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ROLE OF *ULK1B*-REGULATED AUTOPHAGY IN ZEBRAFISH DEFINITIVE HEMATOPOIESIS

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PhD

The Hong Kong Polytechnic University

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Role Of *Ulk1b*-Regulated Autophagy In Zebrafish Definitive Hematopoiesis

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A thesis submitted in partial fulfillment of the requirements for

the degree of

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Kazi Md Mahmudul Hasan

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Abstract

Autophagy is a dynamic and evolutionary conserved lysosomal degradation pathway for cellular remodeling, development and homeostasis, yet its function in definitive hematopoiesis is elusive. Taking advantage of the optically clear and externally fertilized zebrafish (*Danio rerio*) embryos together with the genetic tractability and the availability of pharmacological approaches, here we inhibited autophagy by knocking out uncoordinated-51-like autophagy activating kinases 1a (*ulk1a*) and 1b (*ulk1b*) to investigate the role of autophagy in definitive hematopoiesis.

The overall autophagy processes were monitored by western blot analysis, high-resolution microscopy with zebrafish Lc3 fluorescent transgenic embryo Tg(GFP-Lc3), lysosome dye (Lysotracker red) and autophagy detection dye (CYTO-ID®). Furthermore, hematopoietic phenotypes were examined by whole-mount in situ hybridization (WISH) and flow cytometry-based lineage-specific cell population counts.

Zebrafish deleting *ulk1b* resulted in the inhibition of autophagy activation, but not autophagy flux at the whole embryo protein level. However, both autophagy activation and flux were significantly impaired in neurons, suggesting the tissue-specific requirement of ulk1b in zebrafish autophagy during embryonic development. Further investigation revealed that both chimeric and stable *ulk1b* knock-out significantly decreased the number of hematopoietic stem cells (HSCs) while increased myeloid progenitors, leukocytes and neutrophils in the caudal hematopoietic tissue (CHT). In contrast, both autophagy and hematopoiesis were unaffected upon somatically targated

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$ulk1a^{TAL}$. CYTO-ID® green staining indicated that *ulk1b* knock-out did not affect *coro1a*:DsRed positive leukocyte's autophagy activation but inhibited autophagy flux upon CQ treatment. Chemical inhibition of autophagy using 3-MA treatment recapitulated the hematopoietic phenotypes observed in *ulk1b* mutants, suggesting that the increase in CMPs, leukocytes and neutrophils in the *ulk1b* mutants was likely autophagy-dependent. However, treatment with the autophagy inducer, calpeptin, can only rescue the increased neutrophil population in *ulk1b* mutants. Though calpeptin treatment significantly induced autophagy activation in leukocytes of wild-type and *ulk1b* mutant, the effects of calpeptin treatment on autophagy flux were not examined. It is possible that calpeptin cannot rescue the autophagy defects, particularly the suppressed autophagy flux in all hematopoietic lineages and thus cannot rescue all the hematopoietic phenotypes. Also, maintenance of normal hematopoiesis might require a specific autophagy level under tight regulation.

In this project, we demonstrated that zebrafish is a valuable *in vivo* model for studying autophagy. Our results showed that *ulk1b* knock-out affects zebrafish autophagy in a tissue-specific manner, which is consistent with the current understanding that multiple canonical and non-canonical autophagy pathways involving different subsets of autophagy components are ongoing in different tissues. While our *ulk1b* knock-out models demonstrated the role of ulk1b-dependent autophagy in hematopoiesis, further investigation targeting other autophagy components in a lineage-specific manner is warranted to reveal the complete autophagy network in hematopoiesis.

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2. Xie F, Xu S, Lu Y, Wong KF, Sun L, **Hasan KMM**, Ma ACH, Tse G, Manno S, Tian L, Yue J, Cheng SH. Metformin accelerates zebrafish heart regeneration by inducing autophagy. *npj Regenerative Medicine*. 2021; 6:62, PMID: 34625572

Others:

1. Ma ACH, Mak CCY, Yeung KS, Pei SLC, Ying D, Yu MHC, **Hasan KMM**, Chen X, Chow PC, Cheung YF, Chung BHY. Monoallelic Mutations in *CC2D1A* Suggest a Novel Role in Human Heterotaxy and Ciliary Dysfunction. *Circulation: Genomic and Precision Medicine*. 2020; 13(6):696-706, PMID: 33196317.

2. Chen XK., Yi ZN., Wong GTC., **Hasan KMM**., Kwan JSK., Ma ACH., Chang RCC. Is exercise a senolytic medicine? A systematic review. *Aging cell*. 2020; e13294, PMID: 33378138.

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<u>**Kazi Md. Mahmudul Hasan**</u>, Xiang-Ke Chen and Alvin Chun-hang Ma.

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<u>Kazi Md. Mahmudul Hasan</u>, Xiang-Ke Chen and Alvin Chun-hang Ma.

2. The 14th International Symposium on Healthy Aging, March 16-17, 2019

"Systemic inflammation induces IL-1b-mediated microglia invasion in the Zebrafish brain" (Poster presentation)

<u>X Chen</u>, **KMM Hasan**, JSK Kwan, ACH Ma, RCC Chang.

3. American Society of Human Genetics (ASHG Virtual Meeting), October 27-30, 2020

"Novel damaging mutations in CC2D1A suggest a role in human heterotaxy and ciliary dysfunction"

<u>ACH Ma</u>, CCY Mak, KS Yeung, LCS Pei, D Ying, HC YU, **KMM Hasan**, X Chen, PC Chow, YF Cheung, BHY Chung.

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CMA	Chaperone-mediated autophagy
HSC70	Heat shock protein 70
LAMP2A	Lysosome-associated membrane protein type 2A
ATG	Autophagy-related gene
ULK1	Unc-51 like autophagy activating kinase
BECN1	Coiled-coil myosin-like BCL2-interacting protein
LAP	LC3-associated phagocytosis
LC3	Microtubule-associated protein 1A/1B-light chain 3
mTOR	Mammalian target of rapamycin
AMPK	5' AMP-activated protein kinase or AMPK or 5' adenosine monophosphate-activated protein kinase
PI3Ks	Phosphoinositide 3-kinases
PE	Phosphatidylethanolamine
FIP200	FAK family kinase-interacting protein of 200 kDa
VPS 34	Vacuolar protein sorting 34
BCR	Breakpoint cluster region protein
TALEN	Transcription activator-like effector nucleases
GFP	Green fluorescent protein
CRISPR	Clustered regularly interspaced short palindromic repeats
LPM	Lateral plate mesoderm

ICM	Intermediate cell mass
RBI	Rostral blood island
CMPs	Common myeloid progenitors
hpf	Hours post-fertilization
ALPM	Anterior lateral plate mesoderm
PLPM	Posterior lateral plate mesoderm
VDA	Ventral wall of dorsal aorta
AGM	Aorta-gonad-mesonephros
CHT	Caudal hematopoietic tissue
EHT	Endothelial-hematopoietic transition
GMPs	Granulocyte-macrophage progenitors
PM	Primitive monocyte
CLP	Common lymphoid progenitor
HSPCs	Hematopoietic stem and progenitor cells
ROS	Reactive oxygen species
NK cells	Natural killer cells
ER	Endoplasmic reticulum
ATP	Adenosine triphosphate
PMNL	Polymorphonuclear leukocytes
AML	Acute myeloid leukemia
MDS	Myelodysplastic syndrome
ALL	Acute lymphoid leukemia
MCL	Mantle cell lymphoma

HTLV1	Human T-cell lymphotropic virus type 1
PBS	Phosphate-buffered saline
PTU	1-phenyl 2-thiourea
KOH	Potassium hydroxide
H_2O_2	Hydrogen peroxide
NCBI	National Center for Biotechnology Information
RFLP	Restriction fragment length polymorphism
PCR	Polymerase chain reaction
WISH	Whole-mount in situ hybridization
EDTA	Ethylenediaminetetraacetic acid
3-MA	3-Methyladenine
CQ	Chloroquine
PVDF	Polyvinylidene difluoride
NBT	p-nitroblue tetrazolium chloride
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
PHB	Pre-hybridization buffer
SDS	Sodium dodecyl sulfate
IPTG	Isopropyl β- d-1-thiogalactopyranoside
dNTP	Deoxynucleoside triphosphate
DIG	Digoxigenin
AP	Alkaline phosphatase
$CaCl_2$	Calcium chloride
BCA	Bicinchoninic Acid

MIP	Maximum intensity projection
NaCl	Sodium chloride
DMSO	Dimethyl sulfoxide
PFA	Paraformaldehyde
$MgCl_2$	Magnesium chloride
PH3	Phosphohistone H3
MPN	Myeloproliferative neoplasm
SEM	Standard error of the mean
ANOVA	Analysis of variance

Chapter 1: Introduction

1.1 Autophagy

The term "autophagy" was first coined by Nobel Prize winner in Physiology or Medicine and English-born Belgian cytologist and biochemist Christian de Duve in 1963 [1]. Autophagy is an adaptive and highly conserved metabolic process in eukaryotes consisting of *de novo* synthesis of double-membrane vesicles (autophagosomes), engulfment of cytoplasmic materials and its delivery to the lysosome for degradation under different physiological and pathological conditions such as immunity, hematopoiesis, infections, cancer, aging and neurodegeneration [2-7]. During starvation, stress and amino acid insufficiency, autophagy play a key role in replenishing biosynthetic precursors through the breakdown of cytoplasmic organelles, responding to the stress-mediated cytotoxicity, in particular degrading aggregated proteins, damaged organelles and pathogens [8, 9]. Autophagy is a cytoprotective mechanics in which both too high and too low levels of autophagy may be deleterious to the organisms and leading to cell death [10].

1.2 Types of autophagy

Depending on cargo size and the routes by which cytoplasmic components are managed to degradation, autophagy can be classified into three major categories: microautophagy, chaperon-mediated autophagy and macroautophagy. Microautophagy was first mentioned in yeast and it is the degradation and direct engulfment of organelles such as peroxisome and nucleus by autophagic tubes near the boundary membrane where vacuole invaginates and engulfs cytosolic components directly into the lysosomes [11]. Maintaining cellular homeostasis, cell survival and cargo size under nitrogen limitation are the key roles of microautophagy [12]. Chaperone-mediated autophagy (CMA) does

not involve vesicle formation rather a direct translocation of specific proteins across the lysosome membrane. CMA includes the selective degradation of KFERQ-like motif-bearing substrate proteins to the lysosomes through heat shock protein 70 (HSC70) chaperon and cochaperones like HSP40, the carboxyl terminus of HSC70-interacting protein (CHIP), HSP70–HSP90 organizing protein (HOP) and their engulfment inside the lysosomes through lysosome-associated membrane protein type 2A (LAMP2A) [13]. In contrast, macroautophagy involves double-membrane vesicle formation, encapsulation of cellular organelles, lysosomal fusion and cargo degradation [14].

Autophagy has been previously described as a non-selective or canonical nutrient recycling phenomenon that occurs ceaselessly at the basal level and delineates the random consumption as well as successive degradation of cytoplasmic organelles, aggregated proteins et cetera [15]. Consequently, selective autophagy has been evolved as a discriminant selection, strictly regulated as damaged and cytosolic cargo degradation pathway for the removal of dysfunctional endoplasmic reticulum (ER-phagy) and parts of the ER membranes (reticulophagy), superfluous protein aggregates (aggrepahgy), dispensable peroxisomes (pexophagy), polluted mitochondria (mitophagy), excess ribosomes (ribophagy), lipid droplets (lipophagy), storage or release of ferritin iron (ferritinophagy), stress granules (granulophagy) and intracellular pathogens (xenophagy) to generate certain nutriments in response to environmental stimuli, therefore fostering cell survival and organism health [16-22]. In selective autophagy, the shape and size of the phagophore are regulated by the cargo itself and a variety of adaptor proteins including p62, optineurin (OPTN), nuclear dot protein 52 kDa (NDP52), neighbor of

BRCA1 gene 1 (NBR1), nuclear FMRP interacting protein (NUF1P1) and others to link cargoes to the autophagy machinery [20].

It is well-established fact that canonical autophagy is present at the basal levels and closely associated with the regulation of cellular homeostasis in almost all cell types [23, 24]. Autophagosome formation during canonical autophagy requires the hierarchical activities of different autophagy-related genes (ATGs). Non-canonical autophagy occurred during autophagy initiation, elongation and nucleation [25]. One of the most common forms of non-canonical autophagy is the Beclin 1 *(BECN1*: coiled-coil, moesin-like BCL2-interacting protein) independent autophagy which occurs in the context of cellular survival, proliferation, cell death and the development of immune cells [26]. Another important non-canonical pathway is the LC3-associated phagocytosis (LAP) where LC3 conjugates to the phagosome membrane and plays critical roles in normal cell physiology and disease pathology [27]. Here, the canonical autophagy-dependent and independent roles are summarized (Table 1.1).

1.3 Regulatory mechanism of canonical macroautophagy

Macroautophagy is the most common cytoplasmic cargo clearance mechanism, hereafter, referred to as autophagy. Autophagy prevails at a basal level in most cell types to coordinate cellular homeostasis [14]. Many signaling activities including eukaryotic cell growth and metabolism are regulated by autophagy [28].

Autophagy induction is triggered through distinct signaling cascades under starved conditions or pathogen infection [28, 29]. Under nutrient deprivation and glucose

availability, the mammalian target of rapamycin (mTOR) switches on and prevents ULK1 activation through phosphorylating Ulk1 Ser 757 and disrupting its association with adenosine monophosphate (AMP)-activated protein kinase (AMPK) [30]. During low glucose content, AMPK is activated and mTOR is inhibited, thereby suppressing ULK1 Ser 757 phosphorylation, allowing ULK1 phosphorylation by AMPK interaction and eventually activating autophagy [30]. Next, ULK1 forms a tetrameric complex with FAK family kinase interacting protein of 200 kDa (FIP200), ATG13, and ATG101 to recruit the VPS34 complex for phagophore isolation and autophagosome initiation [31, 32] (Figure 1.1). The class III phosphatidylinositol 3-kinase (PtdIns3K) catalytic subunit VPS34 then interacts with ATG14, VPS15 and BECN1 to form a protein complex (PI3KC3) which is essential for the initiation and expansion of autophagosomes [33]. Furthermore, PI3KC3 synthesizes the lipid phosphatidylinositol-3-phosphate (PI3P), which recruited WD-repeat protein interacting with phosphoInositides (WIPI) proteins and subsequently WIPIs recruit Atg16L1 that conjugates with the autophagosome marker microtubule-associated protein 1A/1B-light chain 3 (LC3; mammalian ortholog of ATG8) through Atg5/12/16L1 complex recruitment [34]. During autophagosome maturation, LC3 translocated from the cytosol to the isolation membrane where the cysteine protease ATG4 cleaved pro-LC3 to generate LC3-I. Then, LC3-I is subsequently transferred by ATG7 to the expanded phagophore membrane where LC3-I travel through Atg3, lipidated to LC3-II and attached to the phagophore membrane [35]. In parallel, ATG5/12/16L complex stimulated the conjugation of LC3-I to phosphatidylethanolamine (PE) to form lipidated LC3 (LC3-II) which binds to receptor molecules such as p62 inside the inner and outer membranes of the autophagosome [36].
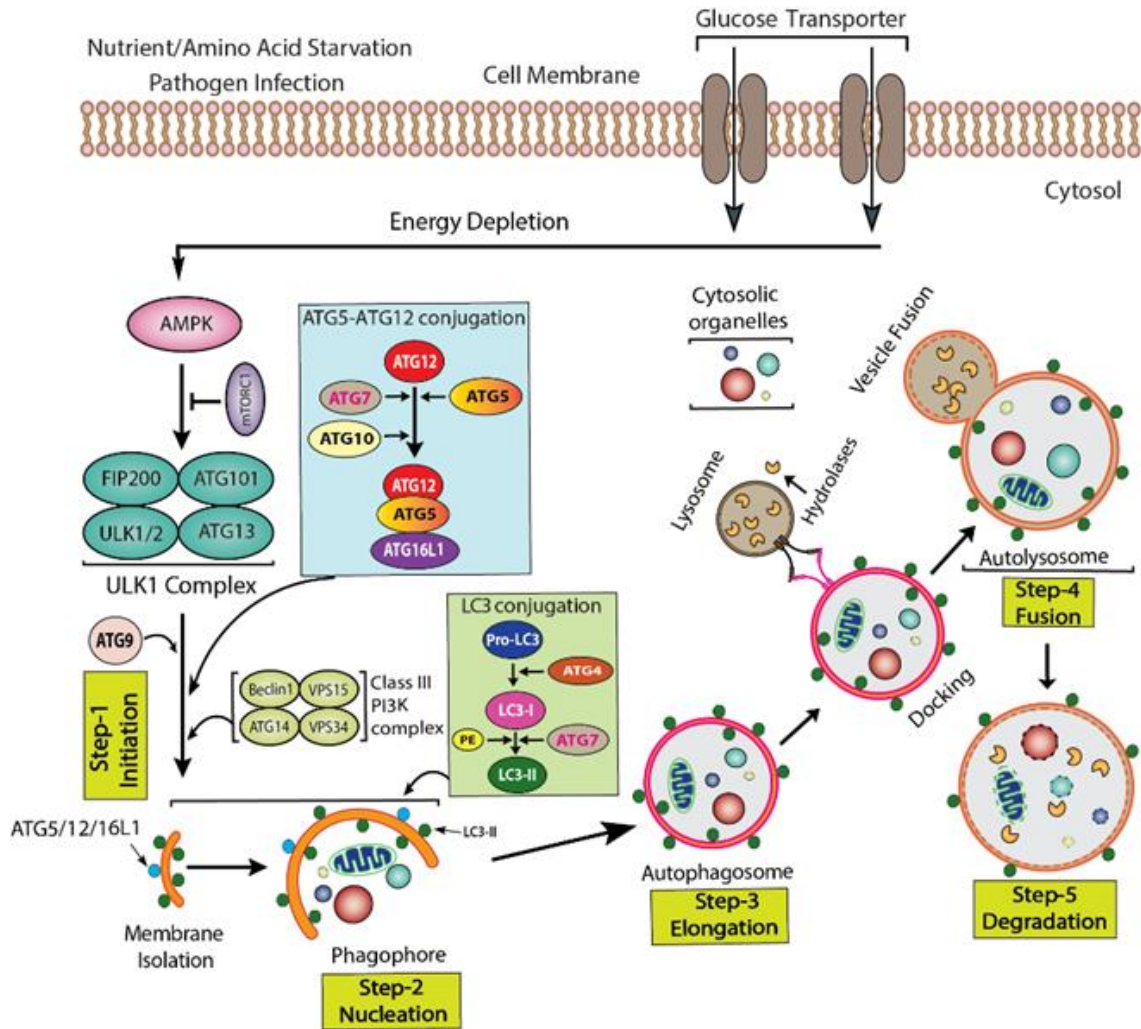
Figure 1.1: Mechanism of autophagy

Autophagy is initiated under nutrient starvation, stress and pathogen infection where the 5' AMP-activated protein kinase (AMPK) activates the ULK1 complex. Autophagy induction results in the recruitment of ATGs to the isolation membrane that forms a cup-shaped structure also termed as phagophore. The isolation membrane then gradually elongated and results in a sphere around the cytosol. Eventually, the isolation membrane seals into a double-membrane vesicle called the autophagosome, where it engulfed cytosolic organelles as autophagic cargo. During phagophore elongation and autophagosome formation, delipidated LC3-I conjugates with the lipid-containing phosphatidylethanolamine (PE) to form lapidated LC3-II. After autophagosome formation, it fuses with the lysosome to form autolysosome where cytoplasmic cargos are broken down by the resident lysosomal hydrolases for cytosolic cargo degradation.

Additionally, LC3-II is also involved in phagophore extension and closure during autophagosome formation. The next step is the fusion of the matured autophagosome with the hydrolase enzyme-containing lysosome, referred to as autophagolysosome to provide its cytoplasmic organelle for degradation (Figure 1.1) [37].

1.4 Importance of the ULK1

ULK1 is a macroautophagy initiatory kinase and paralog of the yeast Atg1 protein. In the human genome, there are five Atg1 orthologues namely ULK1, ULK2, ULK3, ULK4, and STK36 [38]. Among them, only ULK1 and ULK2 have autophagy regulatory functions [39]. Both genes encoded serine/threonine protein kinases having a conserved N-terminal catalytic domain, a serine-proline rich central domain, and a C-terminal interacting region [40]. Although ULK1 and ULK2 are involved in autophagy induction, growing pieces of evidence suggested their functional differences concerning autophagy and other biological properties. Previously it was suggested that autophagy deficiency via ULK1 ablation associated with slow ribosomal and mitochondrial clearance in the reticulocytes while ULK1 is not essential for starvation-induced autophagy in mice [41]. They also found that during erythroid differentiation, ULK1 but not ULK2 upregulated. Another study indicated that functions of ULK2 are more likely to compensate ULK1 ablation in a cell-type manner [40]. ULK1 and ULK2 also played autophagy-independent roles in lipid metabolism [42]. More recently, researchers found that ULK1 and ULK2 have major differences in their transcriptional and post-translational regulators and their autophagy-related interactors [43].

Table 1.1: Canonical autophagy-dependent and independent function(s) of the core ATGs

Protein name	Autophagy step(s)	Canonical autophagy dependent functions(s)	Rf.	Canonical autophagy independent function(s)	Rf.
ULK1 or ATG1	Initiation	Mitochondrial respiration, ATP production, lipid metabolism, mitochondrial and ribosomal clearance	[42, 44]	Cell death and apoptosis, endocytosis, immune signaling, antiproliferative and antineoplastic effects in MPNs, ER-to-Golgi trafficking, cellular homeostasis, ammonia-induced autophagy, endosomal trafficking	[45-52]
ATG2A ATG2B	Elongation	Regulates lipid homeostasis, promotes Atg9-Atg18 interaction, programmed cell size reduction	[53-55]	iDISC dependent caspase-8 activation, apoptosis, lipid droplet localization	[56, 57]
ATG3	Elongation	Induces HIV infection and cell death	[58]	LAP, endosomal trafficking, apoptosis	[59-61]
ATG4B ATG4D	Elongation	Sense balance and Otoconial development induces HIV infection and cell death	[58, 62]	LAP, mitochondrial dysfunction, apoptosis	[59, 63]
ATG5	Elongation	Maintenance of innate lymphocytes, skeletal homeostasis, antiviral immune responses	[64-66]	Immunity, intracellular pathogen killing, apoptosis, adipogenesis	[67-70]
ATG6 or Beclin1	Nucleation	Induces HIV infection and cell death	[58]	Apoptosis, cell death, cancer cell growth, embryogenesis, tumor suppression, STAT3 phosphorylation, DNA damage repair, receptor degradation and cytokinesis, induces viral transmission, improves the life span	[26, 71-78]
ATG7	Elongation	Maintains cellular and behavioral responses, regulates potassium (K^+) level in hypokalemia	[79, 80]	Cell shrinkage, cell cycle arrest, mitochondrial clearance, adipogenesis, ISC integrity maintenance, promotes neuronal health and longevity	[55, 68, 81-83]

ATG8 or LC3	Cargo selection	Maintains tissue homeostasis	[84]	LAP, apoptosis, virus replication, cancer cell survival, lysosome biogenesis, exocytosis	[67, 85-88]
ATG9	Initiation	Pathogenesis of POI	[89]	Maintain lysosomal degradation, axonal degeneration, STING and TBK1 assembly	[90, 91]
ATG10	Elongation	Not known	-	Apoptosis, deficiency leads to ALS and FTD molecular defects, lysosomal degradation, suppress HCV replication	[92-94]
ATG12	Elongation	Mitochondrial homeostasis, cell death, antiviral immune responses, osteoclast secretion, pathogen control	[66, 95, 96]	Endosomal trafficking, mitochondrial apoptosis, endosome to lysosome trafficking	[59, 97, 98]
ATG13	Initiation	Cell cycle progression	[99]	Control virus replication, cardiac development	[100], [101]
ATG14	Nucleation	Autophagosome–endolysosome fusion	[102]	Autophagic cell death	[103]
ATG16L1	Elongation	Urothelial vesicle trafficking	[104, 105]	Apoptosis	[67, 106]
ATG18	Elongation	Programmed cell size reduction	[55]	Neural homeostasis	[107]
ATG101	Initiation	Maintaining respiratory function	[108]	Not known	-
FIP200	Initiation	Maintaining respiratory function	[108]	Control virus replication	[100]
VPS15	Nucleation	Not known	-	Skeletal muscle function, endocytosis, neuronal migration	[76, 109, 110]
VPS34	Nucleation	T-cell homeostasis	[111]	Endocytosis, receptor degradation and cytokinesis	[76]

ULK1: Unc-51 Like Autophagy Activating Kinase 1, LAP: LC3-associated phagocytosis, iDISC: intracellular death-inducing signaling complex, Rf: reference(s), PAS: pre-autophagosomal structure, ISC: intestinal stem cell, STING: stimulator of IFN genes, TBK1: TANK-binding kinase 1, POI: primary ovarian insufficiency, FTD: frontotemporal dementia, ALS: amyotrophic lateral sclerosis, HCV: hepatitis C virus, VPS34: vacuolar protein sorting 34, FIP200: FAK family-interacting protein of 200 kDa

Despite either ULK1 or ULK2 knock-out and their functional differences, both homologs have reasonable importance as knock-out of both ULK1 and ULK2 showed neonatal lethality, which is similar to the deletion of other core ATGs including ATG5 and ATG 7 [41, 46]. Consequently, it remains elusive and challenging to study the general autophagy pathway via ablation of Atg5 or Atg7 or the upstream modulators such as Ulk1, Beclin 1 and Fip200 as the evidence suggested that, loss of core autophagy genes and their key initiatory kinases are often lethal and ultimately causes apoptosis [41, 112-114].

