GENETIC STUDY OF HEMATOPOIESIS DEVELOPMENT BY TWO ZEBRAFISH MUTANTS: UGLY DUCKLING AND TC-244

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Table of Contents

SUMMARY

Hematopoiesis is defined as a biological process that gives rise to all blood lineages in the course of an organism's lifespan. It is widely known that vertebrate hematopoiesis has two phases: a "primitive" (embryonic) phase is followed by the other "definitive" (adult) phase. The transient primitive wave of hematopoiesis initiates the circulation and mainly give rise to primitive erythrocytes and a small portion of macrophages; while the definitive wave of hematopoiesis generate all the blood lineages continuously and gives rise to the fetal and adult peripheral blood cells. However, the molecular mechanisms governing the generation and regulation of these two waves of hematopoiesis are not fully understood. Recently zebrafish (*Danio rerio*) has emerged as a pre-eminent model organism for vertebrate hematopoietic research due to its genetic and embryological advantages.

This dissertation describes the genetic study of two zebrafish hematopoietic mutants: *ugly duckling* (*udu)* and *tc-244*. Phenotypic analysis of udu^{sq1} mutant allele showed that *udu* gene was essential for primitive hematopoiesis development. Loss of *udu* gene in zebrafish led to severe hematopoietic hypoplasia phenotype. Moreover, FACS analysis revealed that this defect in primitive erythroid cells was caused by their abnormal arrest in G2-M phase during cell cycle progression. Besides, transplantation experiment showed that *udu* gene was cell autonomously required for this function in primitive erythroid cells. Of note, a group of molecules that are known to function in cell division or cell cycle regulation were fished out from yeast two-hybrid screen as candidate interaction partners of Udu protein, thus further supporting

the argument that Udu may be required for regulation of cell cycle progression in primitive hematopoietic cells.

Unlike *udu* mutant, *tc-244* mutant was normal in primitive hematopoiesis but had severe defects in generation of definitive hematopoiesis. By whole mount *in situ* hybridization analysis of hematopoietic specific markers, it was found that definitive HSCs were initially specified in *tc-244* mutants, but their further differentiation and development were impaired, leading to the absence of major lineages of definitive hematopoietic cells including erythroid, myeloid, and lymphoid cells. By positional cloning approach, the *tc-244* mutant gene was mapped to a novel zebrafish gene *zgc153228* on linkage group 7. Morpholino knockdown of *zgc153228* showed similar morphant phenotype as *tc-244* mutant, confirming that the mutant phenotype of *tc-244* was indeed caused by loss of function of *zgc153228* gene. Thus, *zgc153228* is identified as a novel factor critical for definitive hematopoiesis development.

In conclusion, by genetic analysis of zebrafish mutants *udu* and *tc-244* in this study, two novel genes (*udu* and *tc-244*) are identified as novel factors involved in the regulation of primitive and definitive hematopoiesis development.

List of Tables

List of Figures

List of Abbreviations

AGM	aorta–gonad–mesonephros
a. a	amino acid
ALM	anterior lateral mesoderm
BAC	bacterial artificial chromosome
bHLH	basic helix-loop-helix
BL-CFC	blast-colony-forming cells
BMP	bone morphogenetic protein
BSA	bulk segregation analysis
CHT	caudal hematopoietic tissue
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNS	central nervous system
ctg	contig
DA	dorsal aorta
dpf	days post fertilization
EGFP	enhanced green fluorescent protein
ENU	N-ethyl-N-nitrosourea
EPO	glycoprotein hormone erythropoietin
ES	embryonic stem
FACS	fluorescent-activated cell sorting
GMP	granulocyte/monocyte progenitor
Hh	hedgehog signaling pathway
hpf	hours post fertilization

HSC	hematopoietic stem cell
ICM	intermediate cell mass
MEP	erythrocyte/megakaryocyte progenitor
MO	morpholino phosphorodiamidate oligonucleotide
PAH	paired amphipathic helix repeat
PBI	posterior blood island
PCR	polymerase chain reaction
PLM	posterior lateral mesoderm
Pre-RC	pre-replication complex
RBC	red blood cell
RBI	rostral blood island
RT-PCR	reverse transcription polymerase chain reaction
SANT-L	SW13, ADA2, N-Cor and TFIIIB-like domain
SNP	single nucleotide polymorphism
SSLP	simple-sequence length polymorphism
SSR	simple sequence repeats
TGF-β	transforming growth factor-β
TILLING	target induced local lesions in genomes
TUNEL	terminal transferase dUTP nick end labeling
udu	ugly duckling mutant
WGS	whole genome shotgun
WISH	whole mount in situ hybridization
Y2H	yeast two hybrid screen
wt	wild type

List of Publications

Jin H, Xu J, Qian F, **Du L**, Tan CY, Lin Z, Peng J, Wen Z. 2006. The 5' zebrafish scl promoter targets transcription to the brain, spinal cord, hematopoietic and endothelial progenitors. *Dev Dyn* 235:60-7

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

Chapter I Introduction

1.1 Current understanding of hematopoietic development

1.1.1 Hematopoiesis and hematopoietic cells

Hematopoiesis is a word derived from ancient Greek (*haima* blood; *poiesis* to make) which means the formation of blood. It is a biological process that produces all types of blood cells throughout the lifetime of an animal. In general, hematopoietic cells can be classified into three types: erythrocyte, leukocyte, and platelet. Erythrocytes or red blood cells are the most abundant cell type, and they stay within the blood vessels and transport oxygen and nutrition. Leucocytes or white blood cells mainly perform the function to combat infection and sometimes phagocytose and digest debris, and there are three categories of leukocytes: granulocyte, monocyte, and lymphocyte. Leucocytes usually migrate across the walls of small blood vessels into tissues to perform their tasks. Beside these two major cell types, blood also contains large numbers of thrombocytes or platelets. They are small and detached cell fragments derived from the giant megakaryocytes and adhere specifically to the endothelial cells, and they help to repair damaged blood vessels and aid in blood clotting. Despite their functional diversity, all these different types of hematopoietic cells are generated ultimately from a common ancestor known as hematopoietic stem cells (HSCs) (Kondo et al 2003).

The hematopoietic system has been extensively studied in the past few decades due to its importance. On the one hand, understanding of hematopoiesis is critical for clinical practice. Human diseases, such as anemia, leukemia,

immunodeficiency, and lymphoma are all caused by defects in this process. Approaches to treat these disorders also require manipulation of hematopoietic cells. For example, bone marrow transplantation or hematopoietic stem cell replacement approach has been used to ameliorate the related diseases (Zon 2001). On the other hand, mechanisms involved in the genesis of hematopoietic system also intrigue scientists from a developmental viewpoint. The hematopoietic system is distinguished from other organ systems by its unique feature that all the mature blood cells are short-lived. Therefore, hematopoiesis needs to be continuous throughout life. This astonishing feature is fascinating topics of developmental biology. Moreover, hematopoiesis studies also set paradigms that are relevant to other parallel organ systems. Therefore, better understanding of hematopoiesis will benefit both clinical application and basic research.

1.1.2 Model organisms of hematopoietic research

The research of hematopoiesis began nearly a century ago and had adopted many different model organisms throughout its history.

Mouse model is the most widely used system in hematopoietic research. The most obvious advantage of the mouse model is because it is mammalian, while other strengths also include the availability of antibodies for the identification and purification of different classes of hematopoietic cells; the variety of *in vitro* and *in vivo* functional assays; and the availability of genetics and genomics tools (de Bruijn 2005). Chicken and frog are the classical non-mammalian model organisms for hematopoiesis research. The chicken embryo has a long-standing history in study of

hematopoiesis, as its accessibility and flat morphology make it well suited for the grafting experiments. By grafting blastoderm of chickens onto the yolk of quail, it was showed that the hematopoietic stem cells that last the lifetime were derived from the mesodermal area surrounding the aorta (Dieterlen-Lievre & Martin 1981). The unique feature that made frog a valuable model organism for developmental hematopoiesis is the clear spatial separation of primitive and definitive hematopoiesis, which takes place in the ventral blood islands and dorsal lateral plate mesoderm respectively (Ciau-Uitz et al 2000). This spatial separation allows for the detailed analysis of the developmental origin of these two distinct cell populations. In addition, similar to the chicken, the externally development of the embryos makes it possible to perform grafting experiments to examine the hematopoietic potential of various mesodermal tissues.

By studying all these model organisms, the major program of hematopoiesis is found to be conserved throughout vertebrate evolution. Details of this conserved process will be reviewed in the following sections to reveal our current understanding of hematopoiesis development.

1.1.3 Origin of hematopoiesis

Early events that lead to hematopoiesis were first revealed by fate mapping in *Xenopus laevis*. It has been shown that the blood fate map domain occupies the vegetal portion of the marginal zone early at 32-cell stage embryo (Lane & Smith 1999). The marginal zone is the equatorial region of the blastula embryo, and, it will

pattern to form mesoderm that later gives rise to tissues such as notochord, somites, kidney and blood during gastrulation. Transplantation experiment between cytogenetically distinct embryos further suggests only ventral marginal zone is induced to form the hematopoietic tissue (Turpen et al 1997). Members of the TGF-β-related BMP family have been shown to be critical for this induction event. Over-expression of *bmp2*, *bmp4*, *bmp7* perturbs normal mesodermal patterning by causing an expansion of ventral mesodermal fates at the expense of more dorsal derivatives (Clement et al 1995; Dale et al 1992; Fainsod et al 1994; Jones et al 1992; Wang et al 1997). Conversely, over-expression of a dominant-negative BMP receptor or inhibition of *bmp4* expression shows an opposite phenotype (Graff et al 1994; Sasai et al 1995; Smith 1995). Similar result was also obtained in *Bmp4* knockout mice (Winnier et al 1995), confirming that the vertebrate hematopoiesis arises from ventral mesoderm.

1.1.4 Hemangioblast

Following gastrulation, a population of ventral mesoderm cells will migrate to the embryonic yolk sac and form blood islands which consist of two lineages of cells, a population of erythroid cells surrounded by a layer of vascular endothelial cells. This phenomenon had lead to the speculation of a common origin for blood and vascular tissue and the existence of a bipotential precursor -- hemangioblast (Pardanaud et al 1989). *In vitro* culture studies of mouse embryonic stem (ES) cells have provided more concrete evidence in support of this hemangioblast concept. Choi and co-workers had described the isolation of blast-colony-forming cells (BL-CFC) from

embryoid bodies which arise during culture of embryonic stem cells (Kennedy et al 1997). When BL-CFCs were cultured in the presence of appropriate cytokines, they could give rise to both hematopoietic and endothelial cells (Choi et al 1998; Robb & Elefanty 1998). Furthermore, hematopoietic stem cells and endothelial cells have similar surface marker and gene expression, including CD34, transcription factors stem cell leukemia gene (*Scl)*, and vascular endothelial growth factor receptor *Flk1* (Amatruda & Zon 1999). *Scl* is known to be essential for embryonic hematopoiesis, but it was also found to play a role in angiogenesis through analysis of chimeric $Scl^{-/-}$ mice expressing a transgene targeting lacZ to vessels (Visvader et al 1998). Mice bearing null mutations of *Flk1* also have profound defects in both hematopoiesis and vasculogenesis (Shalaby et al 1997). Although all these finding support the existence of hemangioblast, the unequivocal proof for this hypothesis awaits the further evidence such as cell tracing experiment. Moreover, isolation of these cells and characterization of their potential will be necessary to formally demonstrate that they represent the hemangioblasts (Keller et al 1999).

1.1.5 Distinct waves of hematopoiesis

In vertebrate, development of hematopoietic lineage is complex, as it occurs in two waves: a primitive or embryonic wave of hematopoiesis is followed by the other definitive or adult wave of hematopoiesis.

In mammal and avian, the first blood cell population is formed in yolk sac, an extra-embryonic structure, which is the site of primitive hematopoiesis. The analogous region in amphibians is the ventral blood island. This transient wave of

hematopoiesis will initiate the circulation and mainly give rise to primitive red blood cells and a small portion of macrophages. These primitive erythrocytes differ from those found in the fetal liver and adult bone marrow in that they are large, mainly nucleated, and produce the embryonic forms of globin (Barker 1968; Brotherton et al 1979). The only other hematopoietic cells present in the early yolk sac are macrophages (Cline & Moore 1972). They mature rapidly and express lower levels of some marker genes suggesting that they could represent a unique population of macrophages (Faust et al 1997; Keller et al 1999).

During further development, the primitive wave is replaced by the definitive wave of hematopoiesis, which can generate all the blood lineages including erythroid, myeloid and lymphoid lineages and gives rise to the fetal and adult peripheral blood cells (Amatruda & Zon 1999). It is widely accepted that definitive hematopoiesis is derived from an intraembryonic location known as the aorta–gonad–mesonephros (AGM) region. Classical experiments in the chick-quail transplantation system, complemented by those that used diploid-triploid Xenopus chimeras, has traced the origins of definitive hematopoiesis to the cells of the AGM region (Chen & Turpen 1995). Transplantation of quail yolk sac to chick embryo showed that definitive hematopoiesis was exclusively of chick origin, thereby establishing the principle of intra-embryonic hematopoiesis (Lassila et al 1982). In the *Xenopus* embryo, transplantation of the dorso-lateral plate (the equivalent of the AGM) from a diploid to a reciprocal triploid embryo found diploid cells in the larval and adult blood (Chen & Turpen 1995). Although similar transplant studies are not feasible in mouse model, HSC activity is detectable after embryonic day 11 (E11) in the AGM region of mice upon transplantation of these cells to an irradiated adult recipient (Medvinsky et al

1993). Definitive hematopoietic cells do not differentiate *in situ* in AGM, but travel to a fetal site of hematopoiesis. In mammals, fetal blood formation occurs in the liver and spleen while the adult site is bone marrow.

However, the relationship between the primitive and definitive hematopoiesis remains unclear and has been the subject of intense investigation. Several groups using *in vitro* differentiation model has suggested that these two lineages share a common precursor (Keller et al 1998; Kennedy et al 1997; Turpen et al 1997). Recent finding showing that yolk sac can contribute to the definitive hematopoiesis *in vivo* also support this argument (Samokhvalov et al 2007). But the fate mapping analysis in *xenopus* clearly indicated that embryonic and adult blood was derived from distinct blastomeres in the 32-cell embryo (Ciau-Uitz et al 2000). Thus, the origin of two waves of hematopoiesis is still controversial. Besides their origination, primitive and definitive hematopoiesis development also seems to be controlled by distinct molecular programs, as gene targeting experiments of two transcription factor *c-Myb*$^{-/-}$ and *Runx1*$^{-/-}$ mice both show normal yolk sac development but severely impaired definitive hematopoiesis (Mucenski et al 1991; Okuda et al 1996). Several other genes are also found to be preferentially utilized by the definitive program as well (Kitajima et al 1999; Porter et al 1997). In contrast, our understanding of the molecular mechanism that specify the primitive hematopoiesis development is poor, as analysis of definitive hematopoiesis in mice with targeted alleles of essential primitive specific genes could be complicated by early embryonic death, due to lack of primitive hematopoiesis (Keller et al 1999).

1.1.6 Hematopoietic stem cell

The most significant feature of definitive hematopoiesis is to generate all the lineages of blood cells continuously. And this ability depends very much on the hematopoietic stem cells (HSCs), which are pluripotent cells capable of self-renewal and differentiation into all hematopoietic lineages (Weissman 2000). The original pool of HSCs is formed during embryogenesis in a complex developmental process that involves several anatomical sites, which finally colonize the bone marrow at birth. In mouse, one and a half days after blood islands appear, at about the time the yolk-sac blood vessels connect to the embryo proper, blood formation in the embryo is first observed at or near the ventral wall of dorsal aorta in the AGM region (Cumano et al 2001). Blood-forming activity attributed to haematopoietic stem cells (HSCs) is evident in this region at around E10.5 (Medvinsky & Dzierzak 1996). Although the AGM region is recognized as the site where definitive HSC activity is detected, the origin of definitive HSCs is still unclear: whether they are produced *de novo* in this intraembryonic region or they are yolk sac origin and are induced to become definitive HSCs after they enter the AGM. The later possibility is supported by the recent finding that *Runx1*-positive yolk-sac cells do form at least part of the definitive HSCs that can contribute to the adult hematopoiesis by using an elegant cell labeling approach (Samokhvalov et al 2007). More complexly, mammalian placenta has been recently recognized as a niche for definitive HSCs. A striking feature of placental HSCs is the rapid expansion of HSC pool between E11.5 and E12.5. As a result, the placenta harbors over 15-fold more HSCs than does the AGM region or the yolk sac, suggesting that the placenta may provide a unique microenvironment for HSC

development (Gekas et al 2005; Ottersbach & Dzierzak 2005). However, the origin of placental HSCs remains mystery.

HSCs first appear in the fetal liver at E11.5. Fetal liver is the primary fetal hematopoietic organ and the main site of HSC expansion and differentiation. However, fetal liver does not produce HSCs *de novo*, but it is believed to be seeded by circulating hematopoietic cells (Johnson & Moore 1975; Mikkola & Orkin 2006). During postnatal life, the definitive HSCs colonize the bone marrow, in which most adult HSCs are quiescent and divide only rarely to maintain an appropriate quantity of differentiated blood cells and to renew the HSC pool (Cheshier et al 1999). Thus, the migration path of definitive HSCs also highlights the importance of the environmental influence on maturation of HSCs.

1.1.7 Hematopoietic lineage commitment

Hematopoietic stem cells do not jump directly from a pluripotent state into a commitment to just one pathway of differentiation; instead, they go through a series of progressive restrictions. HSCs first give rise to multipotent progenitors, cells that lack the capacity for self-renewal but retain multipotent. These progenitors in turn differentiate into precursors, which are committed to a specific hematopoietic lineage. Finally, these precursors will differentiate into different types of mature blood cells. The tentative stepwise hematopoiesis commitment route is summarized in Figure 1.1. Details commitment process of major hematopoietic lineages will be discussed in the following subsections.

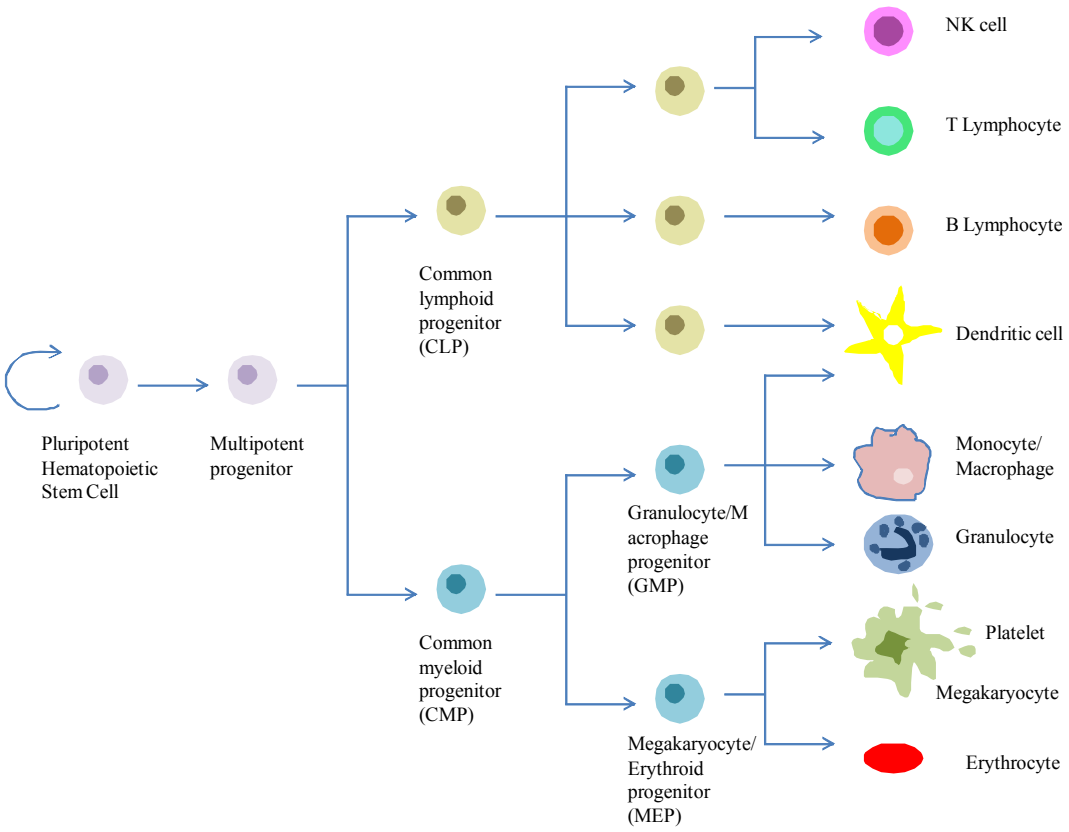


Figure 1.1 Tentative schemes of hematopoietic lineage commitment. HSCs can be subdivided into pluripotent HSCs and multipotent progenitors. The circular arrow indicates self-renewal ability. They give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), which further differentiate into different types of progenitors and mature blood cells. This figure is adapted from (Reya et al 2001).

1.1.7.1 Erythropoiesis

Production of red blood cells (erythropoiesis) is critical for the survival and development in vertebrate embryos (Orkin & Zon 1997), as red blood cells are involved in transporting oxygen and nutrition during embryogenesis. In general, the commitment from HSC to terminally differentiated erythrocyte involves a concerted progression through a common myeloid progenitor (CMP), a megakaryoctye /erythroid progenitor (MEP), an erythroid precursor, a proerythroblast, and an

erythroblast. Early erythroid progenitors are not recognizable morphologically, but can be detected by in vitro colony assays. The first morphologically identifiable precursor cell is the proerythroblast, which distinguished by its large nucleus and basophilic cytoplasm as revealed by Wright-Giemsa staining (Orkin & Zon 1997). Upon its differentiation, the proerythroblast matures progressively to the late erythroblast stage, characterized by a condensed nucleus and pink, hemoglobin-containing cytoplasm.

Despite of intensive studies on erythropoiesis, the molecular events governing this process are far from being understood. However, there is substantial evidence for involvement of some key molecules including transcription factors and cytokines in erythropoiesis (Baumann & Dragon 2005).

SCL, LMO2, GATA2, c-MYB are four broad spectrum factor critical for early hematopoiesis and erythropoiesis. The stem cell leukemia (*Scl*) gene, originally identified in a chromosomal translocation in T-cell acute lymphoblastic leukemia (ALL), encodes a basic helix-loop-helix (bHLH) transcription factor (Begley et al 1991). Gene targeting in mice has demonstrated that SCL is required for early hematopoiesis. $Scl^{-/-}$ embryos are entirely bloodless (Shivdasani et al 1995), and $Scl^{-/-}$ ES cells are unable to generate all the lineages of hematopoietic cells (Porcher et al 1996). Moreover, SCL can build multimeric DNA-associated complex with many other factors to either block or enhance erythroid differentiation (Visvader et al 1997; Vitelli et al 2000; Wadman et al 1997). Thus, *Scl* is critical for both early hematopoiesis commitment and erythroid development. The LIM-domain-only protein 2 (LMO2) has similar functions as SCL in early hematopoiesis and erythroid

lineage specification. But it does not bind DNA by itself, rather, it acts as a bridge between DNA-binding transcription factors such as SCL and GATA1. GATA2 is a transcription factor belongs to the GATA-family. Over-expression of *Gata2* in erythroid precursors block their differentiation (Shivdasani & Orkin 1996). When *Gata2* expression decreases, *Gata1* expression increases to enable erythroid differentiation. *Gata2* null mice are embryonic lethal due to severe anemia of early yolk sac hematopoiesis (Tsai et al 1994), and multipotent progenitors arising from *Gata2* null ES cells proliferate poorly and undergo excessive apoptosis (Tsai & Orkin 1997). Therefore, GATA2 is essential for expansion and survival of HSCs or early hematopoiesis progenitors. The proto-oncogene *c-Myb* is abundantly expressed in immature hematopoietic cells of erythroid, myeloid and lymphoid lineages but decreases as they differentiate (Perry & Soreq 2002). Forced expression of *c-Myb* inhibits erythroid differentiation, thus like *Gata2* it needs to be down-regulated to allow terminal differentiation (Clarke et al 1988). *c-Myb* null mice exhibit normal primitive but impaired definitive hematopoiesis in the fetal liver, result in death at E15 (Mucenski et al 1991), suggesting that c-MYB is required for the expansion of definitive progenitors.

GATA1, FOG1 and EKLF are three important erythroid specific transcription factors. As an abundant hematopoietic-restricted factor, GATA1 serves as a central regulator for erythroid gene transcription and development (Orkin & Zon 1997). *Gata1* null mice show complete ablation of embryonic erythropoiesis due to arrested maturation and apoptosis of erythroid precursors at the proerythroblast stage (Fujiwara et al 1996). However, *Gata1* negative ES cells are capable of giving rise to other hematopoietic lineages, supporting its key role in erythroid commitment. Friend

of GATA (*Fog*) gene, encoding a zinc-finger protein, is isolated as a cofactor for GATA1. It co-expresses and cooperates with *Gata1* gene to promote erythroid and megakaryocytic differentiation (Crispino et al 1999). Mice lacking FOG exhibit blocked erythropoiesis, similar to GATA1-deficient mice (Perry & Soreq 2002). The erythroid Kruppel-related factor (EKLF) is a zinc finger protein. EKLF recognizes a CACC-motif that is found in the regulatory elements of many erythroid-specific genes, including adult *β-globin* gene. *Eklf* null mice die before E16 of severe anemia and β-globin deficiency (Nuez et al 1995; Perkins et al 1995). *Eklf*$^{-/-}$ erythroid cell are exceedingly pale with markedly reduced hemoglobin, and are extremely fragile (Nuez et al 1995; Perkins et al 1995). These features are very similar to those patients with β-thalassemia. But embryonic erythropoiesis and embryonic ε and ζ globin expression in *Eklf* null mice is normal (Perkins et al 1995), showing the critical role of EKLF in the activator of the adult *β-globin* gene in the late stages of erythropoiesis (Perry & Soreq 2002).

The most important growth factor of mammalian erythropoiesis is glycoprotein hormone erythropoietin (EPO), which binds a distinct transmembrane receptor (EPOR) expressed by erythroid precursors. This intracellular signaling up-regulates the expression of globins, transferring receptor and some membrane proteins that are characteristic of erythrocytes (Kendall 2001). In *Epo* and *EpoR* knockout mice, death occurs on embryonic days 11–13.5, owing to failure of definitive erythropoiesis in the liver (Rich 1994; Suzuki et al 2002; Wu et al 1995). The existence of erythroid progenitors in livers of these mutants indicated that EPO-EPOR is not required for the commitment of hematopoietic progenitors to the erythroid

lineage. Rather, they are crucial for proliferation, survival, and differentiation of erythroid precursors (Baumann & Dragon 2005).

1.1.7.2 Myelopoiesis

Granulocytes and monocytes are key mediators of innate immunity and the inflammatory response. Collectively, these cells and their committed progenitors are referred to as myeloid cells. The development path from HSCs to differentiated myeloid cells is orchestrated by several steps: common myeloid progenitors (CMPs) derived from HSCs first give rise to separate progenitors for cells of the granulocyte/monocyte (GMPs) and erythrocyte/megakaryocyte lineages (MEPs). Then GMPs further differentiate into either granulocytes or monocytes. There is accumulating data showing that transcription factors are the key determinants in the orchestration of myeloid identity and fate during this process. Consistently, the defects seen in myeloid development of mice with targeted deletions in genes encoding such key transcription factors are for the most part much more severe than the phenotypes seen in mice deficient in most other myeloid genes, such as growth factors and their receptors (Rosenbauer & Tenen 2007). Compared with erythropoiesis, there is a relatively small number of transcription factors involved in regulating myelopoiesis. Among them, PU.1, CCAAT/enhancer binding proteins (in particular C/EBPα) are two critical ones.

PU.1 (or SPI1) is a member of ETS transcription factors family, and its expression is restricted to blood cells (Klemsz et al 1990). PU.1 is detectable in HSCs, CMPs, CLPs and B cells, but is present at high levels in mature myeloid cells (Back

et al 2005; Nutt et al 2005; Rosenbauer et al 2006). Binding sites for PU.1 are found on almost all myeloid specific promoters (Tenen et al 1997). Thus, it is believed that PU.1 plays an important role in myeloid development by regulating various downstream targets. Loss of *Pu.1* in mice leads to absence of mature granulocytes and macrophages. These mice also exhibited a complete block in B cell differentiation (McKercher et al 1996; Scott et al 1994). More recent analysis has shown that the absence of PU.1 impairs HSC repopulation capacity and precludes differentiation into CMPs and CLPs when PU.1-deficient fetal liver cells were used to generate chimeric animals (Dakic et al 2005; Iwasaki et al 2005; Scott et al 1997). Beside its role in specification of early myeloid progenitor, in vitro culture assay suggests that PU.1 also functions in cell fate decision between granulocytes and macrophages: high levels PU.1 favor the formation of macrophages, whereas low levels PU.1 seem to support granulocyte production (Dahl et al 2003).

C/EBPα is a critical transcription factor involved in the granulopoiesis. It belongs to the leucine-zipper family, and its expression is found in HSCs, myeloid progenitors and granulocytes, but not macrophages (Akashi et al 2000; Radomska et al 1998). C/EBPα knockout mice have normal numbers of CMPs but lack GMPs and all subsequent granulocytic stages (Zhang et al 1997; Zhang et al 2004). But conditional deletion of C/EBPα in GMPs allows normal granulopoiesis *in vitro*, which suggest it is only required before GMP stage (Zhang et al 2004). Therefore, C/EBPα is indispensable for the early granulocytic commitment.

1.1.7.3 Lymphopoiesis

In mammals, the common lymphoid progenitors (CLP) derived from HSCs in the bone marrow are thought to first differentiate into a bipotent T/NK cell progenitor and another B cell progenitor (Rodewald et al 1992; Sanchez et al 1994). The bipotent T/NK cell progenitor will then migrates to the thymus, and give rise to another two subsequent lineages – NK cell lineage and T cell lineage. In thymus, T cell progenitors undergo a precisely orchestrated series of developmental steps to become mature T lymphocytes including the sequential rearrangement of antigen receptor gene segments, as well as change in the expression of cell-surface and intracellular proteins (Charles A et al 2001). B cell lineage is generated and developed in the specialized microenvironment of bone marrow in a similar process as T cells. B cell progenitors proceed through stages that are marked by the sequential rearrangement of immunoglobulin gene segments to generate a diverse repertoire of antigen receptors. This developmental program also involves changes in the expression of other cellular proteins such as CD45R (B220 in mice), CD19, CD43 (Charles A et al 2001). Therefore, the whole process of lymphocytes differentiation is marked by successive steps in the rearrangement of antigen-receptor genes and expression of their protein product, as well as by change in the expression of other cell-surface and intracellular proteins. Moreover, these events are tightly regulated by a series of transcription factors to ensure the normal specification process. One of the early genes known to be critical for initiation of lymphoid program is *Ikaros*, which encodes a family of lymphoid-restricted zinc-finger transcription factors. *Ikaros*$^{-/-}$ mice lacked all T, B, and NK cells and their earliest defined progenitors (Georgopoulos et al 1994),

which strongly argues its important function in early lymphoid progenitor specification.

Transcription factors including PU.1, E2A, EBF, and PAX5 all play distinct but essential role in the B cell commitment. Loss of PU.1 results in complete absence of B cells. And the mechanism by which PU.1 regulate early B-cell development has now been show to be at least partially owing to its ability to transcriptionally activate the gene encoding the α chain of the receptor for cytokine interleukin-7 (IL-7α) in lymphoid progenitors, and IL-7 signaling is essential for the survival, proliferation and differentiation of B cells (DeKoter et al 2002). E2A and EBF belong to the basic helix-loop-helix family transcription factors. They act coordinately to activate B cell specific genes and are necessary but not sufficient to generate pro-B cells (Sigvardsson et al 1997). Pax5 functions downstream of E2A and EBF as both a transcriptional activator of B-cell lineage specific genes and a repressor of genes associated with myeloid and other hematopoietic lineages (Wang & Spangrude 2003). GATA3, a zinc-finger family transcription factor, has been defined as the earliest transcription factor required for T cell lineage commitment. Genetic studies in mouse have shown that GATA3 is required for the development and survival of the earliest committed double negative thymocytes or their precursors (Kuo & Leiden 1999; Ting et al 1996). Notch signaling has also been demonstrated to be important for lymphoid development (Anderson et al 2001; Hoyne 2003; Radtke et al 2004). NOTCH1 is implicated in the determination of T versus B cell lineages. Targeted deletion of *Notch1* resulted in a block of T-cell development, accompanied by the presence of B lymphocytes in the thymus (Pui et al 1999; Radtke et al 1999). And Notch ligands are

shown to provide key signals for T cell differentiation in the absence of an intact thymus (Jaleco et al 2001).

1.2 Zebrafish as a model organism for hematopoietic research

1.2.1 Zebrafish as a new vertebrate model organism

Developmental biologists often rely on the use of animal models to understand the complex mechanism of embryology and pathogenesis of human diseases. There is a greater than 100-year history in utilizing mice as a model organism, primarily because of the striking homology between mice and human from anatomy to cell biology and physiology. However, early developmental events are hard to explore in mice model because of their intrauterine growth and small litter size. In this regard, mice model is only ideal for study of late-acting genes (Driever & Fishman 1996). Although forward mutagenesis screen is feasible in the mouse, it is prohibitively expensive in the way of saturation scale due to long generation times, large space requirements and laborious handling required in maintenance and breeding of animals. In contrast, saturation mutagenesis screens were successfully applied in invertebrate models such as the fruit fly (*Drosophila melanogaster*), and had led to the discovery of many developmentally important genes with broad-ranging vertebrate counterparts (Nusslein-Volhard & Wieschaus 1980). However, invertebrates lack many structures and organ systems that are unique in vertebrates, such as notochord, neural crest, multi-chambered heart, endocrine and exocrine pancreas, and multi-lineage hematopoiesis system (Haffter et

al 1996). In this context, zebrafish (*Danio rerio*), a fresh water tropical cyprinid fish, has come to attention recently as a pre-eminent vertebrate model organism.

