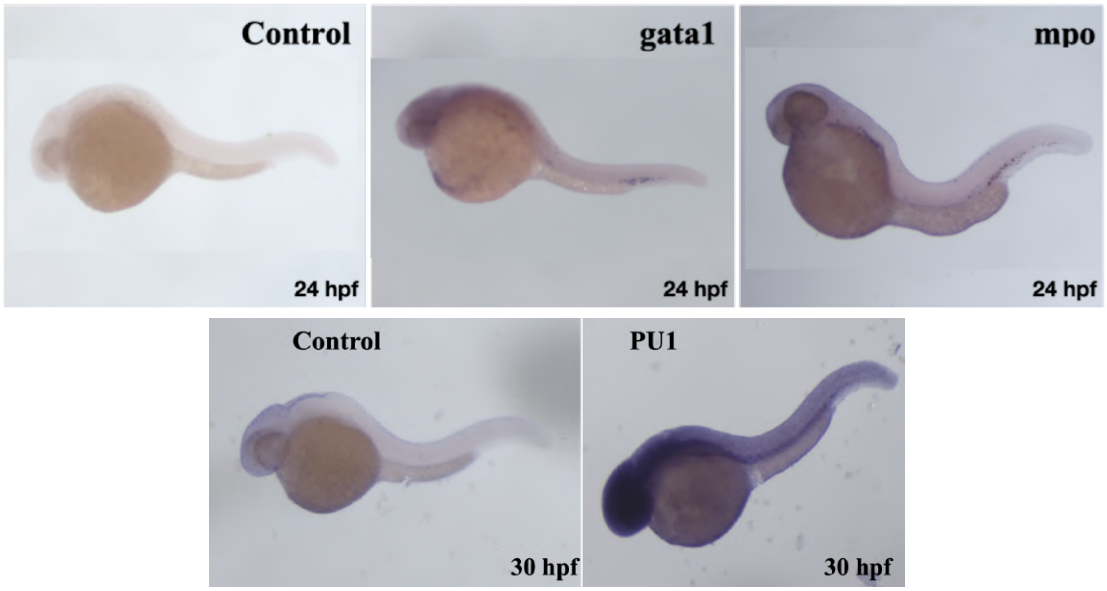


Fig 3.1 Localization of gata1 and mpo transcript at 24 hours post fertilization. Whole-mount in situ hybridization was performed at 24 hpf. Staining was done using BCIP/NBT staining solution for 6 hours.

Pu1 expression at the six somite stage in the anterior mesodermal region of the zebrafish embryo marks the first myelopoiesis (Lieschke et al., 2002). Whole-mount in situ hybridization at 30 hpf showed the pu1 transcripts in the posterior intermediate cell mass and anterior head region.

3.2 The expression pattern of *fli-1* and *flk-1* in hematopoietic differentiation

fli1 overexpression has been shown to play a role in erythroleukemia, suggesting its involvement in hematopoietic development (Ben-David & Bernstein, 1991). Whereas *flk1* (huKDR) is a VEGF receptor (vascular endothelial growth factor), and its expression is known to be tightly regulated in endothelial cells. Previous In-situ hybridization during zebrafish development identified *fli1* transcripts in mesodermal regions, especially in areas where early hematopoiesis initiatives. As it is known that expression patterns for fli1 and gata1 overlap during early stages of development, we wanted to check the expression profile of *fli1* and *flk1* during slightly later stages, i.e., 36 hours and 72 hours post fertilization. At 36 hours hpf, *fli1* expression increases significantly in the lateral mesoderm along the posterior axis. By 36 hours, segmentation development is complete, and fli-1 expression is maintained through intermediate cell mass (posterior compartment) and endothelium. After this, *fli1* and *flk1* expressions are downregulated progressively as cells differentiate (Fig 3.2).

