Zebrasfish bloodthirsty:
Developmental Expression and Identification of the Mammalian Ortholog

A thesis presented

by

Mo Hu

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ABSTRACT OF THESIS

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Abstract

Blood disorders, such as anemias, are serious diseases in humans. Because the hematopoietic program is highly conserved among vertebrates, we are using fish models to isolate novel erythropoietic genes and to elucidate the functions of the encoded proteins. In this thesis, I describe experiments to examine the developmental expression patterns of the novel zebrafish gene, bloodthirsty (bty), and to identify its probable mammalian ortholog.

The novel gene bloodthirsty (bty) was discovered in a subtractive screen that isolated hematopoietic genes that were differentially expressed by the pronephric kidneys of the red-blood Antarctic rockcod, Notothenia coriiceps, and its white-blood relative, the icefish Chaenocephalus aceratus. Zebrafish bty encodes a 532 residue protein (Bloodthirsty, Bty) that belongs to the TRIM (TRIpartite Motif) protein family. Bty contains an N-terminal RING finger, two B-boxes, a coiled-coil region and a C-terminal B30.2 domain. Suppression of translation of the bty mRNA by morpholino oligonucleotide (MO) treatment suggests that Bty is involved in regulation of the terminal steps of the erythropoietic program.

Although bty plays a role in erythropoiesis, it is widely expressed during zebrafish embryogenesis. bty mRNA is expressed in multiple tissues including gut, brain, spinal cord, etc. In contrast, it is not expressed in somites, white matter or matured erythrocytes.
Bioinformatic analysis suggested two candidates for the mammalian ortholog of zebrafish Bty: TRIM27/ret finger protein (33% identity) and TRIM39 (32% identity). To identify the probable ortholog, TRIM27 and TRIM39 mRNAs were tested for their ability to rescue erythropoiesis in zebrafish bty morphant embryos. My results demonstrate that TRIM27 mRNA is able to restore erythropoiesis, whereas TRIM39 mRNA does not, thereby providing strong evidence that the former corresponds to zebrafish bty.

My research advances our understanding of vertebrate erythropoiesis, and therefore may provide new avenues for therapeutic intervention in human diseases of the red blood cell.
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<tr>
<td>AGM</td>
<td>Aorta-gonad-mesonephros</td>
</tr>
<tr>
<td>ALM</td>
<td>Anterior lateral mesoderm</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>bty</td>
<td>bloodthirsty gene</td>
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<tr>
<td>Bty</td>
<td>Bloodthirsty protein</td>
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<tr>
<td>Ca(NO₃)₂</td>
<td>Calcium nitrate</td>
</tr>
<tr>
<td>CHT</td>
<td>Caudal hematopoietic tissue</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilization</td>
</tr>
<tr>
<td>EKLF</td>
<td>Erythroid krüppel-like factor</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ICM</td>
<td>Intermediate cell mass</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>klfd</td>
<td>Krüppel-like factor d</td>
</tr>
<tr>
<td>MEL</td>
<td>Murine erythroleukemia</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLM</td>
<td>Posterior lateral mesoderm</td>
</tr>
<tr>
<td>RBCC</td>
<td>RING finger, B-Box, coiled-coil</td>
</tr>
<tr>
<td>RBI</td>
<td>Rostral blood island</td>
</tr>
<tr>
<td>RDA</td>
<td>Representational difference analysis</td>
</tr>
<tr>
<td>Real time RT-PCR</td>
<td>Real time reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCL</td>
<td>Stem cell leukemia protein</td>
</tr>
<tr>
<td>scl</td>
<td>stem cell leukemia gene</td>
</tr>
<tr>
<td>TRIM</td>
<td>Tripartite motif</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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**Introduction**