1.5 Hematopoiesis

Hematopoiesis is the process by which hematopoietic stem cells (HSCs) give rise to all blood cell types such as leukocytes, red blood cells and platelets [115]. Physiologically, all these blood cells reside inside the bone marrow microenvironment known as the HSC niche [116]. In mammals, hematopoiesis occurs sequentially in four distinct areas including the yolk sac, aorta-gonad mesonephros (AGM), fetal liver and bone marrow [115]. The first wave of blood cell production inside the yolk sac is called "primitive". Primitive hematopoiesis generates red blood cells for tissue oxygenation as the embryo grows quickly. Hematopoiesis in the primitive state is transient and is quickly replaced by adult-type "definitive" hematopoiesis. Throughout embryogenesis, definitive hematopoiesis is associated with hematopoietic stem cells and multipotent progenitors (HSCs/MPPs), which later become mature blood cells and immune cells [117].

During steady-state and under stress conditions, a complex network of HSC-intrinsic mechanisms including transcriptional regulation, metabolic adaptation and epigenetic modification as well as extrinsic factors such as local signals inside the bone marrow microenvironment and long-distance signals from outside the bone marrow regulate the balance between HSC self-renewal and differentiation to maintain homeostasis [116]. Throughout vertebrate evolution, hematopoiesis has generally been conserved [118]. Studies of human hematopoiesis have been complemented and significantly extended by the manipulation of animal models, such as the mouse and zebrafish.

1.6 Autophagy in hematopoiesis

Autophagy is the major lysosomal degradation pathway and it plays a key role in maintaining hematopoiesis. The autophagic process causes the breakdown of cellular organelles and is required for cellular homeostasis, including differentiation processes, such as adipogenesis, erythropoiesis, and lymphopoiesis [119]. To identify the role of autophagy in hematopoiesis, different types of conditional *Atg* knock-out mice models have been used to avoid embryonic and neonatal lethality [120-123]. Autophagy is required for HSCs differentiation and maintenance. For instance, selective degradation of mitochondria (mitophagy) is crucial for regulating HSCs identity [124]. FIP200, a critical regulator of autophagy induction, led to the proper functioning and in vivo maintenance of HSC [112]. Furthermore, *Atg7* deficiency in the hematopoietic system led to the loss of HSC function and dysregulated myeloid cell proliferation [125]. Therefore, autophagy is required for a variety of HSC functions including quiescence, self-renewal, and differentiation [126].

Autophagy is also essential for the development and differentiation of other blood cell types such as B cells, T cells and erythroid cells [127-131] (Figure 1.2). Red blood cell (RBC) differentiation provided the first evidence that autophagy may be involved in hematopoietic differentiation. Additionally, most of the cellular organelles in peripheral tissues are removed when the cells are terminally differentiated into red blood cells. Autophagy is induced in polychromatic erythroblasts at the same time that several autophagy genes are expressed at high levels [132].

1.6.1 Autophagy in HSCs maintenance

During cell differentiation, mTOR pathway inhibition predominantly induces autophagy and potentiates HSC quiescence and self-renewal activities by suppressing mitochondrial functionality [133-135]. Mice with HSC-specific deletion of core autophagy gene *Atg12* resulted in a significant increase in apoptosis [136]. Lack of autophagy by conditionally deleted *Atg7* in the HSCs resulted in disrupted megakaryopoiesis and thrombopoiesis as observed by enlarged platelets with excessive functional abnormalities during platelet activation and aggregation [137]. On the other hand, deletion of FOXO3A, a putative transcription factor of the HSC, and further crossing with GFP-LC3 mice demonstrated that *Foxo3a* regulates pro-autophagy gene expression in HSCs and maintains the quiescence of HSCs to protect them from starvation-induced metabolic stress [136, 138].

HSCs-specific autophagy-deficient mice showed the expansion of hematopoietic stem and progenitor cells (HSPCs), leading to bone marrow failure and severe anemia. Afterward, those mice undergone complete loss of HSC compartment and eventually die

[139, 140]. Knock-out of *Atg5* in mouse hematopoietic cells also resulted in lymphopenia and progressive anemia [140]. However, whether this cellular proliferation occurs either cell-autonomously or due to cytopenias is elusive. Moreover, it has been shown that mice with *Atg5* deficiency in HSCs resulted in survival defects including severe anemia, lymphopenia and significant reduction of HSCs [141]. Similarly, mice with autophagy deficiency via FIP200 ablation inside fetal HSCs were embryonically lethal [112].

Although, it has been reported that *Atg7* is curial for HSC maintenance but the overall autophagy data including how *Atg7* knock-out influence the autophagy activation and how it is regulating autophagy flux were missing [142]. The maintenance of young and aged HSCs compartment was clearly shown by another study while isolating HSCs and granulocyte/macrophage progenitors from the GFP-LC3 transgenic mice bone marrow and measuring p62 and GFP-LC3 levels [136]. Later, it has been suggested that *Atg7* deficiency-induced aberrant megakaryocyte differentiation [137]. This study measured the LC3-II protein levels to observe autophagy activation upon conditionally knocking out *Atg7* from the hematopoietic system. However, they did not show the autophagy flux changes and autophagy drugs (such as rapamycin, calpeptin, 3-MA, chloroquine) induced modulatory effects in *Atg7* knock-out mice, which were essential to confirm such phenotypes were due to *Atg7* regulated autophagy or Atg7 independent autophagy. One study did not interpret the autophagy level upon *Atg5* knock-out [141], while another study investigated the autophagy by only observing p62 level accumulation [112], which is not sufficient to monitor autophagy according to the autophagy guideline [143]. In any experimental setup, measurement and detection of LC3-II level and detection of

autophagy flux by using chloroquine or bafilomycin A1 are critical to assess autophagy more precisely. In further research study by using *Atg5* and *Atg7* deficient mice model, autophagy has been assessed more precisely, demonstrated that high autophagy flux is essential for the maintenance of HSCs [144]. Nevertheless, all of these studies indicated that autophagy is essential for HSPCs homeostasis.

1.6.2 Autophagy in progenitor cells

Likewise the HSCs maintenance and normal functioning, autophagy also played essential roles for the maintenance of hematopoietic progenitors including the common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), megakaryocyte erythrocyte progenitors (MEPs), NK cell progenitors (NKPs) and granulocyte-macrophage progenitors (GMPs) in bone marrow (BM). Mice with the ablation of *Atg7* in the HSPC compartment resulted in significantly reduced CMPs and CLPs in the BM and consequently induced severe myeloid proliferation, likely to human acute myeloid leukemia (AML) [7]. Furthermore, since *ATG5* or *ATG7* knock-down resulted in decreased HSPC frequencies, high autophagic flux is essential for the maintenance of myeloid and erythroid progenitor cells function in vitro and in vivo [144]. Recently, it has been suggested that chemically induced *Atg5* deletion upon tamoxifen administration resulted in a significant reduction in CMPs, MEPs and GMPs in the BM [145]. Previously it has been reported that the mTOR signaling pathway modulated the myeloid progenitor-derived macrophage differentiation by autophagy inhibition [146]. Conditional deletion of *Atg7* in the mice hematopoietic system showed a significant reduction in CMPS, CLPs

and NKPs inside the BM [142], although, this study did not measure or detect the autophagy status in the *Atg7* deficient mice hematopoietic system.

1.6.3 Autophagy in lymphoid maturation

Autophagy renders pivotal roles in lymphoid differentiation and maturation (Figure 1.3). Mechanistic insights using experimental mouse models suggested that deletions of selected *Atg*s such as *Atg5* or *Atg7* inside the T-cell and B-cell compartments involved fundamental autophagy process and regulating cellular renewal, differentiation and immune cell functions during lymphoid maturation.

1.6.3.1 Role of autophagy during B lymphocyte development

During early embryogenesis, B lymphocytes development initiated in a stepwise manner such as pro-B cells, pre-B cells, and immature B cells from HSCs inside the bone marrow while autophagic activity including *Beclin 1* expression and autophagosome abundance was first reported during pro-B cell time point [147-150]. Afterward immature B cells migrate and secrete antibodies to the secondary lymphoid organs where they get fully matured. B lymphocytes are categorized into B-1 and B-2 lymphocytes depending on their cell surface marker expression properties [151].

The first evidence concerning the necessity of autophagy during B-cell development comes from the conditional knock-out of the *Atg5* mice model [129]. *ATG5* deletion in B lymphocytes resulted in defective B cell development during pro-B cell to pre-B cell transition stages with a substantial decrease in B-1 lymphocytes inside bone marrow [129]. Cell death occurs more frequently in peripheral B-1 cells, which reduced their

15

numbers in contrast with the unaffected peripheral B-2 lymphocytes, indicating a relative autophagy dependency [129]. Matured B cells further differentiated into either quiescent memory B cells or long-lived antibody-secreting plasma cells [152]. Autophagy defective plasma cells and memory B cells are incapable of continuous protein biosynthesis and eventually come across misfolded protein aggregation due to apoptosis [153, 154].

1.6.3.2 Autophagy in T lymphocytes

Autophagy plays important role in T-lymphocyte homeostasis [155]. Deficiency of *Atg5* in mice showed full T lymphocytes maturation but peripheral T and B lymphocytes and total thymocytes were reduced [156]. Targeted deletion of *Vps34* and *Atg16l1* in the T-cell compartment of aged mice models impaired the normal development of innate natural killer (NK) T lymphocytes [157, 158]. Consistent with the aforementioned findings, *Atg7* ablated mice within the T-cell compartment perturbed cellular differentiation of invariant natural killer T (iNKT) cells in peripheral lymphoid organs [159]. Under normal circumstances, iNKT cells display upregulated mitophagy during thymus development. Impairment of *Atg7* led to the accumulation of mitochondrial ROS and apoptosis [159]. Furthermore, targeted deletion of T-cell-specific core autophagy gene *Atg5* or *Atg7* resulted in autophagy deficiency and defective T lymphocyte production [160].

1.6.3.3 Autophagy in natural killer lymphoid cells

NKPs derived from CLPs differentiated into immature or innate NK cells (iNKs) and eventually into mature NKs (mNKs), ensure immune response against viral infections and pathogen attacks [161-163]. NKP specific *Atg5* deficient mice showed severe reduction in iNKs and mNKs within the bone marrow and spleen leading to the accumulation of ROS and damaged mitochondria [164].

1.6.4 Autophagy in myelopoiesis and granulopoiesis

Myelopoiesis is a stepwise differentiation and maturation of HSCs to CMPs by terminal differentiation which led to the production of monocytes and granulocytes including neutrophils, basophils and eosinophils. As a result, myelopoiesis is sometimes subdivided into monocytopoiesis and granulopoiesis [165]. It currently remains elusive that how autophagy mediates the monocyte and granulocyte differentiation.

1.6.4.1 Autophagy in erythropoiesis

Autophagy plays a critical role during erythropoiesis where nucleus removal from the erythroblasts initiates reticulocyte formation inside bone marrow [166]. Deficiency of *Atg 5* and *Atg7* in mice may diminish the mitochondrial clearance but could not abolish total autophagy [167, 168]. One of the previous studies reported that in mice deficient with *Ulk1, Atg5* and *Ulk1/Atg5* at developmental hematopoiesis, *Ulk1* dependent autophagy plays the most predominant role in mitochondrial clearance than the *Atg5* dependent autophagy [44]. Similarly, Fip200 ablation impaired the erythroid maturation and rendered defective fetal HSC maintenance [112].
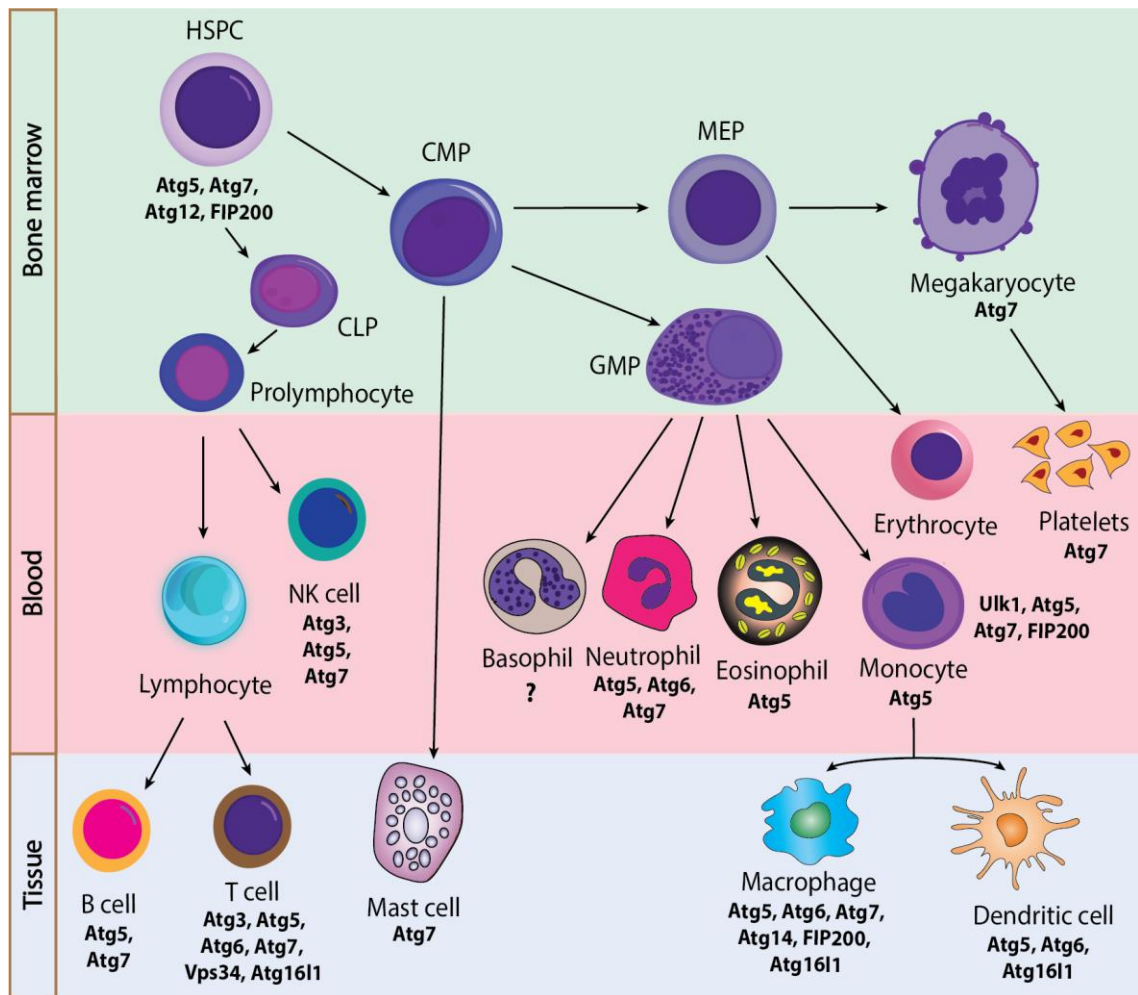
Figure 1.2: *Atg*s **involved in hematopoietic differentiation.**
Autophagy plays a vital role during hematopoiesis indicating that specific stages of hematopoietic cell differentiation require the putative mechanistic involvement of different autophagy genes as well as the multiple autophagy factors. HSPC, hematopoietic stem and progenitor cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; NK cell, natural killer cell.

Studies suggested that mice deficient in *Gata-1* suffered from immature proerythroblast stage and subsequently undergone cell death and apoptosis [169]. *Gata-1* deficient mice exhibited severe anemia and embryonic lethality whereas GATA-1 expression induces

autophagy by upregulating *ATG4B, ATG8* homologs and *ATG12* expression in primary

human erythroblasts by direct transcriptional control [170, 171].

1.6.4.2 Autophagy in neutrophil granulocytes

Granulopoiesis is the sequential differentiation of GMPs to become eosinophils,

neutrophils, basophils and macrophages inside bone marrow [165]. Granulocytes, also

known as polymorphonuclear leukocytes (PMNL) are white blood cells where neutrophil

granulocytes are the short-lived and widely abundant cells of the host immune system

and its functional impairments leading to serious immunodeficiency syndromes [172].

Mice HSC compartments deleted with *Atg7*, displayed myelodysplastic syndrome

(MDS) [139] and targeted deletion of *Atg7* during earliest granulopoiesis leading to

immature precursor accumulation that prevents neutrophil differentiation *in vivo* [173].

Furthermore, *Atg7* ablation led to the defective lipophagy in neutrophil differentiation,

although the exact molecular profiling of how lipophagy directed into the autophagy

machinery was unknown. Moreover, no direct proof found regarding autophagy activity

in basophil or eosinophil granulocytes. During the process of neutrophil development

and maturation, autophagy acts as a defender mechanics to degrade extracellular

materials by canonical autophagy [174], LC3-dependent phagocytosis [175] and

xenophagy [176], while mice with *Atg5* or *Atg7* deficiency led to neutrophil

degranulation and disrupted ROS generation [177].

1.6.4.3 Autophagy in monocyte and macrophage development

Monocytes are bone marrow-derived white blood cells or leukocytes, circulating inside

the blood and upon migrating from the bloodstream into tissues, monocytes are

terminally differentiated into macrophages and dendritic cells [178]. Monocytes are involved in various cytokine induction, antigen presentation while conferring innate immunity and tissue homeostasis [179]. Macrophages are multifaceted innate immune phagocytes that serve as a first-line host defense against intracellular pathogen invasion by mounting pro-inflammatory responses via phagocytosis, releasing cytokines and renovate damaged tissues [180]. Only a few pieces of literature reported the role of autophagy during monocyte to macrophage differentiation. Circulating monocytes derived from CMPs can generate macrophages and dendritic cells inside tissues through cellular differentiation [181]. However, detailed mechanistic insights and molecular events have not been elucidated. Mice lacking *Atg5* inside the granulocytes and monocytes/macrophages resulted in intracellular pathogen infection, indicating an essential role of *Atg5* in cellular immunity and to kill intracellular pathogens via autophagy-independent GTPase trafficking [182].

1.6.4.4 Autophagy in thrombopoiesis and the development of thrombocytes

Thrombocytes or platelets are generated from the megakaryocyte progenitors (MPs) and are colorless, small and flowing cell fragments in the bloodstream that immediately respond to blood vessel injury, form blood clotting, prevent bleeding and assist in hemostasis [183]. Autophagy events also take place in thrombocytes where higher LC3-II turnover is observed in thrombocytes compared to starved thrombocytes [184]. It has been suggested that megakaryocyte and thrombocyte-specific ablation of *Atg7* indicated a markedly reduced LC3-II level, defective platelet aggregation, abrogated granule cargo

packaging, impaired hemostasis and thrombus formation without affecting platelet morphology [184].

In most cases, mice have been used as a common *in vivo* vertebrate model system while targeting different autophagy genes in the hematopoietic systems to delineate the autophagy activities and hematopoietic outcomes (Table 1.2). Nevertheless, the use of transparent *in vivo* models such as zebrafish can be helpful to elucidate the dynamic nature of autophagy in hematopoietic tissues with more details.

1.7 Autophagy and malignant hematopoiesis

The role of autophagy in hematological malignancies is double-faced and paradoxical because of its tumor-suppressive and tumor-promoting properties [185]. Dysregulations of HSPCs self-renewal and differentiation capacity are associated with malignant hematopoiesis. Autophagy is vital for the maintenance of life-long hematopoiesis and deletion of ULK1-interacting partners including FIP200, ATG5 or ATG7 impaired the normal survival capacity of HSCs in mice [112, 139, 140]. In particular, heterozygous deletion of *ATG5* induces the disease progression of acute myeloid leukemia (AML) [140]. Conversely, human AML cells with *ATG7* knock-down have reduced proliferation rate, enhanced chemosensitivity and improved outcomes in AML therapy [140]. Autophagy inactivation by *Atg5* or *Atg7* deletion increased the ROS generation, cell death and survival ability in leukemic mice [186]. Mice with HSC specific deletion of *Atg7* resulted in defective mitophagy and MDS (myelodysplastic syndrome, a complex bone marrow failure disorders associated with aging, impaired clonal hematopoiesis and

anemia)-like phenotypes [142]. In vivo mouse model demonstrated that HSCs isolated from leukemic mice exhibit a higher level of basal autophagy compared to the non-leukemic mice [187]. Deletion of *Atg3* or treatment with autophagy inhibitors to inactivate autophagy from murine bone marrow cells prevented BCR-Abl-mediated leukemogenesis (leukemia induction process that associates multiple genetic and. epigenetic events) *in vitro* [188].

1.8 Zebrafish as a model to study autophagy and hematopoiesis

The optical clarity, high fecundity, externally developed embryos, high throughput chemical screening and the availability of transgenic and mutant strains of zebrafish made them a robust and prime vertebrate animal model for studying developmental biology, disease pathogenesis and more recently to explore autophagy during embryogenesis [189, 190]. Moreover, the revolution in gene editing such as Transcription Activator-Like Effectors Nucleases (TALENs) [191, 192], Clustered Regularly Interspaced Palindromic Repeats (CRISPR) system based genome editing [193] and Tol2 transgenesis were promptly implemented in zebrafish opened up the possibility for cell-type or tissue-specific genetic manipulations [194-200]. Having conserved orthologues of human ATGs such as ULK1 (58%), ULK2 (74%), ATG7 (77%), ATG5 (81%), ATG3 (82%) and human-like sequential multi-lineage hematopoiesis over murine model [201], it may have the likelihood that the results obtained in autophagy research using zebrafish model would be linked to humans as well.

Table 1.2: Autophagy defects during hematopoietic differentiation in mice

Model system	Target cells	Hematopoietic outcomes	Rf.
Fip200$^{flox/flox}$ × Tie2-Cre		Myeloproliferation, HSC apoptosis and severe anemia.	[112]
Atg5$^{flox/flox}$ × Vav-iCre		Anemia.	[202]
Atg5$^{flox/flox}$ × Vav-Cre	HSCs	Lymphopenia, anemia, accumulation of monocytes, macrophages and neutrophils.	[140]
Atg12$^{flox/flox}$ × Mx1-cre		Loss of HSCs.	[136]
Atg7$^{flox/flox}$ × Vav-Cre		Loss of HSC function, severe myeloproliferation.	[139]
Atg5$^{flox/flox}$ × CD19-Cre		Defective antibody responses in B cells, increased cell death in BM and depletion in B-1 B cells.	[129, 203, 204]
Atg5$^{flox/-}$ × Mb1 Cre and Atg5$^{flox/-}$ × CD21 Cre	B cells	Decreased T1 B cells and follicular B-cell numbers, reduced B-1a and B-2 B-cell proportion.	[130]
Atg5$^{flox/flox}$ × Gzmb-Cre Atg7$^{flox/flox}$ × Gzmb-Cre		Loss of T cell function.	[205]
Atg7$^{flox/flox}$ × CD4-Cre		Loss of iNKT in lymphoid organs, lymphopenia, severely compromised CD8$^+$ memory T cells.	[160, 206]
Atg3$^{flox/flox}$ × Lck-Cre		Decreased T cell numbers.	[207]
Atg5$^{flox/flox}$ × Cre-ER^{T2}	T cells	Loss of CD8$^+$ T cells, a severe reduction in lymphoid specific memory T cells.	[208]
Atg5$^{flox/flox}$ × CD4-Cre Atg7$^{flox/flox}$ × Lck-Cre		Significant reduction in iNKT, CD4 and CD8 T cell numbers.	[127, 209]
Vps34$^{flox/flox}$;CD4-Cre		Impaired T cell homeostasis and anemia.	[157]
Atg16l1$^{flox/flox}$;Cd11c-^{Cre}		Expanded T cell proliferation.	[210]
Atg3$^{flox/flox}$ Ubc;cre-ERT2 Nkp46^{cre} × Bnip3l$^{flox/flox}$		Loss of memory NK cells.	[211]
Atg5$^{flox/flox}$;NKp46-Cre FoxO1$^{flox/flox}$;FoxO1$^{AAA/+}$; NKp46-Cre	NK cells	Impairment in NK cell development, reduction in iNKs and mNKs in the spleen and BM.	[164]
CD4 Cre-Atg7$^{-/-}$		Abrogated iNKT development, progressive anemia.	[212]

Atg7$^{flox/flox}$ × Vav-iCre	Erythrocytes	Impaired red blood cell development and severe anemia.	[213]
Becn1$^{flox/flox}$ -Lyz2-cre$^{+/-}$ Fip200$^{flox/flox}$ -Lyz2-cre$^{+/-}$	Monocytes/ Macrophages	Perturbed lymphoid and myeloid cell homeostasis, altered macrophage differentiation.	[214]
Atg7$^{Flox/Flox}$; Mx-Cre	Mast cells	Impairment of mast cell degranulation.	[215]
Vav-Cre × Atg7$^{flox/flox}$ Cebpa-cre × Atg7$^{flox/flox}$ Mx1-cre × Atg5$^{flox/flox}$	Neutrophils and Eosinophils	Impaired neutrophil differentiation.	[173]
Atg7$^{flox/flox}$;Lyz2-Cr		Eosinophilic inflammation.	[216]
Atg7$^{flox/flox}$;LysM-cre		Reduced neutrophil degranulation, increased circulating neutrophil numbers, decreased inflammatory potential of neutrophils.	[177]
CD11c-Cre-Atg5$^{flox/flox}$	Dendritic cells	Reduced migration of DCs.	[217]
Atg7$^{flox/flox}$;PF4-Cre	Megakaryocytes and Platelets	Impaired thrombosis, robust bleeding, platelet aggregation.	[184]
Atg7$^{flox/flox}$;Vav-Cre		Impeded megakaryocyte differentiation, abnormal platelet production.	[218]
Atg5$^{flox/flox}$;PF4-Cre		Delayed thrombus formation, pulmonary thrombosis, significantly reduced thrombin-induced platelet aggregation.	[219]

mNKs, mature natural killers; BM, bone marrow; iNKT, invariant natural killer T; CD, cluster of differentiation; DCs, dendritic cells.

Some of the landscapes already demonstrated that further studies of autophagy in zebrafish could result in a novel understanding of the physiological role of the autophagy mechanism [220-222]. In particular, cloning of zebrafish microtubule-associated protein 1A/1B-light chain 3 (Lc3) targeting autophagosomes and generation of a transgenic green fluorescence protein (GFP) tagged Lc3 fish line (GFP-Lc3) implemented as an excellent platform to monitor autophagy lively under microscopic imaging [223]. The

below table showed the previously identified zebrafish autophagy homologs for different human autophagy proteins (Table 1.3).