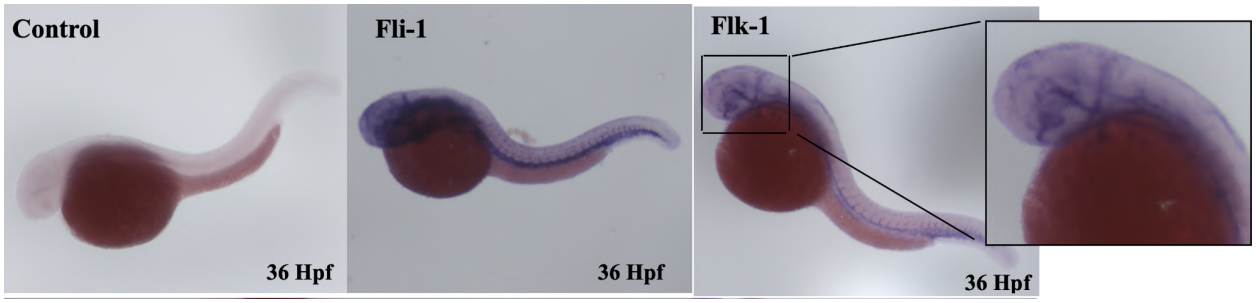


Fig 3.2 Localization of fli-1 and flk-1 transcript at 36 hours post fertilization. Whole-mount in situ hybridization was performed at 36 hpf. Staining was done using BCIP/NBT staining solution for 6 hours.

In line with the previous work, the flk-1 expression pattern overlaps with *fli1*, showing mRNA transcripts in inter somatic vessels and along the posterior axis. By 36 hours post-fertilization, the flk-1 is also expressed in the head region marking endothelial cells

in pharyngeal arches. The comparison of the expression profile of *fli1* and *flk1* at 36 hpf and 72 hpf show the downregulation of both factors, indicating their role specifically in vascular development. By 72 hours hpf, vasculature in zebrafish is established, resulting in reduced signal in WISH. The extended Pro-k treatment gave better staining at 72 hours post fertilization.

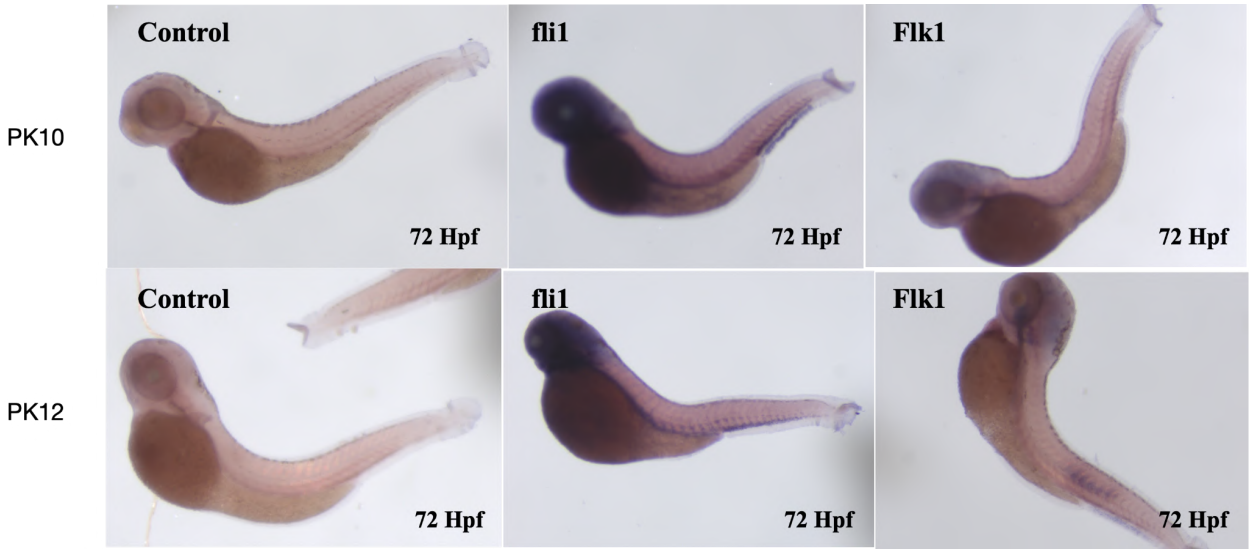


Fig 3.3 Localization of fli-1 and flk-1 transcript at 72 hours post fertilization. Whole-mount in situ hybridization was optimized for the proteinase K treatment for the best expression profile at 72 hpf. Staining was done using BCIP/NBT staining solution for 6 hours.