1. **Zebrafish hematopoiesis**

Zebrafish are an important model system for analysis of hematopoiesis because they have high fecundity (100-200 embryos per female per week by external fertilization), their embryos are optically transparent, and they are easy to use in genetic and chemical screens (de Jong et al. 2005; Lieschke et al. 2007). Like other vertebrates, zebrafish have two waves of hematopoiesis (Galloway et al. 2003). The primitive wave is transient and gives rise to erythrocytes and macrophages (Palis et al. 2001). Primitive hematopoietic stem cells (HSCs) in zebrafish are derived from the intermediate cell mass (ICM) of ventral mesoderm (Detrich et al. 1995) at ~15 hours post-fertilization (hpf). At 24 hpf, the heart starts to beat and the primitive erythrocytes and macrophages begin to enter circulation (Willett et al. 1999). At ~26 hpf, definitive HSCs emerge from the ventral wall of the dorsal aorta and migrate to caudal hematopoietic tissue (CHT) by 48 hpf (Thompson et al. 1998; Burns et al. 2002). Thereafter, the definitive HSCs differentiate, expand and further migrate to seed the kidney marrow (equivalent to mammalian bone marrow) (Murayama et al. 2006). Definitive blood cells enter the circulation at 4-5 days post-fertilization (dpf).

During zebrafish gastrulation, three germ layers are formed within embryo. They are ectoderm, mesoderm and endoderm. The mesoderm is the origin of hematopoietic progenitors including blood and pronephro lineages (Kimmel et al. 1990;
Warga et al. 1999). The anterior lateral mesoderm (ALM), which later develops to the rostral blood island (RBI), is where the primitive myelopoiesis occurs (Paik et al. 2010). The posterior lateral mesoderm (PLM) further forms the ICM, which is the major site of primitive erythropoiesis (Detrich et al. 1995).

There are some crucial transcriptional factors involved in hematopoiesis. From 10 hpf, the stem cell leukemia (scl) gene is expressed together with lmo2, gata2 and fli1 in the hemangioblast, which is the common ancestor of primitive HSCs and angioblasts (vascular progenitors) (Liao et al. 1998). gata1, which is a zinc finger transcription factor, also plays an important role in primitive erythropoiesis (Fujiwara et al. 1996). gata1 is first expressed in a subset of scl+ cells in the PLM and the number of gata1+scl+ erythroid precursors increases with the formation of ICM. (Detrich et al. 1995; Thompson et al. 1998). klf4, together with two other klf family members klf12 and klf4, exhibits high-level expression in primitive hematopoietic system including the ICM at 24 hpf and circulating primitive erythrocytes on the yolk at 48 hpf (Oates et al. 2001). klf4 itself was also detected in definitive hematopoiesis including the pronephros, the circulating definitive erythrocytes in the heart lumen and the caudal hematopoietic tissue (Oates et al. 2001).

2. Discovery of the bloodthirsty gene and its role in erythropoiesis

The bty gene was discovered in a subtractive screen that made use of the Antarctic icefishes (Notothenioidei: Channichthyidae), the only vertebrate taxon whose members
fail to produce erythrocytes or express hemoglobin (Cheng et al. 2007). Yergeau et al. (2005) exploited this phenotype to obtain genes that were expressed by the hematopoietic pronephric kidney of the red-blooded notothenioid, *Notothenia coriiceps*, but not by that of its close relative, the icefish *Chaenocephalus aceratus*. One of these, bloodthirsty, encodes the Bloodthirsty (Bty) protein, which belongs to the TRIM (TRIpartite Motif) family (Reymond et al. 2001). TRIM proteins have been reported to be involved in diverse cellular processes, including transcriptional regulation, cell proliferation and growth, and apoptosis (Motoyoshi et al. 2003). TRIM proteins typically share an N-terminal RING finger, one or two B-boxes, and a coiled-coil region. The distinctive features of TRIM proteins are conferred by their C-terminal domains. Bty contains a B30.2 domain at its C-terminus.

To test the putative erythropoietic function of *bty*, Yergeau et al. (2005) cloned the zebrafish ortholog from a pronephric kidney cDNA library. Using injection of morpholino antisense oligonucleotides (MOs) to suppress the translation of the *bty* mRNA in zebrafish embryos, they found that production of primitive erythrocytes was severely reduced. Control embryos injected with non-binding MOs expressed normal levels of erythrocytes. Furthermore, the MO knockdown phenotype could be rescued by the injection of synthetic *bty* mRNA (Yergeau et al. 2005).