Zebrafish (*Danio rerio*) has emerged as a robust in vivo model for vertebrate hematopoiesis with unique features including high fecundity, optical transparency, dispensable early embryonic hematopoiesis, as well as highly amendable for genetic and chemical manipulation [224, 225]. Zebrafish hematopoiesis has a higher similarity to mammals considering the highly conserved complex regulatory networks and various hematopoietic lineages [115]. Particularly in early embryogenesis, zebrafish embryos do not require a fully effective circulatory system. As a result, it can be utilized as a model to provoke loss-of-function for gene expression. Compare with *in vitro* model, the zebrafish model provides more comprehensive information about the regulation of hematopoiesis, which is dynamic with interactions between hematopoietic cells and their niche at the whole-organism level.

1.9 Zebrafish embryonic hematopoiesis

Zebrafish are being utilized as a perfect model system to study vertebrate developmental hematopoiesis [201, 224, 226-228]. Unlike mammals, zebrafish eggs are fertilized externally and easily available for examining the one-cell embryonic stage. Although invertebrate model organisms such as fruit flies (*Drosophila melanogaster*) have shown their importance while studying embryogenesis [229-231], these models are not quite suitable for working on hematopoiesis and associated mature blood cells because of limited blood cell proliferation or differentiation [232], inappropriate vascular network [233], deficits equivalents of the lymphoid lineages and its mature blood cells [234]. With

the advancing genetic technologies in the past decade, zebrafish models played important roles in modeling hematopoietic defects, which essentially contributes to a comprehensive picture of vertebrate hematopoiesis.

1.9.1 Zebrafish primitive hematopoiesis

During the initial wave of zebrafish primitive hematopoiesis, lateral plate mesoderm (LPM) is the place where the early hematopoietic progenitor as defined by *scl* first appeared in the bud stage at 11 hpf [224]. Afterward, primitive hematopoiesis occurs in the intermediate cell mass (ICM) positioned in the trunk ventral to the notochord and the rostral blood island (RBI) originating from the cephalic mesoderm where it predominantly generates erythrocytes and some primitive macrophages [201, 235, 236].

At 12 hours post-fertilization (hpf) stage, CMPs (*pu.1*/*spi1b*) first arises in the anterior lateral plate mesoderm (ALPM) [237], and differentiate as well as later migrate to the anterior yolk sac in the rostral blood island (RBI) where they expressed the pan-leukocyte marker (*l-plastin*/*lcp1*) after 16 hpf [238-240]. During the embryonic development of both mammals and zebrafish, hematopoiesis occurs in two successive waves namely primitive or embryonic hematopoietic wave and definitive wave [241-243]. Subsequently, pan-leukocyte marker expressed genes subdivided into neutrophil (*mpx/mpo*) and macrophage (*mpeg1.1*) lineages [244, 245] (Figure 1.3).
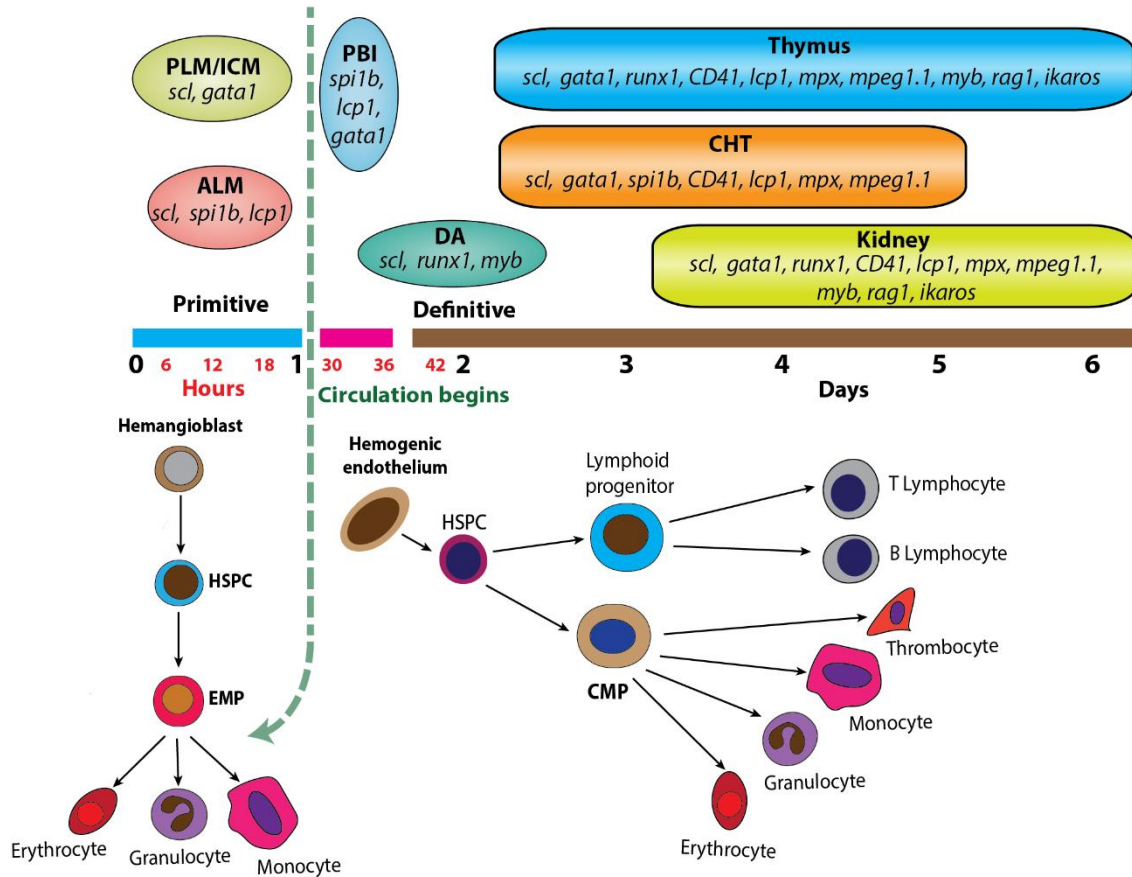
Figure 1.3: Timeline of developmental hematopoiesis in zebrafish.

Around 11 hours post-fertilization (hpf), embryonic primitive hematopoiesis (blue) initiates when the multipotent precursor cells (hemangioblasts, can differentiate into both endothelial and hematopoietic cells) appeared in the anterior lateral mesoderm (ALM) and posterior-lateral mesoderm (PLM). Blood circulation begins at around 24 hpf (pink) and a transient definitive wave initiates in the posterior blood island (PBI) shortly after the appearance of erythro-myeloid progenitors (EMP). Definitive HSPCs arise from the hemogenic endothelial cells of the dorsal aorta (DA) at 26 hpf and migrate to the CHT at 48 hpf. Lymphoid progenitor cells arise at around 54 hpf and seed inside the thymus (site for lymphoid T cells maturation). CMP: common myeloid progenitors.

Table 1.3: Key human autophagy proteins and zebrafish homologs

Human proteins	Zebrafish homolog	Functions in the autophagy pathway	Rf.
ULK1 ULK2	ulk1a, ulk1b ulk2	Initiator of the autophagy pathway, part of the ULK-ATG13-ATG101-FIP200 complex that inhibits mTORC1 complex;	[246]
ATG101	atg101	Part of the ULK complex, responsible for initiating cellular autophagy	[247]
ATG13	atg13	Under nutrient starvation, enhances the kinase activity of ULK complex	[248]
FIP200	Rb1cc1	Novel mammalian autophagy factor that functions together with ULKs and is essential for autophagosome formation	[249]
Beclin 1	beclin 1	Interacts with BCL-2 binding protein; facilitating membrane extension, cargo recruitment and autophagosome maturation	[250]
WIPI1	Wipi1	Major phosphatidylinositol 3-phosphate (PtdIns3P) effectors at the nascent autophagosome	[251]
ATG9A	atg9a	Its phosphorylation renders autophagosome assembly	[252]
ATG3	atg3	The ubiquitin-conjugating enzyme that catalyzes the conjugation of Atg8 and phosphatidylethanolamine (PE) and promotes the lipidation process	[253]
ATG4B	atg4b	A cysteine protease that converts the pro-LC3 to LC3-I; conjugate LC3-I with phosphatidylethanolamine (PE) and yield LC3-II	[254]
ATG5	atg5	Conjugates with ATG12 and assists in autophagy	[255]
ATG7	atg7	Autophagosomal membrane development and fusion within cells	[256]
GABARAP	gabarapa gabarapb	Binds to LC3 and plays a critical role in autophagosome-lysosome fusion	[257]
MAP1LC3B	map1lc3b	Referred to as LC3, the most widely used marker of autophagosomes	[143]
ATG10	atg10	Promoting autophagosome formation	[94]
ATG12	atg12	Facilitates the LC3 lipidation	[258]

| ATG16L1 | atg16l1 | Autophagosome synthesis while binding with the Atg5-Atg12 complex | [259] |

Primitive erythropoiesis first arises in the posterior lateral plate mesoderm (PLPM) at 12 hpf and further migrates to the intermediate cell mass (ICM) at 18 hpf characterized by the expression of *gata1* and embryonic hemoglobin [260]. Cells within the ICM further differentiate into the endothelial cells of the trunk vasculature and proerythroblasts which begin to enter the circulation around 24 hpf. In the intermediate wave of hematopoiesis, the EMPs arise in the posterior blood island (PBI) region autonomously at 30 hpf that generate both myeloid cells (*spi1b*, *lcp1* and *mpx*) and erythrocytes (*gata1*) [261] (Figure 1.3).

1.9.2 Zebrafish definitive hematopoiesis

Zebrafish definitive hematopoiesis arises from the ventral wall of dorsal aorta (VDA) which is similar to the mammalian aorta-gonad-mesonephros (AGM) expressing hematopoietic stem cells *myb* and *runx1* at 36 hpf through endothelial-hematopoietic transition (EHT) [262-265] (Figure 1.3). At 40 hpf, zebrafish thrombocytes arise inside the region of VDA and its circulation begins at 48 hpf characterized by the expression of CD41^{high} in transgenic CD41:GFP zebrafish embryos [266, 267]. Subsequently, it migrates to the caudal hematopoietic tissue (CHT) which is comparable to the mammalian fetal liver. Afterward, the hematopoietic cells come to the kidney and thymus where the multi-lineage hematopoiesis and T lymphocytes are produced and eventually harbored inside the kidney marrow where life-long hematopoiesis takes place [224].

At the end of the primitive wave and the initiation of definitive hematopoiesis, a transient hematopoietic wave arises where the EMPs generate independently from the posterior blood island (PBI) [268] (Figure 1.3).

Upon knock-down or knock-out of different core *Atg*s including *FIP200, Atg5, Atg7, Atg12* and *Atg16* inside the hematopoietic system, it is clear that autophagy plays a critical role while regulating HSCs and its downstream blood progenitors. However, the function of the core autophagy gene and autophagy activating kinase *Ulk1* in the hematopoietic system remaining elusive. In this study, the role of *Ulk1* regulated autophagy in definitive hematopoiesis has been studied using the zebrafish model.

Chapter 2: Objectives

This study made use of the optically clear and robust zebrafish model to investigate the role of autophagy during embryonic hematopoiesis *in vivo*, based on TALEN-mediated knock-out of autophagy initiating kinase ulk1.

Objectives:

1. To develop zebrafish *ulk1* knock-out models.

2. To examine the effects of *ulk1* knock-out in autophagy activation and flux.

3. To investigate the effects of *ulk1* knock-out in definitive hematopoiesis.

4. To examine if the effects of *ulk1* knock-out on hematopoiesis is autophagy-dependent or independent

Chapter 3: Materials and Methods

3.1 Zebrafish strains, feeding and maintenance

Wild-type, Tg(*myb*:GFP) [269], Tg(*mpeg1.1*:eGFP) [270], Tg(*coro1a*:DsRed) [271] and Tg(*GFP-Lc3*) [223] zebrafish lines were kept in normal aquatic conditions. Hatched brine shrimp were fed twice daily to the fish. Natural spawning was used to collect embryos and staged as previously mentioned guidelines [272, 273]. All zebrafish embryonic experiments were performed under the approval and guidelines of the Animal Subjects Ethics Sub-Committee (ASESC), The Hong Kong Polytechnic University.

3.2 Zebrafish embryo collection and staging

To obtain embryos, adult male and female zebrafish were set in a mating tank as 1:3 or 2:4 ratio and separated by a divider one day before embryo collection (Figure 3.1). The next day, the divider was removed and allow the male and female fishes to meet, and embryos were collected from natural spawning. Developmental stages were determined following Kimmel *et al* and staged as mentioned in previous ethical approval [273] (Figure 3.2).

3.3 ULK1 protein sequence alignment

Amino acid sequences for human ULK1 (NP_003556.2), mouse Ulk1 (NP_001334323.1), zebrafish ulk1a (NP_001124103.1) and ulk1b (XP_005161178.1) were obtained from National Center for Biotechnology Information (NCBI) and aligned by ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic tree of ULK1 protein in human, mouse and zebrafish was built up using the neighbor-joining method.
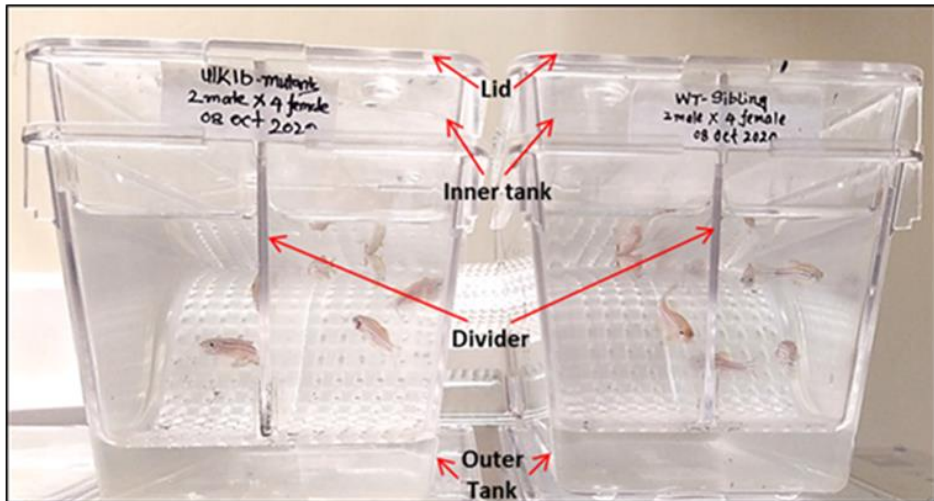
Figure 3.1: Overview of zebrafish husbandry.

(A) The multi-tank aquatic system for zebrafish husbandry in the Department of Health Technology and Informatics, The Hong Kong Polytechnic University. (B) Collection tanks were set to obtain embryos consisting of an outer tank in combination with an inner tank, which contains mesh at the bottom for embryo collection. A divider plate is set inside the inner tank to separate male and female fishes before mating and covered by a lid on the top.
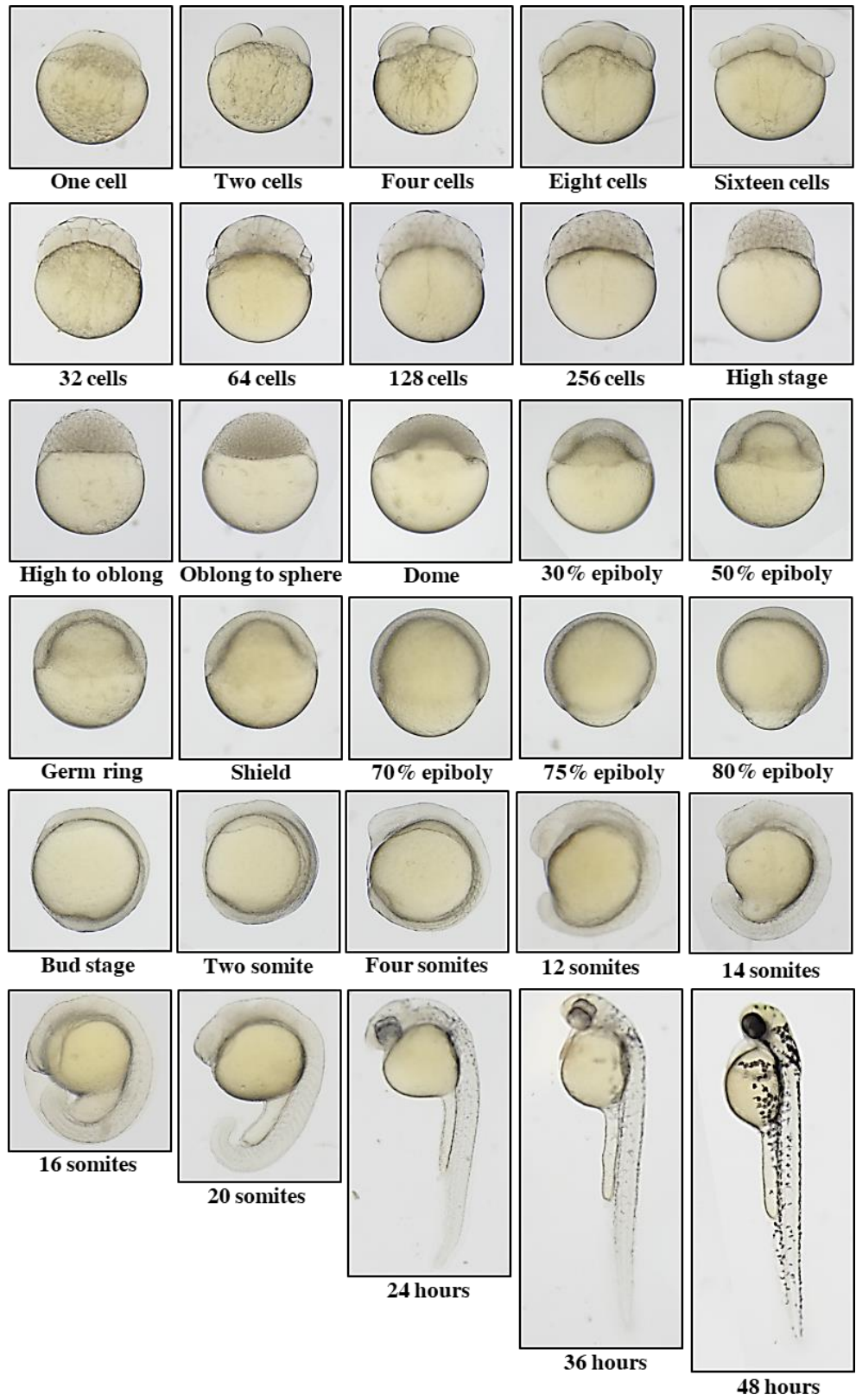
Figure 3.2: Zebrafish embryonic developmental stages.

Stages for zebrafish embryonic development adopted from Kimmel *et al* 1995 [273].

3.4 Generation of the *ulk1* mutants by TALENs

TALEN sequences targeting the genomic region of zebrafish *ulk1a* exon 2 and *ulk1b* exon 4 were designed and synthesized as described previously [274]. Briefly, each TALEN arm was constructed by mixing an appropriate amount of six corresponding plasmids with 1 µL 10x NEBuffer 3.1 (New England Biolabs) and 0.5 µL BsmBI enzyme (Thermo Scientific) mix, gently centrifuged for a few seconds and incubated at 55^0C for 30 minutes.

Afterward, each reaction was added with 1.5 µL 10X T4 DNA Ligase Reaction Buffer (New England Biolabs), 0.5 µL T4 DNA Ligase (New England Biolabs) and 0.5 µL Esp3I enzyme (Thermo Scientific) and made up to 15 µL with deionized water. The whole reaction mixtures were subjected to thermocycler incubations as 37^0C for 5 minutes, 16^0C for 10 minutes for 10 cycles, 37^0C for 15 minutes, 80^0C for 5 minutes and 4^0C forever.

To identify the correct constructs, colony PCR was performed. PCR reactions were then transformed to the TOP10 competent cells following blue/white screening whereas positive white clones were selected and further verified by Sanger sequencing. After confirmed by sequencing results, *ulk1a* and *ulk1b* plasmids were linearized and further purified by PCR purification kit (Qiagen, #28104). Next, every single-stranded DNA was reverse-transcribed to mRNA by T3 mMessage mMachine Kit (Ambion, #AM1348).

3.5 Microinjection

Zebrafish embryos were microinjected as described in previous protocols [275, 276]. Briefly, *ulk1a* left and right TALEN arms and *ulk1b* left and right TALEN arms obtained from T3 mMessage mMachine *in vitro* transcription were mixed respectively to an appropriate amount. One-cell-stage zebrafish embryos were then individually microinjected by 100 pg of *ulk1a* and *ulk1b* TALEN mRNA into the yolk using stereomicroscope (Nikon SMZ800N, USA) and a pressure-controlled PLI-90 pico-liter injector (Harvard Apparatus Limited, USA) (Figure 3.3).

3.6 TALEN mechanism and evaluation of TALEN activities by genotyping

Mechanistically TALEs are genomic tools being used to modify DNA according to their specific loci using customizable DNA binding motifs. DNA recognition domains of TALEs are composed of tracts of identical 33-35 amino acid residues and a partial repeat domain. Within each unit, adenine (NI, NN and HD); guanine (NK, NN and NH for) and thymine (NG) are the repeat-variable di-residues (RVDs) that bind to a specific nucleotide [277, 278]. In the zebrafish model system, TALE nucleases or TALENs have been successfully used to introduce targeted mutations either through non-homologous end joining (NHEJ) or by homology-directed repair (HDR) and homology-independent repair (HIR) using a donor template. With comparable mutagenic activity, TALENs are more specific in binding and have fewer sequence constraints in targeting the genome, compared to other customizable nucleases.

Microinjected embryos from 36 hpf to 2 dpf were collected for restriction fragment length polymorphism (RFLP) genotyping as mentioned previously [279]. To check TALEN mutagenic activities in F0 chimeric mutants, embryos were taken one by one into the PCR strips and digested with genomic DNA (gDNA) extraction buffer (a mixture of KCl, MgCl$_2$, Triton X-100, Tris-HCl of pH 8.0, NP-40 and proteinase K) at 55^0C for 3 to 5 hours with shaking. Afterward, reaction mixtures were incubated for 10 minutes at 98^0C to inactivate the proteinase K and PCR amplified using the genotyping primers (Table 3.1).

Then the PCR products were digested with AciI and NheI restriction enzymes targeting selected bases at the spacer region of *ulk1a* exon 2 and *ulk1b* exon 4 respectively (Figure 3.4). During the imaging of performed agarose gel electrophoresis, an extra undigested PCR band was detected in both somatically targated *ulk1a* and *ulk1b* groups.

Figure 3.3: Microinjector system for zebrafish embryo microinjection.

The system contains a stereomicroscope (Nikon SMZ800N, USA), a gas pressure microinjector (Harvard Apparatus, USA) and a micromanipulator (KANETEC, Japan).
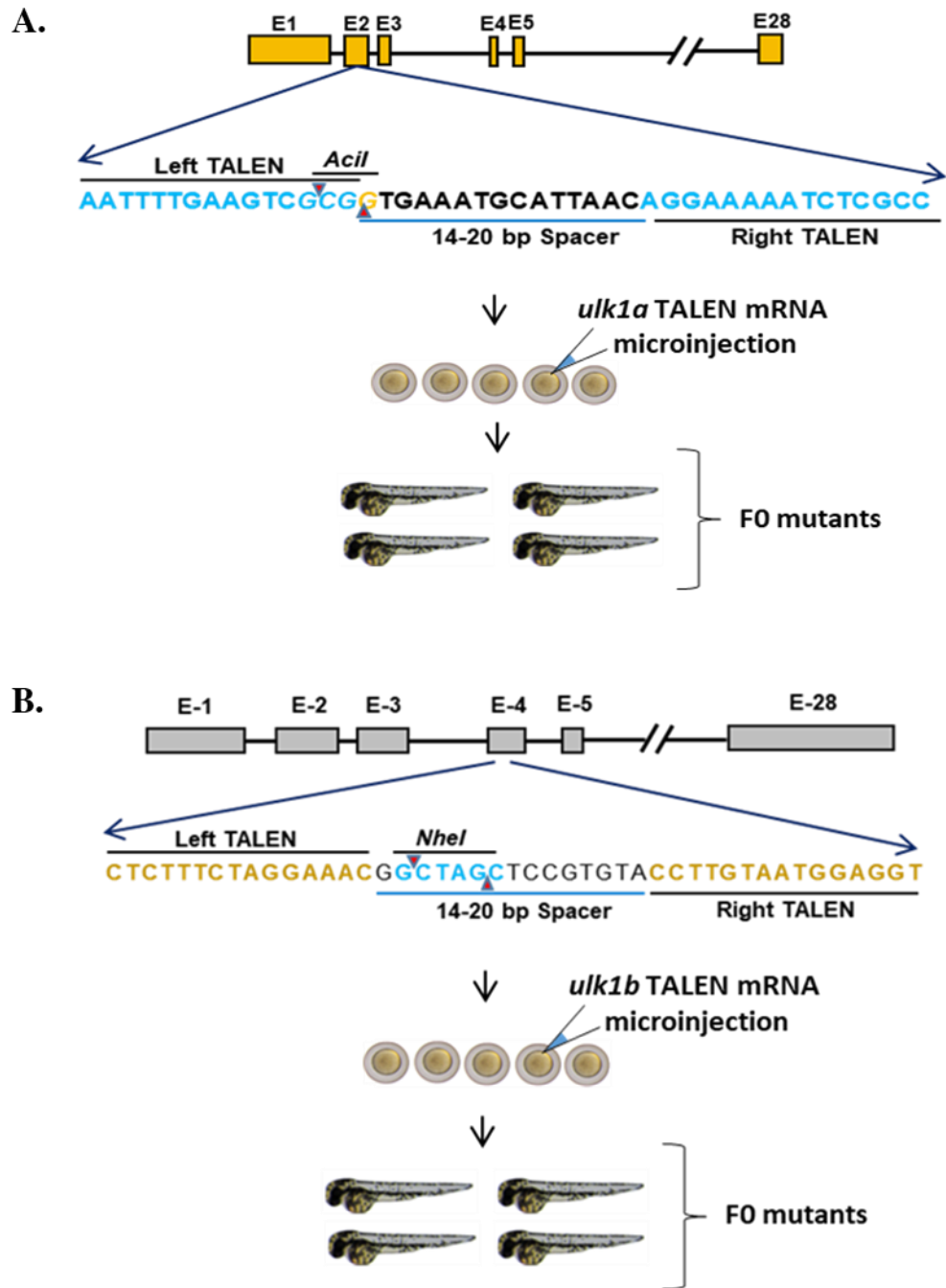
Figure 3.4: Generation of somatically targated *ulk1a^TAL* and *ulk1b^TAL* by TALEN.