3.3 Site-directed mutagenesis for catalytic deactivation of Cas9:

CRISPR mutagenesis has been shown to be efficient in inducing mutations and creating transgenic zebrafish. However, in order to apply the CRISPR system for transcriptional control and epigenetic modulation, the nuclease activity of Cas9 must be inactivated. In the Zebrafish CRISPR toolbox, only a few studies have tested the application of catalytically inactive Cas9 (dCas9). Streptococcus pyogenes Cas9 contains two nuclease domains essential for inducing double-stranded breaks. Two point mutations, D10A and H840A, in these domains, have been shown to create dCas9 (Qi et al., 2013). Here, for CRISPRa/i purpose, we needed to create the codon-optimized version of dCas9 for zebrafish; we used addgene plasmid pME-Cas9 as a template and

performed site-directed mutagenesis (SDM) to induce point mutations in the nuclease domain of Cas9 as mentioned above.

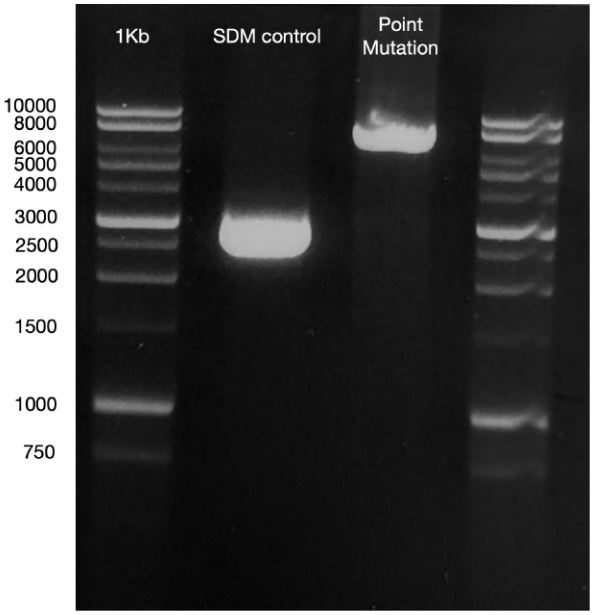


Fig 3.4 Catalytic inactivation of Cas9. Site-directed mutagenesis was performed to mutate the D10A residue in the nuclease domain of Cas9 in pME-nls-Cas9-nls. The mutation in the nuclease domain at D10A inactivates the catalytic activity of Cas9.

We performed SDM with primers specific to D10A mutation and the control plasmid provided with the kit. Expected 7500 band was observed after the SDM PCR, but the reaction did not give any colonies after ligation and transformation. We performed multiple troubleshooting experiments for this. We faced a major problem during ligation as a positive control worked during PCR and transformation. We tried ligation with T4 DNA ligase and quick ligase, with overnight ligation at 16 C and quick ligation at 24C. We also performed prior phosphorylation of vectors and primers to avoid the possibility of dephosphorylated ends essential for ligation.

3.4 Construction of integrable CRISPR vectors

The tol2 technology allows the generation of transgenic by transposase-mediated insertion of CRISPR vectors into the zebrafish genome.

5’ entry clones

The 5’ entry clones are used in the zebrafish tol2 system to supply the enhancer-promoter elements. We are using p5E-hsp70 and p5E-UAS entry vectors with promoters for conditional expression. p5E-hsp70 contains 1500 bp promoter sequences from the hsp70 gene(Halloran et al., 2000). In comparison, p5E-UAS has a multimerized Gal4 upstream activating sequence (10X UAS), which in the presence of Gal4, strongly regulates the expression of downstream elements.

Middle Entry Vectors

The tol2 technology allows the generation of transgenic by transposase-mediated insertion of CRISPR vectors into the zebrafish genome. In this project, we generated tol2 middle entry vectors by cloning codon-optimized dCas9 with the VP160 and VP64 activator domains and KRAB suppressor domains. The VP160, VP64, and KRAB inserts were amplified from pAC149-pCR8-dCas9VP160, dCAS9-VP64_GFP, and dCas9-KRAB, respectively. The inserts were amplified using the primers flanked with the BamH1 and inserted immediately downstream of dCas9 in pME-nls-dCas9-nls. The cloning of VP64 and KRAB inserts with dCas9 was less efficient than VP160 because of the smaller insert size. pME-nls-dCas9-VP160-nls, pME-nls-dCas9-VP64-nls, and pME-nls-dCas9-KRAB-nls constructs were validated by performing PCR with primers specific vector and insert. The clones were later confirmed with sequencing.