In early 1980s, George Streisinger, the founding father of zebrafish research, first introduced zebrafish as a genetic system to the research community (Streisinger et al 1981). The zebrafish embryos are transparent, and their fertilization is external, so that all stages of development are accessible. The zebrafish development is rapid, with a heart beating by the end of the first day and most organs or at least their primordial are in place by five days after fertilization (Kimmel et al 1995). Moreover, these small fish is only 3cm long and can be raised in large numbers and lay hundreds of eggs at weekly intervals. Genetically, zebrafish also maintains the diploid state, an important difference from other fish that can be triploid or tetraploid making genetic analysis difficult (Bahary & Zon 1998). More importantly, many genetic techniques have been developed for zebrafish manipulation (Lieschke & Currie 2007), such as 'cloning' (Streisinger et al 1981), mutagenesis (Chakrabarti et al 1983; Grunwald & Streisinger 1992; Solnica-Krezel et al 1994; Walker & Streisinger 1983), transgenesis (Stuart et al 1988) and genetic mapping (Streisinger et al 1986). All these advantages support and promote the use of zebrafish to question of vertebrate development in the research community.

1.2.2 Hematopoiesis from zebrafish point of view

Given the intense biomedical interest in human organ function and disease, a model system is often judged by how well it predicts human biology (Thisse & Zon 2002). Zebrafish is a vertebrate, so that the genetic program governing organogenesis is more

similar to that of mammals than invertebrate models. The level of evolutionary divergence of fish from the mammalian lineage indicates most organs in the zebrafish appear generally similar as that of mammals, and thus genes involved in zebrafish organogenesis are very likely to have their mammalian counterparts. Yet, they may have difference in their anatomical sites, gene expression and function which can modify the organ in a significant way.

In the following section, recent findings of hematopoiesis development using zebrafish as a model organism will be reviewed. And this can be used as an example to elucidate how the combination of genetic and embryological approaches has proven zebrafish as a powerful model system for developmental studies, and yield insights into the formation and function of vertebrate tissues, organ systems and even into some human disease mechanisms.

1.2.2.1 Primitive hematopoiesis

Successive waves of primitive and definitive hematopoiesis occurring in anatomically different sites are the characteristics of vertebrate embryos (Galloway & Zon 2003). Unlike mammals, primitive hematopoiesis in zebrafish occurs in the intraembryonic region known as intermediate cell mass (ICM) (Willett et al 1999) (Figure 1.2). Fate mapping analyses have demonstrated that ICM origins from the ventral mesoderm of the early gastrulation embryos as other vertebrates (Kimmel et al 1990; Warga & Nusslein-Volhard 1999). Following the extensive morphological movements of gastrulation, the ventral mesoderm occupies the posterior and lateral regions of the

embryos, and this posterior lateral mesoderm (PLM) appears as bilateral stripes, which later migrate to the trunk midline and converge to form the ICM. Similar to other vertebrates, BMP signaling is also primarily responsible for the early patterning event, and this is well demonstrated by the studies of several zebrafish BMP mutant *swirl*(BMP2b) (Kishimoto et al 1997), *snailhouse* (BMP7) (Schmid et al 2000). Recently, a second site of primitive hematopoiesis initiating in the anterior mesoderm has been characterized in zebrafish, and is named as rostral blood island (RBI) (Figure 1.2) (Davidson & Zon 2004; Herbomel et al 1999). However, this region is only responsible for generating primitive myeloid cells.

Many factors that play critical roles in mammalian hematopoiesis initiation and HSC specification have been identified in zebrafish and have spatiotemporal expression pattern comparable to their mammalian counterparts. The stem cell leukemia (*scl*) gene is extensively characterized in zebrafish largely due to its important role found in mammals. The expression of zebrafish *scl* gene is first evident at around 2-somite stage as short bilateral stripes in the PLM (Davidson et al 2003). This expression pattern also overlaps with other transcription factors including *fli1*, *gata2*, *hhex*, *lmo2*. Zebrafish *scl* has similar function as their mammalian counter-parts in HSC specification and erythroid differentiation (Dooley et al 2005; Juarez et al 2005; Liao et al 1998; Qian et al 2007). But whether these scl^+ ICM precursors at this stage represent real hemangioblasts or merely independent populations derived from HSCs and angioblasts that simultaneously develop adjacent to each other is currently unsolved (Davidson & Zon 2004). But one of zebrafish mutant *cloche* may hold answers to this puzzle. *cloche* (*clo*) mutation in zebrafish shows defects in both hematopoietic and endothelial lineages during embryogenesis (Stainier et al 1995).

Expression of multiple hematopoietic stem cell markers and lineage-specific markers is severely reduced or absent in *cloche* embryos; and expression of vascular markers is also significantly decreased (Liao et al 1998; Liao et al 1997; Thompson et al 1998). Therefore, *cloche* gene is considered to be essential for either formation or maintenance of the putative hemangioblasts, and it is also believed that the identification of *cloche* gene should greatly enhance our understanding regarding the signaling events upstream of hemangioblast.

1.2.2.1.1 Primitive erythropoiesis

The initiation of erythropoiesis is unambiguously indicated by the expression of the erythroid-specific transcription factor *gata1* at 4-somite stage (~11 hpf) in a subset of *scl* positive cells in PLM (Davidson et al 2003). These *gata1* positive erythroid progenitor cells undergo proliferation as they migrate towards the midline and "zip-up" along the trunk (Davidson & Zon 2004). Fate mapping analysis using transgenic zebrafish in which the EGFP expression is under the control of *gata1* promoter confirms that those *gata1* positive cells ultimately give rise to the first circulating blood cells (Long et al 1997). The zebrafish *gata1* deficient mutant vlt^{m651} allele also shows a specific defect in erythroid pathway with intact hematopoiesis stem cell, myeloid, and lymphoid development, demonstrating the essential and conserved role of *gata1* gene in erythropoiesis (Lyons et al 2002). Another factor that has been shown to be functionally required for early erythropoiesis is transcriptional intermediary factor 1γ (*tif1γ*) through the study of the zebrafish mutant *moonshine*. The *moonshine (mon)* mutation affects formation of embryonic erythrocytes.

Erythroid progenitor cells in *mon* mutants are initially present but fail to differentiate further and undergo apoptosis (Ransom et al 2004; Ransom et al 1996). Positional Cloning identifies *mon* as the zebrafish ortholog of mammalian transcriptional intermediary factor 1γ *(TIF1γ*) (Ransom et al 2004). Based on the zebrafish data, TIF1γ was later demonstrated to function as an important effecter of the TGFβ/Smad pathway in the control of mammalian erythroid differentiation (He et al 2006).

In zebrafish, blood circulation begins at 25-26hpf, and there are approximately 300 erythroid cells with morphology resembling proerythroblasts in the ICM at that time (Long et al 1997). As these primitive erythroid precursors enter the circulation, they mature into flattened elliptical erythrocytes. Meanwhile, these primitive erythrocytes express various erythroid specific genes necessary for hemoglobin synthesis (α- and β-embryonic globin chains, *alas*, *fch*, and *urod*), iron utilization (*dmt1*), and membrane stability (*band3*, *protein 4.1r*, *β-spectrin*) (Davidson & Zon 2004). Although the exact lifespan of primitive erythrocytes is unknown, transfusion experiment suggested they can persist in circulation for at least 4 days (Weinstein et al 1996).

Besides the essential intrinsic factors, primitive erythropoiesis is also believed to be regulated by extrinsic cues. The zebrafish *bloodless (bls)* mutation has a dominant defect restricted entirely to primitive hematopoiesis (Liao et al 2002). The mutant fish has severe anemia within the first 4 days but completely recovers in the juvenile and adult stages. Erythroid progenitors are specified in *bls* mutant but the number is decreased compared to wild type, and these progenitors also fail to differentiate further and undergo apoptosis, indicating a defect in maintenance or

expansion of the primitive progenitor cells (Liao et al 2002). Transplantation experiment further shows that *bls* gene acts in a non-cell autonomous manner (Liao et al 2002), suggesting that *bls* gene encodes an extrinsic factor specifically required for primitive hematopoiesis development.

1.2.2.1.2 Primitive myelopoiesis

The primitive myelopoiesis takes place in the rostral blood island (RBI) which arises from anterior lateral mesoderm (ALM) (Figure 1.2). Like the primitive progenitors in the ICM, those precursors found in the RBI express similar transcription factors including *fli1, gata2, lmo2, scl* between 3- to 5-somite stage (de Jong & Zon 2005). Soon after that, expression of the myeloid-specific transcription factor *pu.1* in a subset of these precursors marks the initiation of myeloid commitment in this region around 6-somite stage. Using Nomarski microscope, the cells seen in the RBI are identified as macrophages based on their morphology and phagocytosis behavior (Herbomel et al 1999). But unlike monocyte-derived macrophages in mammals, these cells are still able to divide. Although these macrophages are considered as the primitive myeloid cells, it is unknown whether these early macrophages persist in adult zebrafish or whether they are replaced by a second wave of monocyte-derived macrophages (de Jong & Zon 2005). Between 11- to 15-somite stage, the *pu.1* positive cells migrate toward the head midline and form a partially converged mass of cells between the eye and the heart primodium (Herbomel et al 1999; Lieschke et al 2002). These cells then change the direction and migrate laterally, scattering into single cells across the yolk sac. Some of them enter the circulation via the duct of Cuvier, while others disperse

on various tissue surface of the yolk sac outside the blood flow (Davidson & Zon 2004). Although *pu.1* expression levels in these young macrophages decline after 18-somite stage, additional molecular markers for macrophages such as *l-plastin* (an actin-binding protein) and lysozyme C (*lyc*) become expressed at this time (Bennett et al 2001; Liu & Wen 2002). Interestingly, prior to blood circulation, *l-plastin* positive cells are found exclusively in the yolk sac and in the mesenchyme of the head. From 28hpf onwards, *l-plastin* positive cells begin to appear in the ventral region of the tail vein and the surrounding mesenchyme (Davidson & Zon 2004; de Jong & Zon 2005). It will be interesting to find whether these cells represent RBI macrophages brought by the blood circulation, or a second wave of hematopoietic activity initiated from the caudal region.

1.2.2.2 Definitive hematopoiesis

Unlike primitive hematopoiesis, the most significant feature of definitive hematopoiesis is its ability to generate all types of blood cells continuously throughout the lifetime of an animal. And this ability relies on the definitive hematopoietic stem cells (definitive HSCs). In mammals, these definitive HSCs are found in close association with the ventral wall of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region, and then enter the circulation seeding the fetal liver and bone marrow. Gene targeting studies in mice have shown that the *runx1* transcription factor is essential for AGM derived HSC formation (Okuda et al 1996; Wang et al 1996). Similarly, transcripts for the zebrafish *runx1* ortholog are also detected in the ventral wall of the dorsal aorta in the AGM region between 24–48 hpf (Burns et al

2002; Kalev-Zylinska et al 2002) (Figure 1.2), suggesting that this site is analogous to the mammalian AGM region. Moreover, other hematopoietic transcription factor genes including *c-myb* (Thompson et al 1998), *ikaros* (Willett et al 2001), *fli1* (Lawson & Weinstein 2002), *scl* (Davidson & Zon 2004; Zhang & Rodaway 2007) and *lmo2* (Zhu et al 2005) are also found to express in this region during the same time window. Recently, more concrete evidence come from the lineage tracing experiments performed by Jin et al. They showed that the *fli1* positive cells in ventral wall of dorsal aorta were generated *de novo* and can contribute to the T cells found in the thymus, confirming that these cells represent the zebrafish counterparts of definitive HSCs (Jin et al 2007). The molecular mechanisms governing the definitive HSCs formation is also under intensive investigation. By genetic analysis, Notch and Hedgehog (Hh) signaling have been suggested to be critical for the specification of definitive HSCs. Zebrafish mutants in the Hh pathway or wild type embryos treated with the Hh signaling inhibitor display defects in definitive HSCs formation (Gering & Patient 2005). Similar, the Notch signaling mutant *mind bomb* also displays normal embryonic hematopoiesis but fails to specify adult HSCs (Burns et al 2005; Gering & Patient 2005). More strikingly, transient Notch activation during embryogenesis led to a *runx1*-dependent expansion of HSCs in the AGM region. In irradiated adult fish, Notch activity induced *runx1* gene expression and increased multi-lineage hematopoietic precursor cells followed by the accelerated recovery of all the mature blood cell lineages (Burns et al 2005).

In mammals, the definitive HSCs arising from AGM region will first migrate to a fetal site (fetal liver) before colonizing the adult hematopoiesis organ (bone marrow). However, such an equivalent site has not been identified in zebrafish so far.

Recently, the zebrafish ventral tail region has been suggested as a possible candidate. Using the in vivo lineage tracing approach, definitive HSCs generated in the ventral wall of dorsal aorta region are traced to first migrate to this previously unappreciated ventral tail region (named as posterior blood island PBI or caudal hematopoietic tissue CHT, Figure 1.2) before they reach thymus and kidney (Jin et al 2007; Murayama et al 2006). Consistent with this observation, many transcription factors such as *runx1, c-myb*, *scl*, *ikaros* all express in this ventral tail region (Murayama et al 2006). But the exact role that PBI or CHT plays during definitive hematopoiesis is still not clear. Further investigation will be necessary to reveal whether it functions similarly as the mammalian fetal live to promote proliferation and differentiation of zebrafish definitive HSCs.

1.2.2.2.1 Definitive erythropoiesis

In zebrafish, the second wave of erythropoiesis is postulated to begin around 5dpf. This postulation is based on the phenomena that zebrafish *bloodless* mutant with a defect specific in primitive hematopoiesis starts to recovery with circulating red blood cells around 5dpf (Liao et al 2002). The zebrafish definitive erythrocytes are morphologically distinct from primitive erythrocytes; they have less cytoplasm and a large elongated nucleus (de Jong & Zon 2005). Like other higher vertebrates, zebrafish express hemoglobin with a quaternary structure (*α2β2*), and they undergo globin switching from primitive embryonic globin chains to the adult globin chains (Brownlie et al 2003; Chan et al 1997). The molecular program regulating the definitive erythropoiesis is believed to be similar as primitive erythropoiesis.

However, there is a paucity of mutants that are specifically defective in the definitive erythropoiesis. Most blood mutants identified so far have shown defects in embryonic hematopoiesis and are early lethal, thus rare mutant can survive to allow the examination their defects in the late stage.

1.2.2.2.2 Definitive myelopoiesis

The second wave of myelopoiesis in zebrafish generates different myeloid cell types including macrophages and several types of granulocytes (Lieschke et al 2001). However, the origin of definitive myelopoiesis remains unclear.

As mentioned in the previous part, the primitive wave of myelopoiesis gives rise to a special group of macrophages in the RBI region, and it is believed there is a second wave of such activity to generate monocyte-derived macrophages as mammals. Upon initiation of embryonic circulation, a subset of macrophages enter the circulation and are distributed throughout the embryonic tissues. But whether they represent the definitive macrophages awaits further evidence.

Granulopoiesis in zebrafish can give rise to two types of granulocytes: neutrophil and basophils/eosinophil, the later one exhibits characteristics of both basophils and eosinophils (Hsu et al 2001). The cytoplasm of both types of cells is highly granular, but only zebrafish neutrophils have segmented nuclei with typically 2-3 lobes (Lieschke et al 2001). Expression of granulocyte-specific marker *myeloperoxidas*e (*mpo*) is first detected at 18hpf in the presumptive neutrophilic precursors within the caudal ICM. Soon after that, *mpo*-expressing cells also appear as

scattered cell found on the anterior yolk sac (Bennett et al 2001; Lieschke et al 2001). Some of these *mpo* positive cells co-express *pu.1*, while few *mpo* positive cells coexpress *l-plastin*, consistent with these two molecular markers representing two distinct lineages. At 3dpf, *mpo*-expressing cells are scattered throughout the embryos with a prominent aggregation in the ventral tail vein region, but by 4dpf they are distributed throughout the embryos (Bennett et al 2001). It is unknown how long do these granulocytes persist in the embryo and whether they contribute to the granulocyte population that is found in the kidney (Davidson & Zon 2004; Willett et al 1999).

1.2.2.2.3 Lymphopoiesis

Lymphopoiesis is essential for the functional adaptive immune systems in vertebrates. Zebrafish also possesses a similar adaptive immune system as mammals, including having T cells, B cells, antigen-presenting cells, and natural killer-like cells (Davidson & Zon 2004) and similar lymphoid organs, such as thymus.

In zebrafish, the bilateral thymus forms as outgrowths between the third and fourth pharyngeal arches (Davidson & Zon 2004; de Jong & Zon 2005), and first become populated by immature lymphoblasts around 65hpf (Willett et al 1997; Willett et al 1999). Lymphopoiesis is initiated by 92hpf as determined by recombination-activating gene (*rag1* and *rag2*) expression in the developing thymocytes (Willett et al 1997). These thymocytes co-express T-cell specific markers transcription factor *gata3* and T cell receptor alpha (*tcrα*) but not *pax5* (B-cell differentiation marker), suggesting that thymocytes in the thymus at this stage are

restricted to T-cell lineage (Trede et al 2001). As the lymphoid cells differentiate, thymus also continue to increase in cellularity and reach their adult size by 1 month post fertilization (Lam et al 2002). And distinct cortical and medulla region can also be found in the adult thymus, while mature T cell are found in the medulla, immature T cells expressing *rag1* and *rag2* localize to cortex (Lam et al 2002; Schorpp et al 2002). Compare with T lymphopoiesis, B-cell formation is not well known in zebrafish partially due to lacking B cell markers. Based on the *rag1* expression and *rag2* transgenic fish, B cell development is found to be established in the pronephros by 19hpf detected by the expression of *rag1* and *cμ* which encoding the heavy chain subunit of IgM (Danilova & Steiner 2002; Willett et al 1999).

1.2.2.2.4 Thrombopoiesis

Zebrafish thrombocytes are small round nucleated cell. (Jagadeeswaran et al 1999). They have aggregation and adhesion response to platelet agonists, and can be recognized by polyclonal antisera against human platelet markers GpIIb/IIIa and GpIb (Jagadeeswaran et al 1999), thus zebrafish thrombocytes are considered as the functional equivalent to the mammalian platelets (Gregory & Jagadeeswaran 2002; Jagadeeswaran et al 1999). Consistently, many mammalian transcription factors involved in megakaryocyte (platelet precursor) development have also been found in zebrafish, including *fli1*, *gata1*, *nfe2*, and *runx1* (Davidson & Zon 2004). However, little is known about the ontogeny of these thrombytes and the sites of thrombopoiesis in zebrafish (Davidson & Zon 2004; de Jong & Zon 2005). Although circulating

thrombocytes are found in zebrafish embryos as early as 36hpf (Gregory & Jagadeeswaran 2002), their source or their precursor cells have not been discovered.

1.2.2.3. Adult hematopoiesis

Zebrafish kidney functions as the adult hematopoiesis organ, but in the larval stages, only the head kidney (pronephros) is hematopoietic active. Pronephros is first populated by hematopoietic cells at 4dpf (Willett et al 1999). In the next 2 weeks, hematopoietic cells in the pronephros continue to expand but they are mainly restricted to myelo-erythroid cell lineage (Willett et al 1999). The lymphoblasts first appear in kidney approximately 19dpf. In adults, the entire kidney is hematopoietically active and all blood lineages and their precursors can be found. The heterogeneous hematopoietic populations organized into cellular cords surrounding the blood vessels between renal tubules (Davidson & Zon 2004), similar to the organization of mammalian bone marrow. Hematopoietic cells of the kidney marrow can be separated by flow cytometry into four population based on the cell size and granularity (Traver et al 2003). The first population is mainly mature erythroid cells; the second one is a mixture of myeloid cells referred as myelomonocytic cells; the third fraction contain lymphoid cells, polychromatophilic erythroblasts, and mature thrombocytes; the last population is a collection of immature precursors of all lineages. Using in vivo reconsistitution assay, HSCs are found to reside in the third population (Traver et al 2003).

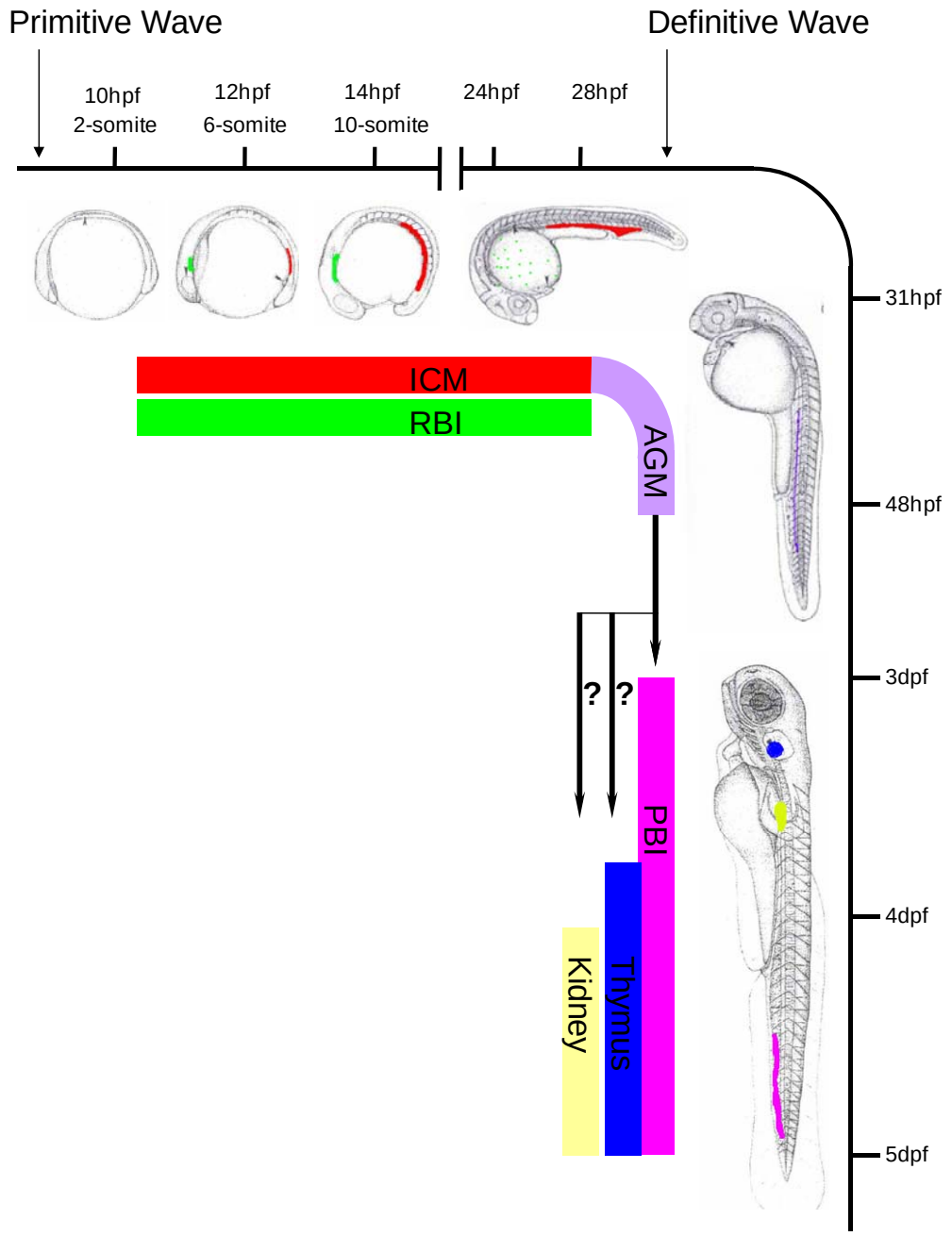


Figure 1.2 Major hematopoiesis sites in zebrafish. The primitive hematopoiesis takes place in the ICM (red) and RBI (green) region respectively. Between 28-31hpf, definitive HSCs are formed in the ventral wall of dorsal aorta of the AGM region (purple), and then they further migrate to PBI (pink), kidney (yellow) and thymus (blue). Question markers indicate that the path is tentative and need further evidence. Adapted from (Hsia & Zon 2005). ICM: Intermediate cell mass; RBI: Rostral blood island; AGM: Aorta-gonad-mesonephros; PBI: Posterior blood island

1.3 Current genetic approaches used in zebrafish hematopoiesis research

1.3.1 Forward genetic approach

1.3.1.1 Mutagenesis screen

In 1993, Nüsslein-Volhard initiated the first large scale forward genetic screen in zebrafish using the chemical mutagen N-ethyl-N-nitrosourea (ENU) at Max Planck institute in Tübingen, Germany (Haffter et al 1996). At the same time, a parallel effort was established by Marc Fishman and his colleges in Massachusetts General Hospital in Boston, USA (Driever et al 1996). Combined together, over 6000 mutation were identified with abnormal embryonic and early larval phenotypes. About 2,000 of these mutations were maintained for further analysis. The genetic and phenotypic analysis of ~1,200 mutations was presented in a series of papers in Journal *Development* (Volume 123) (Driever & Fishman 1996; Haffter et al 1996). Although these two mutagenesis screens had been done for more than 10 years, the resulting large number of mutants that are relevant to many aspects of vertebrate embryonic development became invaluable resource for researchers worldwide, and these efforts also establish zebrafish as a mainstream model system in developmental biology. As an alternative way, insertional mutagenesis screen using retrovirus system in zebrafish was developed by Nancy Hopkins and her colleges at Massachusetts Institute of Technology, Cambridge, Massachusetts, USA in late 1990s (Amsterdam et al 1999; Golling et al 2002). The Hopkins lab had isolated 550 mutants in their screen, representing around 400 different genes, and has cloned more than 300 of these genes

to date (Amsterdam et al 2004). Although the efficiency and coverage of this screen method is much lower than the chemical mutagenesis screening, cloning of the responsible mutated gene is much easier and rapid. Thus, this approach could facilitate the identification of important zebrafish homologues of other invertebrate species and cloning of ENU mutants by candidate gene approach.

Among the available mutants from those screens, approximately 50 independent mutants with defects in hematopoiesis comprising 26 complementation groups were recovered based on the gross inspection for absence of circulating blood or alteration in its color (Ransom et al 1996; Weinstein et al 1996). More importantly, many of these hematopoietic mutants also resemble human blood diseases (Table 1.1), which highlight the conservation between zebrafish and human in the process of blood formation and the potential of zebrafish to model human diseases for the therapeutic benefits.

1.3.1.2 Genetic mapping

The wealth of mutants from the genetic screen is accompanied by the challenge of identification of the affected genes. Positional cloning and candidate gene approach is two commonly used methods for mapping the mutant gene.

Positional cloning is defined as identification of a mutated gene based on its chromosomal map position. This method has two major steps: the first step is to identify a DNA sequence tightly linked to the mutation of interest; and the second step involves assembly of genomic clones spanning the linked region, cataloging the genes in the assembled region, and winnowing this list of genes until

Table 1.1 Zebrafish hematopoietic mutants resembling human diseases.

Mutant	Phenotype	Gene	Human disease	Reference
chardonnay	Hypochromic Anemia	*DMT-1*	Microcytic anemia	(Donovan et al 2002)
Chablis /merlot	Hemolytic anemia	*Band 4.1*	Hereditary elliptocytosis	(Shafizadeh et al 2002)
dracula	Porphyria	*Ferrochel atase (fch)*	Erythropoietic protoporphyria	(Childs et al 2000)
retsina	Hemolytic anemia	*Band 3*	Congenital dyserythropoietic anemia type II	(Paw et al 2003)
sauternes	Hypochromic anemia	*Alas2*	Sideroblastic anemia	(Brownlie et al 1998)
vlad tepes	Early anemia	*Gata1*	Familial dyserythropoietic anemia and throbocytopenia	(Lyons et al 2002)
weissherbst	Hypochromic anemia	*Ferroporti n 1*	Juvenile hemochromatosis	(Donovan et al 2000)
yquem	Porphyria	*Urod*	Hepatoerythropoietic porphyria	(Wang et al 1998)
zinfandel	hypochromic microcytic anemia	*Globin locus*	Hypochromic anemia	(Brownlie et al 2003)

the mutation is identified (Talbot & Hopkins 2000). For first step, a dense genetic map is the key requirement. The current genetic map is comprised of over 2600 simple-sequence length polymorphisms (SSLPs) (Shimoda et al 1999). The zebrafish genome contains ~1.7×10^9 basepairs (Ralph Hinegardner 1972), so the average distance between SSLPs is currently ~650 kb. At this density, many mutations are within a close distance from certain SSLP marker (Talbot & Hopkins 2000). However,

on going efforts to further increase the marker density are still needed and will greatly facilitate the cloning process. The second step of positional cloning relies highly on high quality genome sequence information. In February 2001 the Sanger Institute started sequencing the genome of zebrafish, and two approaches are combined to obtain the sequence information. One is cloning mapping, namely to map and sequence clones from BAC libraries. The finished clones are manually annotated and accessible in Vega (http://vega.sanger.ac.uk/Danio_rerio/index.html), or FPC database (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish/small.shtml). The other approach is whole genome shotgun (WGS), which is faster than the first approach, but its assemblies are automatically annotated, thus are of lesser quality. The sequence and maps assembled by WGS are accessible in Ensembl (http://www.ensembl.org/Danio_rerio/index.html). The position cloning process can be arduous, but it is an unbiased approach that can be applied to any gene whose inheritance can be traced, without prior knowledge of the gene.

For the candidate gene approach, one examines genes with some property expected of the mutated locus to determine if any is disrupted in the mutants. Candidate gene usually is selected based on the expression pattern, knowledge of mutants with similar phenotypes in other organisms, and inferences about the biochemical pathway disrupted by the mutation (Talbot & Hopkins 2000). Especially, when it is combined with positional cloning, it will provide an efficient way of testing candidate genes, as those not linked to the mutation are excluded rapidly. Furthermore, phenotypic rescue assays are also commonly used to test candidate genes, and can be performed with either genomic DNA or in vitro synthesized full-length RNA of the candidates.

1.3.2 Reverse genetic approach

Complementary to forward genetics, the reverse genetic approach is also widely used in revealing the biological function of a specific gene. Currently, antisense morpholino oligos is deemed as the most rapid and efficient gene knockdown technique in the zebrafish research community. Morpholino phosphorodiamidate oligonucleotides (morpholinos or MOs) are synthetic DNA analogues (Ekker & Larson 2001). With high affinity for RNA, MOs serve as excellent antisense reagents. Their efficacy is achieved via a steric block mechanism (RNAseH-independent) (Summerton 1999). MOs can either block translation initiation in the cytosol by targeting the leader and the sequences surrounding the start codon, or they can modify pre-mRNA splicing in the nucleus by targeting splice junctions (http://www.gene-tools.com/node/13). This powerful method has been extensive adopted in studying genes involved in zebrafish hematopoiesis, especially when the mutant of the gene of interest is not available. As examples, knockdown of zebrafish *scl* and *runx1* gene by MOs both produce very specific hematopoietic phenotypes and reveal their conserved roles as mammalian counterparts (Dooley et al 2005; Kalev-Zylinska et al 2002). However, the disadvantage for MOs is that it is a transient method and only suited for very early developmental stages. In addition, their injection can produce dose-dependent toxicity, like the widespread cell death and neuronal degeneration (Heasman 2002). Thus, another reverse genetics method was developed in zebrafish: target induced local lesions in genomes (TILLING) (Wienholds et al 2002; Wienholds et al 2003). This method has been successfully used in other species including plants,

worms, fly, mice and rats (Wienholds et al 2003). The basic procedure of this method involves random mutagenesis of animals which is similar to the forward genetics screening, followed by targeted screening for the induced mutation either by direct sequencing or an enzymatic cleavage method by endonuclease CEL-I (it cleaves the heteroduplex DNA). TILLING can be performed in a high-throughput way for screening of mutation in specific genes of interest. One group reported that 13 different potential knockout zebrafish were generated in a few months using this method (Wienholds et al 2003). Moreover, it may identify multiple mutant alleles for a single gene, which will facilitate the characterization of critical domains and elucidation of downstream signals (de Jong & Zon 2005).

1.3.3 Transgenesis

Transgenesis approach is especially favored in the zebrafish model because of its optical clarity and external development. By linking a fluorescent protein such as GFP or DsRed to an endogenous gene or a promoter of interest, expression can be easily visualized and monitored in the living fish. Currently, various hematopoietic specific transgenic fish have been created (Table1.3), and they have proven to be powerful tools in: (1) in vivo tracing of the specific cell lineage, tissue, or organ of development in the living embryo; (2) identification of regulatory elements; and (3) isolation of lineage specific cell populations by cell sorting (Heicklen-Klein et al 2005). With the development of new lineage specific transgenic fish, the application will be more extensively used in the hematopoietic research.

Table 1.2 Hematopoietic lineage specific transgenic zebrafish lines

Transgenic zebrafish	Expression pattern	Reference
gata1-GFP	Erythroid cells	(Long et al 1997)
gata2-GFP	Hematopoietic progenitors	(Jessen et al 1998)
scl-GFP	Hematopoietic progenitors	(Jin et al 2006; Zhang & Rodaway 2007)
lmo2-dsred	Hematopoietic and vascular tissues	(Zhu et al 2005)
rag1-GFP	Lymphoid cells	(Jessen et al 1999)
rag2-GFP	Lymphoid cells	(Jessen et al 1999)
lck-GFP	T-cells	(Langenau et al 2004)
cd41-GFP	Thrombocytes	(Lin et al 2005)
pu.1-GFP	Myeloid cells	(Hsu et al 2004; Ward et al 2003)
lyc-GFP	Myeloid cells	(Hall et al 2007)

1.4 Aims

Despite the main frame of hematopoietic program has been established by studying the traditional model organisms, many questions remain partially answered. For example, the molecules involved in the regulation of primitive and definitive hematopoiesis have not been fully identified. Toward this purpose, our lab carried out a large scale mutagenesis and followed by a lineage specific genetic screen to search zebrafish mutants that were defective in either primitive or definitive hematopoiesis.

Among the mutants identified, two of them (*ugly duckling* and *tc-244*) are particularly interesting.

This thesis describes phenotype characterization of these two zebrafish hematopoietic mutants: *ugly duckling* (*udu*) and *tc-244*, isolation of responsible gene in one of the mutants (*tc-244*) and functional analysis of *udu* and *tc-244* genes. More specially, the aim of this thesis is to:

(for the *udu* mutant)

1. Perform phenotype characterization of *udu* mutant. The characterization data will reveal the detail hematopoietic defects in the *udu* mutant and the results will indicate *udu*'s role in hematopoiesis development.