(A-B) Schematic illustration representing TALEN mediated genome editing of zebrafish *ulk1a* and *ulk1b*. 14-20bp spacer region containing the mutation initiation site. AciI and NheI restriction enzymes were used in these genotyping assays to cut in a specified location of *ulk1a* and *ulk1b*.

In this study, somatically targated *ulk1a* and *ulk1b* groups were presented as *ulk1a*^{TAL} and *ulk1b*^{TAL} respectively, while their wild-type siblings were introduced as controls. Stable *ulk1b* wild type controls and their mutant siblings were presented as *ulk1b*^{+/+} and *ulk1b*^{-/-}.

3.7 Anti-sense RNA probe synthesis

Complementary DNA (cDNA) sequences of the target genes were PCR amplified and sub-cloned into the pGEM-T-easy vectors (Promega, USA) following the manufacturer's guidelines. The plasmid was ligated and transformed into the One Shot TOP10 chemically competent cells with blue/white screening using isopropyl β- d-1-thiogalactopyranoside (IPTG) and β-galactosidase combination (1:1). Colonies with white appearance were picked for overnight liquid broth culture at 37^0C shaking incubator. The overnight culture was then mini prepped by QIAprep Spin Miniprep Kit (Qiagen, USA) to purify the plasmid DNAs according to the manufacturer's instructions. Purified plasmids were then verified by sequencing using T7 and SP6 primer. Confirmed plasmid DNAs were linearized by specific restriction enzymes and then purified by QIAquick PCR Purification Kit (Qiagen, USA) and concentrations were measured by a spectrophotometer (Thermo Scientific, USA). Purified single-stranded DNAs were next used as the template for anti-sense RNA probe synthesis using DIG RNA Labeling Kit and T7 or SP6 RNA polymerase (Roche Applied Science). To perform the whole-mount *in situ* hybridization (WISH), following plasmids, their linearized restriction enzymes and RNA polymerases were utilized to synthesize riboprobes and to complete the reverse

transcription reaction: *spi1b-* SacI-HF, T7; *myb-* SalI-HF, T7; *lcpI-* NsiI-HF, T7; *mpx-*

PstI-HF, T7 and *ulk1b-* SacII.

Table 3.1: List of primers used in this study.

Primer Name	Primer sequence
Genotyping Primer Sets	
Zf-*ulk1b*-Exon_4-GF	TTCATGGCCCGGGGGT
Zf-*ulk1b*-Exon_4-GR	CTGCTAGGAAGCTCCTCATGG
Zf-*ulk1a*-Exon_2-GF	AGACTTGATCGGACATGGCG
Zf-*ulk1a*-Exon_2-GR	CTCCTGGCAAAGGCACAAAC
WISH Probe Primer Sets	
Zf-*spi1b*-PF	TACATCATCCCACCCCAGCAAAC
Zf-*spi1b*-PR	AATGCTTTCTGTCTGTGTGGCTC
Zf-*lcp1*-PF	GAAGACCAGCGTCCATCTGC
Zf-*lcp1*-PR	CCAGCTCCACCGCATAGTTA
Zf-*mpx*-PF	CTCTGAACCCTGCTTCCCAAT
Zf-*mpx*-PR	TGGAATCTCTATCAGTCTCTTTCCA
Zf-*myb*-PF	CAGCACTCCACCTTAGCACA
Zf- *myb*-PR	TTGGGAGTTCGGAACAGCTC
Zf-*ulk1b*-PF	CCCCTACCCAGGATTCTCCA
Zf-*ulk1b*-PR	GACCACTTGTTTGACGGTGC

GF, Genotyping forward primer; GR, Genotyping reverse primer; PF, WISH probe forward primer; PR, WISH probe reverse primer.

Briefly, 800 ng to 1 μg linearized plasmid DNA, 1 μL RNase inhibitor, 2 μL 10x transcription buffer, 2 μL 10x NTP labeling mixture and 2 μL T7 or Sp6 RNA polymerase were made up to 20 μL with nuclease-free water. The reaction mixture was incubated overnight at 37^0C and 1 μL deoxyribonuclease (DNase) was added, mixed gently and incubated again at 37^0C for 15 minutes.

Then, approximately 60 μL 7.5 M lithium chloride (LiCl) was added to each reaction and incubated overnight at -20^0C for the precipitation of the RNA probe. At 4^0C, the reaction mixture was then centrifuged at 12,000 g for 15 minutes and the upper flow was discarded. Next, ice-cold 70% ethanol was added to wash the pellet, centrifuged for 5 minutes at 7,500 g and the supernatant was removed. Afterward, the pellet was air-dried, resuspended in nuclease-free water. The concentration of the synthesized RNA probes was measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Riboprobes were further 100-fold diluted in pre-hybridization buffer solution and stored under -20^0C.

3.8 In situ hybridization

WISH was performed to determine the spatial and temporal gene expression pattern in zebrafish embryos at the mRNA level during embryonic development [280]. Protocols for WISH have been described previously [275, 276]. Embryos with mentioned developmental stages were collected and fixed with 4% paraformaldehyde (PFA) at room temperature for at least 4 hours. Afterward, fixed embryos were subsequently dehydrated in 25%, 50%, 75% and 100% ethanol for 5 minutes each and stored at -20^0C overnight. Embryos were further rehydrated (100%, 75%, 50% and 25% ethanol) and washed two

times in phosphate-buffered saline with Tween-20 (PBST). To avoid unwanted autophagy induction, 1-phenyl 2-thiourea (PTU) was not used to inhibit the conventional pigmentation in this study [281]. Instead, 4% PFA fixed zebrafish embryos were bleached with 3% H_2O_2 and 1% KOH before WISH. Older embryos other than 24 hpf were penetrated with Proteinase K (20 mg/mL, Roche) at 1:1000 dilution in PBST and re-fixed with 4% PFA for 30 minutes. After subsequent washes in PBST, embryos were soaked in 600 µL pre-hybridization buffer (PHB) (20x SSC buffer, 50% formamide, 50 µg/ml heparin, 5x saline-sodium citrate, 0.1% Tween 20, 5mg/ml rRNA) and incubated in the water bath at 65^0C for 4 hours. Afterward, embryos were hybridized overnight with anti-sense mRNA probes at 65^0C.

Next day, embryos were stepwise washed with 2x SSC and PHB mixture (25% 2x SSC in 75% PHB, 50% 2x SSC in 50% PHB, 75% 2x SSC in 25% PHB, and 100% 2x SSC for 15 minutes each) at 65^0C water bath, followed by 30 minutes 0.2x SSC wash at room temperature. Then subsequently washed in 0.2x SSC and PBST mixture (75% 0.2x SSC in 25% PBST, 50% 0.2x SSC in 50% PBST, 25% 0.2x SSC in 75% PBST and three times in 100% PBST) at room temperature for 5 minutes each. Then, embryos were hybridized with anti-digoxygenin antibody (1:4000) (Roche, Germany) at 4^0C overnight with slow motion shaking.

On day three, embryos were washed four times with PBST each of 20 minutes before washing with AP buffer (100 mM NaCl, 100 mM Tris-HCl with pH 9.5, 0.05% Tween 20 and 5 mM $MgCl_2$,) three times (5 minutes for each wash). Then the embryos were incubated and conjugated to chromogenic alkaline phosphatase that hydrolyzes the 5-

bromo-4-chloro-3-indolyl phosphate (BCIP) to 5-bromo-4-chloro-3-indole and inorganic phosphate. The organic compound is oxidized by nitro blue tetrazolium (NBT) to form an insoluble dark blue diformazan precipitate (NBT/BCIP (Roche, Germany))[282]. After the staining, substrate solution was removed and PBST added before image analysis using a Nikon SMZ1270/1270i stereomicroscope (Nikon Instruments Inc., NY, USA). For long-term storage, stained embryos were immersed in 100% ethanol and stored at -20^0C.

3.9 Flow Cytometry, cell sorting and CYTO-ID® staining

Flow cytometry was performed to enumerate the lineage-specific quantification of hematopoietic populations using transgenic embryos. Embryos as indicated stages were first anesthetized and digested with 0.05% trypsin-EDTA reagent (Gibco™, #25300054) for 50-75 minutes at 28.5^0C. Trypsin digestion was terminated by adding 10mM $CaCl_2$ followed by embryo dissociation into single-cell suspension by pipetting. Dissociated cells were further washed and harvested in 1mL phosphate-buffered saline (VWR™, #E404-200TABS) with 2% fetal bovine serum (Gibco™, #10500-064) and filtered through a 40 µm cell strainer (Falcon®, #352340). Samples were spun down at 1200 rpm for 5 minutes at room temperature and washed two times with PBS having 2% (vol/vol) FBS. Then, the percentages of eGFP^{+} cells from different reporter lines were quantified by BD FACSAria™ III Cell Sorter (BD Biosciences) (Figure 3.5). Data were further analyzed by FlowJo version 7.6 software. To get the net fluorescence intensity, nonspecific immunofluorescent background or autofluorescence (AF) of the fluorescent samples were subtracted from the non-fluorescent wild-type samples. For CYTO-ID®

46
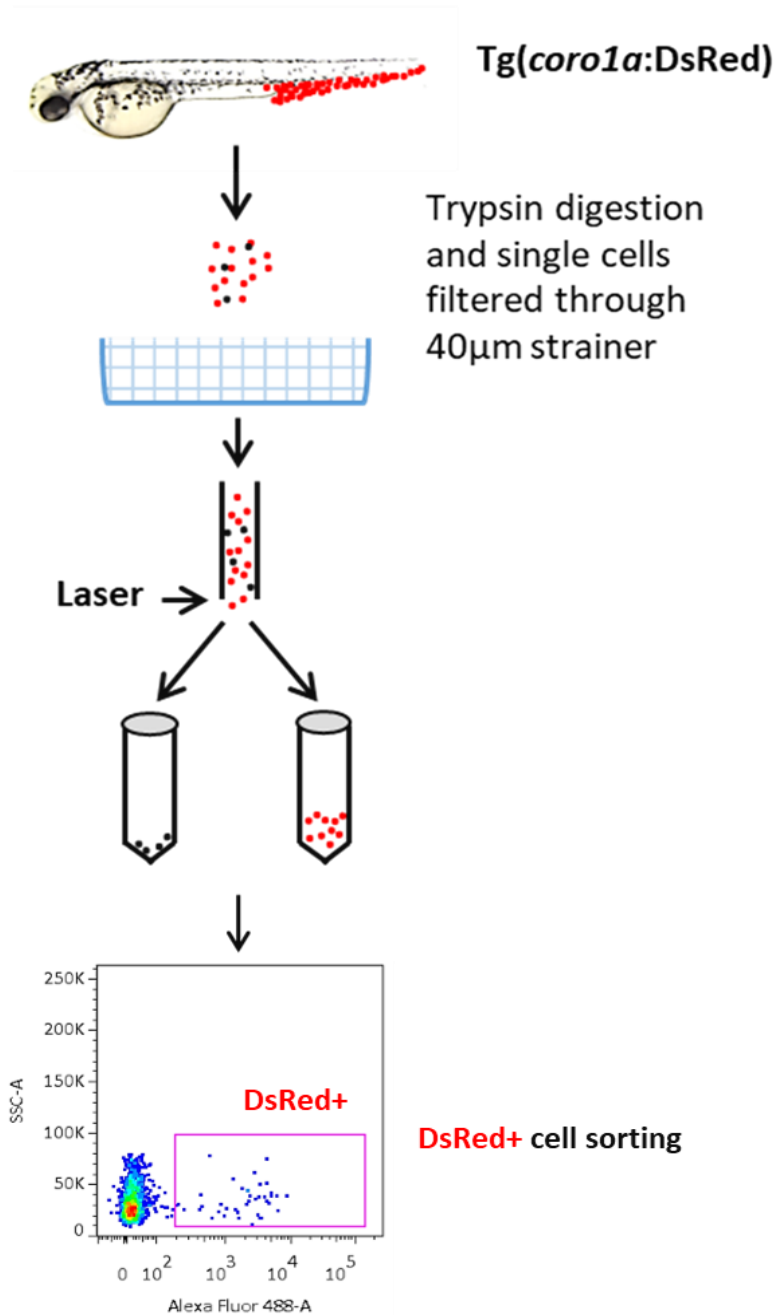
Figure 3.5: Flow diagram for the quantification of myeloid cells by flow cytometry in zebrafish embryos.

Coro1a DsRed positive leukocytes were sorted out by flow activated cell sorting. Tg(*Coro1a*:DsRed) zebrafish embryos were used and the cells were trypsin digested prior to cell sorting.

green that stains acidic autophagosomes, sorted *coro1a*:DsRed positive cells were centrifuged and washed with PBS one time. Then green color CYTO-ID® kit and Hoechst stain added at 1:1 ratio, incubate in dark at 37^0 for 30 minutes. Samples were further washed with PBS and image under the confocal microscope.

3.10 Western blot

CelLytic™ MT Cell Lysis Reagent (Sigma Aldrich, #C3228) was used to deyolk and homogenize the zebrafish embryos. By using BCA assay kits (Thermo Scientific™, # 23225), protein concentrations were determined, and the lysates were mixed with the sodium dodecyl sulfate (SDS) loading buffer and heat-denatured. Then the 12% TGX™ FastCast™ Acrylamide Kit (Bio-Rad Laboratories, Inc., #1610175) was used to electrophorese the protein samples. Afterward, proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, #1620264) and blocked in 5% no fat dry milk (Bio-Rad Laboratories, #1706404). After subsequent washes using Tris-buffered saline (150 mM NaCl, 50 mM Tris base, pH 7.5) with Tween-20 (Bio-Rad Laboratories, #1610781) (TBST), snipped membranes were hybridized with anti-*GAPDH* (Cell Signaling Technology, #2118; 1:20000) and anti-*Lc3b* (Abcam, #ab48394; 1:1000) primary antibodies and incubated overnight at 4^0C. PVDF membranes were further washed in TBST and probed with goat anti-rabbit secondary antibody (Abcam, #ab6721; 1:3000) for 2 hours at room temperature. The membranes were then cleansed with TBST, signals were developed applying Clarity Western ECL Substrate (Bio-Rad Laboratories, #1705061) and imaged under ChemiDoc XRS+ System (Bio-Rad Laboratories).

3.11 Treatment with autophagy modulators and LysoTracker red staining

Zebrafish embryos were treated with 3-MA (Selleckchem, #S2767) which is the class III PI3K inhibitor, calpeptin (Selleckchem, #S7396) which is the calpain protease I and II inhibitor and late stage autophagy degradation blocker chloroquine (Selleckchem, #S4157) which is also known as the autophagosome and lysosome fusion inhibitor, at 10 mM, 50 µM and 100 µM and concentration respectively as previously stated [283]. F1 *ulk1b* mutant Tg(GFP-Lc3) embryos were incrossed to get the wild type, heterozygous and homozygous siblings. To interpret and access the autophagy flux level, 2 dpf zebrafish embryos were treated with CQ to block the autophagy degradation pathway. Lysosome dye, also known as LysoTracker™ Red DND-99 fluorescent dye, was diluted to a 10µM final concentration. Then the Tg(GFP-Lc3) zebrafish embryos at 4 dpf age were incubated with the diluted lysosome dye in dark for 45 minutes at 28.5^0C [220, 284]. Afterward, embryos were carefully washed 3 times before imaging.

3.12 Light-sheet and confocal microscopy

Positive fluorescence signals containing zebrafish embryos were first screened by the Nikon SMZ18 stereomicroscope (Nikon Instruments Inc., NY, USA). Then tricaine (Sigma-Aldrich, # A5040) was used at 0.164 mg/ml concentration to anesthetize the embryos. Afterward, embryos were mounted in 1.5% low gelling temperature agarose (Sigma-Aldrich, # A9045) into a 35 mm glass capillary for light-sheet imaging. Live images were captured by a Zeiss Light-sheet Z.1 Selective Plane Illumination Microscope (Carl Zeiss Microscopy, NY, USA) with a 20X objective lens. On the other hand, confocal images for the CYTO-ID® stained blood cells were taken using a Leica

TCS SPE Confocal Microscope (Leica Microsystems, Wetzlar, Germany) with the 40x objective lens. Furthermore, light sheet and confocal images were processed and analyzed by ZEN imaging and Leica LAS-X imaging software, respectively.

3.13 Phospho-Histone H3 (pH3) immunostaining

Zebrafish Tg(*myb*:GFP) embryos were fixed with 4% PFA for 4 hours at room temperature. Then the embryos were permeabilized with pre-chilled acetone for 20 minutes at -20^0C. After PBST wash, embryos were blocked under 0.1% DMSO, 2% normal goat serum, 0.1% bovine serum albumin and 0.2% Triton-X100 in PBS containing block buffer for half an hour and hybridized with rabbit anti-phospho-Histone H3 (Ser10) polyclonal antibody (Cell Signaling Technology, #9701; 1:1000) for 16 hours or overnight at 4^0C. Embryos were further washed with PBST and incubated with Alexa Fluor 594 goat anti-rabbit secondary antibody (Invitrogen, #A-11012; 1:500) for one hour at room temperature and again washed with PBST before microscopic imaging.

3.14 Statistical analysis

Maximum intensity projections (MIPs) were performed using Z-Stack images (20 out of 100 layers) to analyze the relative number of autophagosomes (GFP-Lc3$^+$), lysosomes (LysoTracker-red$^+$) and merged GFP-Lc3$^+$ and LysoTracker-red$^+$ puncta containing autolysosomes. At least five individual cells were counted per sample and the number of puncta per cell was calculated. ImageJ software version 1.8.0 (NIH) was used to quantify western-blot bands. During WISH analysis, HSCs and myeloid cell numbers were counted manually. Statistical analyses were performed by Mann-Whitney nonparametric U-test and the analysis of variance (ANOVA) with Tukey's multiple comparisons tests

using GraphPad Prism, version 7 (GraphPad Software, CA, USA). In all experiments, results were presented as mean ± standard error of the mean (SEM) and p-value less than 0.05 (p < 0.05) were considered statistically significant.

Chapter 4: Results

4.1 Phylogenetically *ULK1* conserved among vertebrates

Zebrafish *ulk1* is duplicated into *ulk1a* and *ulk1b*. We first examined the phylogenetic relationship among human, mouse, rat, medaka fish and zebrafish *Ulk1* genes (Figure 4.1A). Based on the phylogenetic analysis, zebrafish *ulk1b* is evolutionally more related to human *ULK1,* mouse and rat *Ulk1* and medaka fish *ulk1b* thought multiple sequence alignment showed zebrafish *ulk1a* and *ulk1b* shared very high amino acid sequence similarlity across the whole protein (Figure 4.1B). Overall, zebrafish *ulk1a* and *ulk1b* share 47.85% and 58.02% sequence identity to their human orthologue, respectively (Figure 4.1C).

4.2 Gene synteny of zebrafish *ulk1b* and human *ULK1* are conserved

Gene synteny of human *ULK1,* zebrafish *ulk1a* and *ulk1b* were examined. *Stx2a*, *Sfswap, Mmp17, Ulk1* and *Pus1* are syntenic in both human and zebrafish genome. On the other hand, zebrafish *ulk1a* gene synteny *(fam101a, ncor2, ep400, ulk1a and adgrd1) is* not conserved with the human *ULK1* (Figure 4.2).

4.3 Spatial expression pattern of *ulk1b* during zebrafish embryonic development

Whole-mount in situ hybridization (WISH) was performed to investigate the spatial expression pattern of *ulk1b* during embryogenesis. Early maternal *ulk1b* transcripts were detected at the two-cell stage, suggesting that *ulk1b* may play a role in early embryogenesis (Figure 4.3 i). During the oblong to sphere stage, *ulk1b* expressed ubiquitously (Figure 4.3 ii). Later at 24 hpf and 48 hpf, *ulk1b* was predominantly expressed in the head region, with subtle expression also detected in cells along with the

A.

0.1

ULK1 [*Homo sapiens*]
Ulk1 [*Rattus norvegicus*]
Ulk1 [*Mus musculus*]
ulk1b [*Danio rerio*]
ulk1b [*Oryzias latipes*]
ulk1a [*Danio rerio*]
ulk1a [*Oryzias latipes*]

B.

HS-ULK1	1	MEPGRGGTETVGKFEFSRKDLIGHGAFAVVFKGRHREKHDLEVAVKCINKKNLAKSQTLLGKEIKILKELK	71
Mm-Ulk1	1	MEPGRGGVETVGKFEFSRKDLIGHGAFAVVFKGRHREKHDLEVAVKCINKKNLAKSQTLLGKEIKILKELK	71
Rn-Ulk1	1	MEPGRGGVETVGKFEFSRKDLIGHGAFAVVFKGRHREKHDLEVAVKCINKKNLAKSQTLLGKEIKILKELK	71
Dr-ulk1a	1	-------MESIGKYEFNRKDLIGHGAFAVVFKGRHKQKHNFEVAVKCINRKNLAKSQSLLGKEIKILKELK	64
Ol-ulk1b	1	-------METVGKFEFSRKDLIGHGAFAVVFKGRHREKHEWEVAVKCINKKNLAKSQTLLGKEIKILKELK	64
Dr-ulk1a	1	-------MESVGKFEFSRKDLIGHGAFAVVFKGRHKEKHSWEVAVKCINKKNLAKSQSLLGKEIKILKELK	64
Ol-ulk1b	1	-------METVGKFEFSRKDLIGHGAFAVVFKGRHREKHDWEVAVKCINKKNLAKSQTLLGKEIKILKELK	64

HS-ULK1	72	HENIVALYDFQEMANSVYLVMEYCNGGDLADYLHAMRTLSEDTIRLFLQQIAGAMRLLHSKGIIHRDLKPQ	142
Mm-Ulk1	72	HENIVALYDFQEMANSVYLVMEYCNGGDLADYLHTMRTLSEDTVRLFLQQIAGAMRLLHSKGIIHRDLKPQ	142
Rn-Ulk1	72	HENIVALYDFQEMANSVYLVMEYCNGGDLADYLHTMRTLSEDTVRLFLQQIAGAMQLLHSKGIIHRDLKPQ	142
Dr-ulk1a	65	HENIVSLLDFQEISGCVYLVMEYCNGGDLAEYLHSKGCLSEDTIRVLLQQLAGAMSVLRSKGIIHRDLKPQ	135
Ol-ulk1b	65	HENIVALHDFQETASSVYLVMEYCNGGDLADYLHSKGTLSEDTIRVFLQQITGAMRVLQAKGIIHRDLKPQ	135
Dr-ulk1a	65	HGNIVRLLDYQEIGGCVYLVMEYCNGGDLAEYLHSKGTLSEDTIRIFLQQIAQAMKVLQSKGILHRDLKPQ	135
Ol-ulk1b	65	HENIVALLDFQETVSSVYLVMEYCNGGDLADYLHSKGTLSEDTIRVFLQQIVGAIKVLQSKGIIHRDLKPQ	135

HS-ULK1	143	NILLSNPAGRRANPNSIRVKIADFGFARYLQSNMMAATLCGSPMYMAPEVIMSQHYDGKADLWSIGTIVYQ	213
Mm-Ulk1	143	NILLSNPGGRRANPSNIRVKIADFGFARYLQSNMMAATLCGSPMYMAPEVIMSQHYDGKADLWSIGTIVYQ	213
Rn-Ulk1	143	NILLSNPGGRRANPSNIRVKIADFGFARYLQSNMMAATLCGSPMYMAPEVIMSQHYDGKADLWSIGTIVYQ	213
Dr-ulk1a	136	NILLSYSTGRKSNPNNICIKLADFGFARYLQGNTMAATLCGSPMYMAPEVIMSHNYDAKADLWSVGTIIYQ	206
Ol-ulk1b	136	NILLSHPAGRKSHFNNTCIKIADFGFARYLQNNMMAATLCGSPMYMAPEVIMSQNYDAKADLWSIGTIVFQ	206
Dr-ulk1a	136	NILLCHPEGRKSSSINASIKIADFGFARHLQTNMMAATLCGSPMYMAPEVIMSQNYDAKADLWSIGTIMYQ	206
Ol-ulk1b	136	NILLSYPAGRKSHCNNICIKIADFGFARYLQSNMMAATLCGSPMYMAPEVIMSQNYDARADLWSVGTIVFQ	206