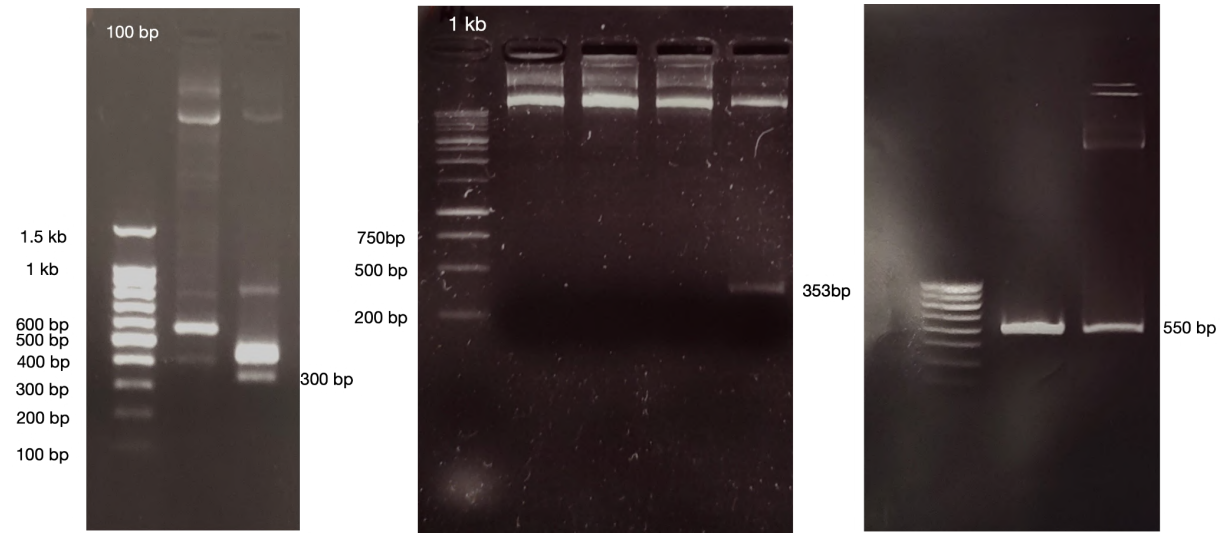


Fig 3:5 Validation of Middle entry clones. PCR validation of pME-nls-dCas9-VP64-nls, pME-nls-dCas9-KRAB-nls, and pME-nls-dCas9-VP160-nls, respectively. Respective size bands of 300bp, 353 bp, and 550 bp were observed for PCR with dCas9-specific primer and insert-specific reverse primers.

3.5 Gateway recombination reaction for expression constructs:

Gateway cloning is mostly the att site-specific recombination system in the lambda phase (Hartley et al., 2000). As shown in Fig., 1 We used three fragments multisite gateway to deliver three key features 1) *5 clones* (p5E-XX), with attL4-attR1 sites flanking the insert, hsp70 or UAS conditional promoter, and 2) *middle clones* (pME-XX), with attL1-attL2 sites flanking zebrafish codon-optimized dCas9 with three different transcriptional regulating domains. 3)*3 clone* (p3E-XX), with attR2-attL3 sites flanking the insert, polyA tail sequence. The three-fragment multisite gateway cloning combines these entry clones with the destination vectors pDestTol2pACryGFP containing to generate the expression vectors as in Fig 3.6.