2. Examine the temporal and spatial expression of *udu* gene, and investigate whether *udu* functions cell-autonomously or non-cell autonomously in hematopoiesis development.

3. Perform domain analysis of Udu protein, and design a yeast two hybrid screen to fish out interaction partners of Udu protein.

4. Examine the potential candidate interaction partners, and reveal the possible pathway or molecular mechanism involved.

(for the *tc-244* mutant)

5. Perform primary characterization *tc-244* mutant using the available hematopoietic markers, and provide information about *tc-244* gene for the candidate gene approach if possible.

6. Use the map-based positional cloning approach to identify the mutated gene in *tc-244* mutant. This is the most critical step in this study.

7. Obtain the full-length sequence of *tc-244* gene, and confirm the mapping result by morpholino knock down or RNA rescue experiment.

8. Determine the expression pattern of *tc-244* gene and investigate *tc-244*'s role in hematopoietic development.

It is believed that cloning and functional study of these two zebrafish mutants may identify novel genes or reveal the novel function of known genes involved in hematopoietic development. Studying of these zebrafish hematopoietic mutants should be of great value in identification of new pathways involved in blood development and improve our current understanding of hematopoiesis.

Chapter II Material and methods

2.1 Zebrafish biology

2.1.1 Zebrafish maintenance and strains used

Both embryos and adult zebrafish used in this study were maintained in the Zebrafish Facility of Institute of Molecular and Cellular Biology. The general maintenance and breeding were carried out under standard conditions at 28.5°C and were kept under the photoperiod cycle set at 14 hours of day (light) and 10 hours for night (dark). Zebrafish strain AB was used as wild type strain for normal crossing, while WIK was used as mapping strain or polymorphic strain in this study.

To prevent the pigmentation in larvae, 0.003% (30 mg/L or 0.2 mM) PTU (1-phenyl-2-thiourea) in egg water (60 μg/mL sea salt in distilled water) is used to replace the normal egg water for embryos between 12-24hpf. Normally, embryos for manipulation are staged by the morphological criteria as described by (Kimmel et al 1995).

2.1.2 Positional Cloning

2.1.2.1 Mapping cross

In order to perform positional cloning, polymorphic hybrid strains was created by crossing the heterozygous AB(mutant)/AB founder with WIK/WIK wild type fish. The offspring were then raised as the F1 family, and half of them would be

heterozygotes. These heterozygotes were identified by multiple random intercrosses, and confirmed pairs were set aside to generate the F2 or mapping family (Figure 2.1). However, for long-term maintenance, the mutation was usually kept on the same strain in which mutagenesis was originally performed (in this case AB strain).

2.1.2.2 Preparation of genomic DNA

Once mapping cross was created and heterozygote mapping pairs had been identified, it was important to tail clip and store the DNA from the grandparents and parents. They are useful for analyzing polymorphisms in the subsequent mapping. In this study, grandparents were sacrificed after obtaining the F2 family, and their genomic DNA was extracted and purified by QIAGEN Genomic-tip 100/G kit (QIAGEN, USA) according to the manufacturer's instruction. Usually 10ng genomic DNA of grandparents was used in each PCR reaction.

When working with embryos or tail clips from parents, they were placed individually into individual well of the 96-well plate. And then 50µl fish lysis buffer (10mM Tris pH8.0, 2mM EDTA, 0.2% Triton X-100) or 1×TE buffer (10mM Tris-Cl, 1mM EDTA, pH8.0) with 0.5mg/ml Proteinase K was add to each well and kept incubated at 55°C overnight. Followed by incubation at 98°C for 10 min to destroy Proteinase K. Briefly spin down lysed embryo debris and 1µl of the supernatant was enough for PCR analysis or can be diluted as necessary.

2.1.2.3 SSLP marker and PCR reaction

To determine the genetic distance, single-sequence length polymorphism (SSLP) or simple sequence repeats (SSRs) microsatellite markers were used as the major type of mapping marker in this study. They usually have varied number of short repeat sequence, thus their size difference can be visualized by running the PCR products on the agarose gel. Most SSLP markers used in initial mapping are available in the MGH/CVRC Zebrafish Server (http://zebrafish.mgh.harvard.edu/zebrafish/index.htm). In the fine mapping stage, most polymorphic SSRs or SSLPs were identified manually. Fully sequence BACs were submitted to MGH-based SSR searching tool (http://danio.mgh.harvard.edu/userMarkers/usrInputSsr.html), and then potential candidates were screened between AB and WIK strain to check if they are polymorphic.

The PCR reaction used to amplify the SSR or SSLP markers:

Component	Volume / 20 µl reaction
Template (genomic DNA)	1ul of crude lyses (~10ng)
10×PCR Buffer	2 µl
10mM dNTP	0.4µl
Forward Primer (10 µM)	0.4µl
Reverse Primer (10 µM)	0.4µl
Taq polymerase	0.4µl
ddH2O	add to 20µl

2.1.2.4 Initial mapping and bulk segregation analysis (BSA)

Initial mapping or linkage scanning is to determine the chromosome or linkage group on which the mutation locates. For this purpose, bulk segregation analysis (BSA) approach was adopted. Genomic DNA from 24 homozygous mutant embryos or sibling embryos of the F2 family were pooled together to make mutant and sibling pools respectively. Then two independent mutant and sibling pools were used to test the linkage by PCR analysis of 300 SSLP markers distributed on the 25 linkage groups (Table 2.1). Linkage was assumed when a WIK band present in the sibling pools was faint or absent in the mutant pool (Figure 2.1). Those linked SSLP markers isolated from BSA were further tested on individual mutant embryos to confirm the linkage, and then the the genetic distance between markers and mutation can be evaluated.

Genetic distance (centimorgan) = recombinant number/ total meiosis events × 100

= recombinant number/(total embryo number×2) ×100

2.1.2.5 Fine mapping

Generally speaking, the first step of fine mapping is to position the mutation between two closely linked flanking markers. Based on recombination mapping principle, markers that are far away from the mutation should yield more recombinants than markers that are close to the mutation. Markers that are on opposite sides of the mutation should give different sets or recombinants, and markers that are on the same side of the mutation should share recombinants. Utilizing this principle, starting from

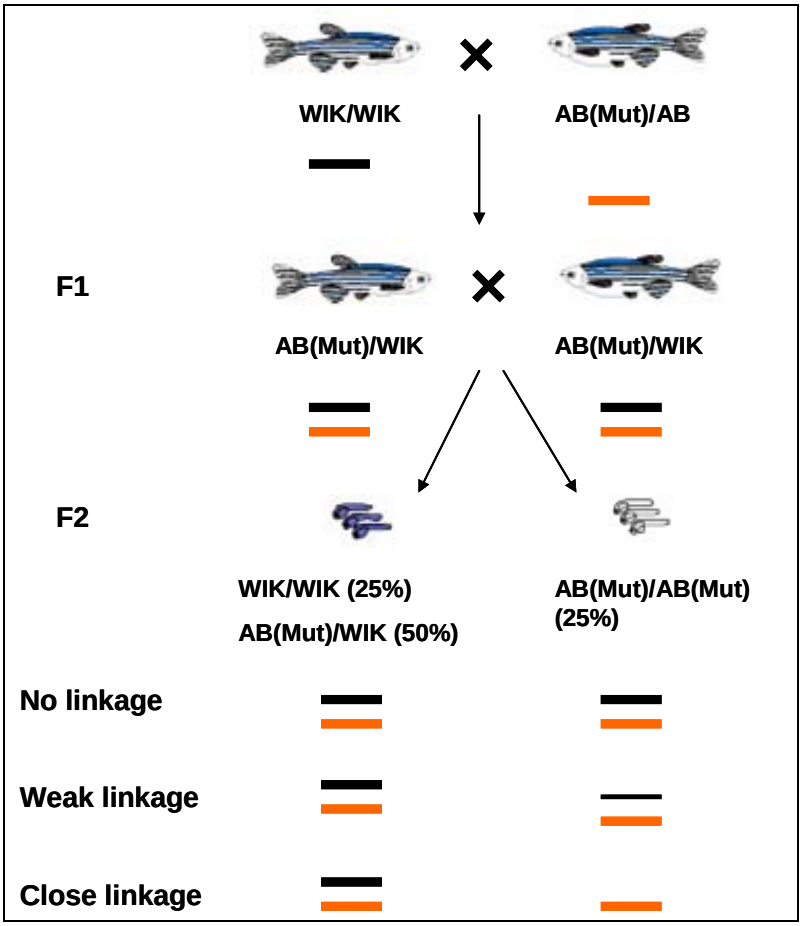


Figure 2.1 Principle of bulk segregation analysis (BSA) used in initial mapping. The black and orange bands indicate the agarose scorable PCR bands amplified using SSLP marker. Difference in the WIK band intensity indicates the linkage in this example. (This figure is adapted from one of the figures found in http://www.eb.tuebingen.mpg.de/departments/3-genetics/zebrafish/robert-geisler/)

Table 2.1 SSLP marker panel for initial mapping (linkage screening)

1	2	3	4	5	6	7	8	9	10	11	12	Linkage Group
>z4593	>z9394	*z3705*	>z1705	>z1351	>Z9704	*z5508*	>Z6802	>Z22347	>z1781	*z6974*	*z13296*	LG1
>z4278	>z851	>Z7634	>z4662	*z13620*	>z1406	>z6617	*z7358*	>z1703	>Z20550	*z22747*	*z13475*	LG2
>z872	>Z7506	>Z8208	>z15457	>z9964	>Z11227	>z3725	>z20058	>Z7486	>z1191	>z6019	*z1185*	LG3
>z3275	>z984	>Z10164	>Z7490	>Z21636	*z20450*	>z5112	>z1366	*z20533*	*z9920*	*z3439(L3)*	*z9257 (L3)*	LG4
>z15414	>Z11496	>Z6727	*z6614*	>z1390	>z3804	>Z14143	>z4299	>z1677	>z1202	*z1454*	*z8921*	LG5
z15448	>z13275	>z880	>z6624	*z10914*	>z5294	>Z13614	*z9230*	>z4297	>z1680	*z1701*	*gof2*	LG6
>z3273	>Z10785	>z1206	>z4706	>z1182	*z1059*	>Z8156	*z1239*	>Z13880	>Z13936	>z5563	>z1313	LG7
>z1634	>z1068	>Z7819	*z11001*	*z22270*	>Z21115	>z789	>z4318	*z8770*	*z8703*	*z6818(L2)*	*z20951(L5)*	LG8
>Z20860	>z1777	>z562	>Z6268	>z4673	>z5080	>z1805	>z20031	>Z10789	>z4577	>z1270	*z7564*	LG9
>z9199	>Z6410	>Z8146	*z3835*	>z1145	*z1191*	*z13219*	>z9701	>z3260	*z14193*	*z7262*	*z6427*	LG10
>z1590	>z3527	>Z13666	>z4190	>z1393	>6909	>z13411	>z3362	>Z10919	*z8032*	*z10727*	*z13395*	LG11
>z1778	>z1225	>Z21911	>z1473	>z4188	>z4830	*z1400*	>z1358	*z7576*	*z22103*	*z6920*	*z1312*	LG12
>z1531	>z5643	>z6104	>Z13611	>z5395	>z1627	>Z7102	>z6657	>z1826	>z6007	*z9951*	*z17223*	LG13
z9366	>z5436	>z1536	>z5435	>z4203	>Z22107	>z1226	>z6666	>z3984	>z1801	*z11837*	*z22144(L20)*	LG14
>Z10289	>Z6312	>Z6712	>Z21982	>z4396	>Z11320	>Z13230	*z7216*	>Z7381	>z6024	>z5223	*z21165*	LG15
>z3741	>z1837	>Z21155	>z6365	*z15453*	>Z10036	>z1215	>z4670	>z1786	>z4175	*z6921*	*z13202*	LG16
z9692	>z4268	>z1490	>Z22083	>Z22674	>z9847	*z21703*	>z4053	>z3491	>z1928	*z3165*	*z6010*	LG17
>z1136	>z1144	>Z13329	>Z8488	>Z10008	*z8343*	>z9154	>z5321	*z11944*	*z7654*	*z11685*	*z20046(L20)*	LG18
>z4009	>z160	>Z7450	>z3782	>z3816	>Z11403	>Z6661	>Z7926	>z1803	*z5183*	*z7686*	*z4825*	LG19
>z4329	>Z8554	>z3954	>Z22041	>Z7158	>z3964	>Z11841	>Z10056	>Z9334	>Z9708	>Z6804	*z7171*	LG20
>z3476	>z1274	*z10508*	>z4492	>Z10960	*z6089*	>z4425	>z1497	>z4074	*z7925*	*z7405*	*z13719*	LG21
>z1148	>Z10673	*z11752*	>z230	>Z10321	>Z21243	*z9516*	>Z9817	>z4682	*z3093*	*z13223*	*z3286*	LG22
>z5683	>Z8945	>z4003	>z15422	>z4421	>z3157	>z1773	>z176	*z20039*	*z5141*	*z20133*	*z3211(L20)*	LG23
>z5075	>z1584	>z5413	>Z23011	>z3399	*z7627*	>z5657	>z3901	>z157	*z13229*	*z9325*	*z11862*	LG24
>z1378	>z13232	>z3490	>z3528	>z5669	>z1462	>z9290	*z11092*	*z8224*	*z1213*	*z8780*	*z1431*	LG25

linked markers in the BSA, the region between two flanking markers can be narrow down until they are 10cM (or less) apart.

With two good flanking markers, 1500~2000 embryos can be tested for recombination events with ease. Upon finishing this step, a number of recombinants were identified. These recombinants were re-arrayed on a new plate to create the recombinant panel. The recombinant panel was then utilized for further mapping analysis. Primer pairs for fine mapping are listed in Table 2.2.

2.1.2.6 Genomic walking

Blast the two nearest flanking markers in Zebrafish Genome Fingerprinting Project (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish/small.shtml) and Ensembl zebrafish genome server (http://www.ensembl.org/Danio_rerio/index.html) database. If both markers located in the same Contig (a collection of assembled BACs), then more markers between the two flanking markers are tested in order to find new flanking markers that are on the same BAC. If not, more markers will be searched on the other contigs which were supposed to be more close to the mutation until they fall into the same contig. Once two flanking markers are on the same BAC, depending on the size of the BAC, one can either directly sequence the coding region of candidate genes in the BAC, or further narrow the distance using new markers until there is one gene left between flanking markers.

2.1.2.7 Sequencing mutation

For genes with known information and small exons, primers can be designed to amplify the exons from genomic DNA templates and proceed to sequence the PCR product. Alternatively, RT-PCR products form homozygous wild type and mutant embryos can be used for the sequence purpose as well.

Table 2.2 Mapping primers list for *tc-244* mutant

Marker Type	Maker Name	Sequence
SSLP	Z4706 (MGH)	5’-TGCATATAAAGGCACCTCCA-3’/5’-TTTCCCCATCAACAAAGCTC-3’
SSLP	Z1059 (MGH)	5’-CACAGCATCACGCTCTCACT-3’/5’-TATACCGTGGAAATTTCGGG-3’
SSLP	z43308 (MGH)	5’-TTACCCTTCCAACCCCTTTC-3’/5’-CCCTGTTACAGCCACTGGTT-3’
SSLP	zK183N2-118 (BX004863_2734)	5’-TGCAAATACAATGCATACCG-3’/5’-TGCGTACAAGACGGTAACAAA-3’
SSLP	zC226L4-1693 (BX511061_2)	5’-GTGATGTCTCTCGAGCTAA-3’/5’-TTGATTGTCTTTTGTTGCTCT-3’
SSLP	CR318603_141264	5’-GCCACCTGCTGGATGATTAC-3’/5’-GGTTTGAGTTTGGCCTAAATTGT-3’
SSLP	CR318603_55485	5’-CCAGGAGAGCTCGCACTAAG-3’/5’-GACCATTGGAAAAGGGCTTC-3’
SSLP	CR376795_108221	5’-ATGTTAATTTGAGCTCTTGA-3’/5’-GGGCGCATTTTCGTAGCTTT
SSLP	CR376795_97384	5’-AAGCTCTCAGATCTACTATC-3’/5’-GGCCCTCCCACCTGTAGAAC-3’
SSLP	CR376795_28662	5’- AACAGTGACACCCTCCTTCG-3’/5’-CTAGAATGTGGCCTCCCTGA-3’
SSLP	CR318672_119281	5’-GCATTCAGATTCTGGGGTGT-3’/5’-CGGATGAACCCATCAATCTC-3’
SNP	CR318672-101395	5’-AACATTTGGGACTCAATCCTA-3’/5’-TGTTGCCCACTCGTCTA
SNP	CR318672-72280	5’-AATGAACTATAAGTGTAGGCT-3’/5’-CATCACCACGACGACTAA-3’
For mutation sequencing	-	5’-GAGAACTCATTTGTGTCACCA-3’/5’-TTTACTGCATTTATCCCTC-3’

2.1.3 Whole mount *in situ* hybridization (WISH)

Whole mount *in situ* hybridization (WISH) is a method used to view the location of nucleic acids (RNA in this case) *in situ*. The tissue is gently fixed so as not to disrupt the RNA, and then it is hybridized to a chemically or radioactively labeled RNA probe that is complimentary (anti-sense) to the desired mRNA of interest. The chemical or radioactive probe can then be detected in the tissue by antibodies to the chemical label or by autoradiography. When using the chemically labeled probe the antibody may be fluorescently labeled or can have an enzyme associated with it that when exposed to substrate generates a colored precipitate so that localization in cells is easily visualized. The WISH method for zebrafish embryos using chemically labeled probes in this study is described in the following paragraph.

Zebrafish embryos at the desire stage were collected and dechorinated by forceps or pronase treatment. For embryos that were old than 1dpf, the working concentration of pronase is 1mg/ml in PBS. After dechorination, embryos were washed briefly by PBST (PBS with 0.1% Tween 20) and then fixed in 4% PFA (Paraformaldehyde in PBS) for 2 hours at RT (room temperature) or overnight in 4ºC. Embryos were than washed for 3 times, each time 5 minutes (3×5min) by PBST, and then equilibrate in 100% methanol for 5 minutes. Replace with fresh methanol and put at -20°C for at least 20 minutes. Rehydration was performed in 50% and 30% Methanol/PBST for 5 minutes each and followed by 2×5min washing in PBST. Embryos old than 1dpf were digested with Proteinase K (10μg/ml in PBST) at RT according to age (4-5dpf embryos need around 20min treatment, 2-3dpf embryos need around 10min or less). After a brief washing in PBST, embryos were re-fixed in 4% PFA for 20 minutes and washed by PBST for 2×5min. Then pre-hybridization was

performed by incubating embryos in HYB+ (50%formamide, 5XSSC, 50μg/ml Heparine, 500μg/ml tRNA, 0.1% Tween20, adjusted by citric acid to pH6.0) at 65°C for at least 1 hour. After pre-hybridization, HYB+ was replace by Digoxigenin(DIG)-labled RNA probe (0.5-1μg/ml) in HYB+ solution and incubated at 65°C overnight. Upon finishing this step, the RNA probe was removed, and embryos were washed as following: 2x30 min in 2×50%formamide/2×SSCT at 65°C, 3×20 min in 2XSSCT at 65°C, 2×30 min in 0.2×SSCT at 65°C, and 2x 5min in MABT (100mM maelic acid, 150mM NaCl adjust to pH7.5, 0.1% Tween20) at RT. After this intensive washes, embryos were blocked by in blocking buffer (2% lamb serum, 2%BMB in MABT) for at least 1 hr at RT, and followed by incubation with anti-DIG-AP antibody solution (1:5000 dilution in the blocking buffer) O/N at 4°C. Subsequently the embryos were washed with MABT for 6x20 min and buffer9.5T (0.1M Tris-HCl, pH9.5, 50mM MgCl2, 10mM NaCl and 0.1% Tween20) for 2x10 min. The embryos were stained with NBT/BCIP substrate solution (one tablet in 10ml H_2O or NBT 0.30mg/ml, BCIP 0.15mg/ml) in the dark from 1/2 hr to O/N at 4°C depending on the probe used. The reaction was stopped by washing the embryos twice with PBST for 10 min each, followed by fixing with 4% PFA for 1 hr. After 1 hr washes with PBST, the embryos were equilibrated in 70% glycerol at 4°C O/N for imaging.

2.1.4 Hemoglobin staining with o-dianisidine

Hemoglobin can function as pseudo-peroxidase, in the presence of H_2O_2, colorless o-dianisidine can be quickly oxidized and turn to brownish color. 2dpf live embryos were incubated with o-dianisidine staining buffer [0.6mg/ml o-dianisidine (2,3-Diphosphoglyceric acid fast blue B, free base, Sigma), 0.01M sodium acetate (pH 4.5),

0.65% H_2O_2, and 40% (v/v) ethanol] in dark for 15 min (Detrich et al 1995). The staining was then observed under the light microscope. For imagining purpose, embryos were fixed by 4%PFA and then equilibrated in 70% glycerol.

2.1.5 Microinjection of Morpholino

Morpholino oligos (MO) are short chains of Morpholino subunits comprised of a nucleic acid base, a morpholino ring and a non-ionic phosphorodiamidate intersubunit linkage (Figure 2.2). With high affinity for RNA, MOs serve as excellent antisense reagents. Their efficacy is achieved via a steric block mechanism (RNAseH-independent) (Summerton 1999). Normally, 18-25mer morpholinos complementary to sequence between 5'UTR through the first 25 bases 3' of the AUG start site or spanning certain exon/intron boundaries is designed to either inhibit translation or modify pre-mRNA splicing.

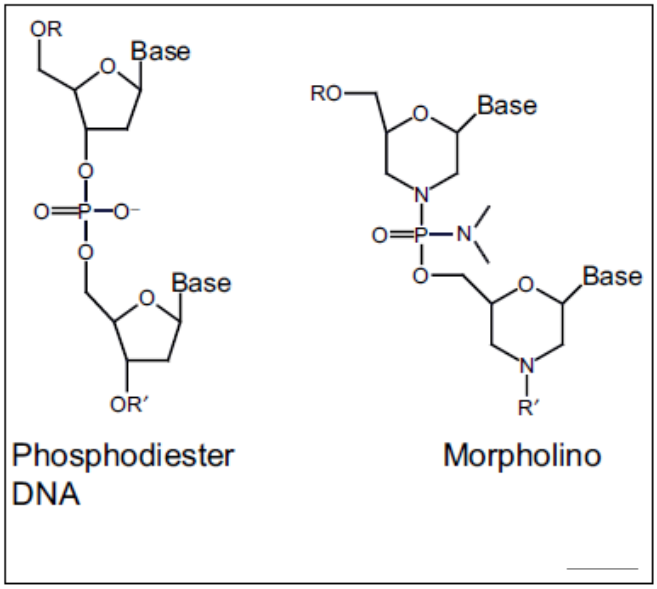


Figure 2.2 Structures of DNA and morpholino oligonucleotides. R and R' denote continuation of the oligomer chain in the 5' or 3' direction respectively.

Morpholino powder (300 nanomoles) (Gene Tools, USA) was usually dissolved in the 60 µl sterile water to make 5mM stock solution. For injection purpose, the 5mM stock solution was diluted into different working concentration, which varied for different MO. Then 1-2nl of MO in working concentration was injected into one-cell stage embryos by microinjection.

Table 2.3 Morpholinos used in this study

Gene name	Morpholino name	Morpholino sequence	Injection quantity
zgc153228	zgc153228-i4e5	5'AGCTCCTAAACAGAAAGACCGGATA 3'	1nl of 0.1mM (equal to around 0.1nm)
N/A	Standard control	5'CCTCTTACCTCAGTTACA ATTTATA 3''	1nl of 0.1mM (equal to around 0.1nm)

2.1.6 Transplantation

Donor embryos were injected with 5% rhodamine-dextran (dextran, tetramethylrhodamine and biotin, 10,000 MW, lysine fixable, Molecular Probes) in 0.2M KCl at one-cell stage. Injected embryos were allowed to develop at 28.5°C for around 3 hours and then dechorionated manually with forceps in an agarose ramp, covered with 1X Danieau buffer (5.8mM NaCl, 0.07mM KCl, 0.04mM $MgSO_4$, 0.06mM $Ca(NO_3)_2$, 0.5mM HEPES pH7.6). 15-30 donor cells from each embryo were transplanted into the dechorionated host embryos of the same stage by micro manipulator. The manipulated donor embryos were saved for genotyping analysis. Contribution of the rhodamine-dextran-labeled donor cells to the circulating blood cells in the host embryos was scored at around 30hpf under fluorescent microscope.

2.1.7 Cryo-section

Fixed or stained embryos were first transferred into molten agar-sucrose solution (1.5% agar, 5% sucrose) in a detached cap of eppendorf tube. Needles were used to adjust samples in desired orientation in the slowly hardening agar. After the agar block solidified, a small block was cut with razor to mount the sample in proper position. The block was then transferred into 30% sucrose solution and incubated at 4 °C overnight. Subsequently, the block was placed on the frozen surface of a layer of tissue freezing medium cryostat on the prechilled tissue holder. The block was then coated with one drop of cryostat and frozen in liquid nitrogen until the block had solidified completely. The frozen block was placed into a cryostat chamber for 30 minutes to be equilibrated with chamber temperature of -25 °C. Normally, 10-15 micron-thick sections were made and placed on poly-lysine coated slides. The slides were dried on a 42 °C hot plate for about 30 minutes. The sections were fixed briefly with 4% PFA/PBS for 10 minutes and washed 3 times in PBS for 5 minutes each. Afterwards, the sections can be processed for further procedures or embedded in several drops of glycerol and covered with cover slip for observation.

2.1.8 Whole mount cell death assay

2.1.8.1 Acridine orange staining

Apoptotic cells can be stained by the addition of DNA fluorochromes which are able to cross the intact plasma membrane, such as acridine orange, a nucleic acid selective fluorescent cationic dye.

Zebrafish embryos (younger than 2dpf) were dechorionated and placed in 5 µg/ml of acridine orange in egg water (Abdelilah et al 1996). After 30 minutes of staining embryos were washed with egg water and viewed under fluorescence microscope.

2.1.8.2 TUNEL assay

Terminal transferase dUTP nick end labeling (TUNEL) is a common method for detecting DNA fragmentation that results from apoptosis. The assay relies on the presence of nicks in the DNA which can be identified by terminal transferase, an enzyme that will catalyze the addition of dUTPs that are labeled with fluorescence or secondarily antibody. This method is also used in zebrafish, but some modifications are made for whole mount assay.

Embryos were fixed in 4% PFA and dehydrated in PBS/methanol series: 50%, 70%, 95%, and 100% followed by incubation in 100% acetone at –20ºC for 10 minutes. And these embryos were rinsed with PBST for 3×5 min, and then they were treated by fresh permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 15 minutes and proteinase K treatment (10µg/ml, 20-30 min). After these steps, embryos were fixed again in 4% PFA followed by three rinses in PBST (5 min each). Embryos were then assayed by using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Switzerland) according to the instruction manual.

2.1.9 Whole mount immunofluorescent staining

Embryos were fixed in 4% PFA/PBS for 2-4 hours at room temperature, and then they were rinsed PBS. To improve the penetration of antibodies, fixed embryos were treated with cold acetone (-20ºC, to remove lipids from cell membrane) for 5-10 minutes followed by washes with deionised water once and PBS three times for 10 minutes each. To block non-specific sites, embryos were incubated in PBDT (PBS with 0.1% TritonX-100 and 1%DMSO) plus 2% lamb serum for 1hr at RT. The embryos were then incubated with primary antibody in the same blocking buffer (1:400 dilution of polyclonal rabbit anti-βe1-globin serum in this study) for overnight at 4 ºC. After removing the primary antibody, embryos were washed in PBST briefly for 2 times and additional 4 times for 30 minutes each. The embryos were then incubated with an appropriate secondary antibody (1:400 diluted fluorochrome-conjugated Alexa Fluo 488 goat anti-rabbit IgG antibody in this study) for overnight at 4ºC. After incubation with the secondary antibody, the embryos were washed with PBDT and analyzed with fluorescence microscope.

2.1.10 Embryos preparation for flow cytometry analysis

The 24hpf *udu*$^{sq1-/-}$ mutant and sibling embryos (300-400/each pool) obtained from crosses of adult *udu*$^{+/-}$/Tg(-5.0scl:EGFP)^{sq1} and *udu*$^{+/-}$ fish were separated based on the morphological phenotype and disaggregated in cold 0.9X PBS with 5% FBS. The cell suspensions were passed through a 70 µm-pore size filter and spun at 1000 rpm for 5 min. The pellets were re-suspended in 1ml Dispase (1U/ml in PBS) (Gibco, USA) and incubated at 37 °C for 20 min. After addition of 1ml washing buffer (Hanks buffered saline solution containing 20% calf serum, 5mM $CaCl_2$ and 50µg/ml DNAse), the cell suspensions were spun at 1000rpm for 5 min and re-suspended in PBS. After passing

through a 40 μm-pore size filter, 10^6 cells/ml samples were applied for FACS analysis (Beckton Dickinson).

For cytology analysis, about 1-2 x 10^5 sorted GFP^+ cells were cyto-centrifuged at 500rpm for 5 min onto glass slides and subjected to May-Grunwald/Giemsa staining. For cell cycle analysis, cell suspensions (1 x 10^6 cells) were spun at 1000rpm for 5 min, re-suspended in 1ml warmed (37 °C) DMEM (Gibco, USA) supplemented with 2% Fetal Calf Serum, 10mM HEPES buffer (Gibco, USA), and 5μg/ml Hoechst 33342 (Sigma, USA), and incubated at 37 °C for 1hr. The cells were then put on ice immediately, spun down at 1000rpm for 5 min at 4 °C, re-suspended in ice-cold HBSS (Hanks Balanced Salt Solution from Gibco) with 2% Fetal Calf Serum and 10mM HEPES buffer (Gibco, USA), and finally subjected to cell cycle analysis by FACS.

2.1.11 May-Grunwald/Giemsa staining

The cytospined slides was covered with 1ml May-grunwald stain solution [May-grunwald solution (BDH Chemicals Ltd, UK):ethanol = 1:3] for 5 minutes followed by staining with 1ml Giemsa stain solution [Giemsa solution (BDH Chemicals Ltd, UK):Phosphate buffer = 1:20] for 20minutes with gently shaking. Then the slides were washed by slow running water and dried in the air.

2.2 General DNA application

2.2.1 Restriction endonuclease digestion

Restriction enzyme digestion was used to screen recombinant clones and release specific DNA fragments. All the restriction enzymes used in the study were purchased from New England Biolabs, Promega, (USA). All digestions were performed at 37 °C or 25°C for 2 hours or overnight, with proper restriction buffers respectively.

2.2.2 Recovery DNA fragment from agarose gel

QIAquick Gel Extraction Kit (QIAgen, USA) was used to recover or purify DNA fragments ranging from 100 bp to 10 kb from agarose gel according to manufacturer's instructions. The DNA fragment could be PCR products, plasmids and DNA after RE digestion. Briefly, the gel slide containing the interested DNA band was cut from the gel and transferred into Buffer (1g to 3ml) and melted at 50 °C for 10 minutes and then loaded into a QIAquick spin column. The column was centrifuged at 14,000 rpm for 1 minute, washed by adding 0.75 ml of Buffer PE, and spun again. The residual Buffer PE was removed by spinning at 14,000 rpm for another 1 minute after discarding the flowthrough. Then 30 µl of H_2O was added to the column and then incubated at room temperature for 2 minute. DNA fragment was eluted into a 1.5-ml centrifuge tube by centrifugation at 14,000 rpm for 1 minute.

2.2.3 Ligation and transformation

DNA ligation reaction was carried out typically in 10 or 20 μl of volume, containing 10X ligation buffer (New England Biolabs or Promega, USA), insert DNA, vector DNA and T4 DNA ligase. The molar ratio of insert-to-vector DNA was usually 3:1. Ligation reaction was incubated RT for 15min if using quick ligation system, other at 4 °C overnight or 16°C for 4 hours.

The *E.coli* DH5α competent cells were thawed on ice and usually 2μl out of 10ul of ligation reaction was added and mixed by gentle pipetting. This mixture was then incubated on ice for 30 minutes. After being heated at 42 °C for 60 seconds, the tube was cooled immediately on ice for 2 minutes. 900μl of LB medium was then added into the mixture. After being incubated at 37 °C for 45 minutes with shaking at 200 rpm, 1/10 and 9/10 of the transformation reaction mixture was spread onto two separate LB plates supplemented with appropriate antibiotics in order to produce proper density of transformant colonies. The plates were incubated at 37 °C overnight.

2.2.4 Plasmid DNA preparation

Small-scale preparation of plasmid DNA was carried out using QIAgen Miniprep kit (QIAGEN, USA), with the processing of alkaline lysis followed by the binding of plasmid DNA to a positive charged silica-based resin. DNA was then re-dissolved and eluted in low salt buffer or water. Firstly, the bacteria in LB liquid medium with appropriate antibiotics were harvested by centrifugation at 5,000 rpm for 5 minute. The bacterial pellet was then re-suspended in 250 μl P1 Cell Resuspension Solution (containing RNaseA). 250 μl P2 Cell Lysis Solution was added to the bacterial suspension and mixed by gently inverting the tube for several times. Then this

mixture was neutralized by adding 350 µl of N3 Neutralization Buffer and inverting the tube 4–6 times. After being centrifuged at 13,000 rpm for 10 minutes, the supernatant was transferred into a fresh mini-column provided in kit and was centrifuged at 13,000 rpm for 1 min. The flow-through was discarded. After adding 750µl Buffer PE, the column was centrifuge at 13,000 rpm for 1 minute. Then the flow-through was discarded again and the column was centrifuge at 13,000 rpm for additional 1 minutes. Then the column was transferred to a new microcentrifuge tube. To elute plasmid DNA, 100µl water was added into the column for 1 minute's incubation at room temperature, followed by centrifugation at 13,000 rpm for 1 minute.