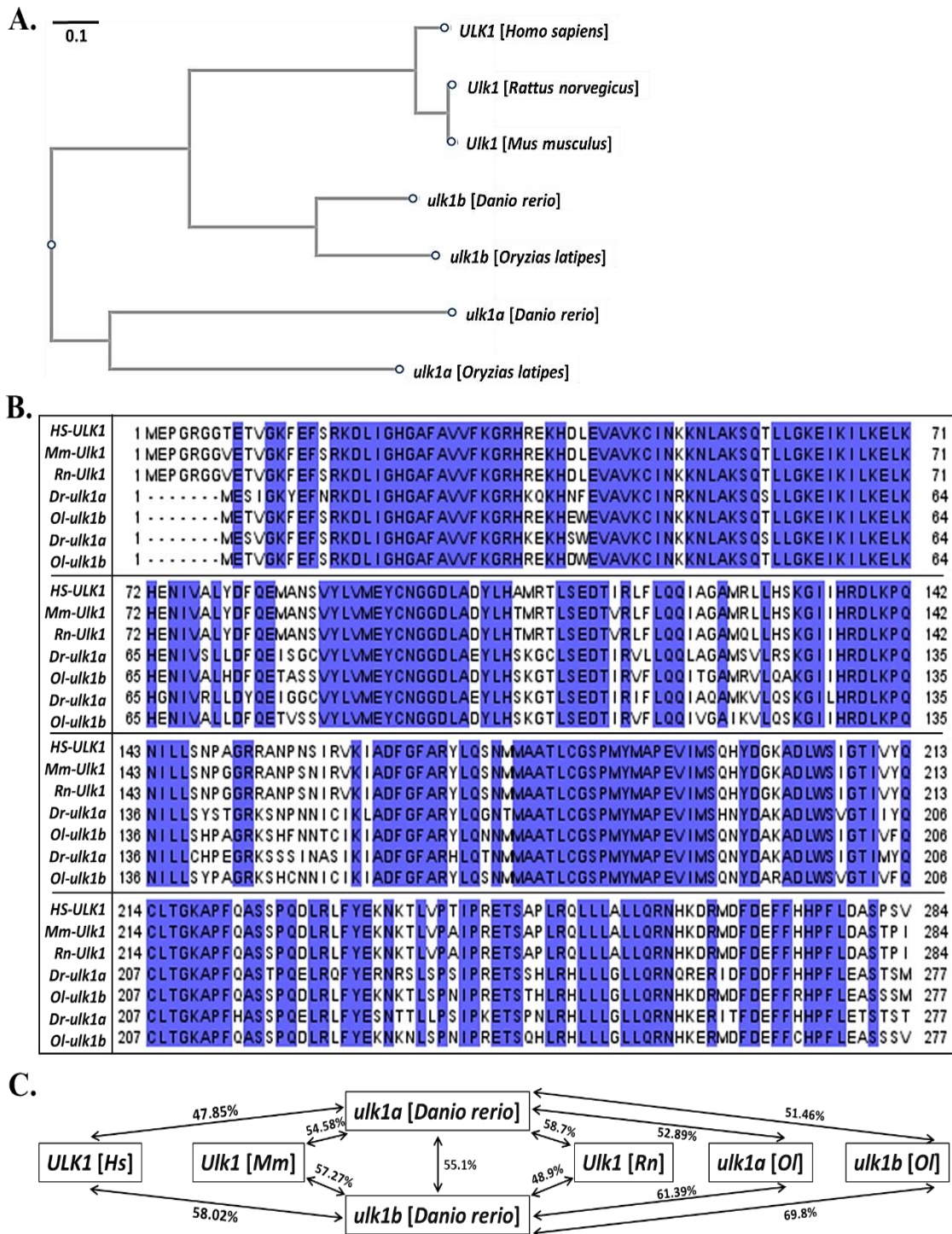|			
HS-ULK1	214	CLTGKAPFQASSPQDLRLFYEKNKTLVPTIPRETSAPLRQLLLALLQRNHKDRMDFDEFFHHPFLDASPSV	284
Mm-Ulk1	214	CLTGKAPFQASSPQDLRLFYEKNKTLVPAIPRETSAPLRQLLLALLQRNHKDRMDFDEFFHHPFLDASTPI	284
Rn-Ulk1	214	CLTGKAPFQASSPQDLRLFYEKNKTLVPAIPRETSAPLRQLLLALLQRNHKDRMDFDEFFHHPFLDASTPI	284
Dr-ulk1a	207	CLTGKAPFQASTPQELRQFYERNRSLSPSIPRETSSHLRHLLLGLLQRNQRERIDFDDFFHHPFLEASTSM	277
Ol-ulk1b	207	CLTGKAPFQASSPQDLRLFYEKNKTLSPNIPRETSTHLRHLLLGLLQRNHKDRMDFDEFFRHPFLEASSSM	277
Dr-ulk1a	207	CLTGKAPFHASSPQELRLFYESNTTLLPSIPKETSPNLRHLLLGLLQRNHKERITFDEFFHHPFLETSTST	277
Ol-ulk1b	207	CLTGKAPFQASSPQDLRLFYEKNKNLSPNIPRETSQHLRHLLLGLLQRNHKERMDFDEFFCHPFLEASSSV	277

C.

Figure 4.1: Phylogenetic relationship, multiple sequence alignment and amino acid sequence homology of Ulk1 orthologues.

(A) Diagram showing the evolutionary relationship among zebrafish (Dr) *ulk1a* and *ulk1b* human (*Hs*) *ULK1,* mouse (*Mm*) *Ulk1*, rat (*Rn*) *Ulk1*, Japanese medaka (*Ol*) *ulk1a* and *ulk1b*. Scale bar represented 0.1 (10%) of genetic variation. (B) Multiple sequence alignment showing the conserved N terminus domain of zebrafish *ulk1a* and *ulk1b* human *ULK1,* mouse *Ulk1*, rat *Ulk1*, Japanese medaka *ulk1a* and *ulk1b* (C) Amino acid sequence similarities between zebrafish (*Dr*) *ulk1a* and *ulk1b* with human (*Hs*) *ULK1;* zebrafish *ulk1a* and *ulk1b* with mouse; zebrafish *ulk1a* and *ulk1b* with rat; zebrafish *ulk1a* and *ulk1b* with Japanese medaka *ulk1a* and *ulk1b* after alignment with protein BALST (Basic Local Alignment Search Tool).
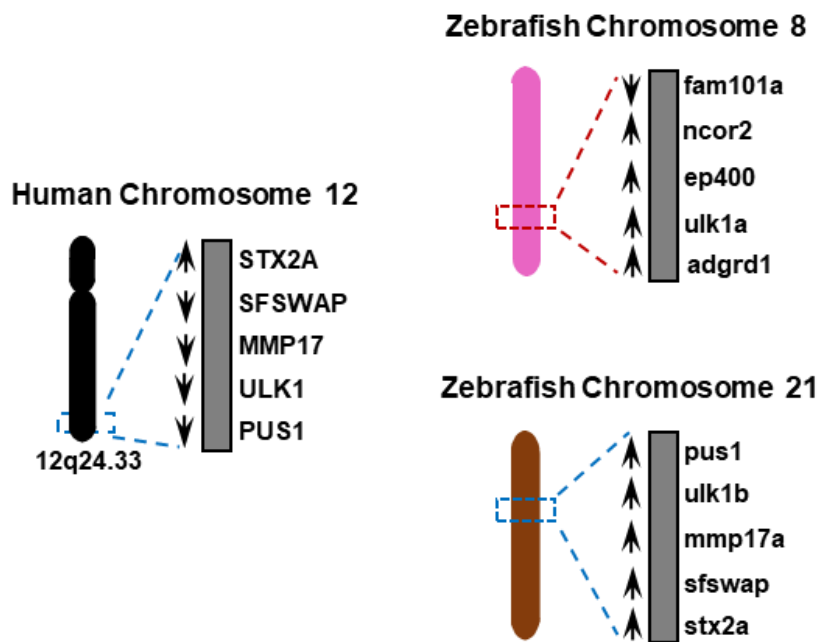

Figure 4.2: Human *ULK1,* zebrafish *ulk1a* and *ulk1b* synteny.

The gene synteny of human *ULK1* surrounding q24.33 position in chromosome 12, zebrafish *ulk1b* gene synteny in chromosome 21, and zebrafish *ulk1a* synteny in chromosome 8. In both human *ULK1* and zebrafish *ulk1a* and *ulk1b*, solid arrows represented the specified and fixed position of a particular gene on the chromosome, and the arrow orientations indicated the RNA/transcript direction.
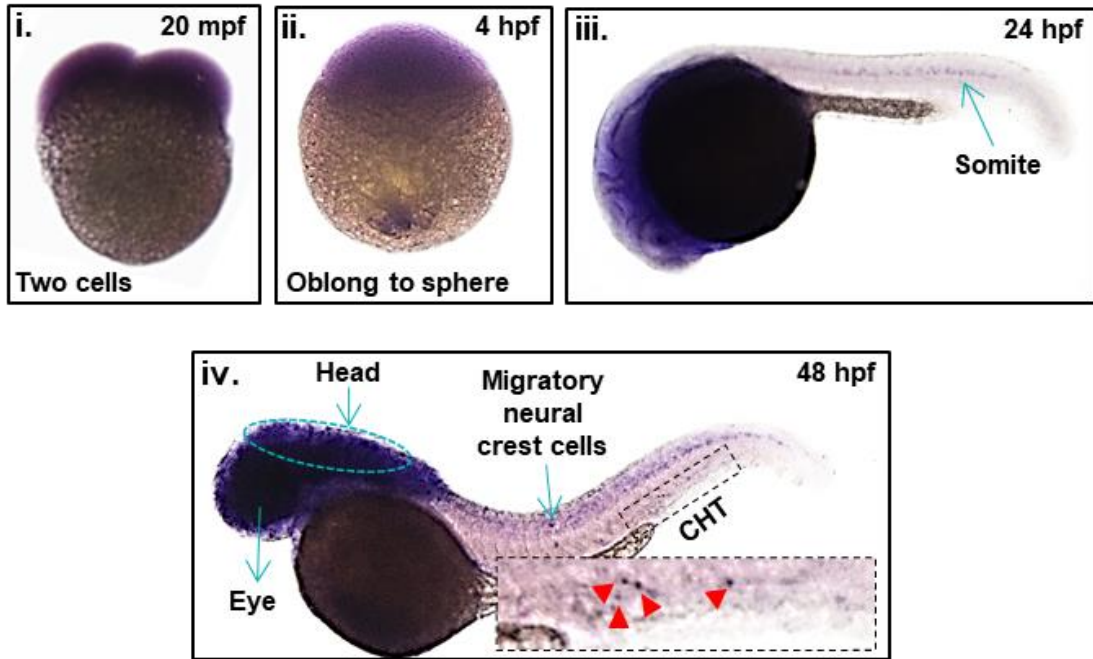
Figure 4.3: Spatial expression pattern of zebrafish *ulk1b*.

Representative lateral views indicating the spatial expression pattern of the zebrafish *ulk1b* using antisense probe at two-cell (i), oblong to sphere (ii), 24 hpf (iii) and 48 hpf (iv) stages. mpf, minutes post fertilization; hpf, hours post-fertilization.

somite and caudal hematopoietic tissue (CHT) (Figure 4.3 iii and iv).

4.4 Gene targeting of zebrafish *ulk1a and ulk1b*

Next, we targeted zebrafish *ulk1a* and *ulk1b* through TALEN-mediated genome editing. TALENs successfully generated small indels at *ulk1a* and *ulk1b* loci in F0 as shown by the uncut bands in restriction fragment length polymorphism (RFLP) genotyping (Figure 4.4). Somatically targeted F0 *ulk1b* embryos were raised and out-crossed with wild-type where further RFLP genotyping and DNA sequencing confirmed a 5-base-pair (bp) deletion in *ulk1b* stable mutants (Figure 4.5 A to C).

4.5 *ulk1b* ablation perturbed autophagy activation, but not flux at the protein level

To assess the autophagy activation, immunoblot analyses were performed with whole embryo lysate to measure the autophagosome-associated Lc3-II protein level. Immunoblot analyses showed that the Lc3-II level was unchanged in somatically targeted *ulk1a* groups while significantly decreased in *ulk1b* mutants compared to the controls, respectively (Figure 4.6 A and B). Next, to examine and visualize whether the somatically targeted *ulk1a* groups and *ulk1b* knock-out affect autophagy flux, zebrafish embryos were treated with CQ. As shown by the significantly increased level of Lc3-II protein, CQ treatment significantly blocked autophagy flux in wild-type, *ulk1a^{TAL}* and *ulk1b* mutants (Figure 4.7 and 4.8). However, the fold-increased in Lc3-II observed in both *ulk1a^{TAL}* and *ulk1b* mutants after CQ treatment were similar to the wild-type controls, indicated that autophagy flux was not affected by somatically targeted *ulk1a* and *ulk1b* knock-out.
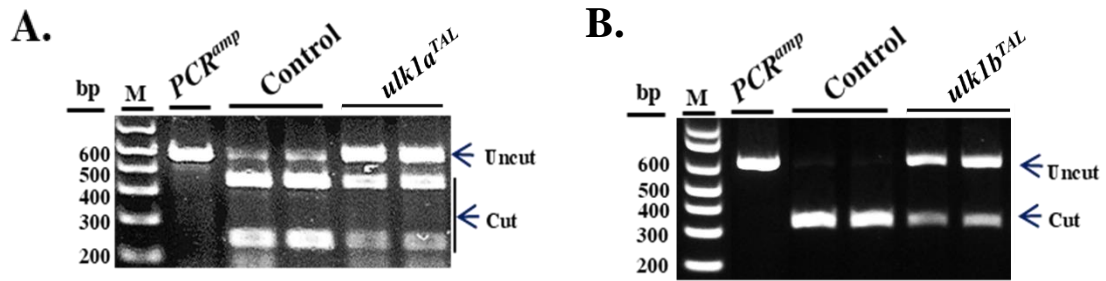
Figure 4.4: RFLP assay for zebrafish *ulk1a* and *ulk1b*.

(A-B) Genotyping performed by RFLP assay after *ulk1a* and *ulk1b* TALEN mRNA microinjection. bp, base pair; M, marker; wild type siblings as control; *ulk1a*^{TAL}, *ulk1a* with TALEN mRNA injected; *ulk1b*^{TAL}, *ulk1b* with TALEN mRNA injected; *PCR*^{amp}, PCR amplicon; Uncut, restriction enzyme undigested PCR product; Cut, restriction enzyme digested PCR product
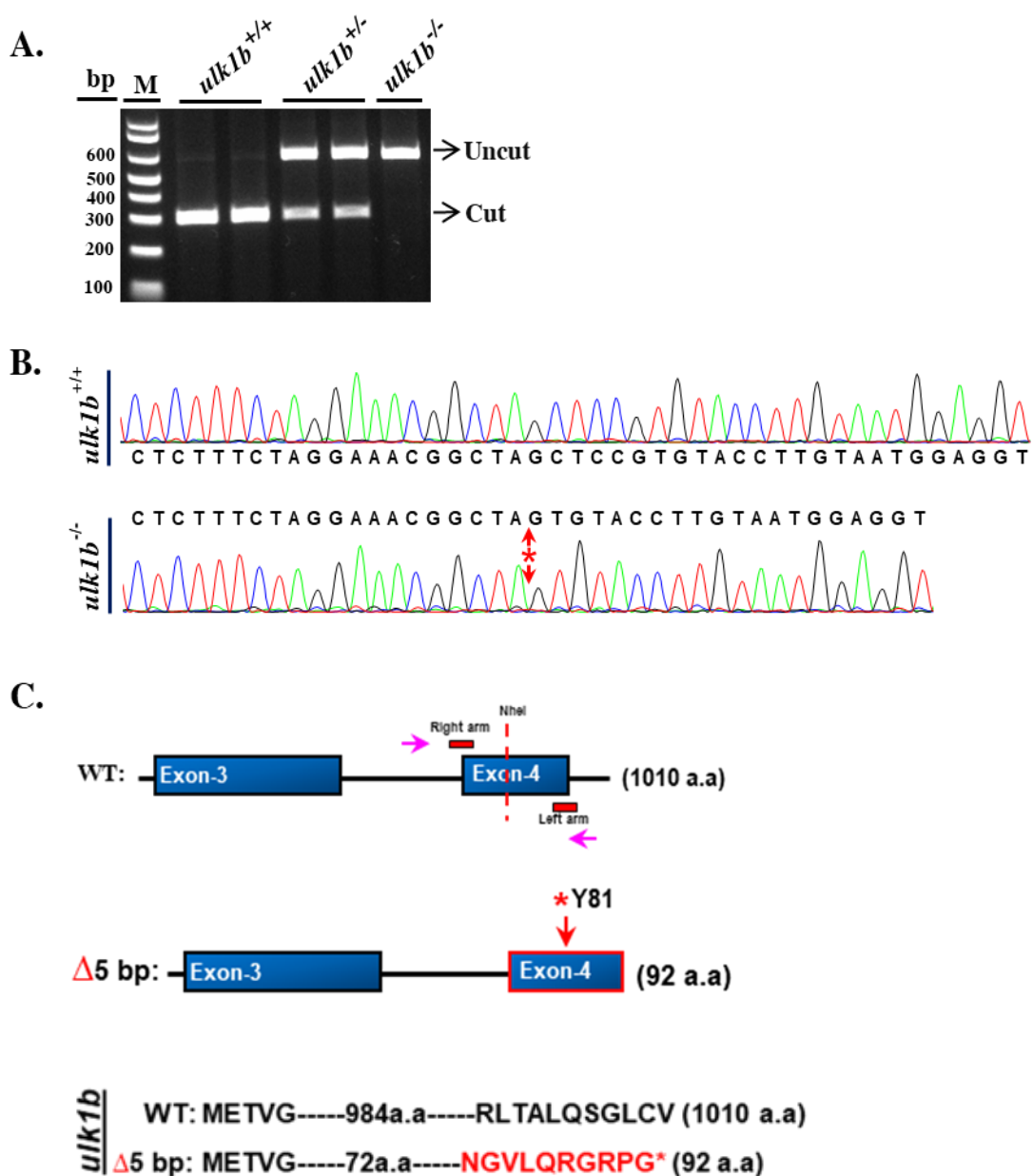
Figure 4.5: Generation of zebrafish *ulk1b* mutants by TALEN.

(A) Genotyping for *ulk1b* F1 siblings done by the RFLP assay. bp, base pair; M, marker; *ulk1b*^{+/+}, wild type sibling as control; *ulk1b*^{+/-}, heterozygous mutant; *ulk1b*^{-/-}, homozygous mutant. (B) A 5bp (Δ5 bp) mutation was confirmed by Sanger sequencing in zebrafish *ulk1b* exon 4. Red arrowheads and stars indicating the TALEN target site to induce possible mutation. (C) Computer-aided ExPASy translator tool given ulk1b truncate protein sequence corresponding to 92 amino acids (a.a).
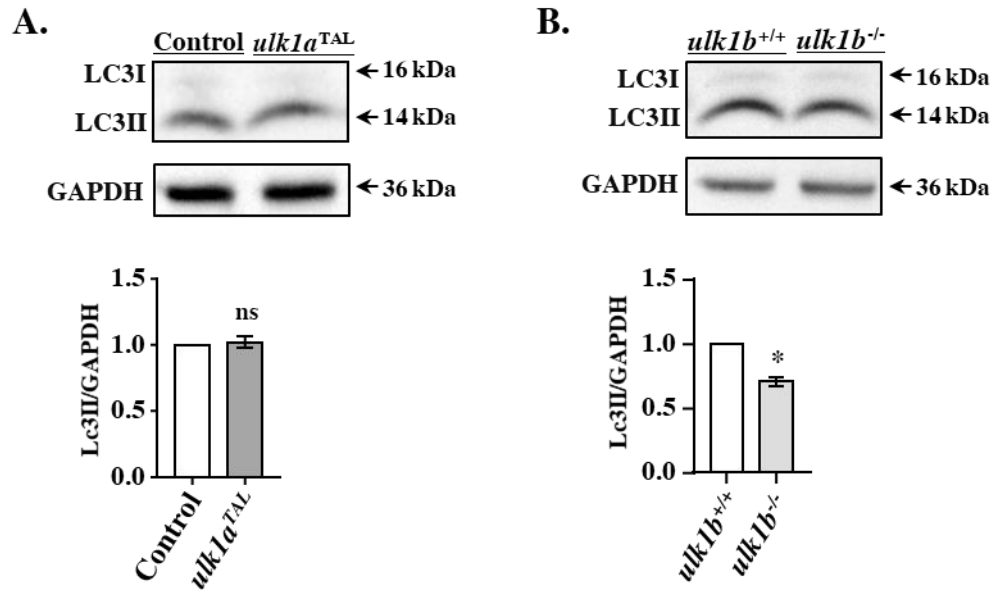
Figure 4.6: Autophagy levels in somatically targeted *ulk1a* groups and stable *ulk1b* mutants.

(A) Western blot results showing that Lc3-II protein level in TALEN mediated somatically targeted zebrafish *ulk1a* chimeric mutants *(ulk1a^{TAL})* compared to *ulk1a* wild type siblings as control. (B) Western blot results also showing the Lc3-II protein level in zebrafish *ulk1b* stable mutants *(ulk1b$^{-/-}$)* compared to the *ulk1b* wild type control siblings *(ulk1b$^{+/+}$)*. Relative Lc3-II protein levels were normalized by GAPDH while setting up the wild-type control value as 1.0. Each control and mutant group comprising in a total of 75 embryos for three independent experiments. Statistical analyses were performed by Mann-Whitney U test in between control siblings and mutants. Error bars were presented here as mean ± standard error of the mean (SEM). *, $p < 0.05$; ns, not significant.
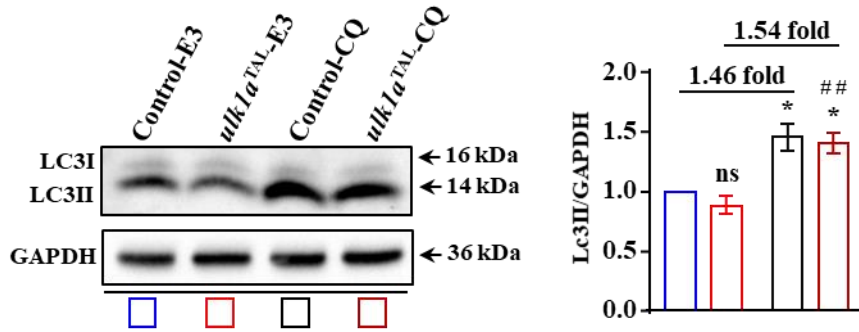
Figure 4.7: TALEN mediated somatically targated *ulk1a* did not affect autophagy.
Representative immune blot images showed the compasrison of Lc3-II protein level in somatically targated *ulk1a* groups (colum bar 2) compared to wild type controls (colum bar 1) treated with E3 fish water. To visualize the autophagy flux or Lc3II accumulation, both *ulk1a*TAL groups (colum bar 4) and wild type control groups (colum bar 3) were treated with CQ. In the bar chart, relative Lc3-II protein levels were normalized by GAPDH while setting up the *ulk1a* control sibling's (colum bar 1) value as 1.0. Each group comprising in a total of 75 embryos for five independent experiments.

Statistical analysis was performed by two-way ANOVA using Tukey's post-hoc method and error bars were presented here as mean $\pm$ standard error of the mean (SEM). *, $p < 0.05$; ns, not significant and ##, $p < 0.01$ compared to the CQ untreated *ulk1a*TAL.
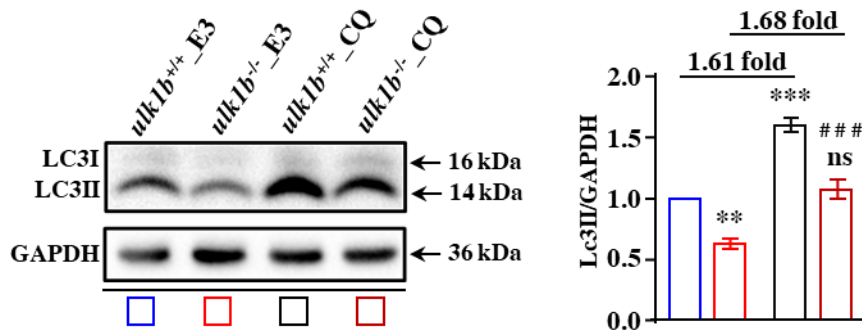
Figure 4.8: Autophagy activation, but not autophagy flux was affected in zebrafish *ulk1b* mutants.

Western blot showed the compasrison of Lc3-II protein level in *ulk1b* mutants (colum bar 2) compared to wild type controls (colum bar 1) treated with E3 fish water. To visualize the autophagy flux or Lc3II accumulation, both *ulk1b* mutants (colum bar 4) and wild type controls (colum bar 3) were treated with CQ. Relative Lc3-II protein levels were normalized by GAPDH while set up the E3 fish water treated *ulk1b*$^{+/+}$ (colum bar 1) value as 1.0. Each group comprising in a total of 75 embryos for three independent experiments.

Statistical analysis was performed by two-way ANOVA using Tukey's post-hoc method and error bars were presented here as mean ± standard error of the mean (SEM). **, $p < 0.01$; ***, $p < 0.001$; ns, not significant and $^{###}$, $p < 0.001$ compared to the CQ untreated *ulk1b* mutants.

4.6 Autophagy activation and flux were affected in the neurons of *ulk1b* mutants

Since autophagy is highly active in embryonic neurons [285], further analyses were performed to examine the effects of *ulk1b* knock-out on autophagy initiation and flux level in neurons of *Tg*(GFP-Lc3) using high-resolution microscopy. Light-sheet imaging showed that autophagosome (GFP-Lc3$^+$), lysosome (LysoTracker-red$^+$) and autolysosome (GFP-Lc3$^+$ and LysoTracker-red$^+$) puncta significantly reduced in the neuron cells of zebrafish *ulk1b* mutants (*ulk1b$^{-/-}$*) compared to the wild type siblings (Figure 4.9 and Figure 4.10). CQ treatment further reduced the number of autolysosomes significantly in both control and *ulk1b* mutants. Importantly, autophagosome (GFP-Lc3$^+$) puncta significantly increased by 1.64 fold after CQ treatment in control groups, but not in *ulk1b* mutants (1.01 fold), suggesting that autophagy flux were inhibited in the neuron cells of *ulk1b* mutants (Figure 4.9 and Figure 4.10).