The specificity of *bty* function was analyzed by wholemount *in situ* hybridization of *bty* morphant embryos against a panel of vascular, myeloid, and erythroid marker genes. Expression of the early and late erythroid precursor markers *gata1* and *gata2,*
the myeloid marker *pu1*, and the vascular marker *flk-1* was not affected by MO treatment, consistent with the normal formation of erythroid, myeloid and vascular progenitor cells. By contrast, the levels of *α-globin*, *β-globin* and *klfd* mRNAs were suppressed by MO treatment, which implies that *bty* regulates the terminal stages of red blood cell differentiation (Yergeau et al. 2005).

3. **TRIM/RBCC proteins and ubiquitination.**

Zebrafish Bty belongs to the TRIM/RBCC protein family which has been characterized as a tripartite motif containing a RING finger, one or two B-box domains and a coiled-coil region (Reymond et al. 2001) and has been reported to be involved in diverse cellular processes (Motoyoshi et al. 2003). During ubiquitination, a multi-step post-translational modification mechanism which controls the protein level via proteolysis (Pickart 2001; Meroni et al. 2005), ubiquitin is activated and transferred to an E2 ubiquitin-conjugating enzyme by E1 ubiquitin-activating enzyme. A specific E3 ubiquitin ligase interacts with both the target substrate and its E2 partner and mediates the transfer of ubiquitin from the E2 conjugase to the target substrate protein (Joazeiro et al. 2000; Glickman et al. 2002). The interaction of E3 ligase with both E2–Ub and a substrate mediates the transfer of ubiquitin from E2 to the substrate and further leads to either protein degradation by the 26S proteasome or other nonproteolytic regulation such as subcellular localization (Verma et al. 2004; Kerscher et al. 2006). Depending on the type of ubiquitin linkages, which is decided by E2, the ubiquitination leads to different
modification of target substrate (Pickart et al. 2004). The TRIM/RBCC proteins serve as E3 ligases via binding E2s through their RING finger domains.

The RING domain was first described as a conserved cysteine-rich amino acid sequence motif, chelating two zinc atoms in the cross-brace structure, which resembled the zinc finger domain (Freemont et al. 1991). The specific sequence of the RING finger domain is C-X_2-C-X_9,39-C-X_1,3-H-X_2,3-C-X_2-C-X_4,48-C- -P-X_2-C (Deshaiies et al. 2009). The conserved cysteine and histidine residues help to maintain the three-dimensional structure of the RING finger (Deshaiies et al. 2009). Bioinformatics analysis has indicated there are over 300 genes encoding the RING domain and nearly half of them were reported to function as E3 ubiquitin ligases (Li et al. 2008; Deshaiies et al. 2009). The TRIM/RBCC protein family is the largest subfamily among the variety of RING finger E3 ubiquitin ligases with the different subdomains within the RING motif (Li et al. 2008).

In contrast, little is known about the function of B-boxes. The structural study of human MID1 (midline-1; TRIM18) revealed that both its B-box 1 and B-box 2 coordinated with two zinc atoms in a cross-brace scheme similar to the E3 RING finger domain, which indicated the possible E3 ubiquitin ligase activity of B-box (Massiah et al. 2006; Massiah et al. 2007). Further evidence also showed that the binding between B-box1 of MID1 and alpha4 leads to the recruitment of its target protein phosphatase 2A (PP2A). This indicated that the B-box domains either facilitated or possessed the RING finger domain E3 ligase activity (Massiah et al. 2008; Massiah et al. 2011). The B-box
domain together with the first coiled-coil region was also required in the interaction of RFP and four bHLH proteins (Bloor et al. 2005).

The coiled-coil region was reported to be necessary and sufficient for homo-interactions of TRIM proteins and to make them form high molecular weight complexes (Pelicci et al. 2001). The coiled-coil region was also reported to influence the antiviral specificity of TRIM5α capsid recognition (Trono et al. 2010) and be essential to proteasome-mediated degradation in PML (promyelocytic leukemia) proteins, which belong to the TRIM/RBCC family (Fanelli et al. 2004).