2.2.5 PCR and sub-cloning

Standard PCR was performed in a volume of 50µl reaction. Each reaction included 5µl of 10X PCR buffer, 0.5µl of 10 mM dNTP, 0.5µl of 10uM forward primer, 0.5µl of 10uM reverse primer, 1- 5U Taq polymerase and template DNA. A typical program used for amplifying 1 kb DNA product was as follows: denaturation at 94 °C for 3 minutes for 1 cycle, followed by 35 cycles of (denaturing at 94 °C for 30 seconds, annealing at 60 °C for 1 minute and extending at 72 °C for 1 minutes) and final extension at 72 °C for 10 minutes. The extension time was increased 1 min per 1 kb if the desired product was larger than 1 kb. Usually, the recovered PCR products can be directly cloned into the pGEM T-Easy or pDrive vector system (Promega, USA) by T-A cloning. Alternatively, the restrict enzyme digestion sites can be also designed in the primer sequence, then PCR product is directly cloned into digested vector accordingly.

List of Primers and constructs used in this study

pGBKT7-Fishbait	5’-CGGAATTCCCTCCTCCGCGCAGTTT-3’ /5’-CGGGATCCACGGCAGGGTTCAGTCC-3’
pGBKT7-FishSANT	5'-CGGAATTCAAGCACAGCCGAGATG-3' /5'-CGGGATCCCGGCAGGGTTCAGTC-3'
pGBKT7-FishPAH	5’-CGGAATTCCCTCCTCCGCGCAGTTT-3’ /5’-CGGGATCCTCTTCATCTCGGCTGT-3’
zgc153228-T-easy	5’-GAATTGTTTTTACTG0ACGTCC-3’ /5’-ACAATGGTGAGGCCTAA-3’

Zgc153228-e4i4-FP: 5’ GTG GCG TAC TCG GAC ATT GT 3’

Zgc153228-e4i4-RP: 5’-AGGGTGGCACTGCGTTCAAG-3’

ELF1α-FP: 5’-CTTCTCAGGCTGACTGTGC-3’

ELF1α-RP: 5’-CCGCTAGCATTACCCTCC-3’

2.3 RNA application

2.3.1 RNA extraction from zebrafish embryos

Total RNA was isolated from dechorionated zebrafish embryos using RNeasy mini kit (Qiagen, Germany). Samples were first lysed and homogenized in denaturing guanidine isothiocyanate (GITC) containing buffer which inactivates RNases and thus ensure isolation of intact RNA. Ethanol was added to the lysate to provide appropriate binding condition and then applied to RNeasy mini column for further purification. The zebrafish embryos were collected at desired stages and placed in 1.5 ml Eppendorf tube. Excess liquid was siphoned out from the tube. 350 µl of RLT buffer

was added into the tube and the embryos were pulverized. The lysate was then spun down at 14000 rpm, RT, for 3 mins. The supernatant was decanted into a sterile 1.5 ml Eppendorf tube. 350 µl of 70% ethanol was added into the clear lysate and mixed well. This mixture was transferred to an RNeasy mini spin column sitting in a 2 ml collection tube. The column was then spun at 10000 rpm for 15 sec. The flow through was discarded. 350 µl of RW1 buffer was pipetted into the RNeasy column to wash, the column was centrifuged at 10000 rpm for 15 sec. The flow through was discarded. 80 µl of RNase-free DNAse (Qiagen, Germany) incubation mix was then added directly onto the RNeasy silica-gel membrane and allowed to stand on the bench at room temperature for 15 mins. Afterwhich, another 350 µl RW1 buffer was added into the RNeasy column and spun down at 10000 rpm for 15 sec. The flow through was discarded. 500 µl of RPE buffer was pipetted on the column and spun down at 10000 rpm for 15 sec. The flow through was discarded and the washing step was repeated with another 500 µl of RPE buffer. The column was then spun at 10000 rpm and for 2 mins and the flow through discarded. The column was then spun down for an additional 1 min to remove any residual trace of ethanol. Column was then transferred to a sterile 1.5 ml Eppendorf tube. Total RNA was eluted by the addition of 40 µl of RNase-free water onto the RNeasy membrane and spun for 2 mins.

RNA was quantified by optical density reading at 260 nm and 280 nm using UV- 1601 spectrophotometer (Shimadzu, Japan). One unit of OD260 is equivalent to 40 ug/ml of RNA, OD260:OD280 ratios >2.0 indicate good quality of RNA products.

2.3.2 Reverse transcription and cDNA synthesis

Reverse transcription was performed using SuperScript™ II RNase H Reverse Transcriptase (Invitrogen). A 20 µl reaction volume was prepared by adding following components into a nuclease-free microcentrifuge tube: 1 µl Oligo(dT)$_{12-18}$ (500 µg/ml), 1 ng to 5 µg total RNA, 1µl dNTP Mix (10 mM each), and top up with distilled water to 12µl. Heat mixture to 65 °C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation. 4µl 5×First-strand buffer, 2µl DTT(0.1M) and 1 µl (200 units) of SuperScript II RT were added and mixed gently, followed by incubation at 42°C for 1 hour. Inactivate the reaction by heating at 70°C for 15 min. The cDNA can now be used as a template for amplification in PCR. However, amplification of some PCR targets (>1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, add 1 µl (2 units) of E. coli RNase H and incubate at 37°C for 20 min.

2.3.4 *In vitro* transcription

Plasmid DNAs were linearized by the appropriate restriction enzyme and then purified using the PCR purification kit (Qiagen). 1µg linerized plasmid DNA was used for a 20ul-reaction of in vitro transcription, which also contained 1µl RNA polymerase (SP6 or T7 or T3 from Stratagene), 1µl RNase inhibitor (Roche), 2µl 10X DIG RNA labeling mix (Roche), 4µl 5X reaction buffer (Stratagene), and nuclease free water. After incubation in a 37°C water bath for 2 hr, 1µl RNase-free DNase I (Roche) was added to the reaction for further incubation at 37°C for 20 min. The reaction was stopped by 1µl 0.5M EDTA (pH 8) and the RNA products were precipitated with 2.5µl 4M LiCl and 75µl 100% ethanol at -80°C for 30 min followed by centrifugation at 4°C for 30min at 12,000 rpm. After washed with 70% cold

ethanol, the RNA pellet was air-dried and resuspended in 20µl sterile DEPC water. 0.5µl of the RNA probe was loaded onto a 1% agarose gel to determine its quality and quantity. Finally, the probe was dissolved in WISH hybridization buffer (50% formamide, 5XSSC, 0.92mM citric acid, 0.1% Tween20, 50µg/ml heparin and 500µg/ml tRNA) at a final concentration of 0.5µg per ml and stored at -20°C.

Table 2.4 RNA probes generated by *in vitro* transcription in this study and their constructs information

Marker gene	**Vector**	**Linearizing enzyme**	**RNA polymerase**	**Region of cDNA sequence**
rag1	PBluescript KS+	BamHI	T7	1426-2944
foxn1	pGEM-Teasy	StyI	SP6	131-828
scl	pGEM-Teasy	PstI	T7	4-1125
lmo2	pGEM-Teasy	SalI	T7	872-1643
band3	pGEM-Teasy	BamHI	SP6	1174-3378
βe1-globin	PBluescript SK+	XbaI	T7	30-584
pu.1	pGEM-Teasy	SacII	SP6	1-1005
mpo	pGEM-Teasy	ApaI	SP6	44-2325
l-plastin	pBluescript KS+	ApaI	T3	404-800
lysozyme C	pBluescript KS+	xhoI	T7	-
udu	pcDNA3.1(-)	Asp718	T7	30-6708
c-myb	pGEM-Teasy	SalI	T7	-
runx1	pDrive	XbaI	T7	91-1384
zgc153228	pGEM-Teasy	SalI	T7	25-2229

2.4 Yeast Biology

2.4.1 Yeast transformation

2.4.1.1 Small scale yeast transformation

Colonies (2–3 mm diameter) from YPDA or SD plate were inoculated into 1 ml YPDA or SD medium. After vortexing it vigorously for 5 min, the mixture was transferred into a flask containing 50 ml of YPD or the appropriate SD medium. This liquid culture was incubated at 30ºC for 16–18 hr with shaking at 250 rpm to stationary phase (OD600>1.5). Then overnight culture was diluted as 1:10 to bring the OD600 up to 0.2–0.3. Incubate the new culture at 30ºC for 3 hr with shaking (230 rpm). At this point, the OD600 should be 0.4–0.6.Place cells in 50-ml tubes and centrifuge at 1,000 x g for 5 min at room temperature. Discard the supernatants and thoroughly resuspend the cell pellets in sterile TE or distilled H2O. Centrifuge at 1,000 x g for 5 min at room temperature and decant the supernatant. The cell pellet was resuspended in 1.5 ml of freshly prepared, sterile 1X TE/1X LiAc. 0.1 ml of these yeast competent cells was added to a new 1.5 ml tube with 0.1 µg of plasmid DNA and 0.1 mg of herring testes carrier DNA, followd by vortexing to mix well all the component. Add 0.6 ml of sterile PEG/LiAc solution to the same tube and vortex at high speed for 10 sec. Keep the tube at 30ºC for 30 min with shaking at 200 rpm. Then 70 µl DMSO was added and mixed well by gentle inversion. The mixture was heat shocked for 15 min in a 42ºC water bath and chilled on ice for 1–2 min. Centrifuge the cells for 5 sec at 14,000 rpm at room temperature. Remove the supernatant. Re-suspend cells in 0.5 ml of sterile 1X TE buffer. 100 µl was used for

plating on SD agar plate that will select for the desired transformants. Incubate the plate up-side-down at 30ºC until colonies appear (generally, 2–4 days).

2.4.1.2 Library scale yeast transformation

Confirmed bait colony from glycerol stock were inoculated into 150ml SD-Trp medium. This liquid culture was incubated at 30ºC for 16–18 hr with shaking at 250 rpm to stationary phase (OD600>1.5). Then overnight culture was diluted as 1:10 to bring the OD600 up to 0.2–0.3. Incubate the 1L new culture at 30ºC for 3 hr with shaking (230 rpm). At this point, the OD600 should be 0.4–0.6.Place cells in 500ml tubes and centrifuge at 1,000 x g for 5 min at room temperature. Discard the supernatants and thoroughly resuspend the cell pellets with 500ml sterile TE or distilled H2O. Centrifuge at 1,000 x g for 5 min at room temperature and decant the supernatant. The cell pellet was resuspended in 8 ml of freshly prepared sterile 1× TE/1×LiAc. Mix the 0.1-0.5mg Library DNA with 20mg of herring testes carrier DNA, followd by adding 8ml competent cell, mix well by vortexing. Add 60 ml of freshly prepared sterile PEG/LiAc solution to the same tube and vortex at high speed for 10 sec. Keep the tube at 30ºC for 30 min with shaking at 200 rpm. Then add 7ml of DMSO and mix well by gentle inversion. Heat shock for 15 min in a 42ºC water bath and Chill cells on ice for 1–2 min. Centrifuge cells for 5 minutes at 1000 rpm at room temperature. Remove the supernatant. Re-suspend cells in 10 ml YPDA. To test the transformation efficiency, spread 100 ml of a 1:1,000, 1:100, and 1:10 dilution onto 100-mm SD/–Leu/–Trp plates. The rest of transformation culture was spread to around 50 150mm SD/-Trp/-Leu/-His/-Ade plates (~200µl/plate). Keep all the plates up-side-down at 30ºC. After 3-5 day, the colonies began to appear, re-streak them to

the new SD/-Trp/-Leu/-His/-Ade/X-α-gal. Allow the plates to grow for about two weeks for weak interaction colonies.

Calculation of transformation efficiency:

Count colonies (cfu) growing on the SD/–Leu/–Trp dilution plate that has between 30–300 cfu.

$$\frac{\text{cfu} \times \text{total suspension vol. (µl)}}{\text{Vol. plated (µl)} \times \text{dilution factor} \times \text{µg DNA used*}} = \text{cfu/µg DNA}$$

cfu/µg x µg of library plasmid used = # of clones screened

2.4.2 Yeast Colony PCR and sequencing of library inserts

A medium size colony (2 – 3 mm) on the SD/-Trp/-Leu/-His/-Ade/X-α-gal plate was transferred into 30µL 0.2% (w/v) SDS and then vortex for 15 seconds. Then it was heated at 90°C for 4 mins and spin down at 14000 rpm for 1min at RT. 1 µl of the supernatant is enough for 50 µL PCR reactions. The T7 sequencing primer was used to sequence the PCR products.

2.4.4 Yeast plasmid isolation and transformation into E.coli

Select the colony of interest (result from screening), and inoculate single colony into 3ml of SD/-Leu and grow it for 16-24 at 30ºC. The culture was harvested by centrifugation at 5000×g for 5 min, and resuspend in 250 µl Buffer P1 containing 0.1 mg/ml RNase A. Then the cell suspension was transfer to a 1.5 ml microcentrifuge tube containing 50–100 µl of acid-washed glass beads (Sigma G-8772), and vortexed vigorously for 5 min. Let the tube stand to allow the beads to settle and transfer supernatant to a fresh 1.5 ml microcentrifuge tube. Next 250 µl lysis buffer P2 was

added to the tube and invert gently 4–6 times to mix. The mixture was incubated at room temperature for 5 min. After that, 350µl neutralization buffer N3 was added to the tube and invert immediately but gently 4–6 times. The whole lysate was centrifuged for 10 min at maximum speed in a tabletop microcentrifuge (13,000 rpm or ≥10,000 x *g*). The cleared lysate from the above step was transfer to QIAprep Spin Column and centrifuged for 30–60s. Then QIAprep Spin Column was washed by 0.75 ml of Buffer PE and centrifuging 30–60s. After discard the flow-through, the column was centrifuged for an additional 1 min to remove residual wash. To elute DNA, 25µl water was added into the column for 1 minute's incubation at room temperature, followed by centrifugation at 14,000 rpm for 1 minute. Although the typical yield is up to 1 µg, it is hard to check the plasmid DNA on the gel directly.

The plasmid prepared can be directly used for normal transformation into ultracompetent DH5α E.coli cells, usually 2–5 µl of elute is enough to yield tens to hundreds of clones.

Chapter III The novel zebrafish *udu* gene is essential for primitive hematopoietic cell development

3.1 Characterization of hematopoietic defects in *udu*^{sq1} mutant

3.1.1 General morphological phenotype of *udu*^{sq1} mutant

To study the genetic programs governing vertebrate hematopoiesis, our lab had carried out a forward genetic screen in search of zebrafish mutants having defects in hematopoiesis. Among the hematopoiesis-defective mutants recovered, one mutant line *wz260* exhibited a morphologic phenotype similar to that of zebrafish mutant *ugly duckling* (*udu*^{tu24}) isolated from the Tübingen large-scale screen (Hammerschmidt et al 1996). Complementation analysis further confirmed that *wz260* was a new allele of *udu*^{tu24}, thus this mutant allele was renamed as *udu*^{sq1}. As both *udu* alleles displayed similar phenotype, only *udu*^{sq1} was used for detail analysis in this study.

The *udu*^{sq1} mutant embryo began to show morphological phenotype from 20hpf. At 24hpf, it can be clearly distinguished from the wild type siblings, as its body axis became short and its tail was not properly extend but bent downwards (Figure 3.1A-B). Besides, *udu*^{sq1} mutant could not initiate the circulation, and the cardiac edema was obvious in 2dpf mutant embryos although heart beating was largely normal in the *udu*^{sq1} mutant (Figure 3.1C-D). The severity of phenotype increased over the course of time leading up to embryonic lethality of *udu*^{sq1} mutant at around 7-10dpf. Although *udu*^{tu24} was isolated from Tübingen screen as a mutant

defective in morphogenesis and tail formation, the hematopoietic defects in the *udu*^{sq1} mutant are the major focus in this study.

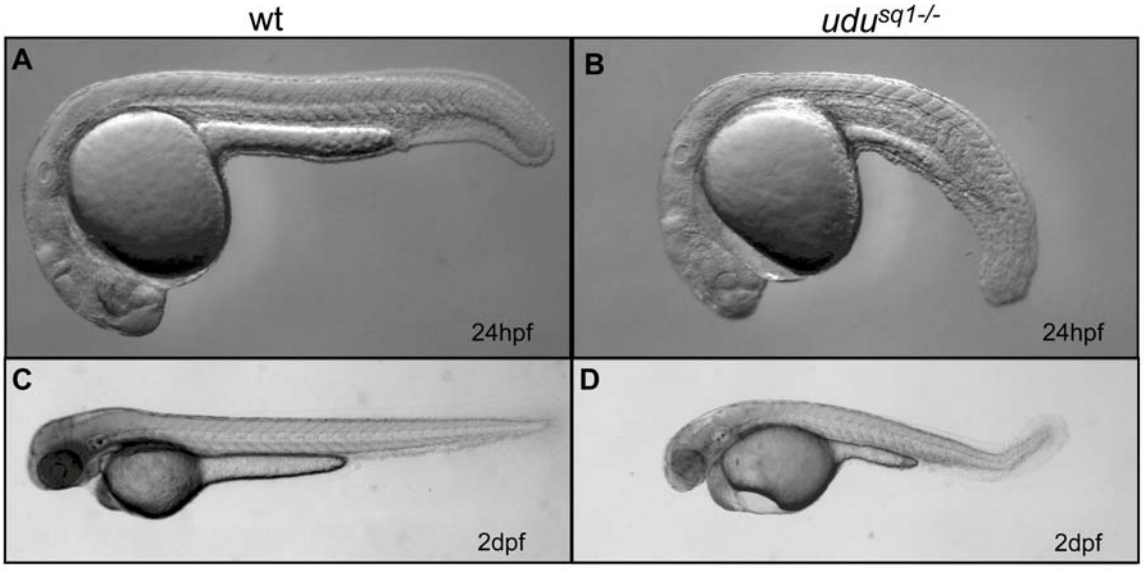


Figure 3.1 Morphology of *udu*^{sq1} mutant. (A-D) Lateral views of live wild type sibling (A, C) and the *udu*$^{sq1-/-}$ mutant (B, D) embryos of 24hpf (A, B) and 2dpf (C, D).

3.1.2 Primitive hematopoietic hypoplasia in *udu*^{sq1} mutant

Visual inspection under microscope revealed that there were very few blood cells in the *udu*^{sq1} mutants. Moreover, they never initiated the circulation, which normally occurs around 26hpf. This hypoplasia phenotype was further confirmed by hemoglobin staining with *o*-dianisidine. Figure 3.2M-N clearly shows that the hemoglobin level in the 2dpf *udu*^{sq1} mutant is severely reduced, and majority of the blood cells found in the heart, tail and yolk surface of wild type embryos are absent in the *udu*^{sq1} mutants.

To explore the primitive hematopoietic defects in depth, expression of early hematopoietic marker gene *scl* was examined by whole mount *in situ* hybridization

(WISH) in *udu*^{sq1} mutant. As shown in Figure 3.2A-B, there is no difference of *scl* expression in both ICM and RBI region between *udu*^{sq1} and wild type siblings before 10-somite stage. This result indicates that *udu* gene is dispensable for the initiation of primitive hematopoiesis. However, from 18-somite stage onwards, expression of *scl* gene in *udu*^{sq1} mutant began to decrease. By 22hpf, primitive erythropoiesis was significantly affected in *udu*^{sq1} mutants shown by the expression of erythroid specific transcription factor *gata1* (Figure 3.2C-D). Notably, the *band3* transcript, encoding an erythrocyte-specific membrane protein critical for erythrocyte maturation (Paw et al 2003), was almost undetectable in *udu*^{sq1} embryos (Figure 3.2E-F). Intriguingly, it was found that *βe3-globin* expression remained relatively normal or only slightly reduced in 22hpf *udu*^{sq1} embryos (Figure 3.2G-H). These observations indicate that the ICM region of 22hpf *udu*^{sq1} embryos contains hematopoietic cells expressing hemoglobin, but they are not normally differentiated erythrocytes. Thus, aberrance in erythrocytes differentiation in *udu*^{sq1} mutants is suggested to be the main reason that causes dramatic decrease of *band3* expression and eventually leads to red blood cell (RBC) hypoplasia at later stages of development as indicated by reduced *βe3-globin* expression from 30hpf onwards in *udu*^{sq1} mutants (Figure 3.2I-L).

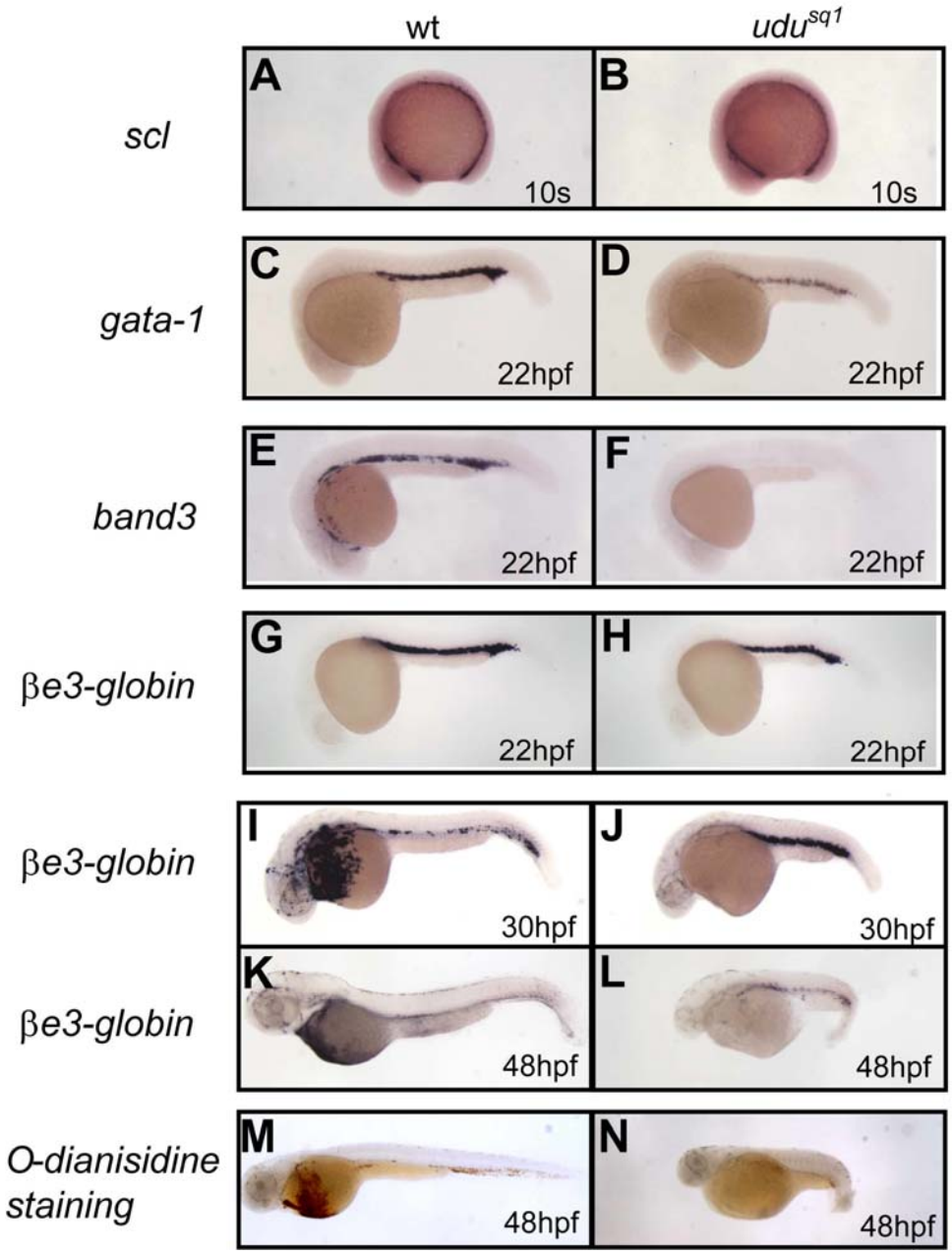


Figure 3.2 Primitive erythroid hypoplasia of *udu*^{sq1} mutant. WISH of *scl* (A, B), *gata1* (C, D), *band3* (E, F), and *βe3-globin* (G-L) expression in wild type siblings (A, C, E, G, I and K) and *udu*^{sq1} mutants (B, D, F, H, J and L). O-dianisidine staining is performed in 2dpf wild type (M) and *udu*$^{sq1-/-}$ (N) embryos. All embryos are in lateral views with anterior to the left.

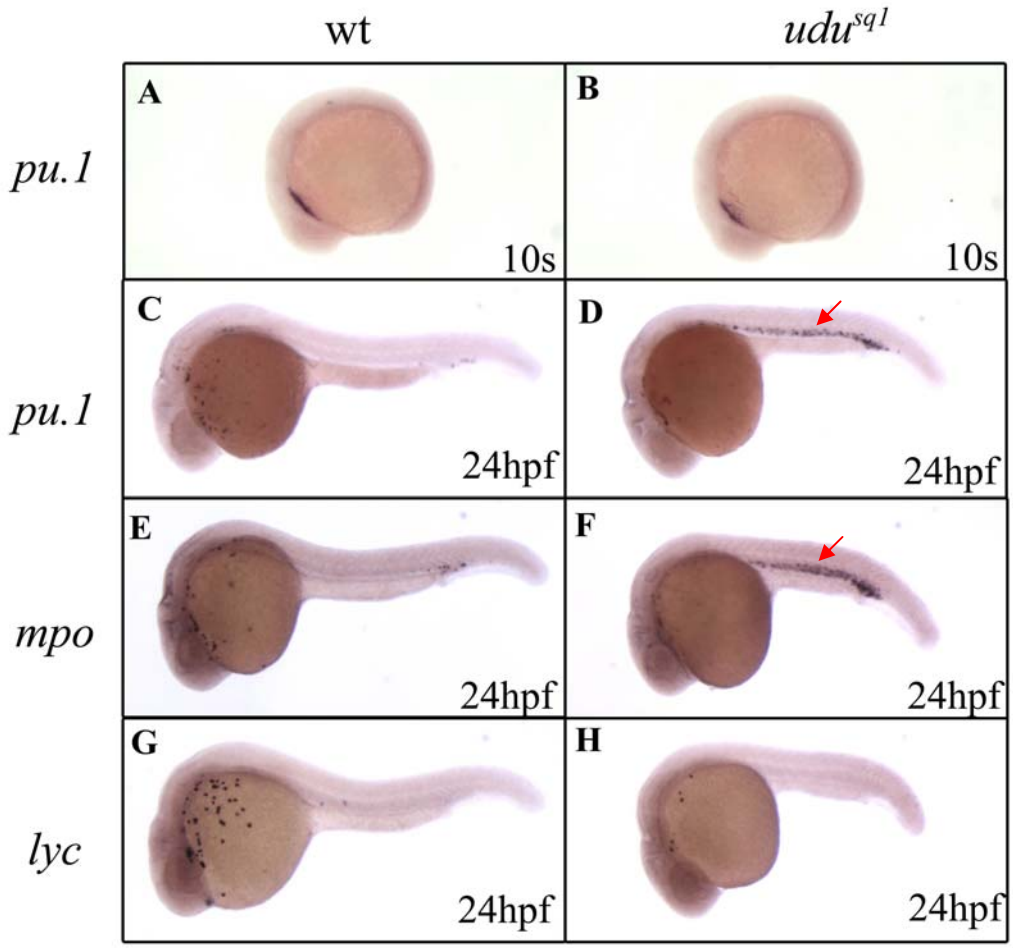


Figure 3.3 Primitive myelopoiesis is defective in the *udu*^{sq1} mutant. WISH of *pu.1* (A, B, C and D), *mpo* (E and F), and *lyc* (G and H) expression of 10-somite (10s) (A and B) and 24hpf (C-H) wild type (A, C, E, G) and *udu*^{sq1} mutant embryos (B, D, F, H). All embryos are in lateral views with anterior to the left. The red arrows indicate the ectopic expression in ICM region.

The initiation of primitive myelopoiesis was also not affected in the *udu*^{sq1} mutant indicated by the normal expression of *pu.1* in RBI before 10-somite stage (Figure 3.3A-B). However, as the development proceeds, the expression of myeloid markers exhibited significant reduction. At 24hpf, WISH of *pu.1*, *mpo*, and *lyc* showed the number of primitive myeloid cells was dramatically reduced in the yolk sac surface (Figure 3.3C-H). Of note, both *pu.1* and *mpo* expressed ectopically in the

ICM region between 24hpf and 30hpf (Figure 3.3C-F). However, such ectopic expression was not seen for two other myeloid markers *lyc* (Figure 3.3G-H) and *l-plastin* (data not shown). In zebrafish, there is a reciprocal negative regulatory relationship between *gata1* and *pu.1* (Rhodes et al 2005). Pu.1 and Gata1 proteins antagonize the expression of each other, thus the transient ectopic expression of *pu.1* and *mpo* is very likely due to decreased *gata1* expression in the ICM region. The absence of ectopic *l-plastin* and *lyc* expression in *udu*^{sq1} mutants further supports the argument, showing that those hematopoietic cells in ICM region expressing *pu.1* and *mpo* did not alter their fate but remained in the early immature erythroid precursor stage.

3.1.3 Primitive *udu*$^{sq1-/-}$ erythroid cells have impaired proliferation and differentiation abilities.

To further illustrate the cellular defects of the hematopoietic hypoplasia in *udu*^{sq1} mutant, TUNEL assay and acridine orange staining were used to check whether this phenotype was due to abnormal apoptosis in the *udu*$^{sq1-/-}$ mutant. As shown in Figure 3.4, there was extensive cell death occurred in the CNS region in *udu*$^{sq1-/-}$ mutant from 18hpf onwards. However, no significant increased apoptosis was observed in the ICM region (Figure 3.4A-F). Thus, this result demonstrates that hematopoietic hypoplasia in *udu*^{sq1} mutant is not due to the abnormal apoptosis of hematopoietic cells. Rather, it is very likely that aberrance in cell proliferation may account for this hematopoietic phenotype found in *udu*^{sq1} mutant.

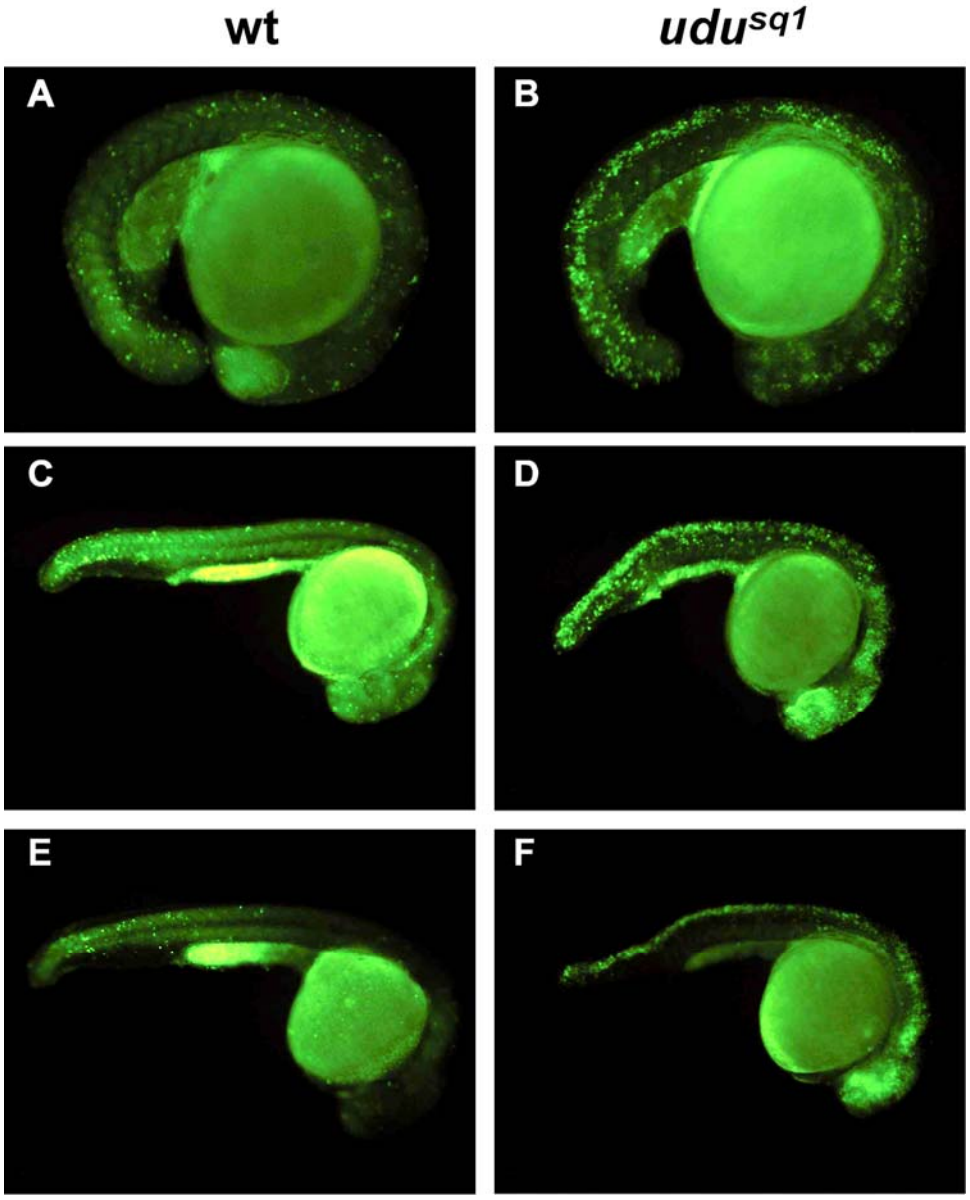


Figure 3.4 Acridine orange staining of cell death in *udu*^{sq1} embryos. Acridine orange staining of 18hpf (A-B), 24hpf (C-D) and 30hpf (E-F) wild type siblings (A, C, and E) and *udu*^{sq1} embryos (B, D, and F). All embryos are orientated with anterior to the right.