4.7 *ulk1b* mutants did not affect leukocyte autophagy activation

To measure autophagy in hematopoietic cells, leukocytes were isolated from the 3 dpf *ulk1b$^{+/+}$* and *ulk1b$^{-/-}$* in leukocyte-specific fluorescent reporter background Tg(*coro1a*:DsRed) by fluorescent-activated cell sorting (FACS). Sorted *coro1a*:DsRed positive cells were stained with autophagy detection kit CYTO-ID$^®$ green that can measure autophagic vacuoles. To validate the CYTO-ID$^®$ assay, treatment with autophagy inhibitor, 3-MA was performed, which significantly reduced the green autophagic vacuoles compared to the E3 treated *ulk1b$^{+/+}$* leukocytes (Figure 4.11).
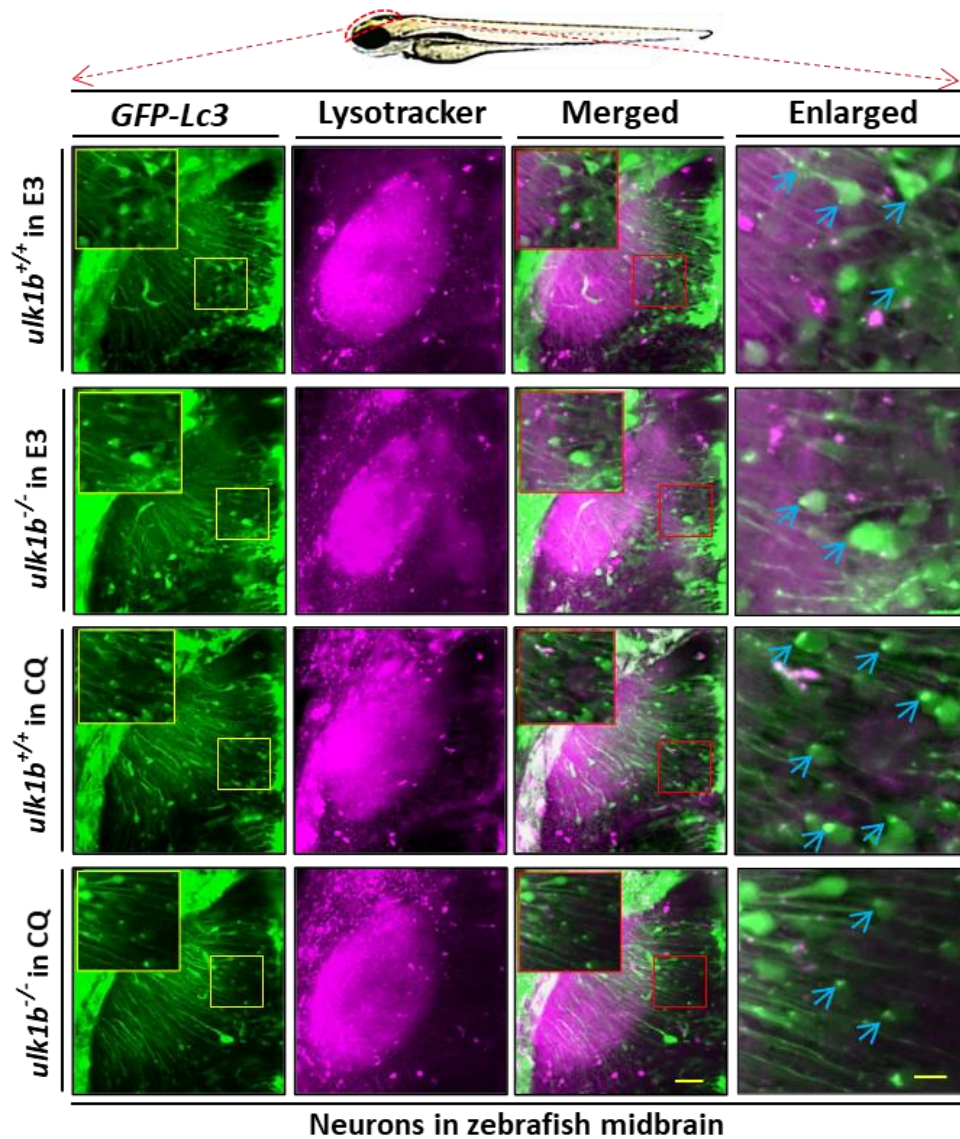
Figure 4.9: *ulk1b* deletion inhibits autophagy flux in *ulk1b* zebrafish mutants.
Schematic illustration showing the imaging of Lc3 positive cells in the midbrain section. The relative number of autophagosomes (GFP-Lc3), lysosomes (LysoTracker-red) and autolysosomes (GFP-Lc3 and LysoTracker-red) puncta per cell in the neurons were counted based on Z-Stack image analysis (20 layers out of 100 layers) with maximal intensity projection (MIP). All four groups individually comprise a total of nine Tg(GFP-Lc3) experimental embryos to complete three biological replicates. Yellow and red boxes showing the autophagosome (GFP-Lc3) and autolysosome (GFP-Lc3 and LysoTracker-red) puncta respectively. CQ: Chloroquine. Scale bar: 40μm (Merged) and 4μm (Enlarged).

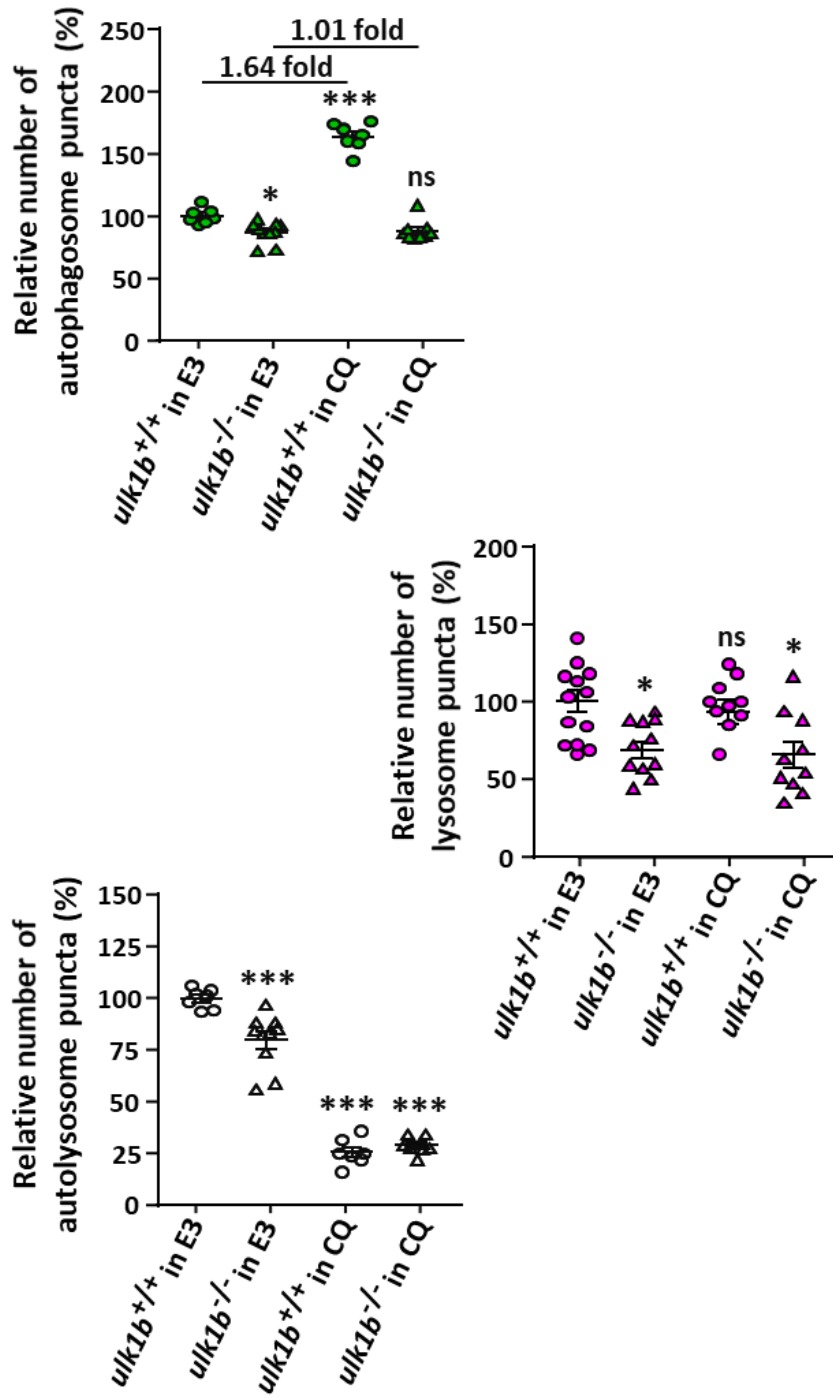**Figure 4.10: Quantitation of the autophagosomes, lysosomes and autolysosomes**

The relative number of autophagosome (A), lysosome (B) and autolysosome (C) was counted from Figure 4.9. Statistical analyses were performed by two-way ANOVA using Tukey's post-hoc method and error bars were presented here as mean ± standard error of the mean (SEM). *, $p < 0.05$; ***, $p < 0.001$ and ns, not significant.

4.8 *ulk1b* inhibition perturbed autophagy flux and most likely autophagy activation in leukocytes

Autophagy flux in zebrafish leukocytes was next examined by treatment with CQ. A significantly increased number of autophagic vacuoles were observed in CQ treated *ulk1b*$^{+/+}$ control with a flux increment of 1.55 fold. In contrast, no changes in the number of autophagic vacuoles were observed in CQ treated *ulk1b*$^{-/-}$ (1.09 fold), indicating that autophagy flux was significantly inhibited in leukocytes of *ulk1b*$^{-/-}$. *ulk1b* knockout likely inhibit autophagy activation because there is no increase in puncta in ulk1b$^{-/-}$ compare with ulk1b$^{+/+}$ (Figure 4.12).

4.9 Calpeptin induces autophagy in leukocytes of wild-type and *ulk1b* mutants

We next examined if treatment with autophagy inducer, calpeptin can induce leukocyte autophagy in zebrafish embryos. In both *ulk1b*$^{+/+}$ and *ulk1b*$^{-/-}$ siblings, autophagic vacuoles in leukocytes were significantly increased after calpeptin treatment (Figure 4.13).

4.10 Somatically targeted *ulk1b*^{TAL} induced HSCs depletion and myeloproliferation

The effect of *ulk1a* and *ulk1b* deficiency on definitive hematopoiesis was examined by WISH. Somatically targeted *ulk1a* (*ulk1a*^{TAL}) had no effects on the expression of HSCs (*myb*), pan-leukocytes (*lcp1*), neutrophils (*mpx*) and myeloid progenitors (*spi1b*) compared to the controls (Figure 4.14 and Figure 4.15). However, somatically targeted *ulk1b*^{TAL} significantly increased the number of *spi1b, lcp1* and *mpx* positive cells,
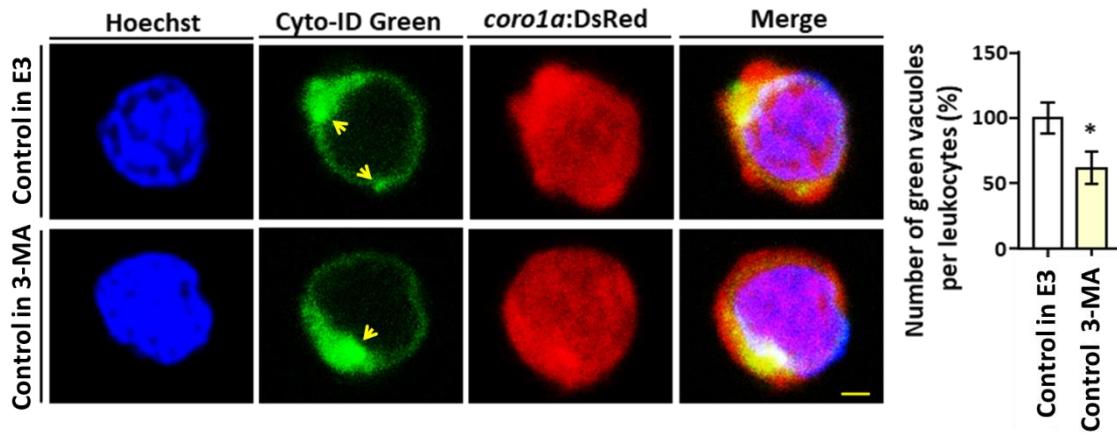
Figure 4.11: Autophagy activation was inhibited in the leukocytes upon 3-MA treatment.

Representative images showing the CYTO-ID® green positive autophagy vacuoles targeting autophagosomes, pre-autophagosomes and autolysosomes on *coro1a*:DsRed positive leukocytes at 3 dpf zebrafish embryos either in the presence or absence of 3-MA. Blue Hoechst dye staining the nuclei. Yellow arrow head indicating the autophagy vacuoles. Scale bar: 4μm. Statistical analysis was performed by Mann-Whitney U test in between control siblings and mutants. Error bars were presented here as mean ± standard error of the mean (SEM). *, p < 0.05; ns, not significant.
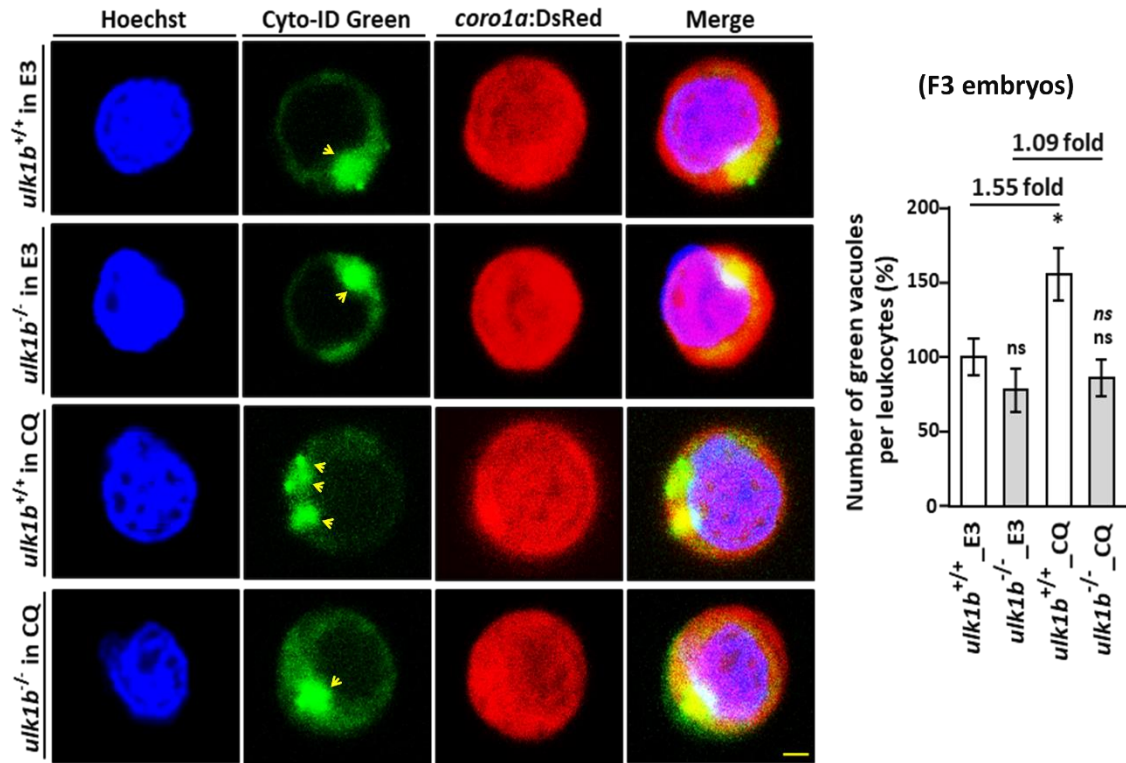
Figure 4.12: Autophagy flux and most likely activation decreased upon *ulk1b* ablation.

Representative images showing the CYTO-ID® green positive vacuoles in leukocytes at 3 dpf *ulk1b*⁺/⁺ and *ulk1b*⁻/⁻ zebrafish embryos either treated or untreated with CQ. Blue Hoechst dye staining the nuclei. Yellow arrow head indicating the autophagy vacuoles. Scale bar: 4µm. Statistical analysis was performed by two-way ANOVA using Tukey's post-hoc method and error bars were presented here as mean ± standard error of the mean (SEM). *, $p < 0.05$; ns, not significant; *ns*, not significant compared to the *ulk1b*⁻/⁻.
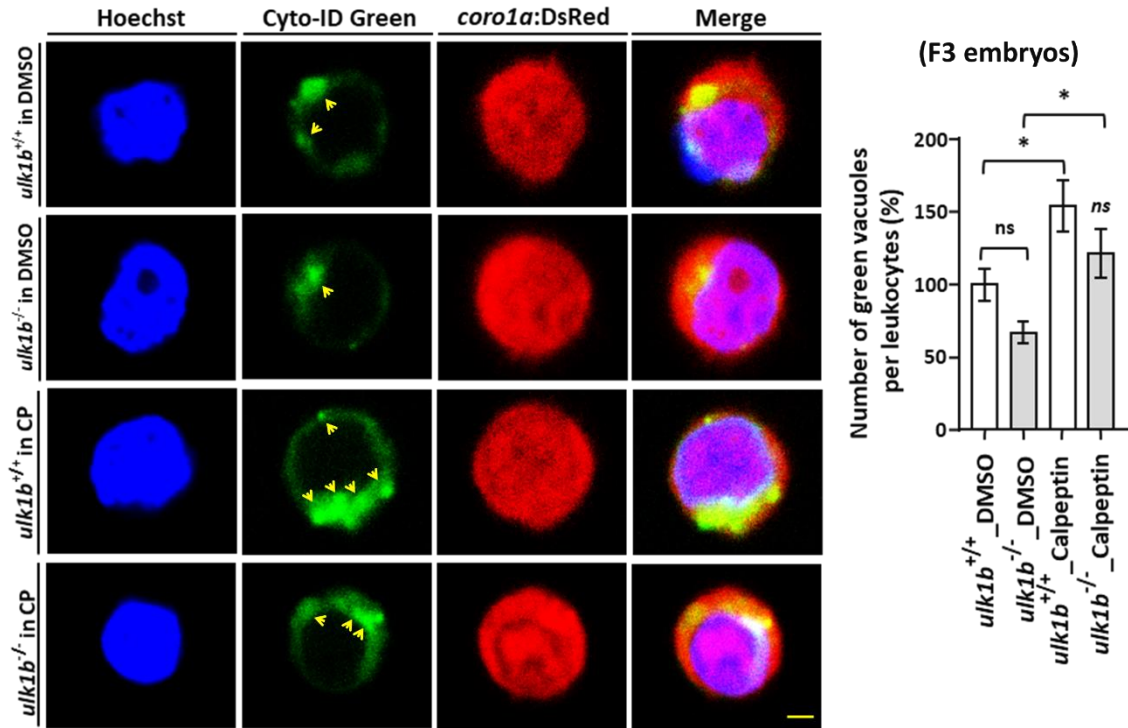
Figure 4.13: Calpeptin-induced autophagy in leukocytes.

Representative images showing CYTO-ID® green positive vacuoles in Tg(*coro1a*:DsRed) positive *ulk1b*$^{+/+}$ and *ulk1b*$^{-/-}$ embryos at 3 dpf either treated with DMSO or calpeptin (CP). Blue Hoechst dye staining the nuclei. Scale bar: 4µm. Statistical analysis was performed by two-way ANOVA using Tukey's post-hoc method and error bars were presented here as mean ± standard error of the mean (SEM). * $p <$ 0.05; ns, not significant; *ns*, not significant compared to the DMSO treated controls.

while decreasing the number of *myb* positive HSCs compared to the wild-type control. (Figure 4.16 and Figure 4.17).

Flow cytometry analysis further demonstrated that knock-out of *ulk1b* increased the number of *coro1a*-positive leukocytes (Figure 4.18). However, no significant difference in the *mpeg1.1*-positive macrophage population was observed in *ulk1b^{TAL}* compared to the wild-type control (Figure 4.19).

Cellular proliferation of the HSC was also examined by immunostaining of phosphohistone H3 (PH3) in Tg(*myb:GFP*) at 72 hpf. The relative number of PH3-positive *myb* significantly increased in *ulk1b^{TAL}* compared to controls (Figure 4.20).

Taken together, these results suggested that *ulk1b^{TAL}* perturbed definitive hematopoiesis, in particular, induced myeloproliferation.
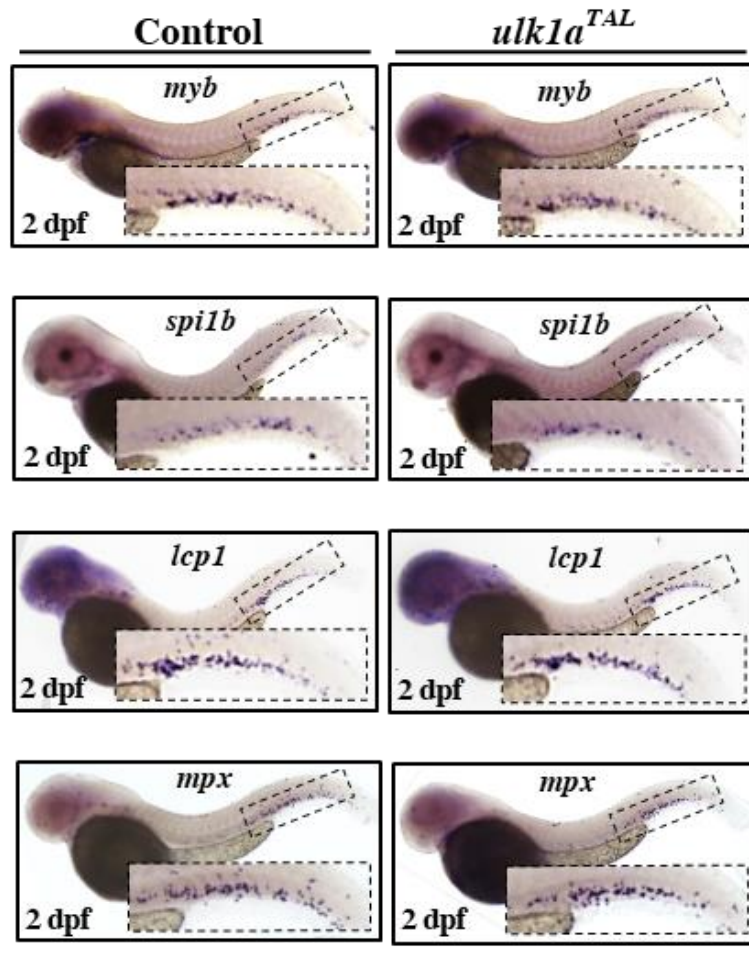
Figure 4.14: Somatically targated *ulk1a^{TAL}* did not affect definitive hematopoiesis.
In situ hybridization indicated the *myb, spi1b, lcp1* and *mpx* gene expression pattern at the CHT region of control and *ulk1a^{TAL}* groups at 2 dpf. Scale bar: 300µm.
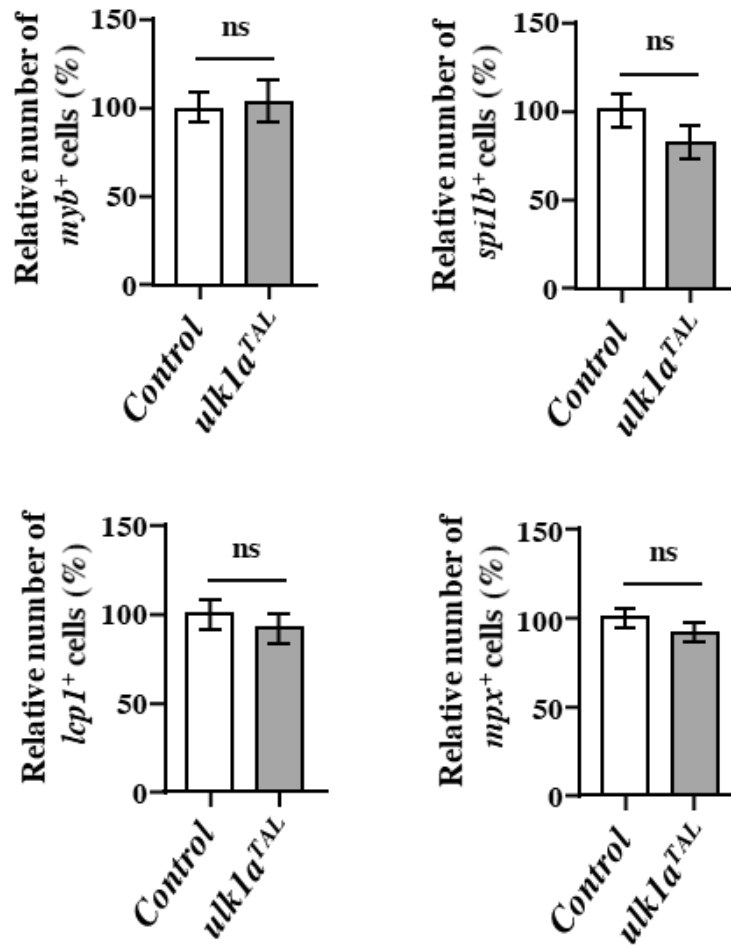
Figure 4.15: Quantitation of hematopoietic cell numbers in *ulk1a* siblings.