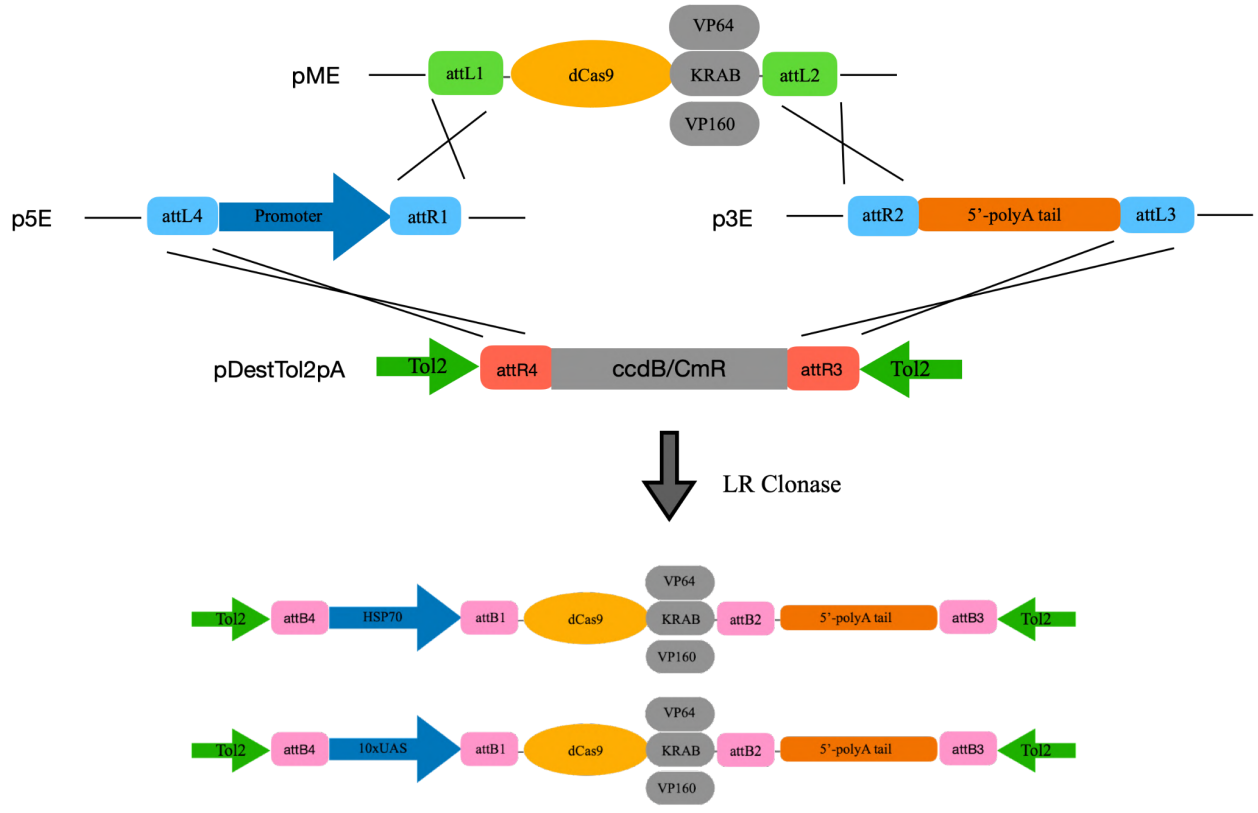


Fig 3.6 Schematic of three fragment assembly Destination vectors. Six destination vectors, three with UAS and HSP 70 promoter followed by dCas9 fused with VP64, VP160, and KRAB.