To investigate this issue, *udu*$^{sq1+/-}$ heterozygous mutant fish was out-crossed with the Tg(-5.0scl:EGFP)^{sq1} (referred to as Tg(5'5kbscl:EGFP) in the original paper) transgenic fish, in which the expression of the enhanced green fluorescent protein (EGFP) reporter gene is under the control of *scl* promoter (Jin et al 2006). The

resulting *udu*$^{sq1+/-}$/Tg (-5.0scl: EGFP)^{sq1} was then mated with heterozygous *udu*$^{sq1+/-}$ fish. Then GFP positive hematopoietic cells from 24hpf *udu*$^{sq1-/-}$/Tg (-5.0scl: EGFP)^{sq1} and sibling/Tg(-5.0scl:EGFP)^{sq1} embryos were collected by the fluorescent activated cell sorting (FACS) (Figure 3.5A and D) and were subsequently subjected to cell cycle analysis. DNA content analysis using Hoechst 33342 staining revealed that, while sibling hematopoietic cells displayed 45.06%, 43.93% and 11.01% of cells in G1/G0, S and G2/M phases, respectively (Figure 3.5B), *udu*$^{sq1-/-}$ mutant hematopoietic cells exhibited abnormal accumulation in G2/M (43.16%) and reduction in G1/G0 (32.13%) and S (24.71%) phases (Figure 3.5E). Moreover, cytology analysis using these sorted cells further showed that wild type GFP+ cells consisted of mainly erythroid cells in various levels of differentiation (more differentiated cell exhibited a round central nucleus, chromatin condensation, and were smaller in size) and a small portion of myeloid cells (characterized by their irregular nucleus) (Figure 3.5C). In contrast, GFP+ cells in the *udu*$^{sq1-/-}$ mutant, which were mainly erythroid cells, were much larger in cell size and lacked chromatin condensation (Figure 3.5F), and were similar to those found at the 16hpf wild type embryos. This feature of nuclear-cytoplasm asynchrony in the mutant cells is a characteristic of abnormal megaloblastic erythroid cells found in human patients with megaloblastic anemia (Wickramasinghe 2006). Taken together, these results demonstrate that primitive *udu*$^{sq1-/-}$ erythroid cells are defective in cell proliferation and differentiation abilities.

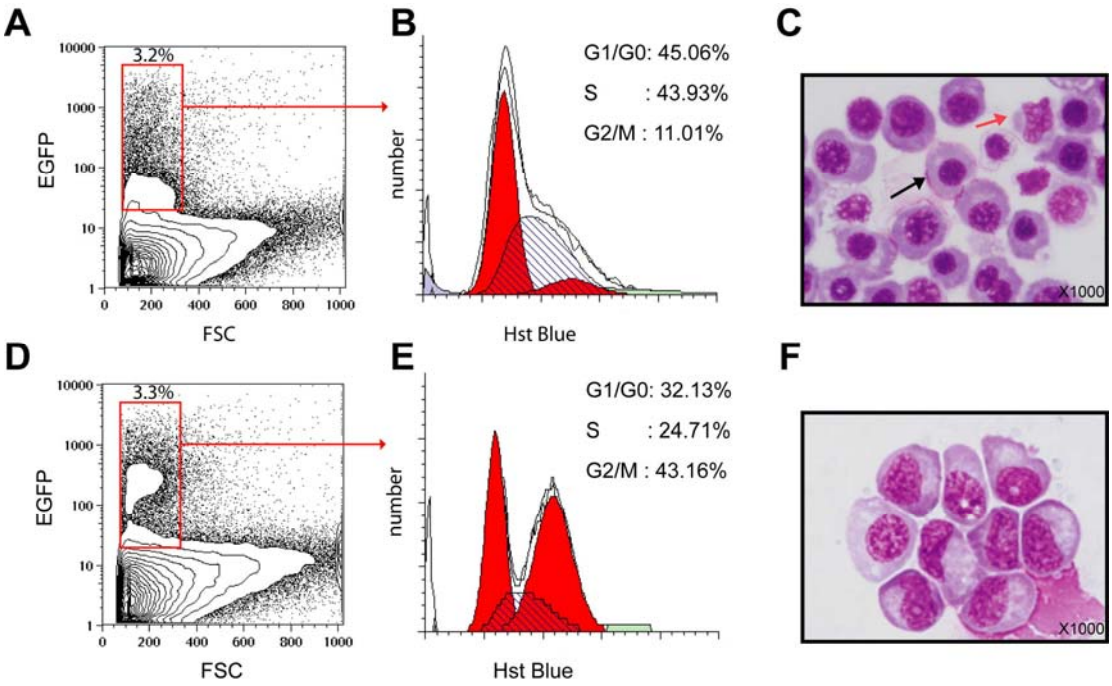


Figure 3.5 Primitive erythroid cells in *udu*$^{sq1-/-}$ mutant are defective in cell proliferation and differentiation. FACS profile of cell suspension from 24hpf wild type sibling (A) and *udu*$^{sq1-/-}$ mutant (D). Y-axis indicates the intensity of the GFP expression and X-axis represents cell size. The GFP-positive hematopoietic cells in A (total of 5,394 cells) (B) and D (total of 6,874 cells) (E) are subjected to DNA content analysis by Hochest33342 staining. The percentages of cells in each phase of cell cycle are given. May-Grunwald/Giemsa staining analysis (magnification x1000) of sorted GFP+ cells in A (C) and D (F). Black and red arrows in C indicate erythroid and myeloid cells respectively.

3.2 *udu* gene functions cell autonomously in primitive erythropoiesis

The mutated gene of *udu*^{sq1} mutant had been mapped to be a novel zebrafish gene (Liu et al 2007). The full-length cDNA of this *udu* gene was used as the template for *in vitro* transcription to make the RNA probe for WISH. Using this probe, *udu* transcript was detected as early as one-cell stage in zebrafish embryos by WISH (Figure 3.6). The maternal *udu* mRNA retained robust expression during blastula stage, and it diminished at the onset of the gastrulation (~6hpf). When segmentation started, the *udu* transcript, presumably the zygotic mRNA, began to re-appear in a ubiquitous manner, and was subsequently enriched in CNS as well as ICM from 18-somite stage onwards (Figure 3.6). At 24hpf *udu* mRNA was also detected in part of the kidney ducts, which was further confirmed by cryo-section (Figure 3.6J).

At 24hpf, although the expression of *udu* gene in ICM region seemed to be weaker than the expression in the nearby kidney, it did co-localize with βE1-globin protein expression (Figure 3.6K-M), confirming that *udu* did express in ICM region. Thus, from the temporal and spatial expressions of *udu* gene, it is very likely that Udu plays a cell-autonomous role during primitive hematopoiesis development. To prove this speculation, early transplantation experiment was performed. Both *udu*^{sq1} mutant and wild type sibling embryos were injected with rhodamine-dextran dye at one cell stage as the donor embryos. When these donors grew to high stage (~3.3hpf), around 15-30 cells were taken out from each *udu*^{sq1} mutant or sibling embryo and then transplanted into the same stage wild type host embryos respectively. Then chimeric host embryos were allowed to develop to around 1.5dpf when blood circulation can be

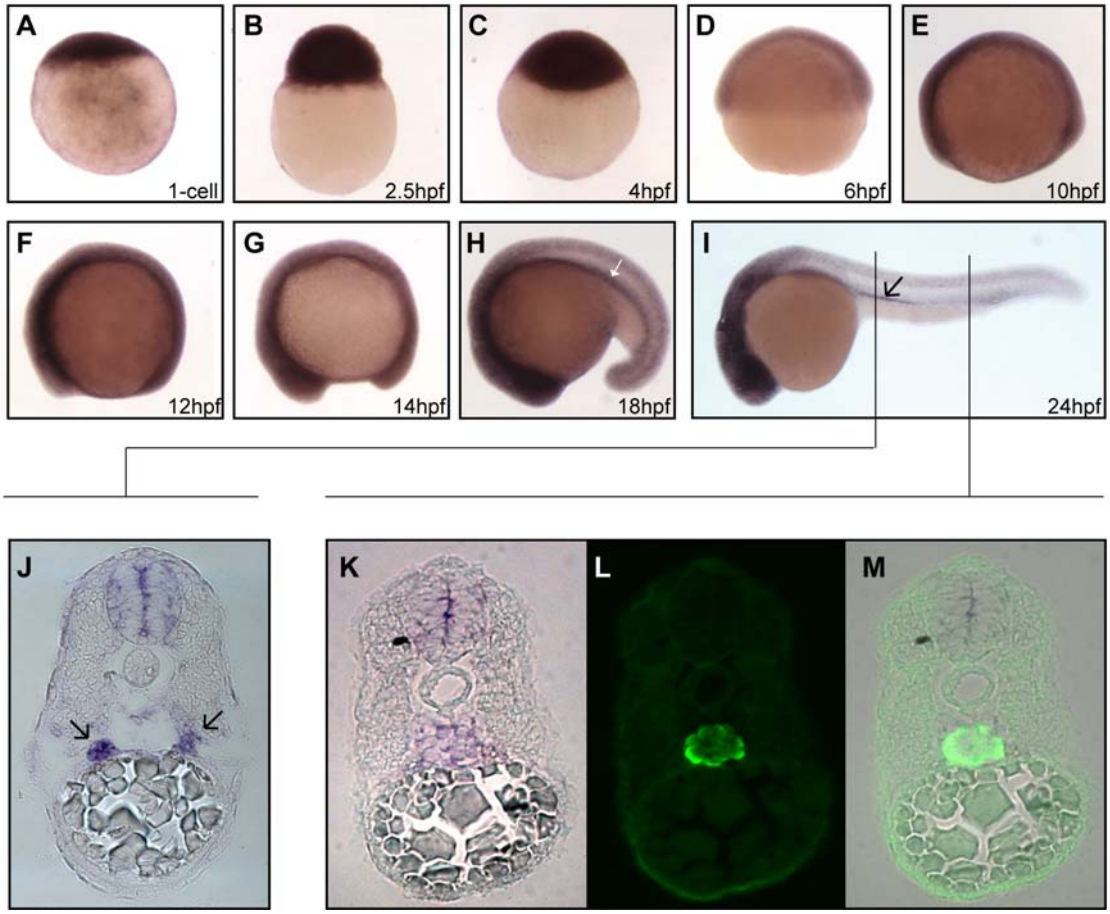


Figure 3.6 Temporal and spatial expression of the *udu* gene during early zebrafish development. (A-I) Lateral views of WISH of *udu* expression in one-cell (A), 2.5hpf (B), 4hpf (C), 6hpf (D), 10hpf (E), 12hpf (F), 14hpf (G), 18hpf (H), and 24hpf (I) embryos. White and black arrows indicate ICM region and the anterior region of kidney ducts respectively. Embryos in A-D are orientated with animal pole on top whereas embryos in E-I are orientated with anterior to the left. Cryo-section is also performed on 24hpf embryo in (I) to show its expression in kidney ducts (J) and ICM region (K) more clearly. Moreover, immunofluorescent staining of βe1globin (L) shows co-localization with *udu* expression (L) in ICM region (M).

easily observed under microscope. Contribution of donor cells to the circulating blood cells in the host embryos was scored by counting the number of the rhodamine-dextran labeled cells (with red fluorescence) in the circulation under fluorescent microscope. The transplantation result was summarized in Table 3.1. As shown in this table, when donor cells were derived from wild type sibling, 42% (50/119) of the recipients bared the rhodamine-dextran labeled donor cells in circulation. And of these 42% recipients, 20% (10/50) contained 10 to 30 circulating donor cells and 22% (11/50) had more than 30 circulating donor cells. In contrast, if the *udu* ^{sq1} mutant cells were used as donors, only 26.7% (16/60) of the host embryos had the donor cells contributing to the blood circulation. More importantly, none of these embryos contained more than 10 circulating rhodamine-dextran labeled blood cells. However, the ratios of wild type sibling and *udu*$^{-/-}$ mutant donor cells contribute to other tissues such as muscle are similar (72.3% and 76.7% respectively). Thus, this transplantation result demonstrates that *udu*^{sq1} cells can contribute to the wild type hosts, but at a much lower ratio and in much fewer numbers, indicating that *udu*^{sq1} cells have the ability to specify into erythroid cells but fail to proliferate and differentiate further. This data is also consistent with the previous characterization result that *udu* is dispensable for the initiation of hematopoiesis, but necessary for the differentiation and maturation of hematopoietic cells. From this transplantation experiment, it is concluded that *udu* gene does play a cell-autonomous role in the development of primitive erythropoiesis.

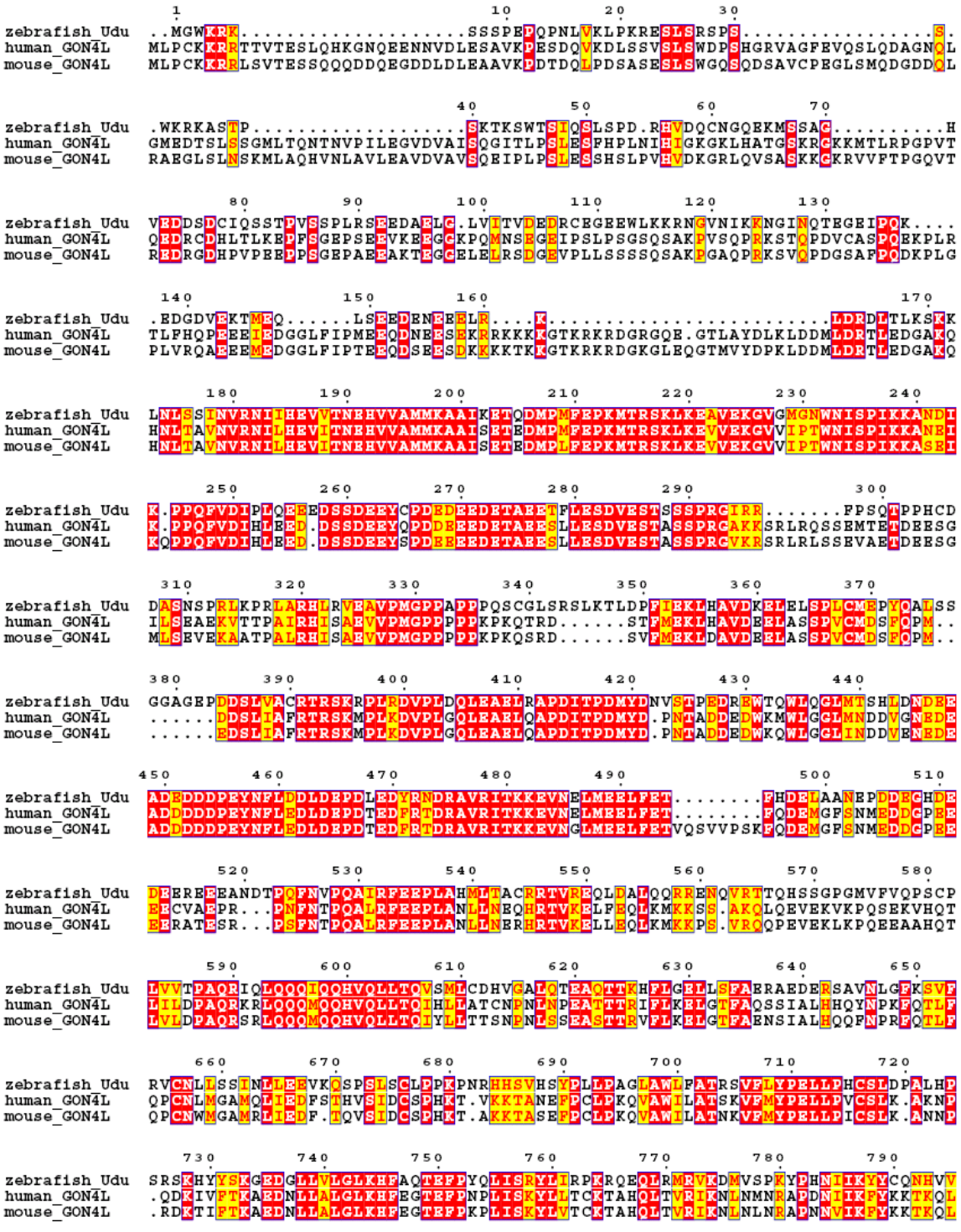
Table 3.1 Summary of cell transplantation analysis

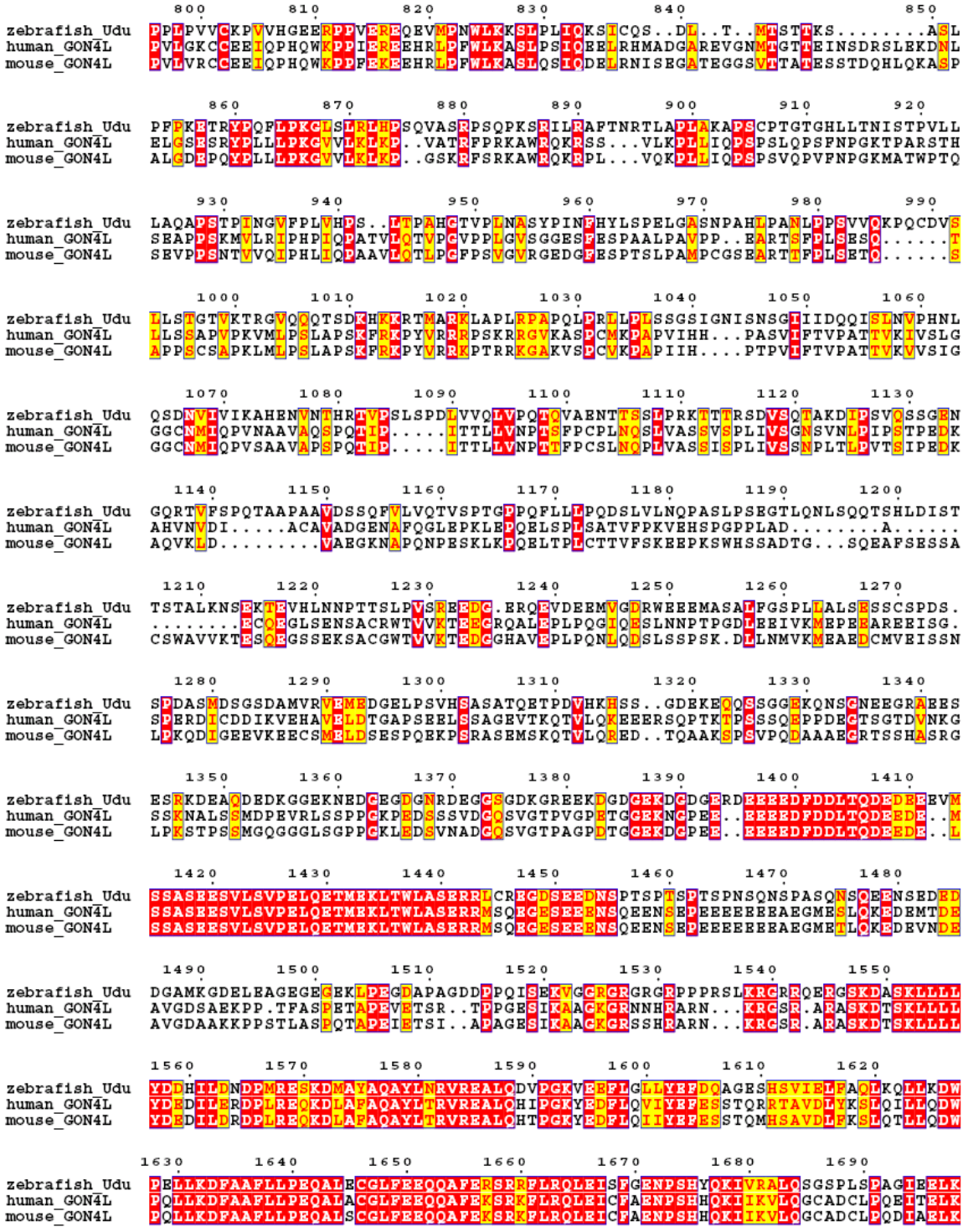
Donor	Number of hosts	No. (%) of hosts with donor-derived tissue				
		Muscle contribution	Blood contribution			
			Total	>30 blood cells	11-30 blood cells	1-10 blood cells
Sibling	119	86 (72.3%)	50(42%)	11	10	29
udu$^{sq1\ -/-}$	60	46(76.7%)	16(26.7%)	0	0	16

3.3 Fishing out the interaction partners of Udu protein

In order to find the mammalian homologues of zebrafish Udu protein, BLAST search was performed in NCBI database, and the BLAST result revealed that zebrafish Udu protein had the highest homology to human and mouse GON4L protein (Figure 3.7). Sequence alignment between these three homologues showed that there were several highly conserved regions (Figure 3.8). The first three conserved regions were named as CR-1, CR-2 and CR-3, because they shared no obvious similarity to any of the known domains although they were highly conserved among three species. The fourth and fifth regions (from a. a 1538 to 1740) were predicted to consist of four α-helices respectively, and their protein structure was very similar to the PAH domain found in yeast SIN3 protein (Wang et al 1990), and thus were designated as PAH-like (PAH-L) 1 and 2 domain. PAH domain is known as Paired Amphipathic Helix Repeat and is composed of four paired amphipathic α-helices. This domain is distantly related to the helix-loop-helix motif, which mediates protein-protein interaction (Spronk et al 2000). The yeast SIN3 protein and its mammalian homologues SIN3A and Sin3B interact

through the PAH domain with numerous sequence specific transcription factors and recruit histone deacetylases to suppress downstream target gene transcription (Silverstein & Ekwall 2005). Thus, zebrafish Udu protein probably form complexes with interaction partners via PAH-L domain similarly. The solution structure (IUG2_A) of the last conserved region of the mouse GON4L (www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=1ug2) revealed that this conserved region resembled the SANT (SW13, ADA2, N-Cor and TFIIIB-like domain) or Myb-DNA binding domain. The SANT and Myb-DNA binding (Myb-DB) domains have a similar overall structure but confer distinct functions (Boyer et al 2004). The Myb-DB domain usually contains two to three tandem repeats, and it recognizes and binds specific DNA sequence; whereas SANT domain usually consists of one to two repeats and is found in the subunits of many chromatin remodeling complexes as the histone-tail-binding module. Considering the fact that the Udu protein contains only one repeat of this domain, this conserved region in Udu protein may resembles the SANT domain in its biological function, and was named as SANT-L domain.


zebrafish_Udu
human_GON4L
mouse_GON4L


zebrafish_Udu
human_GON4L
mouse_GON4L

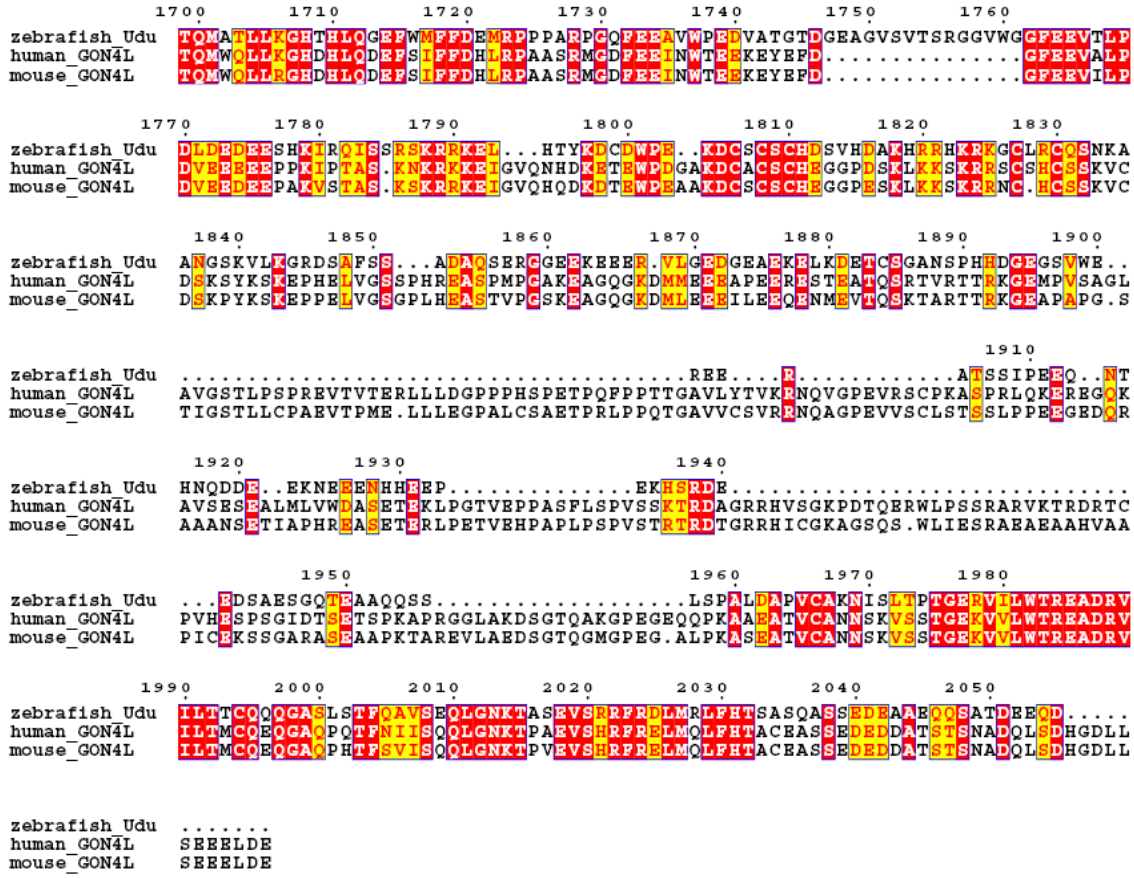


Figure 3.7 Alignment of zebrafish Udu, human and mouse GON4L proteins. Identical amino acids are labeled in red whereas homologous residues, which are scored if they are within a single group of neutral (A, C, G, P, S, T), hydrophobic (I, L, M, V), acidic (D, E, N, Q), basic (H, K, R), or aromatic (F, W, Y), are indicated in yellow.

Figure 3.8 Domain structure of zebrafish Udu protein. Zebrafish Udu protein (2055a.a) contains six conserved regions: CR-1, CR-2, CR-3, PAH-L1, PAH-L2 and SANT-L.

Based on the sequence alignment and domain analyses result one may expects that two PAH-L and SANT-L domain in Udu protein can function as the interaction domains. Thus, in order to find interaction partners of zebrafish Udu protein, yeast two hybrid screen (Y2H) was performed. The bait plasmid containing both PAH-L and SANT-L domain of zebrafish *udu* gene was constructed (pGBKT7-fishbait) and transformed into yeast strain AH109. The toxicity and transcriptional activation test of this bait protein was negative. The library used in this screen was a zebrafish cDNA library generated from a pool of 18hpf, 28hpf, and 48hpf wild type embryos. The efficiency of this screen was 6.25×10^3 cfu/μgDNA. Total clone number screened was ~2×10^6, and around 300 clones were positive for the high stringency test (base on the -trp-leu-his-ade selection). To minimize the false positive clone number, all of them were further inoculated onto the new plates with X-α-gal for the lacZ reporter, which added another layer of stringency control. And more than 95% of the clones were still positive. Thus, a total of 276 positive clones were obtained from this screen. By sequencing and blast analyses, these positive clones represent ~150 unique cDNAs.

To facilitate identification of candidate partners among this large number of positive clones, all the known unique clones were examined in detail and were further classified into five major groups based on their cDNA information. As shown in Table 3.2, the first group of molecules is basically composed of enzymes or molecules involved in metabolisms. But their relationship with Udu protein is not clear. The second group of Y2H result is known as neuronal factors; a total of 15 molecules belong to this group. They are all related with neuronal development and differentiation. As shown in the previous data (Figure 3.4), udu^{sq1} mutant had very obvious cell death and degeneration phenotype in the CNS region, therefore some of the molecules in this group may be the key interaction partners responsible for the

neuronal phenotype, and thus warrant for further analysis. The third group has only 4 molecules, they are known to function in cell division and cell cycle regulation, and 3 of them were picked repeatedly from the screen, indicating the strong interaction between them and Udu protein. Of note, this finding is consistent with the characterization result that *udu*$^{sq1-/-}$ erythroid cells are found to be severely impaired in the cell cycle progression. Thus, it is reasonable to speculate that this group of molecules may function as the interaction partner of Udu protein in maintaining the normal blood cell proliferation and differentiation. Moreover, among them, minichromosome maintenance protein Mcm3 and Mcm4 have similar expression pattern as Udu in zebrafish (expression data from ZFIN http://zfin.org/cgi-bin/webdriver?MIval=aa-xpatselect.apg), suggesting that they are very likely to interact with Udu protein in vivo. The fourth group is mainly composed of molecules known as muscle factors, many of which are giant structure proteins. Although *udu* mutant has somite and muscle defect, whether Udu plays a role in muscle development through interaction with these molecules is currently unclear. The rest of candidates found in this screen belong to the last group; they are either unknown protein or known protein with functions not belonging to any of the above groups. Thus they are classified as the last group.

As the bait protein used for this two hybrid screen was designed to include both PAH-L and SANT-L domain, it is also important to determine whether the candidate molecules interact with one of them or both of them. Several molecules from different groups in Table 3.2 were selected to do individual hybrid test with bait construct containing only SANT-L domain (pGBKT7-fishSANT) or PAH-L domain (pGBKT7-fishPAH). Interestingly, all the candidate molecules tested so far interacted only with PAH-L domain but not SANT-L domain. Thus, this result appears to

confirm the domain analyses result that PAH is the major protein interaction domain in Udu protein.

Table 3.2 Summary of clones from yeast two hybrid screen

	Identity of the clone	**Clone number**
Enzyme or molecules involved in metabolism	PREDICTED: Danio rerio similar to diacylglycerol kinase, delta	1-1,
	Danio rerio propionyl-Coenzyme A carboxylase, alpha polypeptide(pcca)	1-27, 3-49, 3-50, 4-6, 4-34, 4-36, 4-52, 6-34
	Danio rerio 3-hydroxyisobutyrate dehydrogenase (hibadh)	2-19, 2-30, 2-42, 3-3, 3-4, 3-21
	PREDICTED: Danio rerio similar to butyrobetaine (gamma) 2-oxoglutarate dioxygenase 1 (gamma-butyrobetaine hydroxylase) transcript variant 3	1-30, 2-28, 3-22, 4-37-1
	Danio rerio AU RNA binding protein/enoyl-Coenzyme A hydratase (auh)	6-10,
	Danio rerio ADP-ribosylation factor 1 like	6-12,
	PREDICTED: Danio rerio similar to Protein kinase C-binding protein NELL2 precursor (NEL-like protein 2) (Nel-related protein 2)	6-35,
	Danio rerio NAD(P)H dehydrogenase, quinone 1 (nqo1)	6-39,
Neuronal Factors	Danio rerio midkine-related growth factor b (mdkb) or pleiotrophin 2	1-29,
	Danio rerio green-sensitive opsin 1 (grops1)	1-19,
	Danio rerio nocA related zinc finger 1 (nlz1)	2-1, 2-3, 2-43, 5-19, 5-38, 6-48
	Danio rerio ribonucleoprotein (elrD) OR Danio rerio ELAV (embryonic lethal, abnormal vision, Drosophila)-like 4 (Hu antigen D) (elavl4)	2-39,

	Danio rerio dachshund c (dachc)	3-27,
	PREDICTED: Danio rerio similar to centrosome protein cep290 (LOC560588)	3-36,
	Danio rerio ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 (Hu antigen C) (elavl3)	5-8,
	Danio rerio thrombospondin 4 (thbs4)	6-13,
	Danio rerio laminin, alpha 1 (lama1)	6-15,
	Danio rerio retinoschisis (X-linked, juvenile) 1 (rs1)	6-20,
	Danio rerio reticulon 1-c (rtn1), alternatively spliced	6-22,
	PREDICTED: Danio rerio similar to Protein kinase C-binding protein NELL2 precursor (NEL-like protein 2) (Nel-related protein 2)	6-35,
	Danio rerio odd Oz/ten-m homolog 4 (odz4)	6-21,
	Danio rerio HuG (hug)	6-46,
	Danio rerio ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	7-30,
Molecules related with cell division	Danio rerio similar to Kinesin-like protein KIF2C (Mitotic centromere-associated kinesin) (MCAK)	1-5, 2-7, 2-47, 4-18, 4-47
	Danio rerio MCM4 minichromosome maintenance deficient 4, mitotin	1-6, 3-12, 3-30, 5-3,
	PREDICTED: Danio rerio similar to BUB1 budding uninhibited by benzimidazoles 1 homolog beta	1-20, 1-32, 2-25, 4-46, 4-49, 5-27, 5-35, 7-9, 7-25, 2-9
	Danio rerio MCM3 minichromosome maintenance deficient 3 (S. cerevisiae) (mcm3)	6-23,
Muscle Factors	Danio rerio profilin 2 like	1-3,
	PREDICTED: Danio rerio similar to Nebulin (LOC555656)	2-17-1, 5-7, 5-18, 5-37, 7-17, 5-21, 6-31, 5-37, 4-37-2

	PREDICTED: Danio rerio similar to titin isoform N2-B	1-31, 2-21
	Danio rerio tropomyosin 4 (tpm4), transcript variant 1	2-29,
	PREDICTED: Danio rerio myosin, heavy polypeptide 1, skeletal muscle (myhz1)	2-36, 4-3
	Danio rerio myosin, light polypeptide 2, skeletal muscle (mylz2)	5-6,
	Danio rerio myosin, light polypeptide 9, like (myl9l), mRNA	6-37,
	Danio rerio troponin I type 2 (skeletal, fast) (tnni2)	5-5,
	PREDICTED: Danio rerio similar to Myomesin 2 (LOC571300)	5-11,
	PREDICTED: Danio rerio similar to Filamin A (Alpha-filamin) (Filamin 1) (Endothelial actin-binding protein) (Actin-binding protein 280) (ABP-280) (Nonmuscle filamin)	6-14, 5-20
	PREDICTED: Danio rerio similar to gamma filamin	4-10,
	PREDICTED: Danio rerio similar to myosin binding protein C, fast-type	7-19,
Others and unknown	Danio rerio hairy/enhancer-of-split related with YRPW motif 2 (hey2)	3-17, 1-15
	Danio rerio calponin 2 (cnn2) mRNA	1-33, 2-18, 2-33, 2-48, 3-23, 4-19, 5-26
	Danio rerio protein arginine methyltransferase 1 (prmt1)	1-24, 2-10, 2-14, 2-20, 2-23, 2-41, 3-11, 3-34, 4-24, 5-16,
	Danio rerio coactivator-associated arginine methyltransferase 1(carm1)	1-14, 2-2
	PREDICTED: Danio rerio similar to STAT6	1-34,
	Danio rerio heterogeneous nuclear ribonucleoprotein	2-15, 2-35, 3-37, 4-

	U-like 1(hnrpul1)	51
	PREDICTED: Danio rerio similar to ankyrin repeat domain 15	3-1,
	PREDICTED: Danio rerio similar to striated muscle preferentially expressed protein	4-8, 4-40, 3-35
	Danio rerio transferrin-a (tfa)	3-43,
	Danio rerio bcl2-like (bcl2l)	4-2,
	Danio rerio SNW domain containing 1 (snw1) or ski interactiong protein	4-14, 5-9
	Danio rerio digestive-organ expansion factor (def)	5-13,
	Danio rerio bromodomain containing 8	5-22, 5-30,
	Danio rerio thrombospondin 4 (thbs4)	6-13,
	Danio rerio upstream transcription factor 2, c-fos interacting (usf2)	7-7,
	PREDICTED: Danio rerio similar to CXXC finger 6	1-35, 3-31, 4-7, 2-6, 2-11, 3-20, 4-44
	PREDICTED: Danio rerio similar to elastin microfibril interfacer 2 (LOC571391)	6-24, 5-39, 3-33
	Danio rerio ripply3 (ripply3) possiblely	3-24,
	PREDICTED: Danio rerio similar to oxysterol-binding protein-like protein 6	3-40, 4-48
	Danio rerio actin related protein 2/3 complex, subunit 5A	3-45,
	Danio rerio TRK-fused gene	3-52, 4-41
	Danio rerio bcl2-like (bcl2l)	4-2,
	Danio rerio polymerase (RNA) I polypeptide C (polr1c) or Danio rerio RNA polymerase I 140 kDa subunit	4-16,
	Danio rerio mitchondrial cyt b gene for cytochrome b	4-33,

	PREDICTED: Danio rerio similar to centrosome-associated protein 350 (LOC564120)	6-4,
	Danio rerio proteasome (prosome, macropain) subunit, beta type, 3 (psmb3)	6-5,
	Danio rerio protein disulfide isomerase-related protein P5 precursor (pdip5)	6-28,
	Danio rerio hypoxia-inducible factor 1, alpha subunit, like (hif1al)	6-33,
	Danio rerio kelch-like 12 (Drosophila) (klhl12)	6-1,
	Danio rerio kinesin-like protein 2 (knsl2)	7-4,

3.4 Discussion

In this study, I have showed that the *udu* gene, which encodes a novel zebrafish protein containing two PAH-L repeats and a SANT-L domain, plays an important role in primitive hematopoiesis development.