The relative number of *myb, spi1b*, *lcp1* and *mpx* positive cells were quantified from WISH results. Statistical analyses were performed by Mann-Whitney U test in between uninjected controls and *ulk1a^{TAL}*. Error bars were presented here as mean ± standard error of the mean (SEM). ns, not significant.
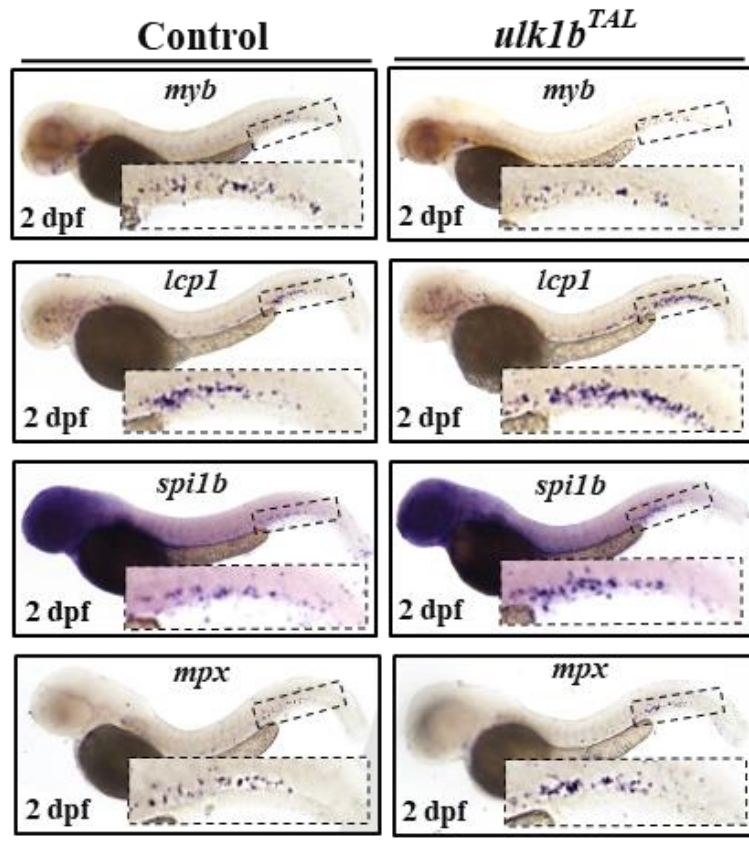
Figure 4.16: Somatically targeted *ulk1b^TAL* perturbed definitive hematopoiesis. Representative images from WISH showed the expression of zebrafish *myb, spi1b, lcp1* and *mpx* gene at the CHT region in controls and *ulk1b^TAL* at 2 dpf. Scale bar: 300μm.
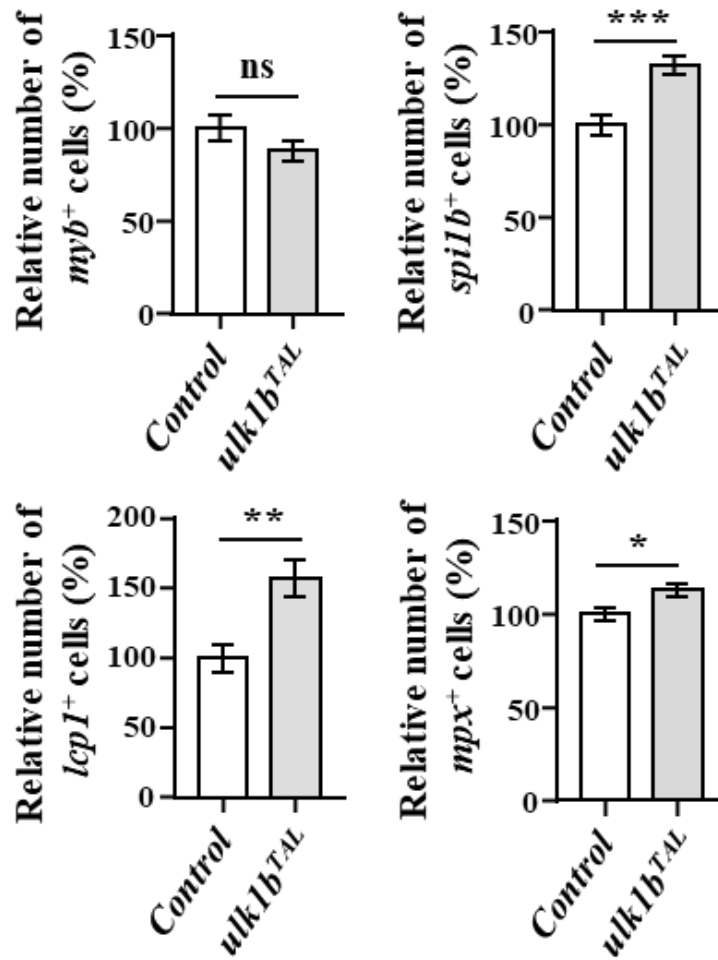
Figure 4.17: Quantitation of hematopoietic cell numbers in *ulk1b* siblings.

The relative number of *myb, spi1b*, *lcp1* and *mpx* positive cells were quantified from WISH results. Statistical analyses were performed by Mann-Whitney U test in between uninjected controls and *ulk1b*[TAL]. Error bars were presented here as mean ± standard error of the mean (SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and ns, not significant.
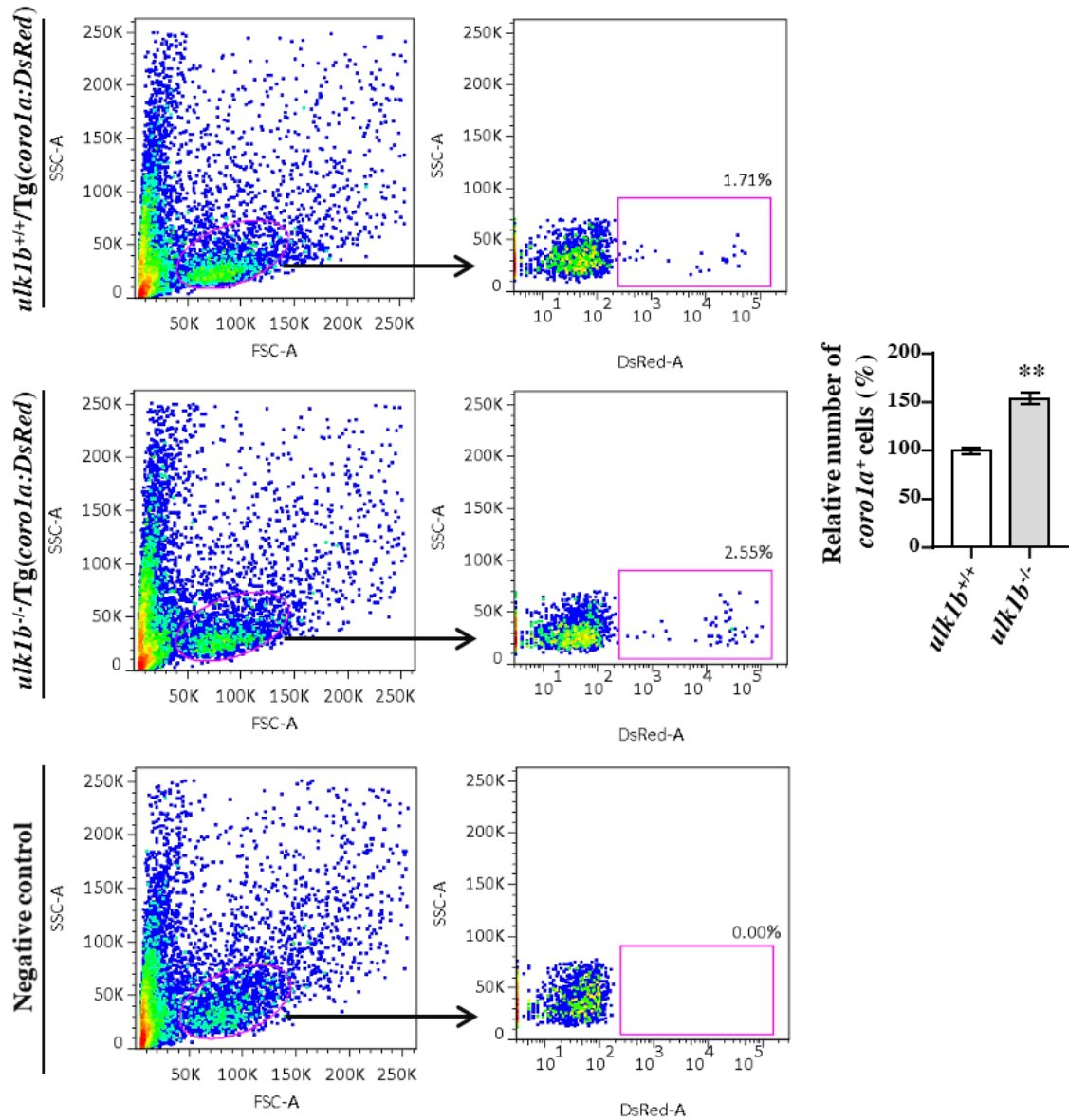
Figure 4.18: *ulk1b⁻/⁻* mutants induces leukocyte numbers.

Flow cytometry-based cellular analysis showing the quantitation of *coro1a*:DsRed positive cells from the wild type controls and *ulk1b* mutants. Statistical analysis was performed by Mann-Whitney U test in between controls and *ulk1b⁻/⁻*. Error bars were presented as mean ± standard error of the mean (SEM). **, p < 0.01.
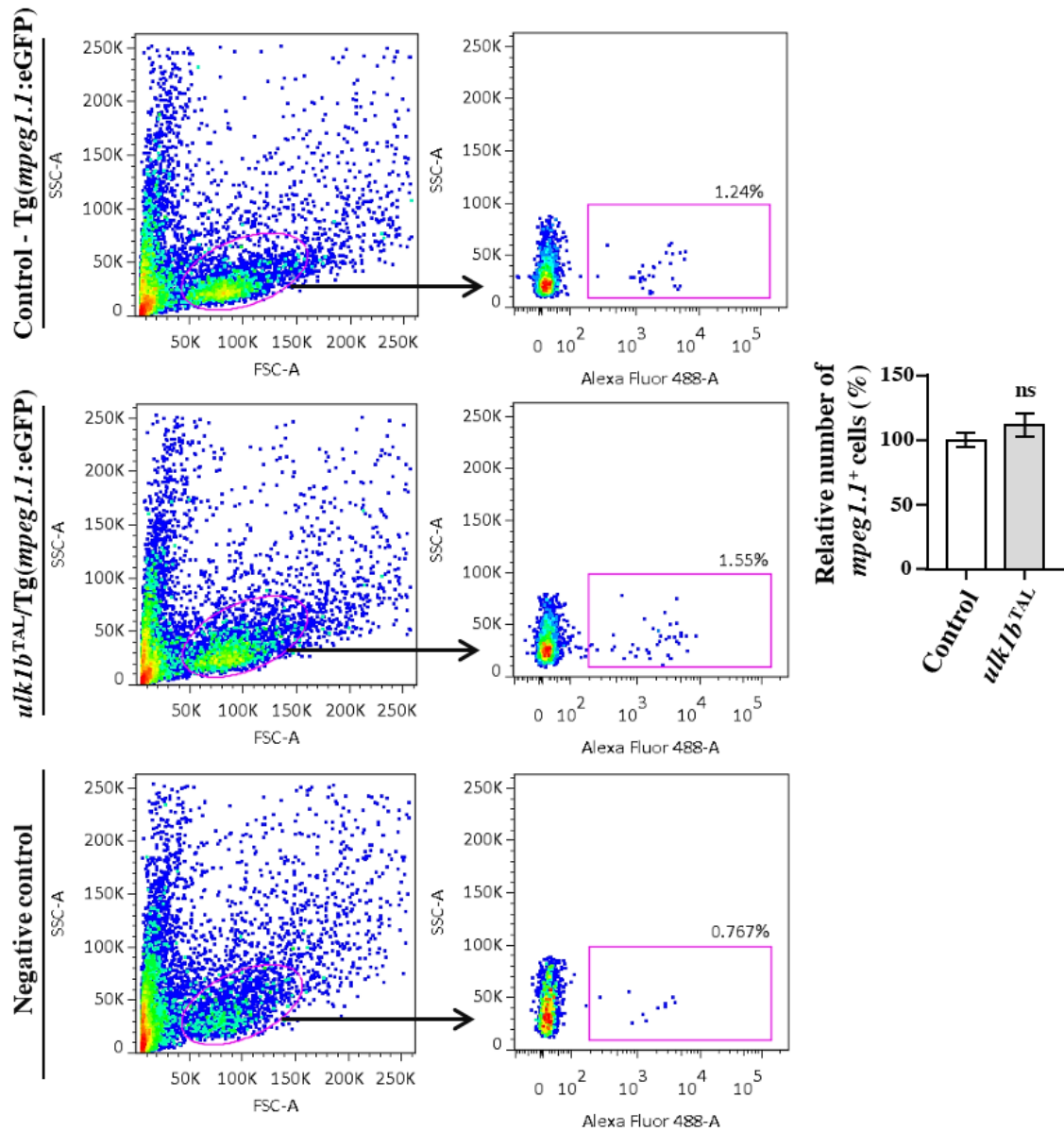
Figure 4.19: Macrophages were unchanged somatically targated *ulk1b*.

Flow cytometry-based cellular analysis showing the quantitation of *mpeg1.1*:eGFP positive cells from the wild-type controls and TALEN mediated *ulk1b^{TAL}* groups. Statistical analysis was performed by Mann-Whitney U test in between controls and *ulk1b^{TAL}*. Error bars were presented as mean ± standard error of the mean (SEM). ns, not significant.
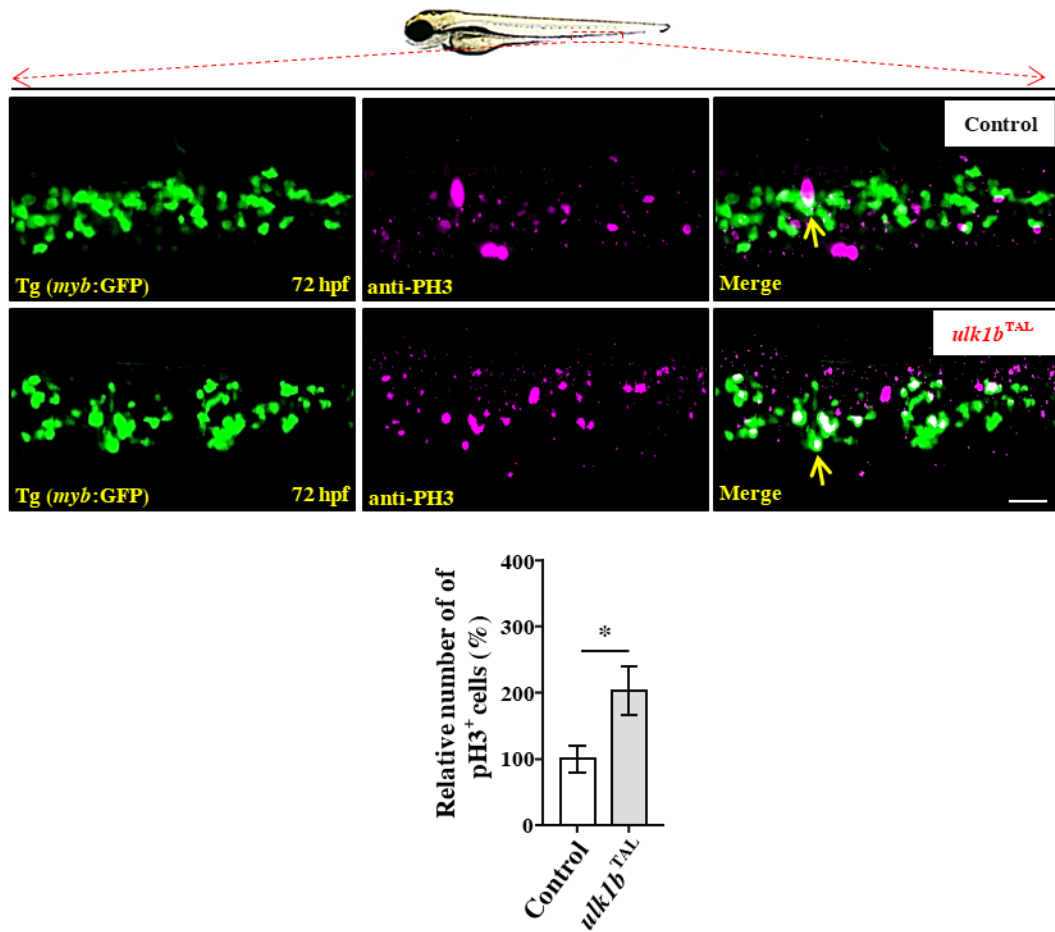
**Figure 4.20: Autophagy deficiency-induced HSC proliferation.**

Staining of GFP-positive HSC with phospho histone 3 (pH3) protein in the CHT of 3 dpf control and *ulk1b*[TAL]. Arrowhead indicates double-positive proliferative HSCs. Statistical analysis was performed by Mann-Whitney U test in between controls and *ulk1b*[TAL]. Error bars were presented here as mean ± standard error of the mean (SEM). Scale bar: 8μm. *, $p < 0.05$.

4.11 Stable *ulk1b* knock-out perturbed definitive hematopoiesis

The potential role of autophagy in hematopoiesis was further examined in stable homozygous *ulk1b* mutant (*ulk1b*$^{-/-}$). Similar to the hematopoietic phenotypes observed in chimeric mutants, the number of *spi1b, lcp1* and *mpx* positive cells significantly increase in *ulk1b*$^{-/-}$ compared with *ulk1b*$^{+/+}$ siblings while the number of *myb* positive HSCs decreased. (Figure 4.21 and 4.22).

4.12 Autophagy inhibitor 3-MA perturbed definitive hematopoiesis in zebrafish

Next, the link between autophagy and the hematopoietic phenotypes observed in *ulk1b* mutants was investigated by treatment with 3-MA. As shown by WISH, the number of myeloid progenitors, leukocyte and neutrophil increase and HSC reduced after 3-MA treatment compared with controls, indicated that 3-MA can recapitulate the hematopoietic phenotypes in *ulk1b* mutants (Figure 4.23 and Figure 4.24).

4.13 Calpeptin only rescued the increased neutrophil numbers in *ulk1b* mutants

To further investigate if hematopoietic phenotypes observed in *ulk1b* mutants are autophagy-dependent, *ulk1b*$^{-/-}$ zebrafish embryos were treated with autophagy inducer calpeptin. However, calpeptin treatment could only rescue the increase in neutrophils, but not other hematopoietic lineages (Figure 4.25 to Figure 4.27). The effects of *ulk1b* knock-out and the autophagy drugs (3-MA and calpeptin) on zebrafish definitive hematopoiesis were also summarized (Table 4.1).
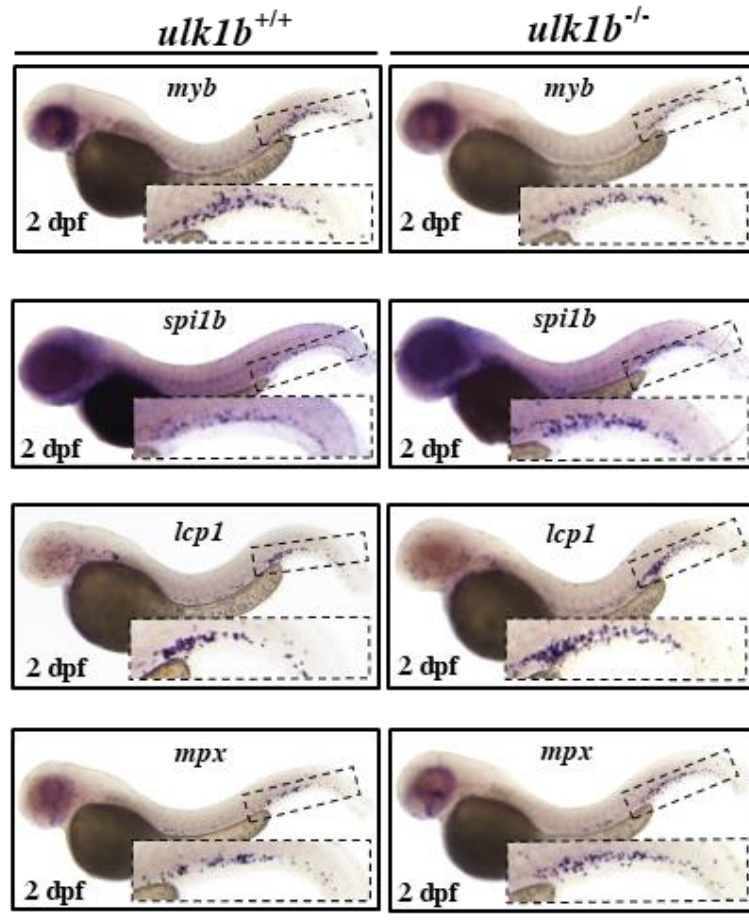
Figure 4.21: Loss of *ulk1b* perturbed definitive hematopoiesis.

Representative WISH images showing the expression of *myb, spi1b, lcp1* and *mpx* gene in *ulk1b*^+/+^ and *ulk1b*^-/-^ siblings at 2 dpf in the CHT region. Scale bar: 300μm.
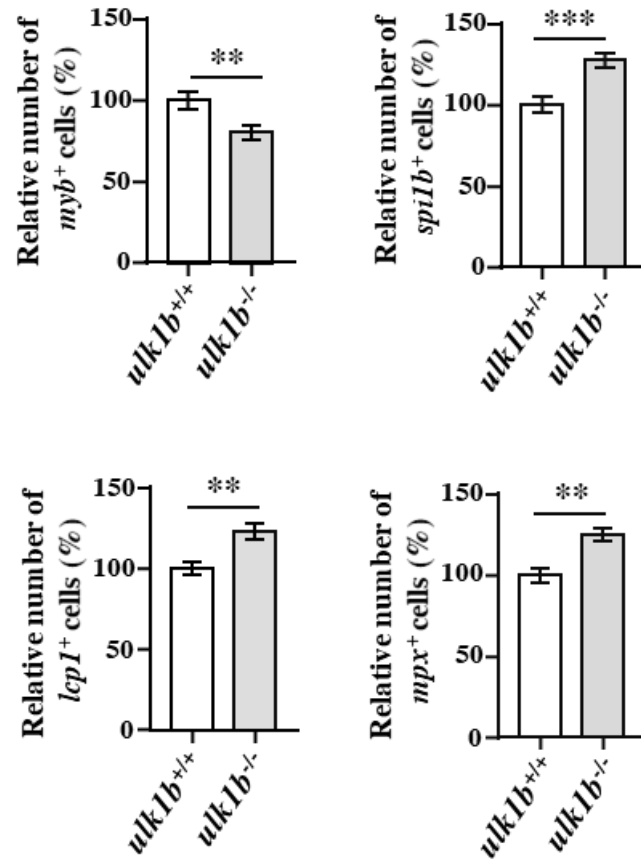
Figure 4.22: Quantitation of hematopoietic cell numbers in *ulk1b* siblings.

The relative number of *myb*, *lcp1, spi1b* and *mpx* positive cells were quantified from WISH results. Statistical analyses were performed by Mann-Whitney U test in between *ulk1b*$^{+/+}$ and *ulk1b*$^{-/-}$. Error bars were presented here as mean ± standard error of the mean (SEM). **, $p < 0.01$; ***, $p < 0.001$ and ns, not significant.
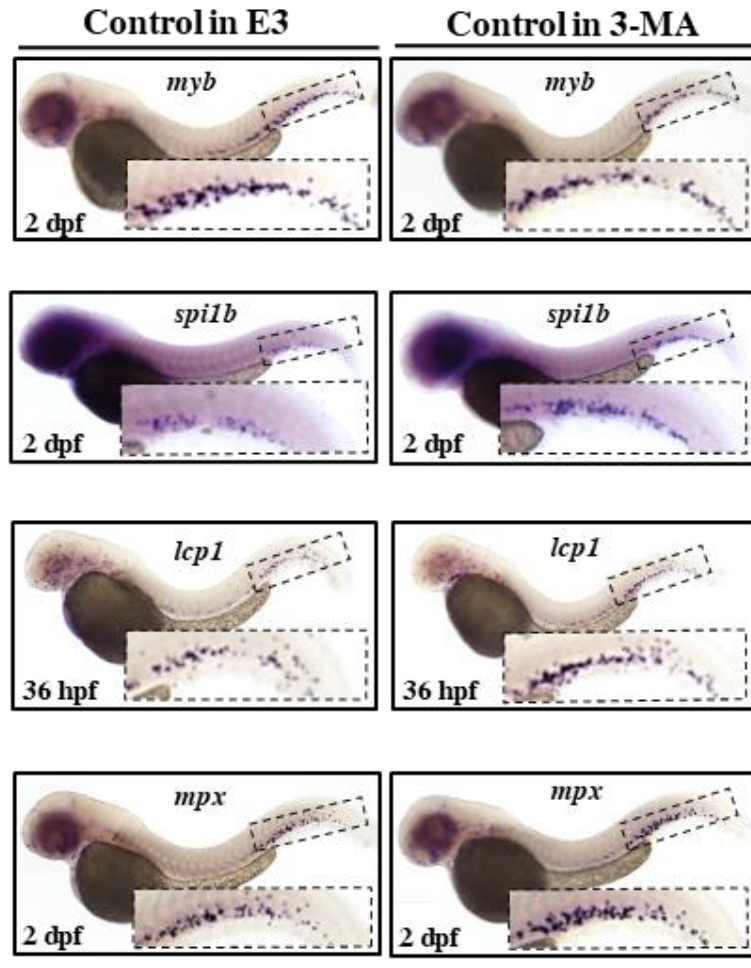
Figure 4.23: 3-MA recapitulated *ulk1b* mutant phenotypes in wild-type siblings.
Representative WISH analysis indicated the expression of *myb, spi1b, lcp1* and *mpx* positive cells in E3 fish water treated and 3-MA treated wild type siblings in definitive hematopoiesis. Scale bar: 300μm.
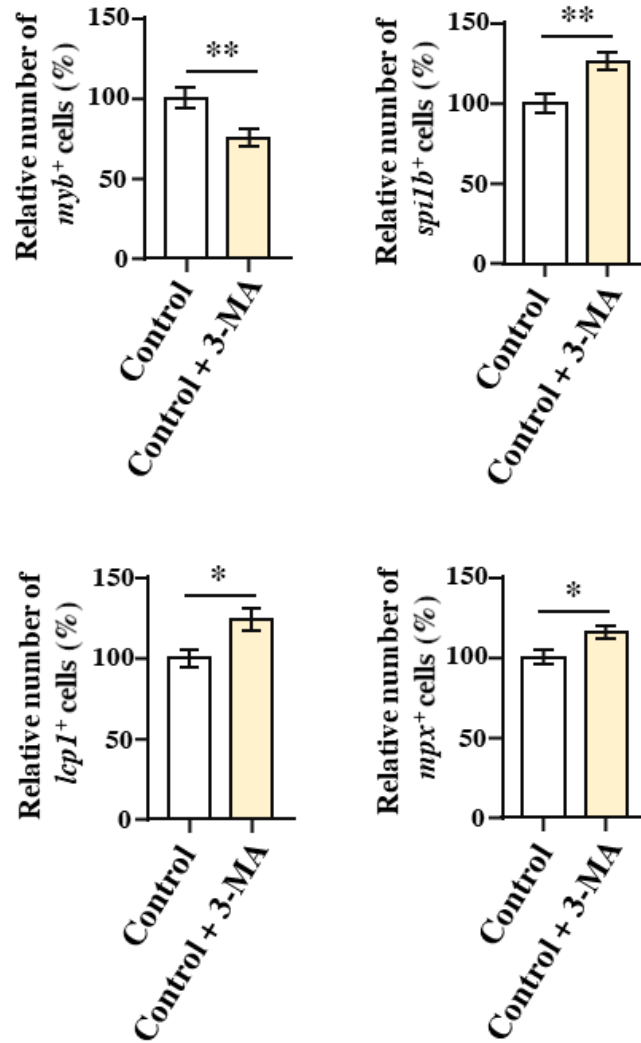
Figure 4.24: Quantitation of HSC and myeloid cell numbers after 3-MA treatment. The relative number of *myb, spi1b, lcp1 and mpx* positive cells were quantified from WISH results. Statistical analyses were performed by the Mann-Whitney U test. Error bars were presented here as mean ± standard error of mean (SEM).*, $p < 0.05$ and **, $p < 0.01$.
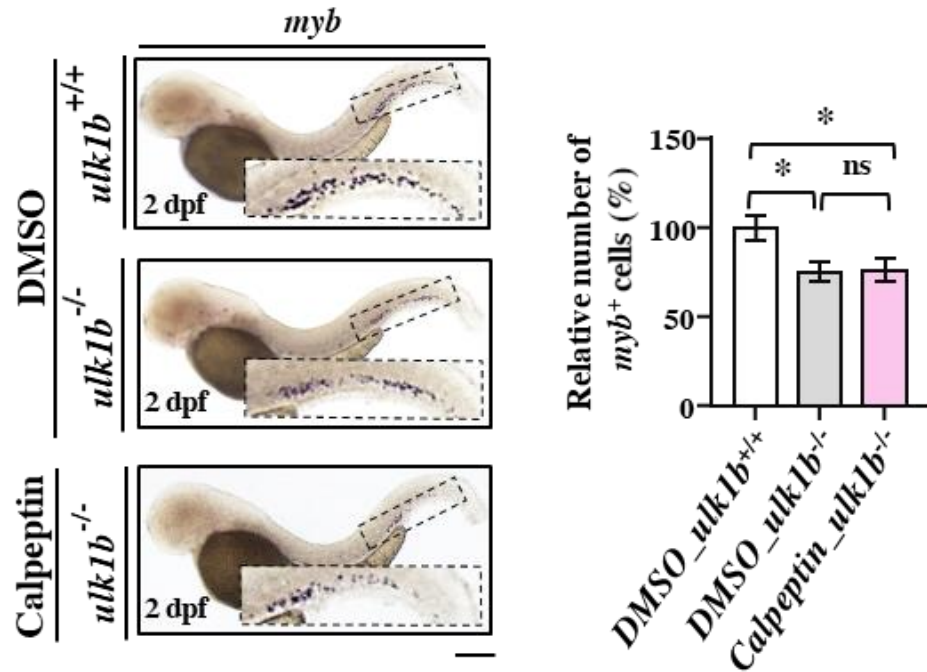
Figure 4.25: Calpeptin unable to rescue the HSCs decrease in *ulk1b* mutants.