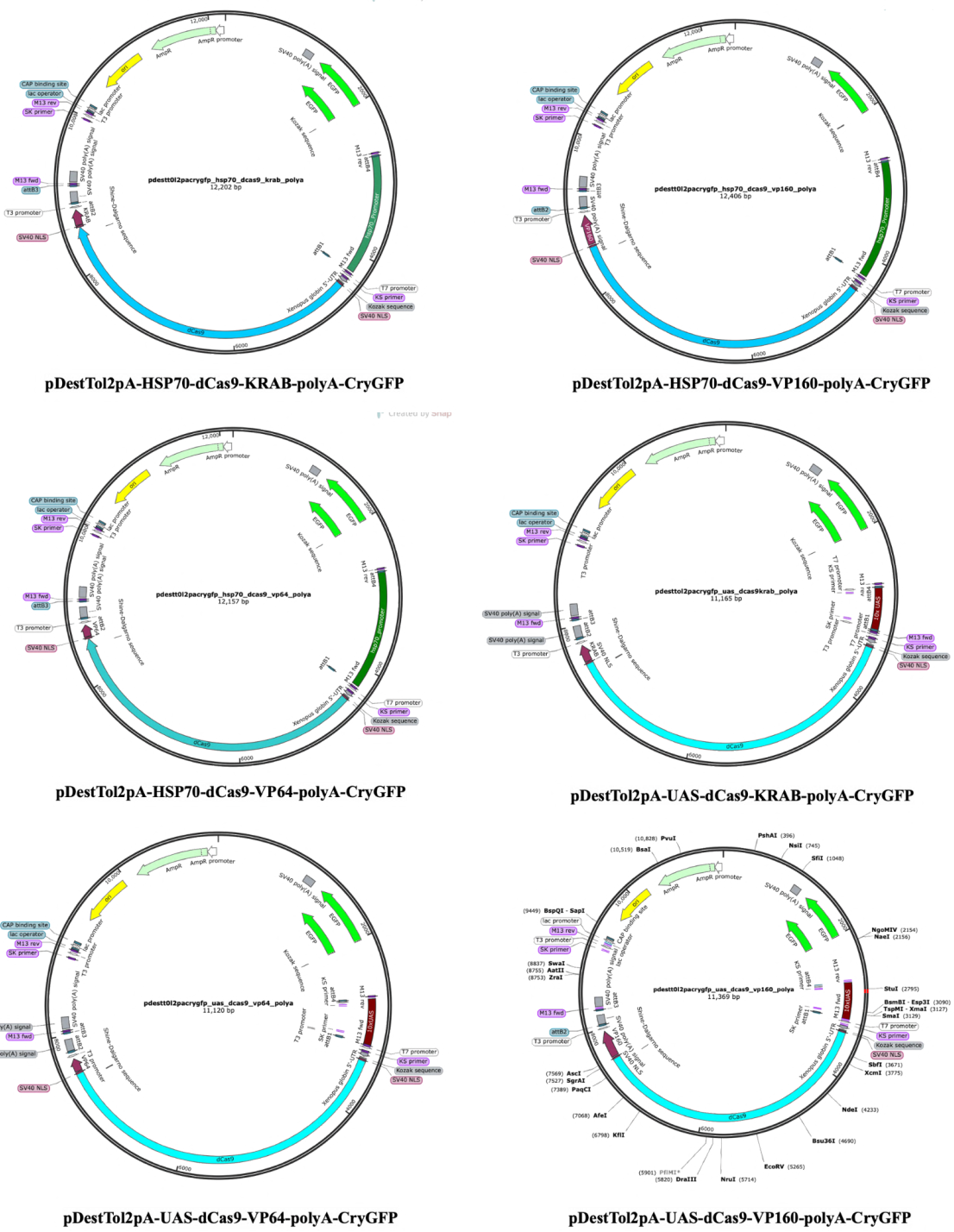
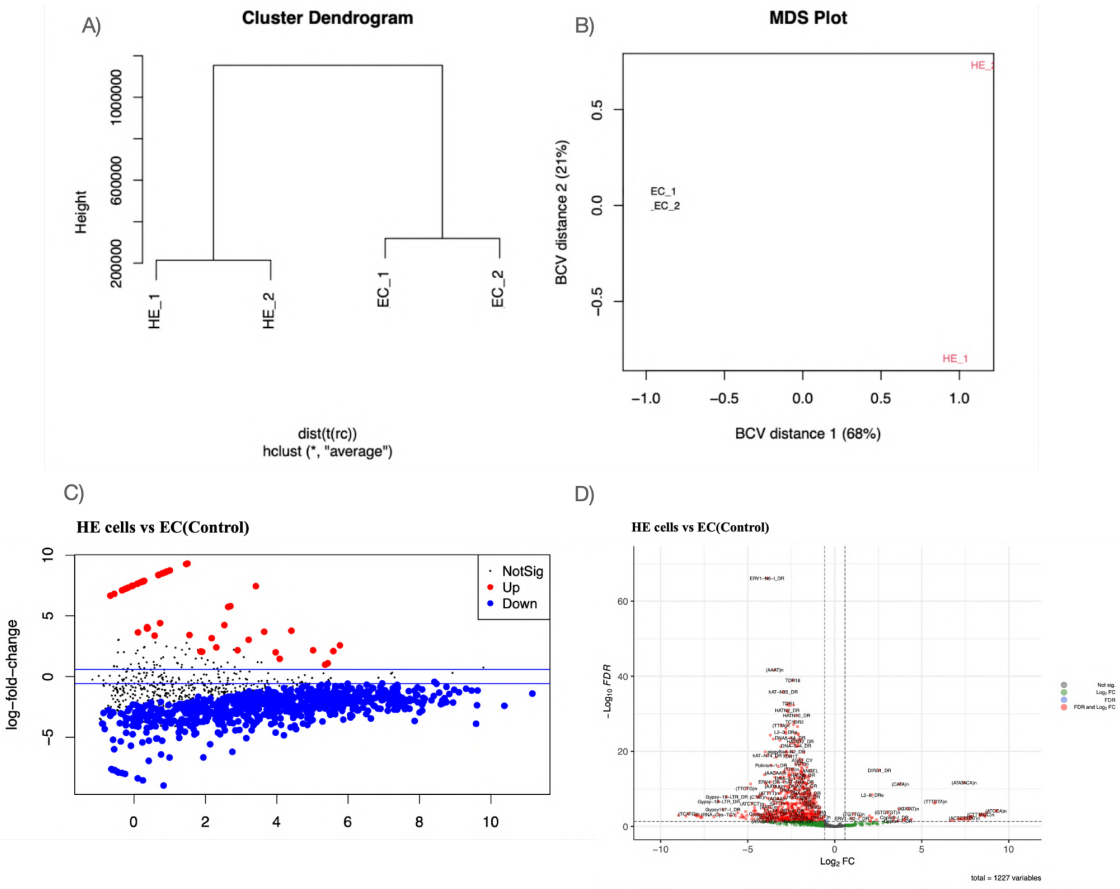