3.4.1 Function of *udu* gene in early embryogenesis

Although WISH showed that *udu* RNA was ubiquitously expressed throughout early development, *udu* gene appeared to be dispensable for early embryogenesis as well as initiation of primitive hematopoiesis. Considering *udu* RNA is maternally deposited in embryos and remains robust until gastrulation stage, absence of early phenotype in the udu^{sq1} mutant is possibly due to the functional compensation of maternal *udu* RNA. In order to test this possibility, one morpholino was designed to block the translation of *Udu* protein, but the morphant phenotype was very similar to the

zygotic mutant and no early phenotype in embryogenesis was observed. One possible explanation for this result is that Udu simply does not play a major part in early development. The second possibility is due to the maternal effect that had been also reported by other group (Gore et al 2005). They found that injection of antisense morpholinos targeting zebrafish Nodal-related morphogen *Squint* (*Sqt*) into fertilized embryos resulted in similar phenotype as zygotic *sqt* mutants. But injection of the same morpholinos into oocytes can cause severe loss of dorsal structures which is not seen in the zygotic *sqt* mutant embryos. Thus, one may expect earlier or more severe phenotypes if maternal *udu* RNA is removed in oocytes. Alternatively, more delicate primodial germ-cell replacement approach described by Ciruna et al can be used to address this issue in the future (Ciruna et al 2002), this approach will generate mutant completely devoid of maternal RNA.

3.4.2 Identification of *udu* as a novel factor essential for primitive hematopoiesis

Characterization data of udu^{sq1} mutant strongly suggests that zebrafish *udu* gene is critical for primitive hematopoiesis. And this requirement of Udu in erythroid development seems to restrict to the lineage differentiation stage. The specification of early hematopoietic progenitors is not affected in the $udu^{sq1-/-}$ mutant, but the subsequent erythroid lineages development is highly dependent on the functional *udu* gene. Hematopoietic specific factors such as *scl*, *lmo2*, and *gata1* all show a gradual decrease after 10-somite stage in $udu^{sq1-/-}$ mutant; while those late hematopoietic markers for more differentiated erythroid cells such as *band3* is more severely affected in $udu^{sq1-/-}$ mutant. FACS and DNA content analysis further confirm that

udu$^{sq1-/-}$ hematopoietic cells are abnormally blocked in the cell cycle progression. Therefore, loss of *udu* gene blocks erythroid cells differentiation and makes them not only abnormally stay at the precursors stages but also with impaired proliferation ability. However, it is not clear whether Udu is only required in a special time window during erythropoiesis or is consistently necessary for all the stages of erythroid development. This is possible to be tested in the mammalian in vitro culture system if the mammalian homologue of zebrafish *udu* functions similarly in hematopoiesis development.

Compared with erythropoiesis, the role of Udu in myelopoiesis is less well understood in this study, especially their defects at the cellular level. The characterization data found that initiation of myelopoiesis was normal in the *udu*^{sq1} mutant, but further development of primitive myeloid cells was also significantly affected shown by severely reduced marker gene expression. However, it is not clear that whether loss of *udu* gene also lead to block of cell cycle progression in myeloid cells. There are several limitations for understanding the cellular defects of primitive myeloid cells in *udu*^{sq1} mutant. The *scl* promoter droved GFP transgenic line is not suitable for FACS analysis in this case, as most GFP positive hematopoietic cells are erythroid cells. Besides, cell number of primitive myeloid lineage is also much less than erythroid lineage, which is another challenge for FACS. Alternatively, *udu* splicing morphants in the myeloid specific transgenic background (such as *pu.1* transgenic line) can be used to explore the cellular defect of *udu*$^{sq1-/-}$ myeloid cells using FACS approach in the future.

3.4.3 Cell autonomous role of *udu* gene in primitive hematopoiesis

In this study, both the expression pattern of *udu* gene and early transplantation experiment suggest that *udu* gene functions cell autonomously to regulate the primitive erythropoiesis. In the transplantation experiment, donor cells taken from both wild type sibling and $udu^{sq1-/-}$ mutant were placed into wild type host embryos, then contribution of the donor cells to the circulating blood cells in the hosts was scored as the main criteria. The conclusion from this transplantation is that *udu* gene is cell autonomously required for primitive erythroid development. However, it is difficult to assay the contribution of donor cells to the host myeloid cells due to lack of myeloid specific transgenic line as the donors and the lower number of myeloid cells. Although whether *udu* gene play a cell autonomous role in primitive myeloid development is not clear at the current stage, similar phenotype found in the myelopoiesis and erythropoiesis in the $udu^{sq1-/-}$ mutants indicates that *udu* gene may function similarly in both process.

The transplantation experiment performed in this study (mutant → wild type) suggests that *udu* gene functions cell autonomously in erythroid development, however, the possibility that $udu^{sq1-/-}$ erythroid cells are defective due to the combination of both cell and non-cell autonomous effects can not be ruled out. This hypothesis can be proved by reverse transplantation, which evaluates the contribution of wild type donors to $udu^{sq1-/-}$ mutant hosts. However, udu^{sq1} mutants have other defects including short body axis and abnormally formed somites and tail, and blood circulation is never initiated though residual blood cells usually can be found in udu^{sq1} mutant. In such a mutant background, it will be impossible to evaluate the contribution of wild type donors to the mutant hosts by observing circulating blood

cells directly. Other evaluation method, such as checking the erythroid markers expression, is not feasible as all the erythroid markers tested have residual expression in *udu*^{sq1} mutant. But it is possible to use the absence of ectopic *pu.1* and *mpo* expression in the ICM region as a criterion for assaying contribution of wild type cells to the mutant blood cells in the future. If *udu* gene function only cell autonomously in the erythroid development, absence of ectopic *pu.1* or *mpo* expression is expected when wild type cells contribute to the *udu*$^{sq1-/-}$ mutant host in the ICM region. Otherwise, non-cell autonomous effect of *udu* gene is also suggested.

3.4.4 The potential interaction partners of Udu protein

Based on the sequence alignment and structure analyses, Udu protein contains a putative SANT-L domain. It is known that most of the SANT domain containing molecules interact with histone tails to achieve their roles in chromatin remodeling (de la Cruz et al 2005). But none of the histone proteins were fished out in the yeast two hybrid screen in this study. There are two possible explanations for this result. One possibility is that the *udu* SANT-L domain does interact with histone proteins, but the interaction ability of PAH-L domain is so strong that somehow it interferes with or masks the function of SANT-L domain. An alternative explanation would be that the SANT-L domain does not function as the real SANT domain and rather it functions as the Myb-DB domain in the Udu case. And this hypothesis needs be further tested.

Among the clones fished out from yeast two hybrid screens, zebrafish Mcm4 and Mcm3 protein emerged as very interesting candidates. The MCM proteins were

originally identified as proteins required for minichromosome maintenance in yeast *Saccharomyces cerevisiae* (Maine et al 1984). In eukaryotes, MCM2 through -7 is a family of six highly conserved and structurally related proteins, which form the key component of pre-replication complex (preRC) as the replication-licensing factors for DNA duplication during the cell cycle (Maiorano et al 2006). Besides as part of the preRC at origins during replication initiation, MCM proteins also act during elongation, presumably as a helicase at replication forks (Labib et al 2000; Moyer et al 2006). But the in vivo analysis of MCM proteins functions in multicellular organism has been scarce. Recently Ryu et al reported isolation of zebrafish *mcm5* mutant (Ryu et al 2005). They found that in retina *mcm5* expression was maintained only in proliferative retinal cells but down- regulated in differentiated cells. Consistently, prolonged S phase and cell-cycle-exit failure have been detected in mcm5 mutant retina cells (Ryu et al 2005). Although detail studies of zebrafish *mcm3* and *mcm4* have not been reported, their expression has been characterized and available in ZFIN expression database. Both *mcm3* and *mcm4* genes have a similar expression pattern as *udu* gene. In early stage, they express ubiquitously but decrease gradually after 1dpf with expression only in the certain region of brain. Moreover, comparison of expression profile between wild type and udu^{sq1} mutant by microarray analysis also identify a series of MCM protein as potential targets including *mcm2*, *mcm4* and *mcm3* (Liu et al 2007). Taken together, zebrafish Mcm3 and Mcm4 protein are very possible to be the real interaction partners of Udu protein. However, their *in vivo* interaction needs to confirmed, for example by immunoprecipitation, in the future. And *mcm3* and *mcm4* morphants can be examined to provide further evidence as well.

Chapter IV Characterization and positional cloning of zebrafish *tc-244* mutant

4.1 *tc-244* mutant exhibits a definitive specific phenotype

Tc-244 is another interesting mutant isolated from our lab's forward genetic screen. By visual inspection under optical microscope, *tc-244* mutant was indistinguishable from wild type siblings before 3.5dpf. But after that homozygous *tc-244* mutants gradually display a phenotype of smaller head and eyes, also with intensified eye color compared with wild type siblings (Figure 4.1A-B). From 5dpf onwards, the speed of blood circulation in some mutant larvae began to decrease and they eventually died by 10-14dpf.

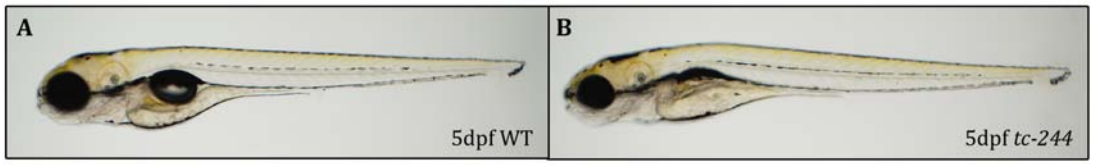


Figure 4.1 Morphology of *tc-244* mutant. Lateral views of live wild type sibling (A) and homozygous *tc-244* mutant (B) larva of 5dpf.

Consistent with the normal morphology and behavior of *tc-244* mutant before 3.5dpf, the expression of hematopoietic marker *βe1-globin* for erythroid cells and *lyc* for myeloid cells also showed no difference between mutants and wild type siblings before 3.5dpf (Figure 4.2), suggesting that primitive hematopoiesis is largely normal in the *tc-244* mutant.

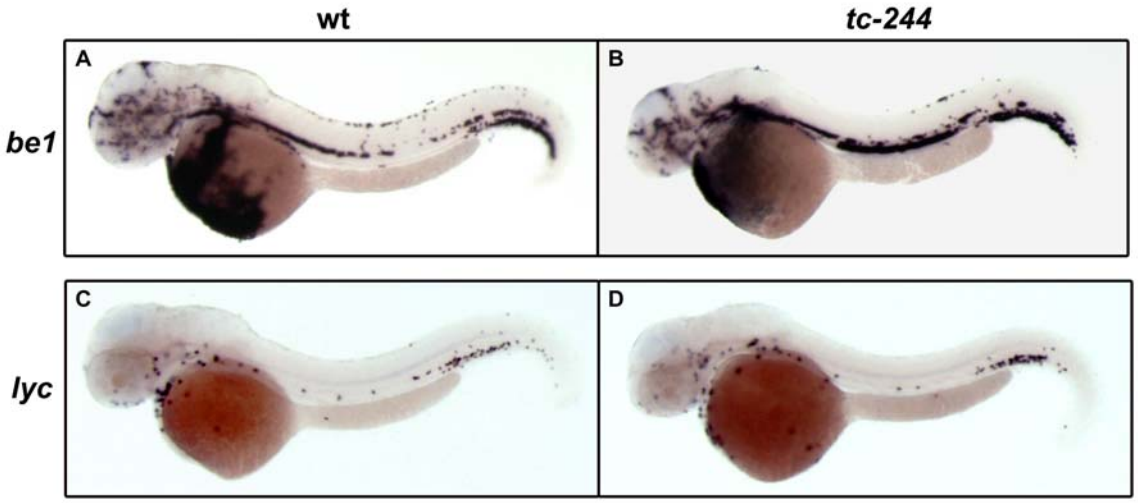


Figure 4.2 Primitive hematopoiesis is normal in *tc-244* mutant. WISH showed that the expression level of hematopoietic markers *βe1-globin* (A, B) and *lyc* (C, D) was similar in 2dpf wild type (A, C) and homozygous *tc-244* mutant (B, D).

From 4dpf onwards, although there was no obvious abnormality found in the *tc-244* mutants by visual inspection of blood circulation, some marker genes expression pattern did reveal significant difference between homozygous *tc-244* mutant and wild type siblings. As shown in the Figure 4.3A-B, the expression of recombination-activating gene1 (*rag1*), which catalyzes the rearrangement of immunoglobulin genes in immature B lymphocytes and T cell receptor genes in immature T lymphocytes respectively (Willett et al 1997), was totally lost in the *tc-244* mutant thymus (Figure 4.3B). *rag1* gene is considered as one of the early markers for lymphoid lineage development, thus this result implies that *tc-244* mutant is defective in lymphoid development. Of note, the specification of T lymphocytes highly depends on signals from their residential environment-thymus, therefore the thymus marker *foxnI* (*whnb*), which is indispensable for thymic epithelial cell differentiation (Boehm et al 2003; Trede et al 2001), was used to examine the thymus development of *tc-244* mutants. The wild type siblings expressed *foxnI* at the third

pharyngeal branchial arch and exhibited a scattered pattern (Figure 4.3C), but in the *tc-244* mutant, *foxnI* expression was severely condensed and was more obvious to detect in the same region (Figure 4.3D). This abnormal condensed expression pattern in *tc-244* mutant indicates the thymic rudiment and epithelial cells are specified but the thymus primordial is not expanded probably due to lack of lymphoid cells colonization in the *tc-244* mutant. Thus based on these results, it is concluded that the lymphoid lineage is not properly specified in the *tc-244* mutants.

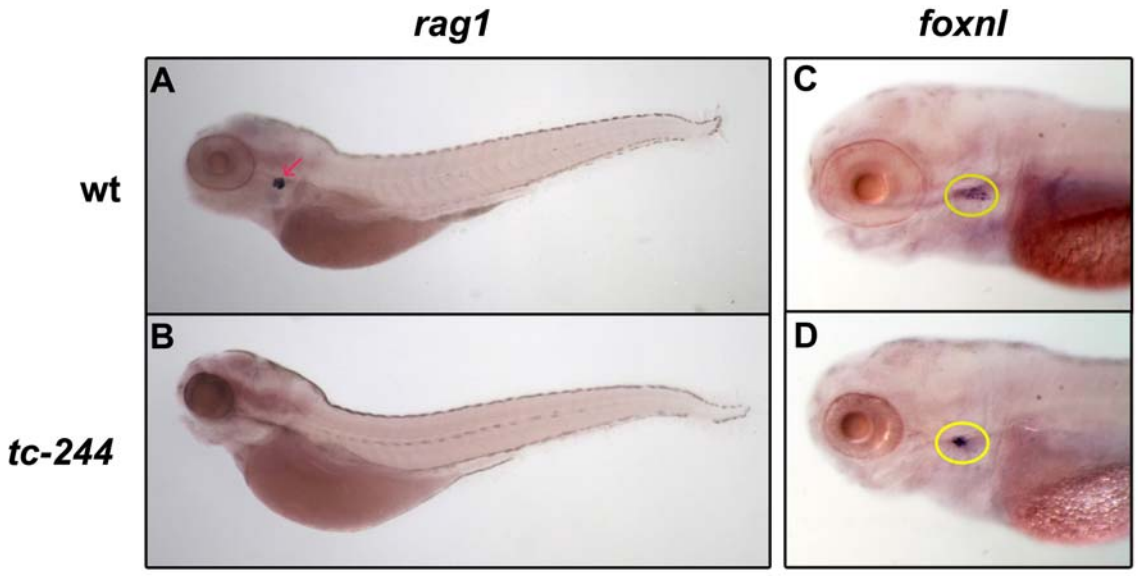


Figure 4.3 Lymphoid lineage development is defective in *tc-244* mutant. *Rag1* expression is detected in 4.5dpf wt (A) but not in the 4.5dpf *tc-244* mutant (B). *foxnI* expression exhibits a scattered pattern in 4dpf wt (C) and a condensed pattern in 4dpf *tc-244* mutant (D). Red arrow indicates the *rag1* expression in thymus. Yellow circles indicate *foxnI* expression in the third pharyngeal branchial arch.

To further examine whether the defects in *tc-244* mutant restrict to the lymphoid lineage or other lineages of definitive hematopoiesis are also affected in this mutant, the expression of erythroid specific *βe1-globin* and myeloid specific *lyc* genes were selected as markers to check the *tc-244* mutants. Indeed, the expression level of

both markers was significantly down-regulated in 5dpf *tc-244* mutants. In wild type siblings, *βe1-globin* marked erythroid cells were found mainly in the pharyngeal arches, PBI, and kidney (Figure 4.4A). But most of these erythroid cells in the *tc-244* mutant were absent, especially in the kidney and PBI region (Figure 4.4B). Similarly, *lyc* marked myeloid cells mainly resided in kidney and scattered across the ventral posterior tail in the wild type (Figure 4.4C), but in *tc-244* mutant *lyc* expression was also lost (Figure 4.4D). Taken together, these data demonstrate that major lineages of definitive hematopoiesis were all severely impaired in the *tc-244* mutants, suggesting that loss of *tc-244* probably disrupts the early events of definitive lineages specification.

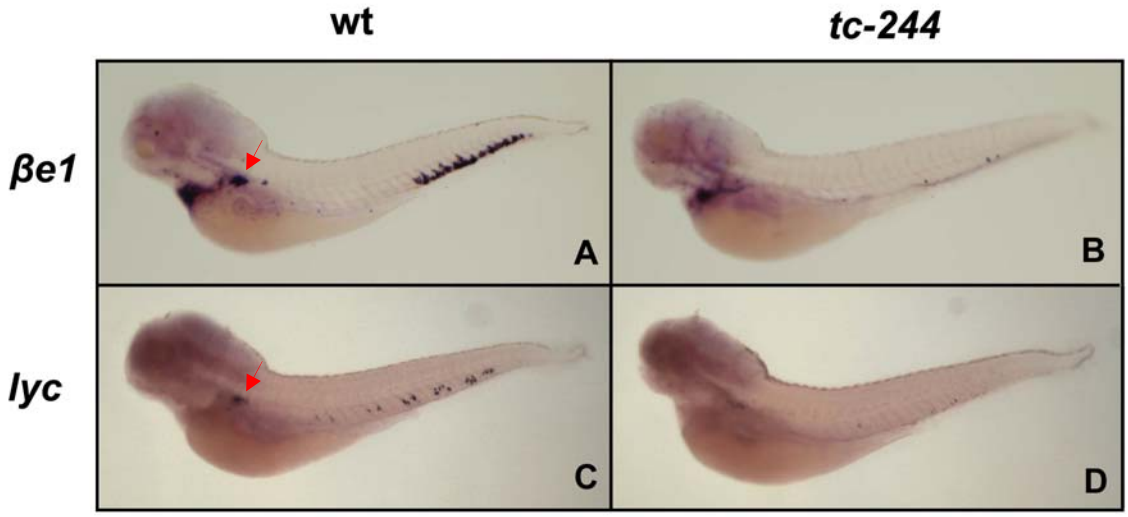


Figure 4.4 Definitive erythroid and myeloid lineage development is defective in *tc-244* mutant. WISH of *βe1 globin* (A-B) and *lyc* (C-D) expression in 5dpf wt (A, C) and *tc-244* mutant (B, D) larvae. The red arrows indicate the expression in kidney.

To investigate this possibility, the expression of *runx1* and *c-myb*, two marker genes of definitive HSCs, was examined. In 35hpf *tc-244* mutant, definitive HSCs associated with the ventral wall of dorsal aorta in the AGM region seemed to be

properly specified in *tc-244* mutant shown by the normal expression of *c-myb* (Figure 4.5A-B) and *runx1* (data not shown, pattern similar as *c-myb*). However, from 3dpf onwards, *c-myb* expression began to decrease significantly in *tc-244* mutants. In 5dpf wild type siblings, *c-myb* was expressed strongly in thymus, kidney, and PBI (Figure 4.5C), but this pattern was not seen in the *tc-244* mutant (Figure 4.5D). Consistently, the expression of transcription factor *scl* which is essential for HSCs specification also showed similar result (Figure 4.5E-F), especially in the PBI region (Figure 4.5G-H). Taken together, these results suggest that although definitive HSCs were initially formed, their survival or further differentiation was impaired in the *tc-244* mutants, which led to the absence of major lineages of definitive hematopoietic cells including erythroid, myeloid and lymphoid cells.

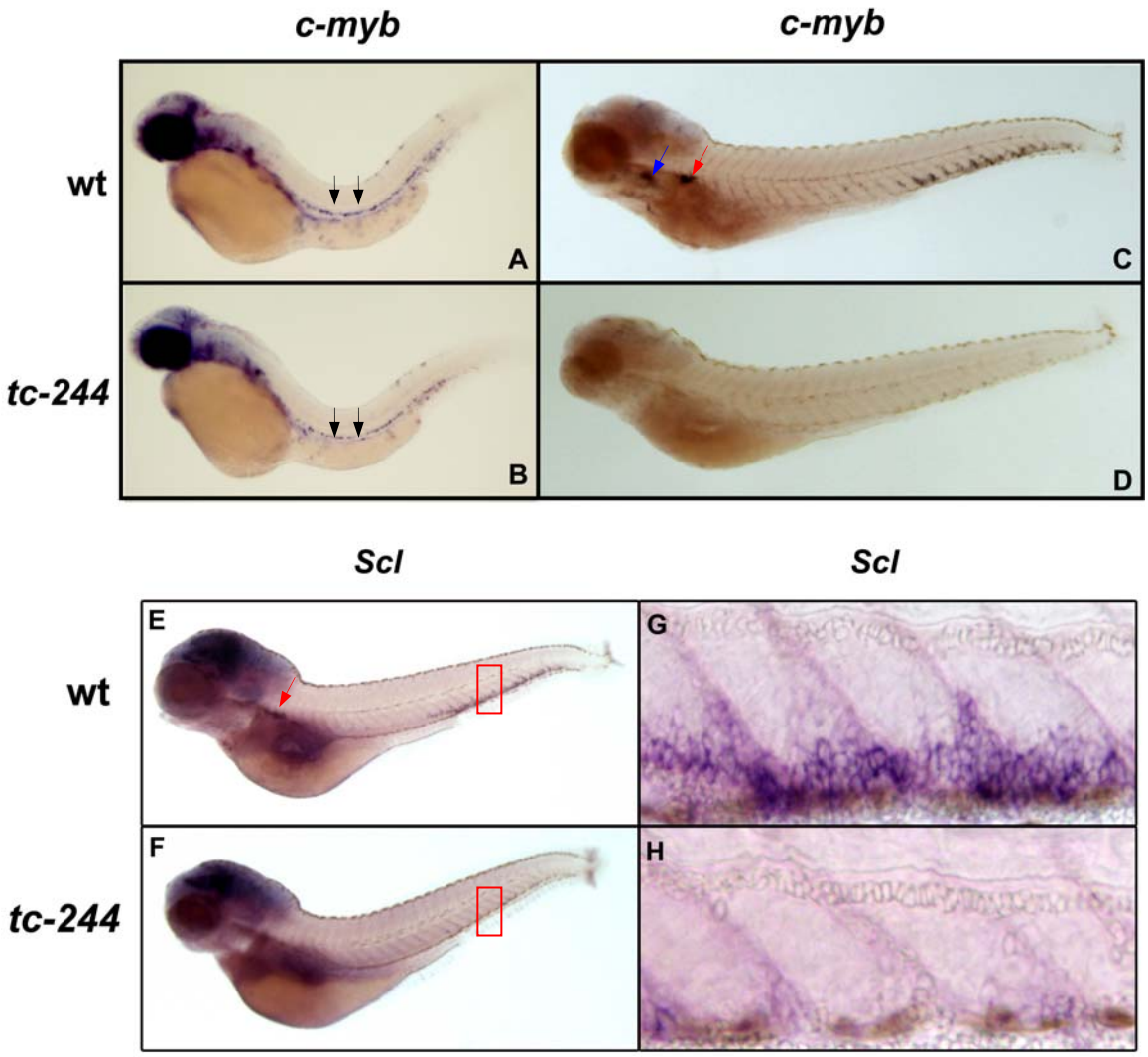


Figure 4.5 Definitive HSCs development is affected in *tc-244* mutant. WISH of *c-myb* expression in 35hpf wild type sibling (A), *tc-244* mutant (B), 5dpf wild type sibling (C) and *tc-244* mutant (D). WISH of *scl* expression in 5dpf wt (E) and *tc-244* mutant (F). Black arrows indicate the ventral wall of dorsal aorta region. Red and blue arrows indicate kidney and thymus region respectively. The red rectangles in PBI region of (E) and (F) are magnified as (G) and (H).

4.2 Positional Cloning of *tc-244* mutant

4.2.1 *tc-244* gene locates on linkage group 7

To clone the *tc-244* mutant gene, positional cloning was used as the major approach in this study. Firstly, a polymorphic hybrid strain was created by out-crossing a female heterozygous *tc-244* fish of AB strain (*tc-244*$^{+/-}$♀) with a wild type male fish (wik9♂) of WIK strain. Their progenies were used as the mapping family. 9 pairs of heterozygous *tc-244* parents in chimeric AB/WIK background from this mapping family were identified by random in-crosses, and then ~ 2000 homozygous *tc-244* mutant embryos (*tc-244*$^{-/-}$) from these 9 pairs of parents were collected and used in the mapping analysis.

As the first step of positional cloning, bulk segregation analysis (BSA) (principle of BSA refer to Figure 2.1) was employed to locate the *tc-244* mutation to a certain chromosome or linkage group. As shown in Figure 4.6A-B, BSA analysis of two independent pools of homozygous *tc-244* mutant and sibling embryos showed that the *tc-244* mutant pools gave rise to the dominant AB band for both SSLP markers z4706 and z1059, indicating that *tc-244* mutation was linked with these two markers which located on linkage group 7. This result was then confirmed by testing individual homozygous *tc-244* embryos from one of the mutant pools of BSA analysis (Figure 4.6C-D). In order to determine the rough genetic distance and direction between these two SSLP markers and *tc-244* mutation on the genetic map, more single embryos were tested. As a result, there were 2 single recombinants identified for z4706 and 26 single recombinants plus 1 double recombinant identified for z1059

in a sample panel of 95 homozygous *tc-244* mutant embryos. Moreover, the recombinants of z4706 and z1059 were not overlapped,

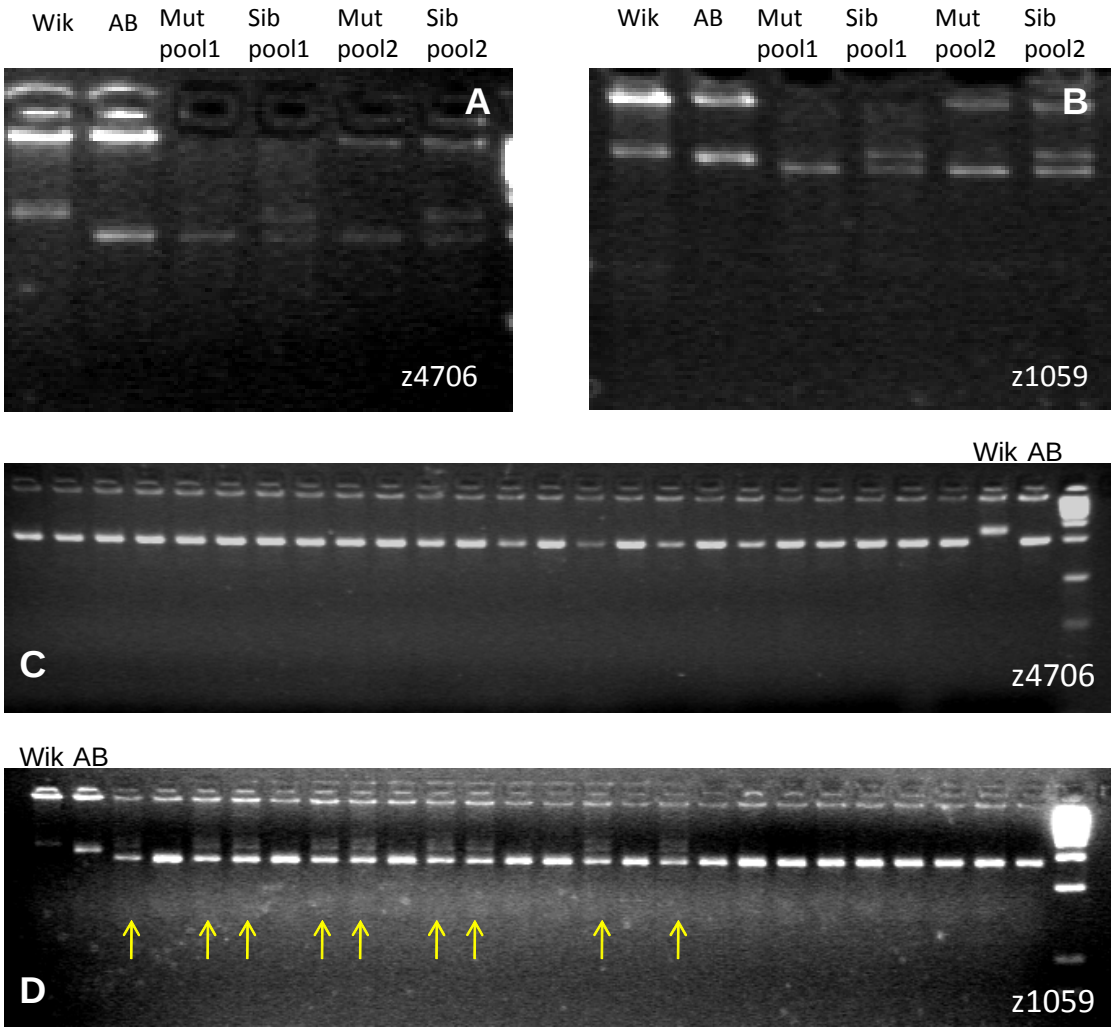


Figure 4.6 Linkage scanning of *tc-244* mutant. Bulk segregation analysis (BSA) result indicates that SSLP z4706 (A) and z1059 (B) on linkage group7 are linked with *tc-244* mutation. Single embryo test confirms the BSA result. There is no recombinants for SSLP z4706 (C) and 9 recombinants for SSLP z1059 (D) in the 24 individual embryos from one the mutant pool used in BSA analysis. Wik indicates the grandparent band of WIK strain, and AB indicates the grandparent band of AB strain. Yellow arrows indicate the recombinants in the single embryos test.

showing that z4706 and z1059 located at the opposite direction to the *tc-244* mutation with a rough genetic distance of 1 centimorgen (cM) and 14.7cM respectively. To facilitate mapping analysis, z4706 was designated as north marker, and z1059 as south marker. And these two SSLP markers can be easily located on the current zebrafish genetic map provided by MGH/CVRC Zebrafish Server (Figure 4.7). Because the genetic distance between z4706 and z1059 was more than 10cM, it was too far to do the fine mapping using these two SSLP markers. Thus all the available SSLP markers between z4706 and z1059 in the MGH map were screened for polymorphism, and only one marker z43308 was found, and this marker was a south marker and was roughly 11cM far from the *tc-244* mutation (Figure 4.7). Unfortunately, no more available SSLPs can be used to further narrow down the distance between north and south markers on the current genetic map. In summary, according to the initial mapping result, *tc-244* gene is mapped on linkage group 7 between SSLP marker z4706 and z43308, with a rough genetic distance of 12cM between them.