The B30.2 domain, also known as PRYSPRY, was first found in human class I MHC (human histocompatibility complex) region (Vernet et al. 1993) and was used to identify divergent members of the B30.2 family (Henry et al. 1998). It was reported that there were a few conserved hot-spot residues dedicated to specific protein-protein interactions that influenced the diverse functions of TRIM proteins (Woo et al. 2006; James et al. 2007). For instance, the B30.2 domain of TRIM5α is essential for retroviral restriction in specificity and potency (Nisole et al. 2005; Stremlau et al. 2005) and a single amino acid change in variable region 2 (V2) of B30.2 domain could affect its anti-HIV2 activity (Kono et al. 2009). A recent study demonstrated that B30.2 domain was also critical to nuclear localization and nuclear bodies (NB) formation in TRIM22 (Sivaramakrishnan et al. 2009).

Numerous studies have shown that members in the TRIM/RBCC protein family mediate ubiquitination as E3 ligases such as TRIM32, TRIM37, TRIM22, etc (Kallijarvi
et al. 2005; Kudryashova et al. 2005; Eldin et al. 2009). For instance, TRIM11, which contains the tripartite motif protein and a c-terminal B30.2 domain, has been demonstrated to interact with and mediate the proteasomal degradation of the protein Humanin (HN) (Niikura et al. 2003). The deletion of the conserved cysteine hampers the effect of TRIM11 on the degradation of HN (Niikura et al. 2003). Another example is the study of MID1/TRIM18 and MID2/TRIM2, both of which have been shown to bind their ubiquitination substrate Alpha 4 (Short et al. 2002). In addition, several TRIM/RBCC proteins have the capacity of auto-ubiquitination (Urano et al. 2009). An immunoblotting experiment showed that higher molecular mass corresponding the mono-ubiquitinated and poly-ubiquitinated forms of TRIM17 were detected in ubiquitination assays (Lassot et al. 2010).

4. Candidate mammalian bty genes

Based on bioinformatic analysis (Yergeau et al., 2005), the two plausible mammalian candidates for zebrafish Bty are TRIM27/ret finger protein (33% identity) and TRIM39 (32% identity). These two TRIM proteins contain N-terminal RING fingers followed by type-2 B-boxes, coiled-coil regions, and C-terminal B30.2 domains. Their domain structures are identical to that of zebrafish Bty, except that they lack a type-1 B-box.

TRIM27 reportedly associates with the nuclear matrix and possesses DNA-binding activity in human and rodent tumor cell lines and in mouse testicular germ
cells (Isomura et al. 1992). It interacts with four different bHLH proteins, including SCL (stem cell leukemia factor), but not with GATA-1 or PU.1 (Bloor et al. 2005). The interaction between TRIM27 and SCL requires the B-box and coiled-coil region of the former and the bHLH region of the latter (Bloor et al. 2005), but the functional relevance of this association is unclear. TRIM27 together with 17 other nuclear proteins have been suggested to affect hematopoietic stem cell activity by functional screen and validation. The 18 genes encoding these factors have high expression levels in HSC-enriched tissues, such as total bone marrow or fetal liver (Deneault et al. 2009). The potential hematopoietic function of TRIM27 makes it a viable candidate as the mammalian ortholog of zebrafish Bty.

The gene encoding TRIM39 (also known as ring finger protein 23) is found in the Class I region of the MHC (major histocompatibility complex) (Orimo et al. 2000). Pan et al. (2011) recently showed that TRIM39 mRNA is abundant in spleen, with lower levels found in liver, brain and lung. These results suggest that TRIM39 may be involved in the innate immune response against infectious disease (Pan et al. 2011).

My research is focused on two major hypotheses:

1) Bty is involved in the erythropoietic program and therefore is expressed in tissues that form red blood cells. This hypothesis will be tested indirectly by analyzing the temporal and spatial patterns of bty mRNA expression in a developmental series of zebrafish embryos from 12 hpf to 4 dpf (days postfertilization) by whole mount in situ hybridization and sectioning of embryos. The presence of bty
mRNA in erythropoietic tissues at appropriate developmental times would provide support for Hypothesis 1.