Fig 3.7 Plasmid maps of the six generated destination vectors. (Generated with Snapgene)

In the recombination reaction making entry clones was efficient and reliable even with the moderately competent bacteria and yielded many colonies. However, making an expression construct with the three-fragment LR reaction was less efficient and time-consuming as DH5 alpha-competent bacteria yielded no colonies. Three fragment LR reactions only yielded colonies when transformed with electro-competent cells. With the Gateway LR reaction, we generated six crispr expression vectors, which can be injected to generate stable lines. (Plasmid maps in Fig 3.6) We performed validation PCR with primers specific to middle elements, i.e., dCas9. While microinjecting, we injected 50 pg of plasmid at the one-cell stage. Around 90 % of the injected embryos died within 24 hours, and those that survived showed no GFP expression in the lens.

3.5 Transposable elements transcriptomic analysis:

To identify the transposable elements expressed differentially in the hemogenic endothelial cells and endothelial cells, we performed the transposable elements transcriptomic analysis on previously published data on zebrafish 26 hours post fertilization embryos (Lefkopoulos et al., 2020). The identified top differentially expressed TEs transcripts, or enriched transposable elements families, will be used as a target to design the guide RNA injected with the CRISPR expression vector. We downloaded the raw data from the SRA database from the previously published dataset (Lefkopoulos et al., 2020). The cluster dendrogram for the sample shows clustering of the respective replicates, but the MDS plot showed some variation between the replicates in hemogenic endothelial cells. The DE analysis with edgeR identified 795 differentially expressed transposable elements transcripts, with 743 being transcriptionally downregulated and 52 upregulated in hemogenic endothelial cells compared to endothelial cells.



Summary of differentially expressed TEs from exact test (FDR < 0.05, FC 1.5)

HE vs EC cells

```
## Down      743
## NotSig    432
## Up         52
```

Fig 3.8 Transcriptomic analysis of Transposable elements between indifferent cell lineages. We compared endothelial and hemogenic endothelial cells from zebrafish 26 hpf embryos to identify top differentially expressed TE transcripts. A) and B) are Cluster dendron and MDS plots showing sample clustering. C) and D) are volcano plots showing DE transcripts. Exact test, FDR < 0.05, FC 1.5).