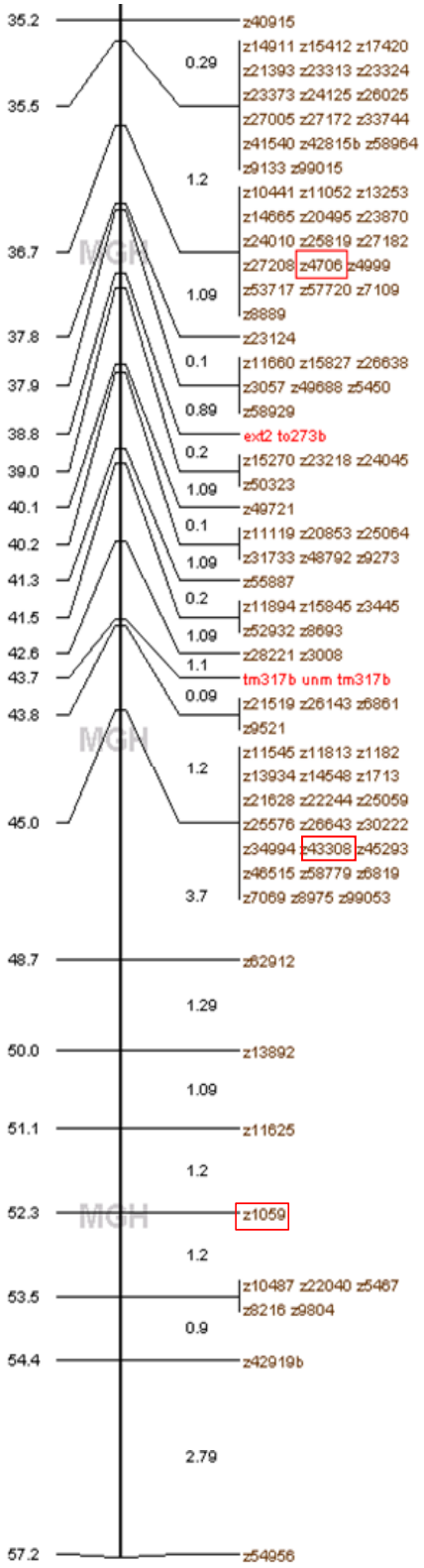


Figure 4.7 Part of the genetic map of linkage group 7. Flanking SSLP markers z4706, z1059 and z43308 are in red rectangles. The *tc-244* mutation locates between z4706 and z43308. (Adapted from MGH server)

4.2.2 *tc-244* gene is mapped to a novel zebrafish gene

Although the zebrafish genome sequencing project has not been finished yet, a large part of the assembled contig (ctg) and scaffold sequence is already available in sanger zebrafish fingerprinting database (FPC) and Ensembl zebrafish genome server (Ensembl). In order to take advantage of these databases, the two flanking markers were used to blast the database, and z4706 was found to hit ctg676 (BAC zK11F12), and z43308 was on ctg697 (BAC zC191G21). Several contigs were found to locate between them, and some fully sequenced BACs on those contigs were used for SSLP searching. One marker (named as zK183N2-118) on the BAC zK183N2/ctg675 was found to be polymorphic, and among the 96 homozygous *tc-244* embryos tested, there were 4 single recombinants with a genetic distances around 2cM south from *tc-244* mutation. Thus, zK183N2-118 and z4706 markers were very closely linked with *tc-244* mutation. Moreover, the genetic distance between these two markers and the *tc-244* mutation was suitable for the further fine mapping as well as the starting point of genomic walk.

Screen of 1910 homozygous *tc-244* embryos using z4706 (North marker) and zk183N2-118 (South marker) yields 34 north recombinants and 74 south recombinants respectively. As the sequencing project is still under construction, there are a lot gaps and mis-assembled region in the current assemblies, and unfortunately, z4706 and zK183N2-118 located on the separated contigs that were not overlapped. Thus genomic walk from the north contig or BAC to the south contig or BAC was necessary. Meanwhile, more fully sequenced BACs in contig 675 and 676 were screened for SSLP markers to further narrow down the distance to facilitate the genomic walk. As a result, zC226L4-1693 on BAC BX511061 was identified as a

south marker more close to the mutation, and it located on the same ctg676 as north marker z4706 in the newly released FPC database. In this latest version of FPC, ctg676 was a very large contig containing hundreds of BACs, but most of them were not fully sequenced yet. In order to find more close flanking markers and begin the genomic walk, a simplified contig composed of only sequenced BACs of ctg676 was constructed by testing the representative markers from each BAC or aligning BAC ends directly (Figure 4.8). A series of SSLP and SNP markers were identified by screening those BACs in this simplified ctg676, and polymorphic markers were further used to test the recombinant rate of homozygous *tc-244* mutants. Finally, *tc-244* mutation was mapped to a region covered by three BACs between markers CR318603_55485 and CR318672_119281 (Figure 4.9). There were 8 candidate genes found in this region according to the Ensembl database. But all of them were not well characterized and candidate gene approach was very difficult to apply in this case with very limited gene information. Thus, more straightforward approach, sequencing the coding region of these candidate genes, was used. Finally, one nonsense point mutation in the novel gene *zgc153228* was identified, and it was a T to G transition causing a premature stop codon (TAT → TAG) in the transcript (Figure 4.10). No other mutation was found in the region. Therefore, the positional cloning result strongly suggests that the mutated gene in *tc-244* mutant is *zgc153288*.

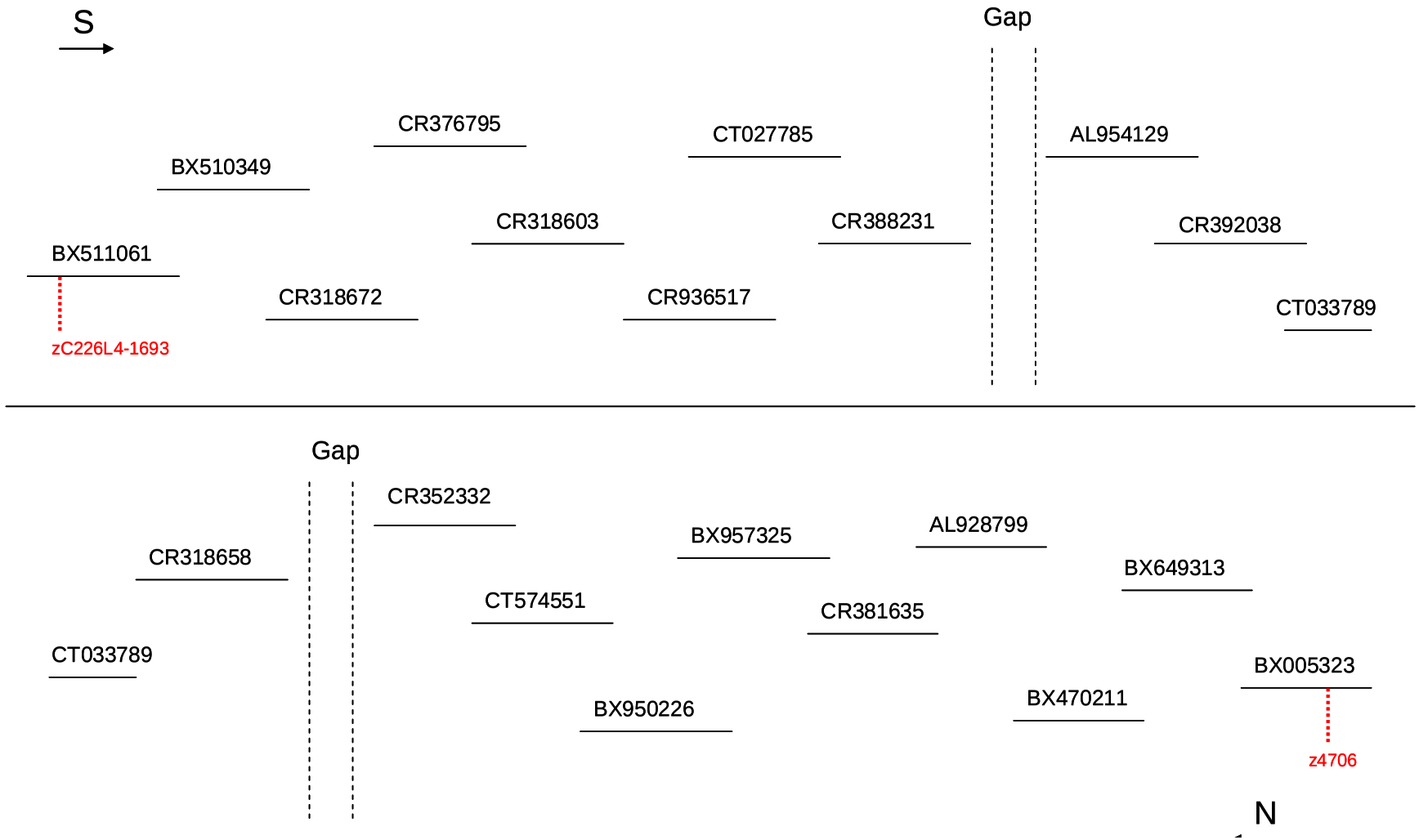


Figure 4.8 Simplified contig676 is composed of a series of fully sequenced BACs. *Tc-244* mutation is mapped within this contig. Two flanking SSLP markers are in red. The BAC is named using their accession number. The region between dashed lines indicates a sequence gap.

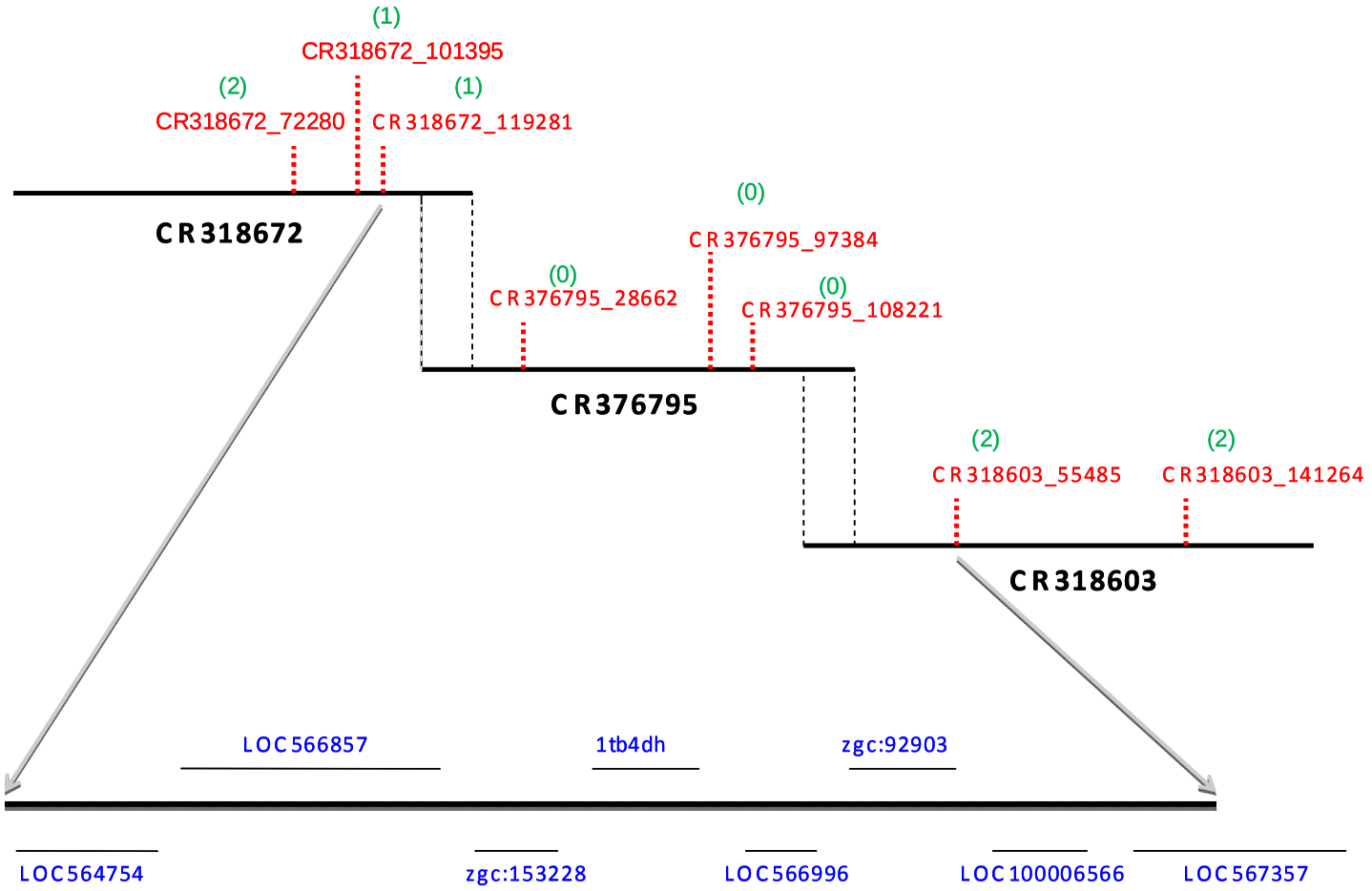


Figure 4.9 The final 3-BAC region of genomic walk. Three BACs CR318672, CR376795, and CR318603 are overlapped. All the SSLP and SNP markers are in red with their respective recombinants number (out of total 1910 homozygous *tc-244* embryos) in green in the brackets. The region between marker CR318672_119281 and CR318603_55485 is magnified and contains 8 candidate genes in blue.

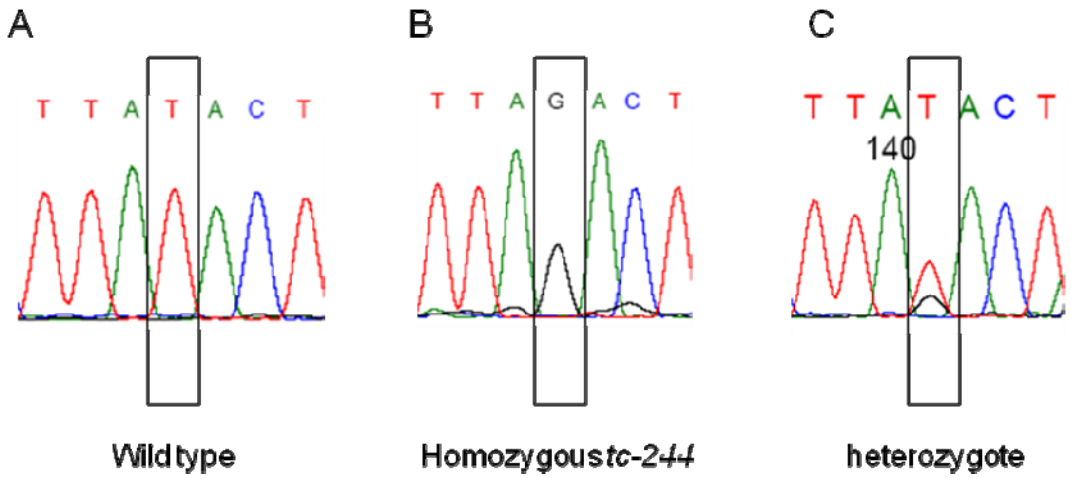


Figure 4.10 A nonsense mutation is detected in *zgc153228* gene. The point mutation is a T to G transition indicated by black rectangles.

4.3 Cloning the full-length of *zgc153228* gene

In the Ensemble database, there are several ESTs mapped in the region of *zgc153228*. Based on the EST information, a full-length cDNA of *zgc153228* was amplified by RT-PCR (*zgc153228-b*). Another full-length cDNA clone of *zgc153288* is also available (*zgc153228-a*) in NCBI database. However, a comparison of these two cDNA sequence revealed a 48 basepairs gap region (Figure 4.11). Therefore, it seems that *zgc153228* has at least two forms of transcripts (*zgc153228-a* and *-b*). Alignment of these two transcripts sequence with the genomic sequence also revealed the gene structure of these two transcripts as shown in Figure 4.12A. *zgc153228-a* was 48bp shorter than *zgc153228-b*. This 48bp gap region was part of the exon4 in *zgc153228-b*, but it became part of intron4 in *zgc153228-a*. RT-PCR using primer pair specifically flanking this gap region (e4i4FP and RP) revealed the expression pattern of these two forms (Figure 4.12B). *zgc153228-a* was the only type of transcript expressed before 6.5hpf, but after that *zgc153228-b* began to express and both transcripts existed in

relatively similar ratio until 3dpf. However, from 3dpf onwards, *zgc153228-b* became the dominant form. Based on this pattern, it is very likely that *zgc153228-a* is the maternal form; while *zgc153228-b* most probably is the zygotic form.

```
zgc153228-a      ------------------------------------------------------------

zgc153228-b      GAATTGTTTTTACTGACGTCCAATTTTGTATATATTTTTATACAATCATACGCTTTATTA 60

zgc153228-a      ------------------------------------------------------------
zgc153228-b      TAATATAATTCCAAGTTTAATGAGAAAAAAATAAGTGTTTGAAATCATTTATTTGTCGAG 120

zgc153228-a      ----------------------------------ATTTTTCGGAAGTGACAAGCGAGG 24
zgc153228-b      GAACCACTTCATGTCACGTGACGAAGGCAAGCGCGGGCATTTCGGAAGTGACAAGCGAGG 180
                                                    ********************

zgc153228-a      CAGATTGAGTGTCTCATGAAAACAATTCTGTTTTCCGAGGGTAACAAACTATCTCTGAAG 84
zgc153228-b      CAGATTGAGTGTCTCATGAAAACAATTCTGTTTTCCGAGGGTAACAAACTATCTCTGAAG 240
                 ************************************************************

zgc153228-a      TGTGAGCCACAACCACATAAGCAGCCATGCTGGACGGGGCTCAGTTTATTGAGGCGTTGT 144
zgc153228-b      TGTGAGCCACAACCACATAAGCAGCCATGCTGGACGGGGCTCAGTTTATTGAGGCGTTGT 300
                 ************************************************************

zgc153228-a      CACGGTTGGGTTACCCACGGGCTTCAATGCTGAAATGTTCAGAGTTTGACTGGTTATTTG 204
zgc153228-b      CACGGTTGGGTTACCCACGGGCTTCAATGCTGAAATGTTCAGAGTTTGACTGGTTATTTG 360
                 ************************************************************

zgc153228-a      ACACAGCACCAGATAACCTGCACCTTTTACGCATTGTCTGCAACCGCCTGAAACGCTGCA 264
zgc153228-b      ACACAGCACCAGATAACCTGCACCTTTTACGCATTGTCTGCAACCGCCTGAAACGCTGCA 420
                 ************************************************************

zgc153228-a      ATGTTTTAGCACCAGAAGAATTGCAAGCGTACAAGTCACTACAAGAGTCTGGGAAACCAA 324
zgc153228-b      ATGTTTTAGCACCAGAAGAATTGCAAGCGTACAAGTCACTACAAGAGTCTGGGAAACCAA 480
                 ************************************************************

zgc153228-a      TTTTAGATGAGGCCACACTGGCTGAGCTGCTTAAAACCTGTTTGCCCGCGGATGGGGGTT 384
zgc153228-b      TTTTAGATGAGGCCACACTGGCTGAGCTGCTTAAAACCTGTTTGCCCGCGGATGGGGGTT 540
                 ************************************************************

zgc153228-a      TCGGGTCACAGAGCTGTTCTTCACTGGGGGGAGAAGGAACTGTTACAATTGAGGAACTTG 444
zgc153228-b      TCGGGTCACAGAGCTGTTCTTCACTGGGGGGAGAAGGAACTGTTACAATTGAGGAACTTG 600
                 ************************************************************

zgc153228-a      AGACAGAACTTCAAGCCCTCCGTAAGGAAAAGCAGCTAAAACAACGCAGACTCAACAAGC 504
zgc153228-b      AGACAGAACTTCAAGCCCTCCGTAAGGAAAAGCAGCTAAAACAACGCAGACTCAACAAGC 660
                 ************************************************************

zgc153228-a      TGCAAATGCAAGCAGCCAGCTGGGGTGCAAACTCCTCTCTGTCCCAAATGCTGCTGCAGG 564
zgc153228-b      TGCAAATGCAAGCAGCCAGCTGGGGTGCAAACTCCTCTCTGTCCCAAATGCTGCTGCAGG 720
                 ************************************************************

zgc153228-a      AAGGAGACAATAAAGTAAAGGATGCCAACTCTGCCCTAGCAGCCGAAAATGCATACACCA 624
zgc153228-b      AAGGAGACAATAAAGTAAAGGATGCCAACTCTGCCCTAGCAGCCGAAAATGCATACACCA 780
                 ************************************************************

zgc153228-a      ATTCAGCTCTAGACAACCTGTCAAAGGAGACCACCAAACTGGCTGGCTTTTTCCAAAGTG 684
zgc153228-b      ATTCAGCTCTAGACAACCTGTCAAAGGAGACCACCAAACTGGCTGGCTTTTTCCAAAGTG 840
                 ************************************************************

zgc153228-a      ATCTTTCTAATTCCCTAAGGAGCAAAGATGGTGCCTCTCCATCTTTAGGTCTTCAATCAG 744
zgc153228-b      ATCTTTCTAATTCCCTAAGGAGCAAAGATGGTGCCTCTCCATCTTTAGGTCTTCAATCAG 900
                 ************************************************************
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zgc153228-a      GGAGCCCAGTGCTCTTCTCTCAGTGTCCTCTAGAGCCTTTCCTCCATCAACAGGAGCAAT 804
zgc153228-b      GGAGCCCAGTGCTCTTCTCTCAGTGTCCTCTAGAGCCTTTCCTCCATCAACAGGAGCAAT 960
                 ************************************************************

zgc153228-a      TTACCAAAACTCTGGCTTCATACACACAGCGTCAATTCTTTCAGGGAATCTCTGACTTAA 864
zgc153228-b      TTACCAAAACTCTGGCTTCATACACACAGCGTCAATTCTTTCAGGGAATCTCTGACTTAA 1020
                 ************************************************************

zgc153228-a      TGGAGACCTCGACTTTCAGTCGCTCCCAGATGACCAATCTGAGCTGTTGCAGTGAGAATG 924
zgc153228-b      TGGAGACCTCGACTTTCAGTCGCTCCCAGATGACCAATCTGAGCTGTTGCAGTGAGAATG 1080
                 ************************************************************

zgc153228-a      GGAAAGAGGTGGATGAAGAACTAGTGGAGCTTAGGAAGAAGGAGATGGCACAACTGCAGT 984
zgc153228-b      GGAAAGAGGTGGATGAAGAACTAGTGGAGCTTAGGAAGAAGGAGATGGCACAACTGCAGT 1140
                 ************************************************************

zgc153228-a      GGGCATTTATAGTGGCTCAATACCAGGTGGTGAAAGAGAAAGCTGAAGAGAATGGTGACA 1044
zgc153228-b      GGGCATTTATAGTGGCTCAATACCAGGTGGTGAAAGAGAAAGCTGAAGAGAATGGTGACA 1200
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zgc153228-a      AGACAGCAAAAGAGTGGCTTATCCAGCGATTGAACAGCAGCTCAGAGGCTGTGCACTACT 1104
zgc153228-b      AGACAGCAAAAGAGTGGCTTATCCAGCGATTGAACAGCAGCTCAGAGGCTGTGCACTACT 1260
                 ************************************************************

zgc153228-a      CGCCGATGAGCAGACTTGAGCCTGACCTACGCTCTGAGATCACATCAGTTCAGTTTGAAA 1164
zgc153228-b      CGCCGATGAGCAGACTTGAGCCTGACCTACGCTCTGAGATCACATCAGTTCAGTTTGAAA 1320
                 ************************************************************

zgc153228-a      TTCAATCTCTTTTGTTAGACCCAGTCCGCTCTGCATTACGAGACTGTGCACGTCTTCTTA 1224
zgc153228-b      TTCAATCTCTTTTGTTAGACCCAGTCCGCTCTGCATTACGAGACTGTGCACGTCTTCTTA 1380
                 ************************************************************

zgc153228-a      ACATCCCTGTGGTGCGTGGTGATCTCGCTCTTCAGGTAGCCAGGCAAAATTACCTCGCTT 1284
zgc153228-b      ACATCCCTGTGGTGCGTGGTGATCTCGCTCTTCAGGTAGCCAGGCAAAATTACCTCGCTT 1440
                 ************************************************************

zgc153228-a      CCAGACAGACTGAGGTTCGTGACCAGCTTCTCCATCAAAAAGCCTTTTTTGAACTTTTAC 1344
zgc153228-b      CCAGACAGACTGAGGTTCGTGACCAGCTTCTCCATCAAAAAGCCTTTTTTGAACTTTTAC 1500
                 ************************************************************

zgc153228-a      GTCTGGCCCAGGATGCAGAGCTTCTGAGAGGAAAGAGGGTGATGGGGCAAATTAATGACA 1404
zgc153228-b      GTCTGGCCCAGGATGCAGAGCTTCTGAGAGGAAAGAGGGTGATGGGGCAAATTAATGACA 1560
                 ************************************************************

zgc153228-a      TTGTGGAGAGGCTGGAGGGAGCAACACAAGATGCTACTCAAAGAGAAAACACTTTGACAC 1464
zgc153228-b      TTGTGGAGAGGCTGGAGGGAGCAACACAAGATGCTACTCAAAGAGAAAACACTTTGACAC 1620
                 ************************************************************

zgc153228-a      AATCTCACTTGACTGAAGACCCCTTCCTTGGTGCTAATGCTAATCAACAGGTCATCAGCT 1524
zgc153228-b      AATCTCACTTGACTGAAGACCCCTTCCTTGGTGCTAATGCTAATCAACAGGTCATCAGCT 1680
                 ************************************************************

zgc153228-a      CCAAGGACACAGCCTTTACCAGGTTGCTCCAGATGCTTGAGTTGGGAAAAGCATCCACAA 1584
zgc153228-b      CCAAGGACACAGCCTTTACCAGGTTGCTCCAGATGCTTGAGTTGGGAAAAGCATCCACAA 1740
                 ************************************************************

zgc153228-a      ACAGAGAGGATCCATTTCAAACTTACAGTAAGCTGGAAACTGCAGCCTCCAAACTACAAG 1644
zgc153228-b      ACAGAGAGGATCCATTTCAAACTTACAGTAAGCTGGAAACTGCAGCCTCCAAACTACAAG 1800
                 ************************************************************

zgc153228-a      AGGATTTGATCACTGTACAAGAGGCTTTGGATGGAGCAAGACAAGAGCAGGCTTATACTG 1704
zgc153228-b      AGGATTTGATCACTGTACAAGAGGCTTTGGATGGAGCAAGACAAGAGCAGGCTTATACTG 1860
                 ************************************************************

zgc153228-a      GAGCTCGATTAGAACGTGACCGAGATGCACTTGACCAAGTGGCGTACTCGGACATTGTGC 1764
zgc153228-b      GAGCTCGATTAGAACGTGACCGAGATGCACTTGACCAAGTGGCGTACTCGGACATTGTGC 1920
                 ************************************************************

zgc153228-a      AGCCTCTCCTGAGGCCGCAGG---------------------------------------- 1785
zgc153228-b      AGCCTCTCCTGAGGCCGCAGGTATGTGCTACAGCCACACCTGCACTGGAGCTCTGCCCAC 1980
                 ********************

zgc153228-a      ---------AGCTCACAATAGTTGTTGATGAACTGGAGGTCAAGCAGAAGACCCTGTATA 1836
zgc153228-b      ATGCACAGGAGCTCACAATAGTTGTTGATGAACTGGAGGTCAAGCAGAAGACCCTGTATA 2040
                          **************************************************
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zgc153228-a      TGCTTCTTCAGGAGATAGTTGGGGACTTGAAAACCAAACGCGGAAGGCTTGAACGCAGTG 1896
zgc153228-b      TGCTTCTTCAGGAGATAGTTGGGGACTTGAAAACCAAACGCGGAAGGCTTGAACGCAGTG 2100
                 ************************************************************

zgc153228-a      CCACCCTGCGGAGAGAGAGGGAACTGTATGTCTATTTTCATCTGGACCCAAGATTACTCA 1956
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                 ************************************************************

zgc153228-a      ACAGAGCAGTAAGAGATATAGAGGCTCAAGCAGGGGTTATGTAGAATCAAATCTCATTTC 2016
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                 ************************************************************

zgc153228-a      TCCACTCCAACCAACATGTACAGCTTCCTGCACAAATTATATAACTGAGAAATCAATGCC 2076
zgc153228-b      TCCACTCCAACCAACATGTACAGCTTCCTGCACAAATTATATAACTGAGAAATCAATGCC 2280
                 ************************************************************

zgc153228-a      CTGTTGGTTAGGCCTCACCATTGTTGCTTCTGTAGGTCTCGTATGTAGATCTGTATTATC 2136
zgc153228-b      CTGTTGGTTAGGCCTCACCATTGTTGCTTCTGTAGGTCTCGTATGTAGATCTGTATTATC 2340
                 ************************************************************

zgc153228-a      TGCTGTTCTTTAGGTATGTTAACAAATTTGTATTCAGTACCTAGATTAACATTAATCTGC 2196
zgc153228-b      TGCTGTTCTTTAGGTATGTTAACAAATTTGTATTCAGTACCTAGATTTACATTAATCTGC 2400
                 ********************************************** ************

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zgc153228-b      AATTGTACTTTAACAGATGTTCCATAATTTGTATAAATCATGTTGTTAGTTTATTTGGTG 2460
                 ************************************************************

zgc153228-a      ATTAACAGATGTATCCTTGTTAATAAATATTTGTTATCTCAAAAAAAAAAAAAAAAAAAA 2316
zgc153228-b      ATTAACAGATGTATCCTTGTTAATAAATATTTGTTATCTCAGCAA--------------- 2505
                 ****************************************  **
```

Figure 4.11 Alignment of two *zgc153228* transcripts. The cDNA sequence of *zgc153228-a* and *zgc153228-b* is aligned. The gap region within coding sequence is highlighted in red.

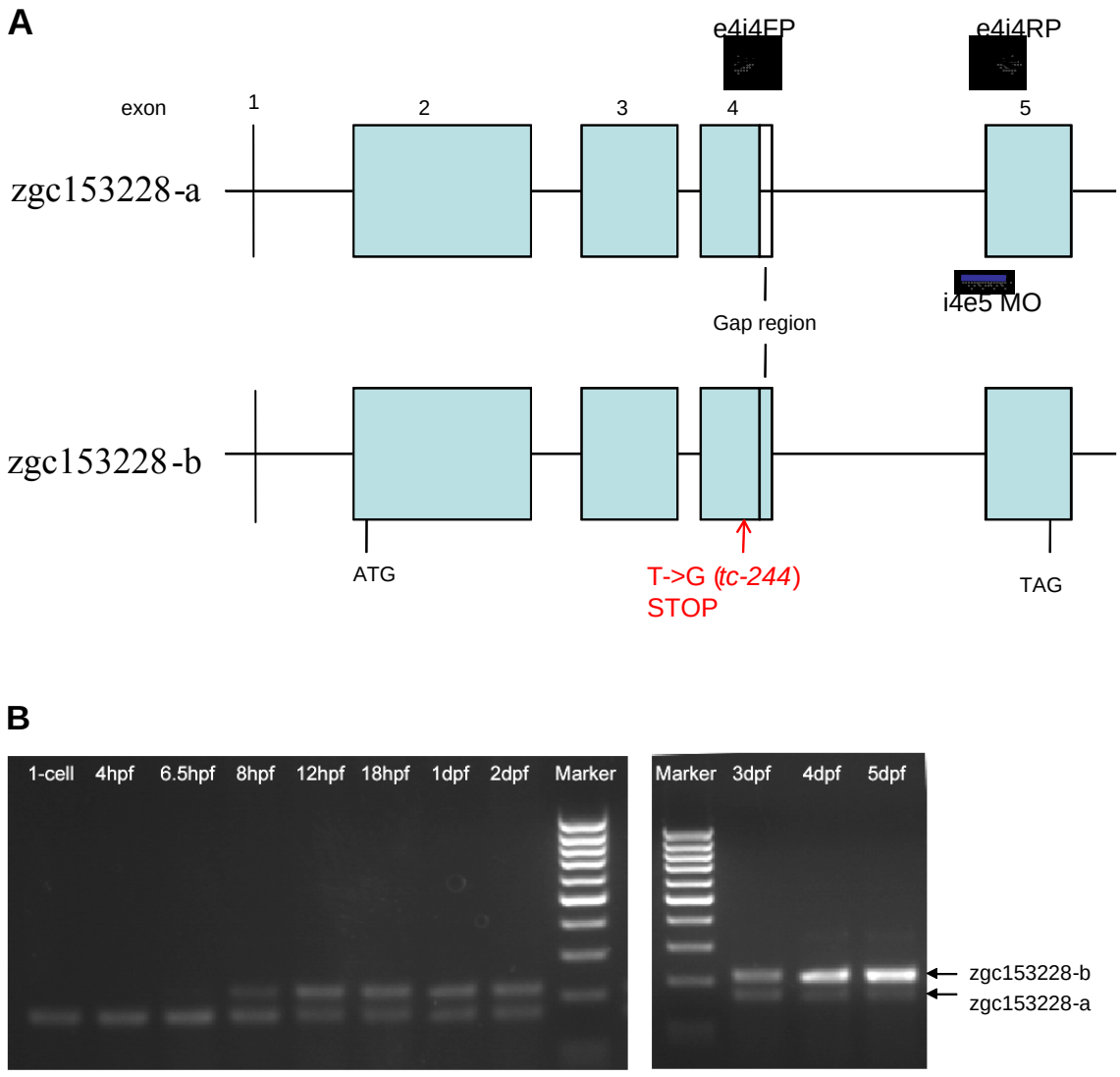
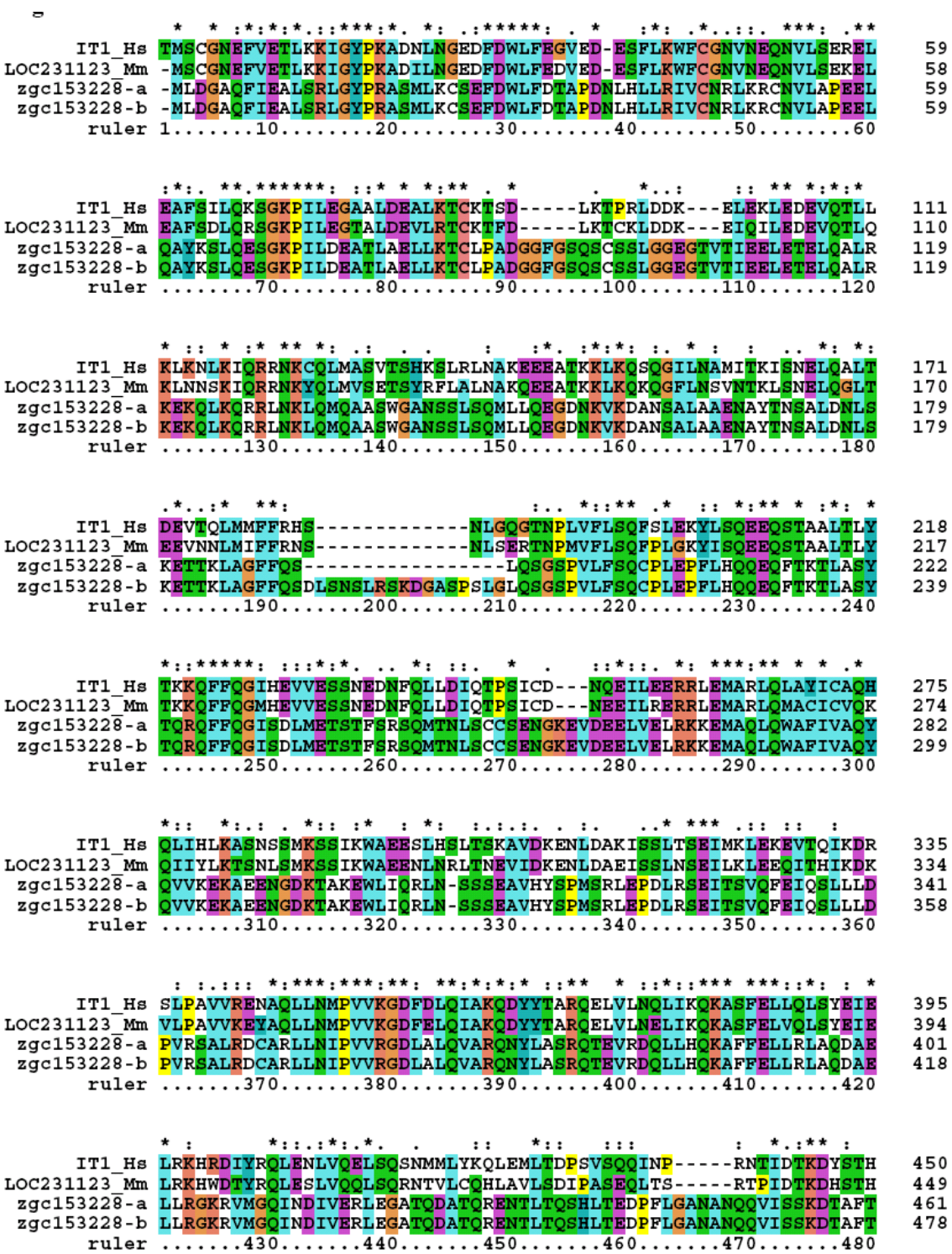


Figure 4.12 *zgc153228* has two forms of transcripts. (A) The gene structure and (B) expression pattern of two transcripts. e4i4FP and e4i4RP indicate the primer pair for amplifying the gap region. i4e5MO indicates the splicing morpholino target region. The mutation site of *tc-244* mutant is marked in red.