2) *The mammalian ortholog of zebrafish bty is TRIM27 or TRIM39.* This hypothesis will be tested by attempting to rescue the *bty* MO knockdown phenotype in zebrafish embryos (morphant = absence of erythrocytes at 32-36 h) by co-injection of murine *TRIM27* or *TRIM39* mRNAs. The ability of one or both of the mammalian mRNAs to rescue the morphant phenotype (i.e., restore red cell production) would provide support for Hypothesis 2.
Materials and methods

1. Zebrafish maintenance

Wild-type zebrafish (Danio rerio) were obtained from EKK Will Waterlife Resources and were kept 100 liter aquaria heated to 28°C. Embryos were obtained by breeding one male and one female zebrafish and collected immediately after fertilization. Embryos were kept in embryo water in 28°C incubator.

2. Whole mount in situ hybridization of embryos and vibratome sectioning

The expression pattern of bty in zebrafish embryos of 8 different time points were examined by whole mount in situ hybridization of antisense RNA probes. Embryos older than 24 hpf were treated with pigment inhibitor [0.003% 1-phenyl 2-thiourea (PTU)]. At 12 hpf, 18 hpf, 24 hpf, 30 hpf, 36 hpf, 48 hpf, 72 hpf (3 dpf) and 96 hpf (4dpf), zebrafish embryos were harvested and fixed with 4% paraformaldehyde in 1x PBS overnight at 4°C. Whole-mount in situ hybridization was performed as described (Thisse et al. 2008). The template pBK-CMV-zbty was linearized by digestion with SpeI and the antisense cRNA probe was transcribed using T7 polymerase and labeled with digoxygeninuridine-5’-triphosphate (DIG-UTP). After hybridization, embryos were transferred into gelatin/albumin embedding solution [2.2 g gelatin (TYPE B) in 450 ml PBS; 135 g chick egg albumin; 90 g sucrose] for several minutes and then into molds containing fresh embedding solution with 2.5% glutaraldehyde fixative. The molds were covered with cellophane, and the samples were kept overnight at 4°C. The blocks
were sectioned at 15-20μm the next day with Leica VT1000 S vibratome. Selected sections were mounted with 80% glycerol and examined using Nikon Eclipse 800 DIC microscope.

3. Knockdown of zebrafish Bty with antisense morpholino oligonucleotides

Two pairs of fluorescein isothiocyanate (FITC)-tagged antisense MOs were generated and tested successfully for the consistency of the morphant phenotype in former experiments. MO Zebb302a, 5’ATATGGAGGTAAACGGTC-3’, targeted the 5’UTR upstream of start codon was injected into the embryos 1 to 2 hpf. The MOs were dissolved in Danieau’s Solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0mM HEPES (pH 7.6)] to a final concentration of 1 mM. Zebrafish embryos were injected between 1 to 4 cell stage with MO Zebb302a and Danieau’s solution as the control (5–10 nl at 1 ng/nl) by use of a PLI-100 Picoinjector (Medical Systems Corporation) and a Narishige Micromanipulator. Embryos were maintained in embryo medium at 28°C. The positive MO injected embryos were selected by examining the green fluorescent signal by using Nikon Eclipse 800 DIC microscope.

4. Rescue of the bty morphant phenotype

The constructs of mouse Trim27 and mouse Trim39 cloned into pCMV-SPORT6 were purchased from Open Biosystems. The constructs were linearized by digestion with NotI and transcribed with SP6 to yield a capped RNA with mMessage mMachin Kit (Ambion). Polyadenylation of 3’-end was performed using the Poly (A) Tailing Kit
(Ambion). The mRNAs were purified from the enzyme with MEGAclear (Ambion). Zebrapine embryos were injected with mouse Trim27 and mouse Trim39 mRNA and MO Zebb302a as following treatment conditions: 1) Zebb302a (5ng); 2) Zebb302a antisense MO (5ng) plus mouse Trim27/Trim39 mRNA (10 and 100pg); 3) Danieau’s solution. Embryos were collected at 32hpf.