Discussion

The primary goal of this project is to generate the CRISPR expression constructs harboring conditional promoters modulating the expression of the dCas9 activator and repressor to generate stable zebrafish transgenic lines. The gateway system using an att-based recombination system for cloning three or more DNA fragments has worked efficiently. The injection of these constructs with tol2 transposes efficiently generated transgenics. (Balciunas et al., 2006; Fisher et al., 2006; Kawakami et al., 2004). To perform gateway assembly of the conditional promoter, dCas9, with transcriptional activator and inhibitor, and polyA tail, we initially designed middle entry vectors by cloning VP64, VP160, and KRAB downstream of dCas9.

CRISPRa/in the system is limited as zebrafish codon-optimized dCas9 expression declines with the lifespan and generations. To fully exploit the potential of CRISPRa/i in zebrafish, in this project, we generated six different expression vectors which can be injected to generate zebrafish transgenesis. We used two conditional promoters, HSP70 and UAS, which will allow us to induce the dCas9 expression with heat shock or the UAS-Gal4 system. As we injected a few of the constructs at one cell stage embryo at 50 pg of DNA, most died within 24 hours. 50pg of DNA content might be toxic for the embryos. We need to optimize the injection concentrations by injecting 25 pg and 12 pg of plasmids. After optimizing the DNA concentration for injection, we can screen the embryos with the GFP reporter expressed in the eye of the zebrafish.

Our final goal is to investigate the role of transposable elements in mammalian developmental hematopoiesis. For this purpose, with the generation of stable zebrafish lines expressing conditional dCas9, we will assess the effect of TEs overexpression or inactivation in the zebrafish embryos by performing WISH, FACS, and RNA-Seq. Hemogenic endothelial cells represent a 1-3 % fraction of endothelial cells, which are the precursors for the definitive hematopoiesis; we hypothesize that differentially expressed transposable elements between these two lineages will likely be involved in HSCs differentiation. We could identify 743 downregulated and 52 upregulated transposable elements from the cell type comparison gene expression analysis between endothelial and hemogenic endothelial cells in zebrafish. However, because of variation

in the HE samples' duplicates, we will perform the RNA-Sequencing by sorting the EC, and HE cells at 36 hours and 48 hours post fertilization in fli1:GFP, Flk-1:CFP, and Gata2-dsRed zebrafish lines. We will design transcripts and families-specific multiplex gRNA against the differentially expressed transposable elements by extracting family-specific TE annotations from RepeatMasker. We will use CRISPOR and CHOP-CHOP gRNA design tools to generate 20 nucleotide-long unique guide sequences for specific loci. The U6x sgRNA multiplex cassette, cloned into pGGDestTol2LC-sgRNA vectors (up to 5), will be injected into the zebrafish transgenic. The single locus of TE transcripts will also be targeted by cloning a single gRNA into the vector or can be injected directly into the 1-cell stage zebrafish embryo.

Many studies have shown that RNA molecules of repetitive elements, mitochondrial RNA, and snRNAs can activate RLR.(Chiappinelli et al., 2015; Rajshekar et al., 2018; Ranoa et al., 2016). Lefkopoulous et al. showed that during endothelial to hematopoietic transition, while cells transition from endothelial lineage to hemogenic endothelial lineage, different TEs are expressed (Lefkopoulos et al., 2020). Also, these TEs are being recognized by RLRs receptors and actively shaping the fate of HSPCs. However, which classes of TEs were responsible for RLR activation is still unexplored. With conditional zebrafish transgenesis, we can target different families of TEs directly involved in hematopoiesis.

To investigate the changes in the expression of hematopoietic markers with enhanced or repressed expression of certain TEs, we synthesized and optimized the WISH protocol for the gata1, mpo, pu1, fli1, and flk1 at 24 hours, 30 hours, 36 hours and 72 hours post fertilization. We speculate that TEs are involved in EHT, any perturbations in the expression pattern of certain TE families will significantly affect the expression of these markers. Further, we can validate any changes in the differentiation of HSCs by performing FACS and CFU assays for different cell lineages.

Previous research provided evidence about the involvement of TEs in the hematopoietic system, exploring their role with CRISPRa/i can answer many open questions in the field. It will be interesting to examine which families of TEs are involved during EHT and explore their mechanism of action. As transposable elements are conserved across

zebrafish and humans, hence similar mechanisms are likely involved in the human hematopoietic system, and investigation in this direction can have clinical applications. Also, the robust zebrafish stable lines described above will be a great addition to the zebrafish resources.

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