The expression of *myb* positive cells in DMSO treated between *ulk1b*^{+/+}, *ulk1b*^{-/-} and calpeptin treated *ulk1b*^{-/-} embryos at 2 dpf. Scale bar: 300μm. Statistical analysis was performed by one-way ANOVA using Tukey's post-hoc method and error bars were presented here as mean ± standard error of the mean (SEM). *, $p < 0.05$ and ns, not significant.
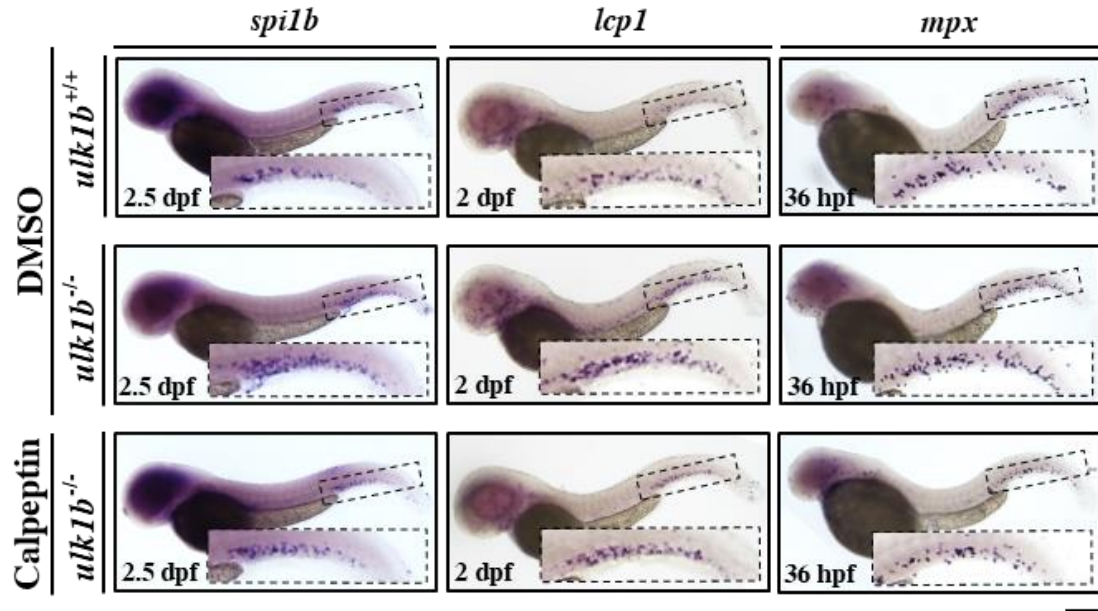
Figure 4.26: Calpeptin only rescued neutrophil cell numbers in *ulk1b* mutants.

Representative images from WISH analysis showed the expression of *spi1b, lcp1* and *mpx* in DMSO treated *ulk1b*$^{+/+}$, *ulk1b*$^{-/-}$ and calpeptin treated *ulk1b*$^{-/-}$ embryos in the CHT. Scale bar: 300μm.
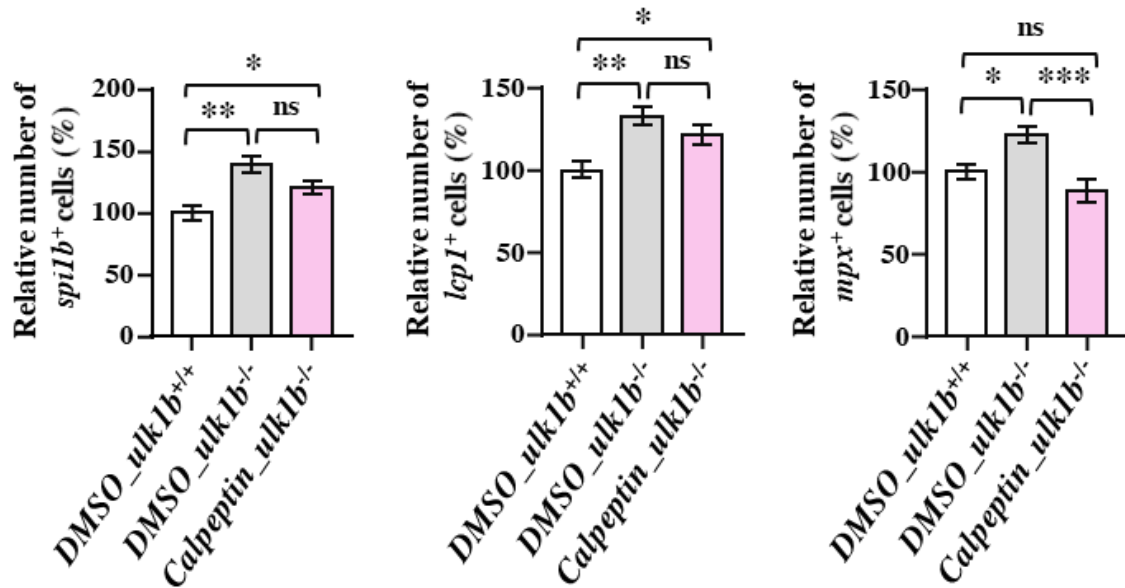
Figure 4.27: Quantitation of myeloid cells in *ulk1b* mutants and siblings.

The relative number of *spi1b, lcp1* and *mpx* positive cells was quantified from WISH results. Statistical analyses were performed by one-way ANOVA using Tukey's post-hoc method and error bars were presented here as mean ± standard error of the mean (SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and ns, not significant.

Table 4.1: Summary of the *ulk1b* autophagy in hematopoietic cells

Conditions	*myb*⁺ cells (HSCs)	*spi1b*⁺ cells (CMPs)	*lcp1*⁺ cells (Leukocytes)	*mpx*⁺ cells (Neutrophils)	Compared to
***ulk1b* knock-out**	Decreased	Increased	Increased	Increased	*ulk1b$^{+/+}$*
Control + 3-MA	Decreased	Increased	Increased	Increased	*ulk1b$^{+/+}$*
***ulk1b* knock-out + Calpeptin**	Not rescued	Not rescued	Not rescued	Rescued	DMSO + *ulk1b$^{-/-}$*

Chapter 5: Discussion

In this study, optically clear zebrafish lines were employed to investigate the role of autophagy in definitive hematopoiesis. In zebrafish, *ulk1* is duplicated into *ulk1a* and *ulk1b*. The phylogenetic tree showed that both zebrafish *ulk1a* and *ulk1b* are the orthologues of human *ULK1*. Synteny analysis indicated that zebrafish *ulk1b* is highly conserved with the human *ULK1* and shared 58% amino acid sequence similarity with the human counterpart. Furthermore, knock-out of *ulk1b* perturbed autophagy and definitive hematopoiesis. Although *ulk1a* shared nearly 48% amino acid sequence similarity with the human *ULK1,* the gene synteny of zebrafish *ulk1a* and human *ULK1* were not conserved. Moreover, overall autophagy and hematopoietic phenotypes were unchanged after somatically targeting *ulk1a*, indicating that *ulk1a* may play either compensatory or redundant roles during autophagy and definitive hematopoiesis. Therefore, this study focused on *ulk1b*-regulated autophagy and its role in regulating zebrafish definitive hematopoiesis.

Zebrafish *ulk1b* was ubiquitously expressed during early embryonic development and later expressed in the head region, somites and CHT. Consistent with the predominant expression of zebrafish *atg5* in the forebrain, midbrain, and hindbrain [286], higher expression of *ulk1b* was also detected in the zebrafish head region. Previously studies reported that zebrafish *ulk1* transcription started from 23 hpf [223] and at the one-cell stage [287], respectively. In this study, ubiquitous expression of zebrafish *ulk1b* was also detected in one to two-cell stage embryos, similar to the high expression of other zebrafish *atg*s including *atg5, atg7, becn1* and *atg12* in one to two-cell stage [286, 287]

and highlighted the importance of *ulk1b*-regulated autophagy during early embryonic development.

Autophagy is an essential process for embryogenesis, whereas autophagy deficiency in zebrafish results in developmental defects including abnormal heart structure, defective cardiac looping, abnormal valve development and reduced survival [286, 287]. As expected, TALEN mediated chimeric knock-out of *ulk1b* leaded to embryonic death and deformities as previously shown by *atg5, atg7* and *becn1* morpholino knock-down [286, 287]. However, more than 50% of the chimeric knock-out *ulk1b* embryos survived with normal morphology. Although both studies suggested that zebrafish *atg5* knock-down mediated embryonic deformities signify *atg5* in body development, stable *atg5* mutant data were missing. Recently, it has been reported that zebrafish embryos with homozygous deletion of *atgs* (*fip200$^{-/-}$, atg101$^{-/-}$, atg13$^{-/-}$, atg9a$^{-/-}$:atg9b$^{-/-}$, atg2a$^{-/-}$:atg2b$^{-/-}$, atg5$^{-/-}$, atg14$^{-/-}$ atg16l1$^{-/-}$*) usually died around 2 weeks-post-fertilization [108]. Moreover, conventional knock-out of the core *Atg*s including *FIP200, Atg9A*, and *Atg13* causes embryonic lethality in mice [288-290] and mice with the deletion of Atg conjugation components including *Atg3, Atg5, Atg7, Atg12,* and *Atg16L1* can survive during embryonic development but die within one day after birth [120-122, 291, 292]. However, knock-out of either *Ulk1* or *Ulk2* in mice displayed normal development and viability [293]. In this study, homozygous *ulk1b* mutants also survived with normal gross development, probably due to the functional redundancy or compensatory effects of *ulk1a* and/or the presence of non-canonical autophagy. Collectively, *ulk1b* knock-out

zebrafish is potentially a better model of autophagy-deficiency than other *atg*s knock-out models in the hematopoietic study because *ulk1b* mutants have normal gross morphology.

At the whole embryo level, loss of *ulk1b* perturbed autophagy activation with markedly reduced Lc3-II protein level while the autophagy activation in somatically targeted *ulk1a* groups was unaffected. Previously, it has been reported that mice with either *Ulk1* or *Ulk2* knock-out and *Ulk1/Ulk2* double knock-out (DKO) were associated with a reduced level of LC3-II protein expression [293, 294]. Decreased Lc3-II protein level was also observed in zebrafish upon *atg5* morpholino knock-down [295]. However, none of the studies reported a complete removal of Lc3II protein upon *Ulk1, Ulk2* or *Ulk1/Ulk2* knock-out, which suggested that a lower level of autophagy is still ongoing. The amount of Lc3-II protein found in *ulk1b* mutants might be generated in *ulk1b*-independent autophagy.

Furthermore, autophagy flux was evaluated by assaying Lc3-II turnover in zebrafish whole embryo tissue lysate by treating with late-step autophagy inhibitor, CQ. CQ treatment indicated that autophagy flux was not significantly affected in both somatically targeted *ulk1a* groups and *ulk1b* stable mutants, unlike the previous study that reported that knock-out of *Ulk1* in mouse embryonic stem cells (ESCs) significantly reduced autophagy flux [296]. Autophagy flux in *ulk1* mutants was further investigated by using Tg(GFP:Lc3) with LysoTracker Red staining. Similar to our previous report [285], autophagy level is largely varied in different embryonic tissues with the highest level detected in skin, muscle and neuron. In *ulk1b* mutant, defective autophagy activation as

well as autophagy flux were found in neurons, suggested that different canonical and non-canonical autophagy pathways, including ulk1b-dependent and ulk1b-independent autophagy, are ongoing in different embryonic tissues.

Despite the well-known effect of autophagy in blood cells [112, 139, 140, 213, 218], the role of *ulk1*-regulated autophagy in hematopoiesis is largely unknown. To study the role of autophagy in embryonic hematopoiesis, we examined the effects of *ulk1b* knock-out on hematopoiesis, in particular, the effects on autophagy status in hematopoietic cells as well as the effects on different hematopoietic lineages. Autophagy vacuoles were measured on *coro1a*:DsRed-positive leukocytes, whereby FACS-sorted *coro1a*:DsRed leukocytes were stained with CYTO-ID® green autophagy detection dye that can visualize autophagic vacuoles (including amphisomes and autolysosomes) and autophagy activation and flux were monitored in presence of either CQ or bafilomycin A1 [297]. Notably, autophagy flux was significantly reduced in *coro1a*:DsRed-positive leukocytes of *ulk1b* mutants compared to wild-type siblings. Also, both chimeric and stable homologous *ulk1b* mutants displayed a decrease in the number of HSC and an increase in the number of myeloid lineages, highlighted the previously unknown role of ulk1b on definitive hematopoiesis. While the connection between the decreased HSC and myeloproliferation requires further investigation, maybe *ulk1b* knock-out increased HSC proliferation, driving the differentiation into progenitor and myeloid lineages while depleting the HSC population. Since *ulk1b*-regulated autophagy knock-out induce myeloproliferation, alterations of this dynamic process might influence the HSC fate and hematopoietic system homeostasis. However, in patient having FLT3-ITD mutated acute

myeloid leukemia, higher expression of ULK1 have found with increased phosphorylation of ULK1 while treating with autophagy inhibitors can ameliorate the ULK1 phosphorylation [298]. In our findings, *ulk1b* mutants with perturbed definitive hematopoiesis were associated with inhibited autophagy flux, whereas higher flux is visualized in the transformation from HSCs to leukemia stem cells (LSCs) [299]. While LSCs and AMLs are malignant cells, here we focused on normal hematopoietic cells in our zebrafish models. Autophagy might have differential role on the basis of cell types, for instance, autophagy could protect AML cells while restricting the proliferation of normal cells.

A significantly lower level of autophagy flux has been detected in *Atg7* deficient aged mice bone marrow macrophages and indicated that higher flux is essential for preventing age-related immune senescence and increased cell death [300]. Higher autophagic flux is also important for the maintenance of HSCs quiescence and stemness, ESCs identity and proper neutrophil differentiation and function in mice [173, 301, 302]. It is also reported that autophagy flux inhibition led to HSPCs proliferation defects in zebrafish mutants [303]. While our study identified *ulk1b*-regulated autophagy flux in zebrafish leukocytes, autophagy in other hematopoietic lineages was not examined due to the limitation in lineage-specific red fluorescent reporter lines, which certainly warrants further investigations.

To investigate if the hematopoietic phenotypes observed in *ulk1b* mutants were autophagy-dependent, we chemically modulated autophagy with 3-MA and calpeptin.

Treatment with 3-MA, which is a PI3K inhibitor blocking early autophagosome formation, recapitulated hematopoietic phenotypes observed in *ulk1b* mutants, strongly suggested that an autophagy-dependent role of *ulk1b* in definitive hematopoiesis. However, treatment with autophagy inducer, calpeptin cannot rescue all hematopoietic phenotypes in *ulk1b* mutants except the increase in neutrophils (Figure 5.1).

There is a possibility that calpeptin cannot restore autophagy to the threshold level in every hematopoietic lineage, or maybe calpeptin cannot rescue *ulk1b*-dependent canonical autophagy. Although calpeptin induces autophagy activation in leukocytes of both wild-type and *ulk1b* mutants, consistent with the previous study [304], its effects on autophagy flux in leukocytes as well as autophagy in other hematopoietic lineages were not examined. While the failure in rescuing hematopoietic phenotypes in *ulk1b* mutant does not support the autophagy-dependent hypothesis, calpeptin might not be able to completely restore the autophagy and thus rescue all hematopoietic phenotypes in *ulk1b* mutants. Also, maintenance of different hematopoietic lineages may require a tightly regulated autophagy level, either increased or decreased autophagy beyond a certain threshold might result in deregulation of zebrafish hematopoiesis.

In current understanding, autophagy is complex and not a linear pathway, there are multiple canonical and non-canonical autophagy pathways and the precise autophagy network in vertebrate hematopoiesis remains largely unknown. One limitation of our study is that we did not use other genetic tools such as antisense oligo or morpholino to knockdown *ulk1* gene function to study autophagy and hematopoiesis, and to justify the
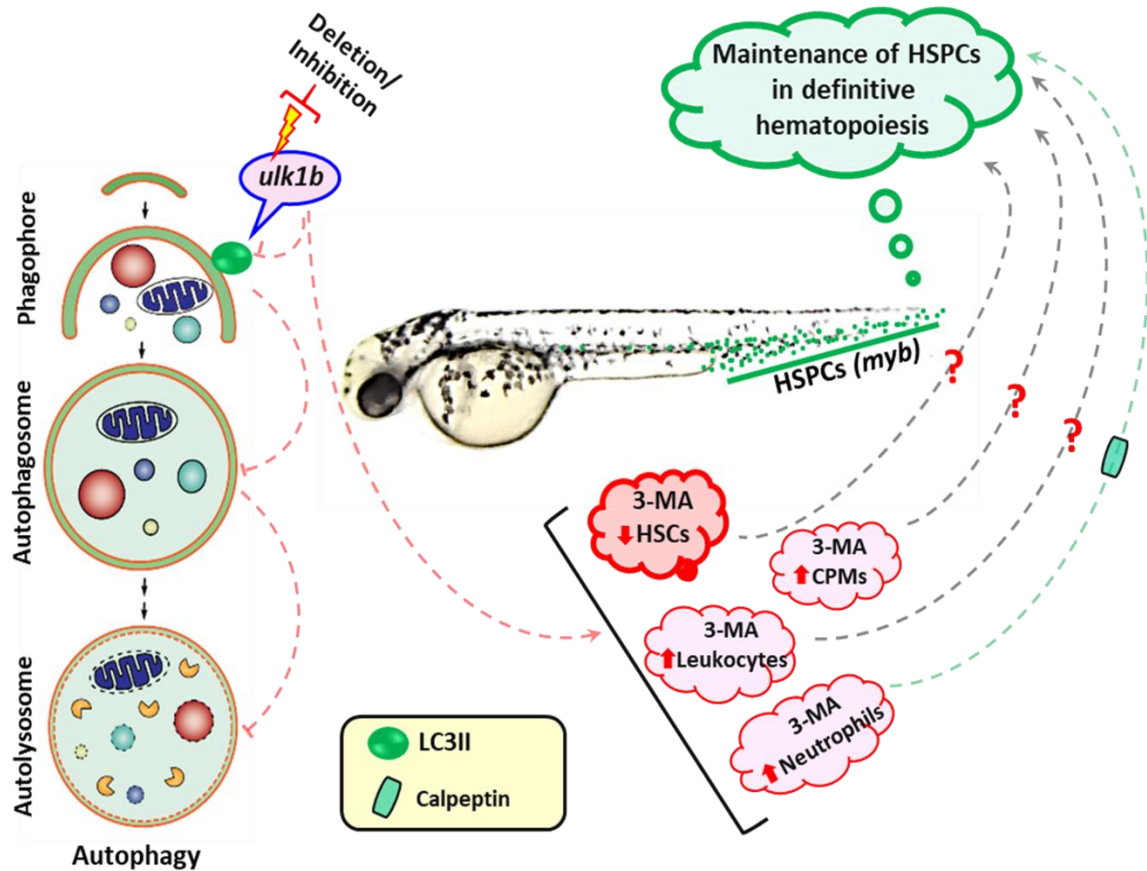
Figure 5.1: Diagram indicating the role of *ulk1b* in zebrafish definitive hematopoiesis.

Zebrafish *ulk1b* was a positive regulator of HSPCs maintenance. Knock-out of *ulk1b* perturbed autophagy and definitive hematopoiesis while generating an increased number of myeloid cells at the expense of HSCs. Some of the hematopoietic defects can be partially rescued by chemically targeting *ulk1b* with the autophagy inducer calpeptin. Red dash arrowheads indicated the suppression of autophagy upon *ulk1b* ablation. Green dash arrowhead indicated the rescue of neutrophil cell numbers upon autophagy induction, while the black dash arrowheads indicated that calpeptin treatment cannot rescue the HSCs, CMPs and leukocytes in the *ulk1b* mutants.

existing data generated by TALEN gene targeting. However, morpholino has its own limitation such as its knockdown efficacy and non-specific gene effects [305]. Conversely, TALEN technology has lower off-target effects, higher specificity and such an approach in this study led to the development of stable mutants of *ulk1* from somatically targeting the F0 embryos. Nevertheless, somatically targeting *ulk1a* and *ulk1b* using other advanced technologies such as CRISPR/Cas9 and TALEN targeting other exons of *ulk1* would justify our data more precisely.

The major limitation in this project is that we only targeted *ulk1a* and *ulk1b* ubiquitously in zebrafish to study the role of autophagy in definitive hematopoiesis. Moreover, we just worked on *ulk1a* chimeric mutants, which possibly carried a mixture of *ulk1a* mutations including in-frame mutation or mutation that does not alter the gene function. This could be the reason *ulk1a* chimeric mutants have very subtle phenotypes concerning autophagy and hematopoiesis. Furthermore, we also did not study the wild-type endogenous *ulk1b* mRNA overexpression experiment in the stable mutants, which might elaborate the function of *ulk1b* in autophagy and hematopoiesis more detail. In particular, with various autophagy assays currently available in the field, the basal autophagy level in blood cells, especially, in leukocytes are nearly undetectable. Further studies targeting different *atg*s in a lineage-specific manner and better autophagy assay with much higher sensitivity are needed. Nevertheless, our study demonstrated that zebrafish is a unique and efficient *in vivo* model with great potential to elucidate the role of autophagy and hematopoiesis.

Autophagy has been reported to implicate in hematological malignancies. While many studies have reported that autophagy inhibition overcomes drug resistance [306, 307] and suppresses leukemia cell growth [308, 309], others have demonstrated that autophagy suppression is important for leukemia development [310, 311]. These contradictive results suggested that autophagy plays paradoxical roles in leukemogenesis depending on cellular contexts. Our results showing that autophagy suppression would lead to deregulation of normal myelopoiesis, demonstrating the important anti-oncogenic role of autophagy during early stage of myeloid malignancies. Further investigation with bona fide models of hematological malignancy and tissue specific knock-out of autophagy from the myeloid cell are warranted. For instance, zebrafish has emerged as an important model organism for human cancer and models of hematological malignancies including myeloproliferative neoplasm (MPN), acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) with highly conserved oncogenic pathways and pharmacologic responses were reported [312-316]. With the well-developed methodologies in autophagy study [223, 281, 284] and genetic engineering [317] zebrafish could be developed into a unique modelling platform to define the role of autophagy in hematological malignancies, which will provide important information for the development of autophagy-related therapeutic strategies against these heterogeneous diseases.

Chapter 6: Conclusion

In summary, using the zebrafish embryonic model, we demonstrated that zebrafish *ulk1b* played essential roles in autophagy and definitive hematopoiesis. While ulk1b-dependent and independent autophagy are ongoing in different tissues during zebrafish embryonic development, *ulk1b* but not somatically targated *ulk1a^{TAL}* induced the decrease in HSC as well as the increase in myeloid lineages, which is likely autophagy-dependent.

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