5. **Staining of hemoglobin with o-dianisidine**

Zebrapine embryos were harvested at 32hpf. The hemoglobin of erythrocytes circulating on the yolk of both MO-injected and non-injected embryos was detected by staining with o-dianisidine. Embryos were incubated in o-dianisidine solution [6.5ml o-dianisidine, 1.63 ml 0.1 M NaOAc pH 4.5, 6.5ml sterile H2O,325 ul H2O2 (30%)] for 5 minutes in the dark and then rinsed with distilled water for three times, 5 minutes each. Embryos were fixed with 4% paraformaldehyde in 1x PBS overnight at at 4°C. Stained embryos were examined in 70% glycerol using Nikon SMZ-U dissecting microscope.
Results

1. The spatial and temporal expression pattern of bty

1.1 The expression of bty at different stages of zebrafish embryos

Figure 1 shows both transverse and sagittal sections of 6-somite stage (12 hpf) embryos. At early somitogenesis, bty transcript was present through the embryo from the anterior to the posterior through the truck region (Fig. 1A-C). Fig. 1D shows the sagittal section cutting through the head region to the trunk region. Fig. 1E shows the sagittal section cutting through the dorsal trunk. Fig. 1F shows the sagittal section cutting the caudal tail region. All panels show that bty mRNA was expressed intensely in endoderm and mesoderm including paraxial mesoderm and lateral plate mesoderm but lightly in ectoderm.

Figure 2 shows that at the 20-somite stage (18 hpf), bty was present expressed rostrally in the central nervous system (CNS) and caudally in the ICM. Figs. 2A-B show the transverse sections that pass through the forebrain region to the hindbrain region. Note the uniform accumulation of bty mRNA in CNS structures but not in the overlying ectodermal tissue. Fig. 2C shows the transverse section cutting from head region to the anterior trunk. bty transcript was present in the ventral CNS and ventral tissues of the trunk. Fig. 2D shows a transverse section cutting through the mid trunk to the yolk. The neural tube and notochord show low level of bty mRNA expression. Consistent
Figure 1 bty mRNA expression in zebrafish 6-somite (12 hpf) sections. (A-C)

Transverse sections cutting from anterior to posterior. (D) Sagittal section cutting through the head region to the trunk region. (E) Sagittal section cutting through the dorsal trunk. (F) Sagittal section cutting the caudal tail region. The zebrafish embryos were sectioned at ~15μm with Leica VT1000 S vibratome. All sections were mounted with 80% glycerol and photographed using Nikon Eclipse 800 DIC microscope.
**Figure 2** *bty* mRNA expression in zebrafish 20-somite (18hpf) sections. (A-B) Transverse sections that pass through the mid and hindbrain regions. (C) Transverse section that passes the anterior region of the head and the anterior trunk. (D) Transverse section cutting through the mid trunk to the yolk.
with the 6-somite (12 hpf) stage, *bty* expression was present in endodermal and mesodermal layers, including the anterior ICM, but lightly in the overlying ectoderm.

Figure 3 shows that *bty* mRNA was present ubiquitously from the head region to the trunk of the embryos at prim-5 stage (24 hpf). Fig. 3A shows the transverse section of the embryo head, *bty* mRNA was present in optic cup, the head mesoderm and CNS tissue including telecephalon, diencephalon, rhombencephalon. Fig. 3B shows the transverse section cutting from the hindbrain to the yolk. The expression of *bty* mRNA was present in the ventral of hindbrain, the undifferentiated head mesoderm and endoderm. There is no expression of *bty* mRNA in the epithelium and lower expression level in several cell layers beneath the epithelium. Fig. 3C shows the transverse section from the mid trunk to the yolk extension. *bty* expression was present in the neural tube and the myotome surrounding the notochord. Fig. 3C also shows strong *bty* expression between notochord and the yolk, where located the differentiated ICM including dorsal aorta, axial vein and bilaterally pronephric ducts. By the prim-5 stage, the pronephric duct has a lumen all along its length (Kimmel et al. 1995). There was no expression of *bty* mRNA in the notochord. Still, the overlying layer of the embryo presented a much lower expression level of *bty* mRNA.