Analysis of zgc153288 protein sequence identified a Signal peptidases I signature 3 pattern (SPASE_I_3) of PeptidaseS26A family (a.a149-162) and two coiled-coil domains (a.a102-139, and a.a 498-529). The function of most peptidasesS26 family proteins is the processing of newly-synthesised secreted proteins. They remove the hydrophobic, N-terminal signal peptides as the proteins are translocated across membranes (Dalbey & Von Heijne 1992). The SPASE_I_3 signature corresponds to a conserved region of unknown biological significance located in the C-terminal section of all these peptidase proteins (http://www.expasy.org/prosite/PDOC00418). Coiled-coil domain is a typical hyper-secondary structure formed by two or more α-helices that are wound around each other in either a parallel or anti-parallel configuration (Lupas & Gruber 2005). Proteins with this domain contribute to the broad range of biological functions such as forming the intermediate filaments in the dynamic skeletal networks of the cytoplasm and nuclear lamina, and mediating the function of transcriptional regulators (Barbara et al 2007).

Mammalian homologues of zebrafish zgc153228 were also identified by blast search in NCBI database. The human protein IT1 (or C4orf15 protein) and mouse hypothetical protein LOC231123 are the most conserved candidates by alignment with the protein sequence of zebrafish zgc153228 (Figure 4.13). However, both of these mammalian homologues are considered as the novel proteins with unknown functions.


IT1_Hs TMSCGNEFVETLKKIGYPKADNLNGEDFDWLFEGVED-ESFLKWFCGNVNEQNVLSEREL 59
LOC231123_Mm -MSCGNEFVETLKKIGYPKADILNGEDFDWLFEDVED-ESFLKWFCGNVNEQNVLSEKEL 58
zgc153228-a -MLDGAQFIEALSRLGYPRASMLKCSEFDWLFDTAPDNLHLLRIVCNRLKRCNVLAPEEL 59
zgc153228-b -MLDGAQFIEALSRLGYPRASMLKCSEFDWLFDTAPDNLHLLRIVCNRLKRCNVLAPEEL 59
ruler 1.....10.....20.....30.....40.....50.....60
IT1_Hs EAFSILQKSGKPILEGAALDEALKTCKTSD-----LKTPRLDDK---ELEKLEDEVQTLL 111
LOC231123_Mm EAFSDLQRSGKPILEGTALDEVLRTCKTFD-----LKTCKLDDK---EIQILEDEVQTLQ 110
zgc153228-a QAYKSLQESGKPILDEATLAELLKTCLPADGGFGSQSCSSLGGEGTVTIEELETELQALR 119
zgc153228-b QAYKSLQESGKPILDEATLAELLKTCLPADGGFGSQSCSSLGGEGTVTIEELETELQALR 119
ruler .....70.....80.....90.....100.....110.....120
IT1_Hs KLKNLKIQRRNKCQLMASVTSHKSLRLNAKEEEATKKLKQSQGILNAMITKISNELQALT 171
LOC231123_Mm KLNNSKIQRRNKYQLMVSETSYRFLALNAKQEEATKKLKQKQGFLNSVNTKLSNELQGLT 170
zgc153228-a KEKQLKQRRLNKLQMQAASWGANSSLSQMLLQEGDNKVKDANSALAAENAYTNSALDNLS 179
zgc153228-b KEKQLKQRRLNKLQMQAASWGANSSLSQMLLQEGDNKVKDANSALAAENAYTNSALDNLS 179
ruler .....130.....140.....150.....160.....170.....180
IT1_Hs DEVTQLMMFFRHS----------NLGQGTNPLVFLSQFSLEKYLSQEEQSTAALTLY 218
LOC231123_Mm EEVNNLMIFFRNS----------NLSERTNPMVFLSQFPLGKYISQEEQSTAALTLY 217
zgc153228-a KETTKLAGFFQS----------LQSGSPVLFSQCPLEPFLHQQEQFTKTLASY 222
zgc153228-b KETTKLAGFFQSDLSNSLRSKDGASPSLGLQSGSPVLFSQCPLEPFLHQQEQFTKTLASY 239
ruler .....190.....200.....210.....220.....230.....240
IT1_Hs TKKQFFQGIHEVVESSNEDNFQLLDIQTPSICD---NQEILEERRLEMARLQLAYICAQH 275
LOC231123_Mm TKKQFFQGMHEVVESSNEDNFQLLDIQTPSICD---NEEILRERRLEMARLQMACICVQK 274
zgc153228-a TQRQFFQGISDLMETSTFSRSQMTNLSCCSENGKEVDEELVELRKKEMAQLQWAFIVAQY 282
zgc153228-b TQRQFFQGISDLMETSTFSRSQMTNLSCCSENGKEVDEELVELRKKEMAQLQWAFIVAQY 299
ruler .....250.....260.....270.....280.....290.....300
IT1_Hs QLIHLKASNSSMKSSIKWAEESLHSLTSKAVDKENLDAKISSLTSEIMKLEKEVTQIKDR 335
LOC231123_Mm QIIYLKTSNLSMKSSIKWAEENLNRLTNEVIDKENLDAEISSLNSEILKLEEQITHIKDK 334
zgc153228-a QVVKEKAEENGDKTAKEWLIQRLN-SSSEAVHYSPMSRLEPDLRSEITSVQFEIQSLLLD 341
zgc153228-b QVVKEKAEENGDKTAKEWLIQRLN-SSSEAVHYSPMSRLEPDLRSEITSVQFEIQSLLLD 358
ruler .....310.....320.....330.....340.....350.....360
IT1_Hs SLPAVVRENAQLLNMPVVKGDFDLQIAKQDYYTARQELVLNQLIKQKASFELLQLSYEIE 395
LOC231123_Mm VLPAVVKEYAQLLNMPVVKGDFELQIAKQDYYTARQELVLNELIKQKASFELVQLSYEIE 394
zgc153228-a PVRSALRDCARLLNIPVVRGDLALQVARQNYLASRQTEVRDQLLHQKAFFELLRLAQDAE 401
zgc153228-b PVRSALRDCARLLNIPVVRGDLALQVARQNYLASRQTEVRDQLLHQKAFFELLRLAQDAE 418
ruler .....370.....380.....390.....400.....410.....420
IT1_Hs LRKHRDIYRQLENLVQELSQSNMMLYKQLEMLTDPSVSQQINP-----RNTIDTKDYSTH 450
LOC231123_Mm LRKHWDTYRQLESLVQQLSQRNTVLCQHLAVLSDIPASEQLTS-----RTPIDTKDHSTH 449
zgc153228-a LLRGKRVMGQINDIVERLEGATQDATQRENTLTQSHLTEDPFLGANANQQVISSKDTAFT 461
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ruler .....430.....440.....450.....460.....470.....480

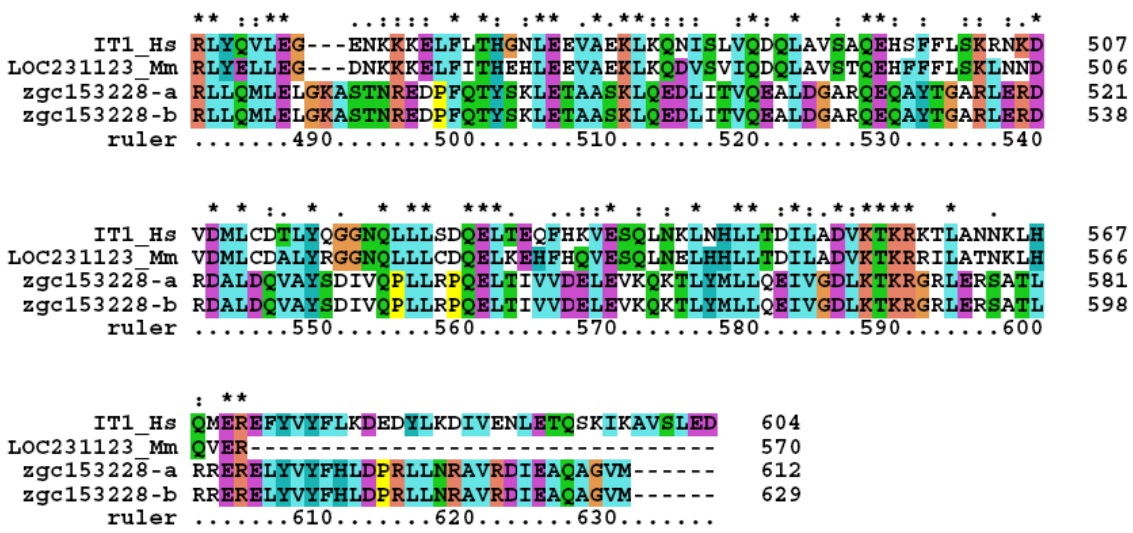

Figure 4.13 Alignment of human IT1, mouse hypothetical protein LOC231123, and zebrafish zgc153228 protein sequence.

4.4 Expression pattern of *zgc153228*

In order to have an idea of the temporal and spatial expression pattern of *zgc153228* gene, full-length zygotic form of *zgc153228* was *in vitro* transcribed to make RNA probe for whole mount *in situ* hybridization analysis. And the expression pattern of zgc153228 by WISH was shown in Figure 4.14. *zgc153228* expressed as early as one-cell stage, and remained robust expression until gastrulation stage, when it seemed to enrich in the embryonic shield region. After that the expression of *zgc153228* was ubiquitously detected with an obvious enrichment in the brain. From 1dpf onwards, the expression of *zgc153228* decreased gradually and restricted mainly in the brain. Thus, this pattern implies that *zgc153228* is an embryonically expressed gene, and its function may be critically involved in embryonic development.

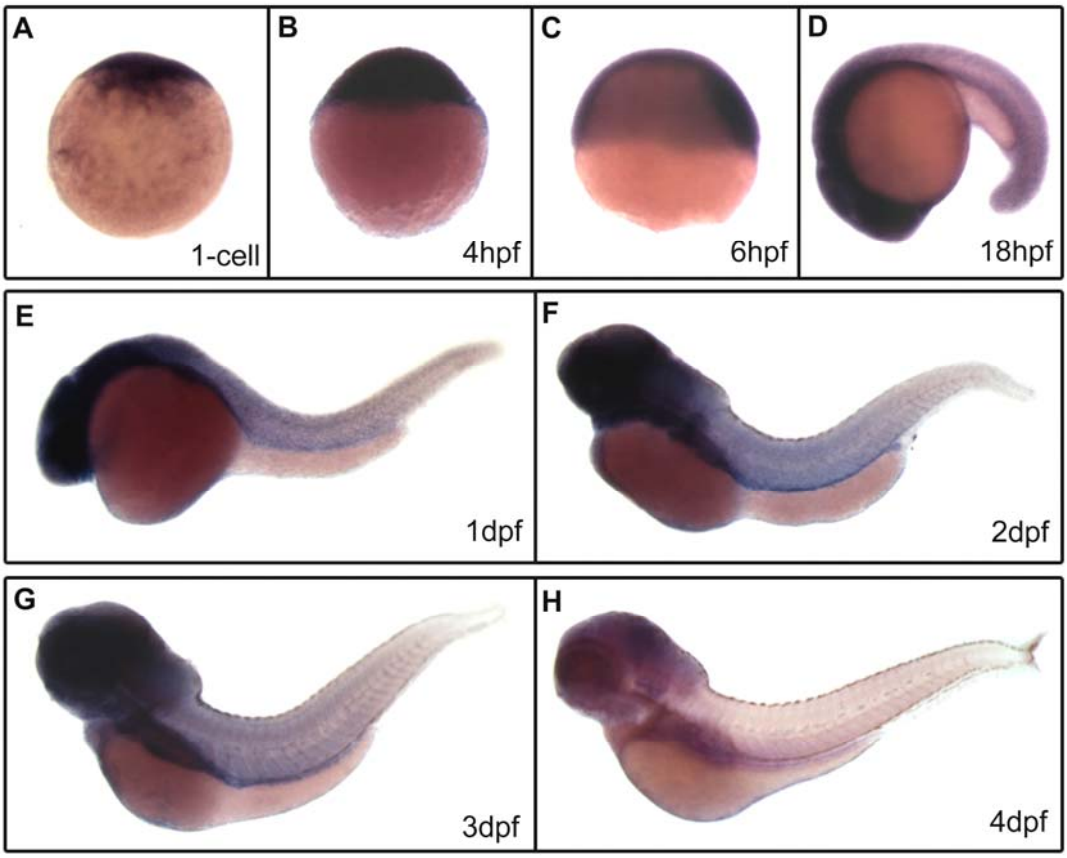

Figure 4.14 *zgc153228* gene expresses ubiquitously during embryonic development. WISH of *zgc153228* expression in 1-cell stage (A), 4hpf (B), 6hpf (C), 18hpf (D), 1dpf (E), 2dpf (F), 3dpf (G), 4dpf (H) wild type embryos. Embryos in A-C are orientated with animal pole on top whereas embryos in D-H are orientated with anterior to the left.

4.5 Morpholino knockdown of *zgc153228*

To confirm that the *tc-244* mutant phenotype was indeed caused by the mutation in *zgc153228* gene, one antisense morpholino oligo (i4e5MO) targeted to modify the splicing of *zgc153228* mRNA was designed at the intron4/exon5 boundary (Figure 4.12A) and injected into the one-cell stage wild type embryos. As expected, the morphant phenotype was very similar to the *tc-244* mutant based on the WISH result of *c-myb* and *lyc* expression (Figure 4.15A-F). In all the injected embryos, 48%

(24/50) of the morphants had almost null expression of *c-myb* in both kidney and PBI region (Figure 4.15B), while 52% (26/50) of them showed decreased *c-myb* expression (Figure 4.15C). Similarly, 46.5% (20/43) of the morphants lost *lyc* expression (Figure 4.15E); the rest 53.5% (23/43) had different level of decreased *lyc* expression (Figure 4.15F). Besides, the neuronal phenotype of small head and small eyes were also seen in these morphants. Thus, the *zgc153228* splicing morphant highly mimicked the *tc-244* mutant phenotype. RT-PCR result confirmed that the zygotic transcript (*zgc153228-b*) in i4e5 morphant was significantly reduced compared with the control morphants (Figure 4.15G). Therefore, loss of function mutation in zebrafish gene *zgc153228* indeed causes the *tc-244* mutant phenotype. Thus, *zgc153228* gene is referred to as *tc-244* gene.

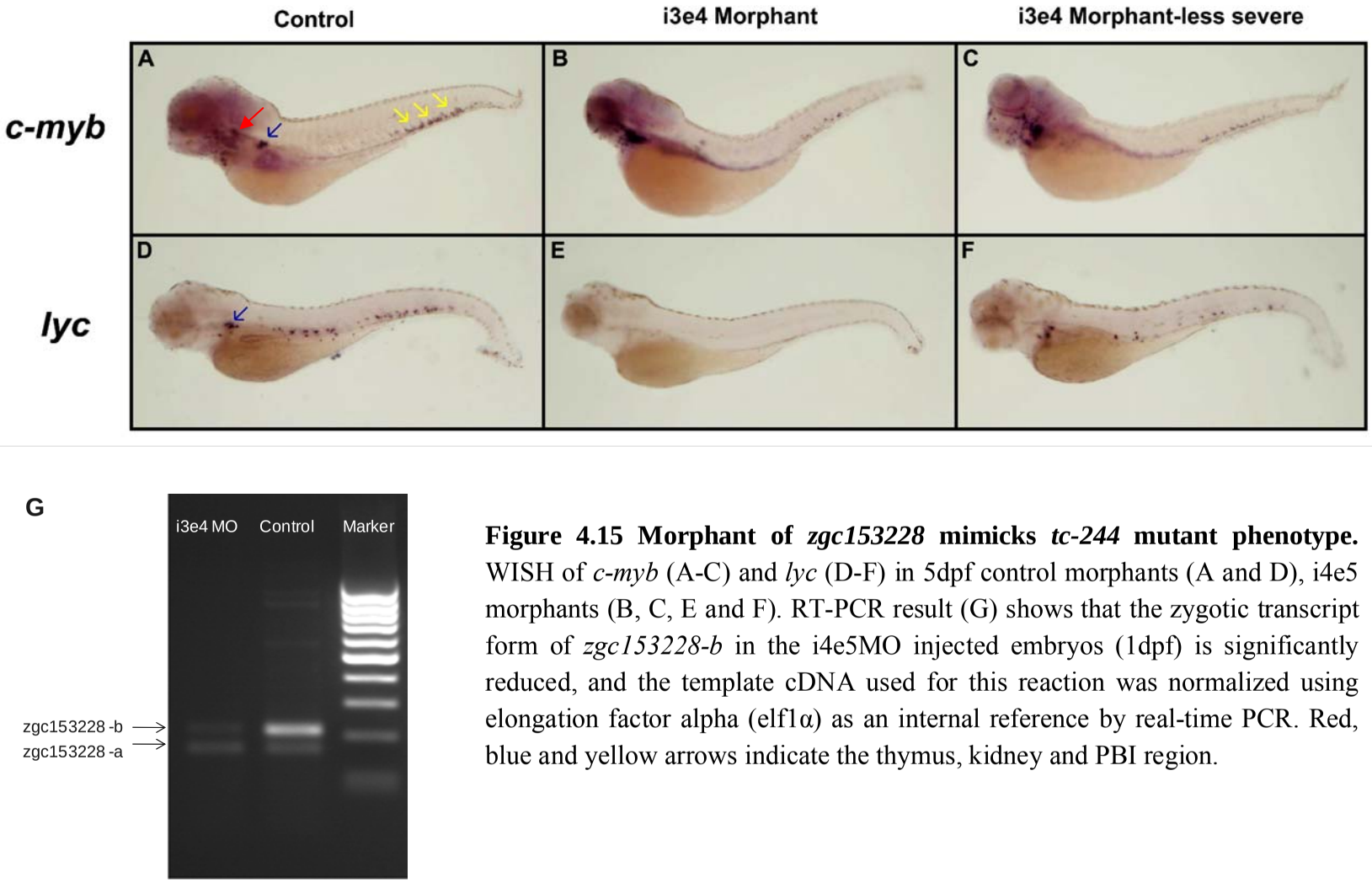

Figure 4.15 Morphant of *zgc153228* mimicks *tc-244* mutant phenotype. WISH of *c-myb* (A-C) and *lyc* (D-F) in 5dpf control morphants (A and D), i4e5 morphants (B, C, E and F). RT-PCR result (G) shows that the zygotic transcript form of *zgc153228-b* in the i4e5MO injected embryos (1dpf) is significantly reduced, and the template cDNA used for this reaction was normalized using elongation factor alpha (elf1α) as an internal reference by real-time PCR. Red, blue and yellow arrows indicate the thymus, kidney and PBI region.

4.6 Discussion

4.6.1 *tc-244* is a definitive hematopoietic mutant

Characterization results in this study suggest that *tc-244* mutant is specifically defective in generation of definitive wave of hematopoiesis, because the early forming tissues and primitive hematopoiesis development is largely normal in the *tc-244* mutants. Majority of the zebrafish hematopoietic mutants isolated so far have defects both in primitive and definitive hematopoiesis; while most of the definitive hematopoiesis specific genes, in contrast, have been identified in the mice by reverse genetic approach. Thus, exploration of molecular mechanism controlling the definitive hematopoiesis development is impeded due to lack of definitive hematopoiesis specific mutants. According to the characterization data, *tc-244* mutant is an interesting mutant model for studying definitive hematopoiesis, especially the *tc-244* gene has been mapped to a novel zebrafish gene.

In this study, it was showed that definitive HSCs in *tc-244* mutant were initially formed in the ventral wall of DA in the AGM region, but their further development was impaired and major lineages of definitive hematopoiesis were absent. There are several possibilities for these defects: one possibility is that loss of *tc-244* gene specifically disrupts the definitive HSCs specification such as affecting its proliferation and survival ability. Currently, Notch-runx and Hedgehog pathway has been implicated in the maintenance and specification of definitive HSCs, thus the relationship between *tc-244* and these known pathways may be explored in detail in the future. Besides, CD41 has been recently reported to be a marker for hematopoietic stem cells in zebrafish (Kissa et al 2008). Thus crossing the transgenic CD41 to *tc-*

244 mutant may help further analyze the HSCs defects in the *tc-244* mutant. The second possibility is that definitive HSCs in *tc-244* mutant can specify into progenitors of different lineages but they are defective in their homing abilities which prevent them from migrating to the proper niches. As we known, definitive hematopoiesis occurs in several different anatomical sites in vertebrates. In zebrafish, definitive HSCs are generated *de novo* in the AGM region and then migrate to PBI, thymus, and kidney for further proliferation and differentiation. Thus, the migration defect may also cause the mutant phenotype if *tc-244* gene is required for such functions. Lineage tracing approach may be used in the future to help to address this possibility. The third possibility is that although definitive HSCs and their progenitors for the subsequent lineages are properly specified and homed, the microenvironments or niches for their growth and differentiation are defective in the *tc-244* mutant. The transplantation experiment can be used to examine whether *tc-244* mutant phenotype is caused by such non-cell autonomous effect. For example, if PBI is defective and cannot provide certain signals required for further development of those progenitors, they may die and the mutant phenotype is expected. In order to define the real cause for the defects found in *tc-244* mutants, more detail characterization is needed in the future.

4.6.2 Cloning of *tc-244* mutant gene

For cloning of chemical mutagenezied zebrafish mutants, there are mainly two approaches: positional cloning and candidate gene approach. In general, cloning by the candidate gene approach begins with an in depth analysis of phenotype, then these phenotypic analysis of gene function will be combined with the information about the

approximate genetic map location. Finally, candidate gene will be confirmed by sequencing or complementary rescue experiment. In the *tc-244* mutant case, this approach had also been tried but it was difficult. Firstly, the current phenotype characterization information of *tc-244* mutant is helpless in predicting the detail gene function. Secondly, few definitive specific genes are reported so far, and many zebrafish homologues of mammalian candidates have not been characterized due to lack of genome sequence information. Thus, positional cloning is adopted as the major approach to map the mutated gene in *tc-244* mutant.

Positional cloning is the classical method of forward genetics, and has been used in both plant and animal models. In zebrafish, many mutants have been mapped using this approach as well. Based on the mapping experience of *tc-244* mutant, marker technology and genome information are considered as most critical parameters for a successful cloning. The marker technology used in the study is SSLP (or SSR) and SNP. SSLP is favored over SNP in most cases, as it is simple to use and relatively cheap. But in the fine mapping stage, SNP is also highly recommended because the chance of finding a polymorphic SNP is larger than finding a polymorphic SSLP, especially in zebrafish due to lack of inbreed strains. For the genome information, although the zebrafish genome sequencing project hasn't been finished yet, a large part of automated or manually annotated sequence is available in the public database. Thus combining information from both genetic map and physical map is especially useful in positional cloning. But it is noted that many gaps and mis-assembled sequence frequently present in the current database, most of which has not been manually annotated. Thus manually verification of the assembly is also necessary in some cases.

It is believed that positional cloning and candidate gene approach will be combined more efficiently in mapping zebrafish mutant in the future as the sequencing project completes and more zebrafish genes are characterized.

4.6.3 Functional implication of *tc-244* gene

By antisense morpholino knockdown approach, it was confirmed that *tc-244* mutant phenotype was indeed caused by loss of function of zebrafish novel gene *zgc153228*. Complementary *zgc153228 RNA* rescue of *tc-244* mutant will also be performed in the future to provide additional evidence. Moreover, as *zgc153228* gene has two forms of transcripts, the relationship between these two forms and their function will be interesting topics in the future, especially regarding to the hematopoiesis phenotype.

Currently, a lot of genes that are critical for hematopoiesis development have shown their conserved roles across the vertebrate species. The human and mouse homologues of zebrafish *tc-244* were identified by blast, but it is not clear whether they are orthologs of *tc-244* since both of them are novel gene with unknown functions. Therefore, functional assay may be applied in zebrafish system or mammalian system to analysis if they have a similar biological function and represent the real orthologs of *tc-244* gene.

Domain analysis revealed a signal peptidases I signature 3 pattern (SPASE_I_3) of PeptidaseS26A family in the zgc153228 protein. Peptidase family S26A is also named as signal peptidase I family. Many signal peptidases identified belong to this family, and their function is the processing of newly synthesized

secreted proteins. Therefore, although the exact function of SPASE_I_3 pattern in this peptidase family protein is unknown, it is possible that zgc153228 also has proteolysis activity as other members of this family. And this possibility will be tested in the future. However, it is not clear whether zgc153228 function as a signal peptidase protein. Signal peptidases are membrane proteins, thus the cellular localization of zgc153228 should be examined to provide more information to help clarifying this issue. Eukaryotic signal peptidases commonly function as oligomeric complexes containing two divergent copies of the catalytic monomer (http://merops.sanger.ac.uk/cgi-bin/merops.cgi?id=S26). Interestingly, two coiled-coil domains were also identified in zgc153228 protein. Many coiled-coil containing proteins are known to form dimers with each other through their coiled-coil regions, thus the coiled-coil region may facilitate its oligomerization if zgc153228 functions as a signal peptidase.

According to the temporal and spatial expression pattern of *tc-244*, it seems that *tc-244* gene ubiquitously expresses in the embryonic stages, and gradually exhibits an enriched pattern in the brain in larvae. Although it does not show a hematopoietic-specific pattern, it is believed *tc-244* gene express in the hematopoietic cells and organs similar as other tissues in the embryonic stages. However, whether *tc-244* plays a cell-autonomous role in definitive hematopoiesis development await transplantation data in the future. Interestingly, the human homologue IT1 has an EST expression profile (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.368454) showing that IT1 expresses in adult blood, kidney, lymph node, spleen, and thymus. Thus, it will be interesting to explore whether *tc-244* gene also has a role in adult hematopoietic system.

Chapter V Conclusion

udu and *tc-244* are two newly isolated zebrafish hematopoietic mutants from our lab's forward genetic screen. This thesis describes the detail phenotype analysis of these two mutants and study of the roles of their responsible genes in hematopoiesis development.

The first part of this thesis is focused on the zebrafish *ugly duckling* (*udu*) mutant allele udu^{sq1}. Characterization results of this mutant allele strongly suggest that loss of *udu* gene leads to the defective proliferation and differentiation abilities in the primitive hematopoietic cells. Moreover, this defect in primitive erythroid cells is revealed due to their abnormal arrest in the G2-M phase during cell cycle progression. The expression pattern of *udu* gene and transplantation experiments further demonstrate that *udu* gene is cell-autonomously required for primitive erythropoiesis. However, non-cell autonomous effect of *udu* gene in primitive hematopoiesis development is not excluded and need further studies.

In order to elucidate the molecular mechanism that how *udu* gene affects the primitive hematopoietic development, yeast two hybrid screen was performed to fish out the candidate interaction partners of Udu protein. As a result, hundreds of positive clones were obtained from this screen, and they were classified into several groups by sequencing and blast analysis. Consistent with the characterization result that $udu^{sq1-/-}$ erythroid cells were found to be impaired in the cell cycle progression, a group of molecules that are known to function in cell division or cell cycle regulation has been found from the yeast two hybrid screen. Interestingly, some of them also had similar

expression pattern as Udu, thus they are very likely to function as the real interaction partners of Udu protein in vivo.

Taken together, these findings reveal that *udu* gene is a novel factor essential for primitive hematopoiesis development in zebrafish, and it plays important roles in regulating primitive erythroid proliferation and differentiation. The candidate interaction partners found by Y2H provide the valuable insight into the molecule mechanism that *udu* gene may regulate the cell cycle progression of primitive hematopoietic cells. Although it is widely accepted that the genetic program of primitive hematopoiesis is established through mesoderm induction and patterning, stem cell specification, and lineage differentiation, critical elements governing these major steps are yet to be identified. Thus identification of zebrafish *udu* gene has added to the existing knowledge about genetic regulation of primitive hematopoiesis development, and provides new information for understanding the proliferation and differentiation of hematopoietic cells.

Although the above results strongly suggest Udu as an important regulator of hematopoietic development, the mechanism of how *udu* gene regulates the primitive erythroid cell cycle progression is still not clear. One of the very promising ways to elucidate the molecule mechanisms of *udu* is to further analysis those candidate molecules (especially mcm proteins) from the yeast two hybrid screen. The in vivo interaction test between them and Udu protein will provide key evidence of Udu's role in regulating cell cycle progression, and morpholino knockdown results of those candidates can be compared with the mutant phenotype to provide further clues. Finally, mutation analysis of critical interaction region will be performed to prove that

the interaction between the candidates and Udu protein is critical for the function of Udu.

In the second part of the thesis, another mutant *tc-244* was examined in detail and found to be specifically deficient in definitive hematopoiesis. The phenotype analysis results showed that *tc-244* mutant was normal in primitive hematopoiesis but had severe defects in generation of major definitive lineages including erythroid, myeloid and lymphoid cells. A closer examination revealed that definitive HSCs initially formed in the AGM region, but their further development was impaired in the *tc-244* mutants, thus leading to the absence of major lineages of definitive hematopoietic cells. Using positional cloning approach, *tc-244* mutant gene was mapped to locate on linkage group 7, and a nonsense point mutation (T to G transition) was identified in a novel zebrafish gene *zgc153228* in *tc-244* mutant genomic DNA. Morpholino knockdown of *zgc153228* showed similar morphant phenotype as *tc-244* mutant, confirming that the mutant phenotype of *tc-244* was indeed caused by loss of function of *zgc153228* gene. Using whole mount in situ hybridization, *zgc153228* was found to express ubiquitously in the early embryonic stages, implying that its function may be critically involved in embryonic development including the hematopoietic system. Interestingly, *zgc153228* has two different transcripts that probably correspond to the maternal and zygotic form of *zgc153228* gene.

According to the characterization data, *tc-244* mutant is an interesting model for the definitive hematopoiesis development research. Currently, few mutants from either ENU or insertional screen are implicated in definitive lineages specification and differentiation, especially the early events of this process. Moreover, *tc-244* mutant gene is identified by positional cloning, thus making functional study of this *tc-244*

gene in zebrafish possible. Traditionally, understanding of definitive hematopoiesis development in zebrafish largely relies on the knowledge from the mammalian model. However, *tc-244* gene is apparently a novel gene firstly identified in zebrafish, and blast analysis revealed that it had mammalian homologues with unknown function as well. Therefore, the work described in the second part of this thesis leads to identification of a novel gene involved in genetic regulation of definitive hematopoiesis development.

As many other novel genes identified in zebrafish, it remains unclear how *tc-244* gene participate in genetic network of definitive hematopoietic development currently. Moreover, although *tc-244* gene was detected to express ubiquitously in the embryonic stage, it is uncertain whether this gene functions cell autonomously or non-cell autonomously in definitive lineage development, transplantation approach wil be used to solve this issue in the future. Besides, domain analysis identified a signal peptidases I signature 3 pattern (SPASE_I_3) of PeptidaseS26A family in tc-244 protein, however it is not clear whether tc-244 protein function as a signal peptidase. The cellular localization of this protein will be explored in the future to provide more evidences for this argument. Finally, functional studies will be performed to reveal the relevance between zebrafish *tc-244* gene and its mammalian homologues, especially their roles in the definitive hematopoiesis development.

In conclusion, by genetic analysis of zebrafish mutants *udu* and *tc-244* in this study, two novel genes (*udu* and *tc-244*) are identified as novel factors involved in the regulation of primitive and definitive hematopoiesis development.

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