Figure 4 shows the *bty* mRNA expression in zebrafish embryo at prim-15 stage (30 hpf). Fig. 4A shows the transverse section that passes through the anterior head region. *bty* expression was present in telecephalon, diencephalon and optic cup. Figs. 4B-C show the transverse sections cutting from the hindbrain to the yolk. *bty* expression was
Figure 3 *bty* mRNA expression in zebrafish at prim-5 stage (24 hpf) sections.  (A) Transverse section of the embryo head region. (B) Transverse section cutting from the hindbrain to the yolk. (C) Transverse section that passes through the mid trunk to the yolk extension.
**Figure 4** *bty* mRNA expression in zebrafish at prim-15 stage (30 hpf). (A) Transverse section cutting the anterior head region. (B-C) Transverse sections pass through from the hindbrain to the yolk. (D) Transverse section from the mid trunk to the yolk. (E) Transverse section cutting from posterior trunk to the yolk extension.
present in the hindbrain, dorsal aorta root, head mesoderm and endoderm. bty mRNA was also expressed in the shallow dome of pectoral bud. Fig. 4D shows the transverse section from the mid trunk to the yolk. The expression of bty was present in the neural tube, somites and gut but not notochord. The expression of bty mRNA was also present in the undifferentiated mesoderm and endoderm. Fig. 4E shows transverse section cutting from posterior trunk to the yolk extension. bty expression was present in the neural tube, dorsal aorta, axial vein and pronephric ducts but not the notochord. From all the transverse sections, there is no expression of bty mRNA showed in the epithelium. Both mesoderm and endoderm layers showed strong bty expression.

Figure 5 shows the bty mRNA expression at prim-25 stage (36 hpf). Fig. 5A shows transverse section of the embryo head region. bty expression was present in telencephalon, diencephalon, mesencephalon and optic cup but not the commissure in the brain. Fig. 5B shows the transverse section that passes through the hindbrain to the yolk through the otic vesicle. bty expression was present in the hindbrain and the branchial arch. Fig. 5C shows the transverse section cutting from the anterior trunk to the yolk. Fig. 5D shows the transverse section cutting from the mid trunk to the yolk extension. bty expression was present in the spinal cord, liver, gut and pronephric ducts. bty expression was not present in the commissure or the notochord. It is worth to notice that the expression level of bty mRNA in the somites decreased at prime-25 stage as shown in Fig. 5D compared with the ones at prim-15 stage shown in Fig. 4E.
Figure 5 bty mRNA expression in zebrafish at prim-25 stage (36 hpf). (A) Transverse section of the head region. (B) Transverse section cutting from the hindbrain to the yolk through the otic vesicle. (C) Transverse section cutting from the trunk to the yolk. (D) Transverse section cutting from the trunk to the yolk extension.
Figure 6 shows *bty* mRNA expression pattern at high-pec stage (48 hpf). The transverse section that passes through the anterior head region (Fig. 6A) shows that *bty* mRNA was present in mesencephalon, diencephalon, telencephalon and optic cup but not the posterior commissure (POC). Fig. 6B shows the transverse section cutting from the hindbrain to the heart. *bty* mRNA was present in myelencephalon, pharyngeal arch, hatching gland and atrium but not the track of posterior commissure (TPOC). Fig. 6C shows the transverse section from the hindbrain to the yolk through the otic vesicle. Besides myelencephalon and pharyngeal arch, *bty* expression was also present in dorsal aortic root and undifferentiated mesoderm and endoderm. Fig. 6D shows the transverse section from the caudal hindbrain to the yolk. *bty* expression was present in caudal hindbrain and dorsal aortic root. In addition, *bty* mRNA was also expressed in the undifferentiated endoderm and lateral plate mesoderm. Fig. 6E shows the transverse section from the anterior spinal cord to the yolk through pectoral fin bud. *bty* expression was present broadly in the spinal cord, pectoral fin bud, pronephric tubules, liver and undifferentiated endoderm but lightly in somites. Fig. 6F shows the section from the spinal cord to the yolk extension in which gut region showed intensive *bty* expression. *bty* mRNA was also present in the spinal cord, somites and pronephric ducts to some extent. In terms of germ layers, the *bty* mRNA appeared to be present in both endoderm and mesoderm (Figs. 6C-E) including the lateral plate mesoderm (Fig. 6D). *bty* expression did not show in the notochord, the commissures or the epithelium. Among
all the differentiated regions *bty* mRNA was present, the CNS tissue and gut showed the highest expression level.

Figure 7 shows *bty* mRNA expression pattern in zebrafish embryo at protruding-mouth stage (72 hpf). The transverse section cutting from the head to the anterior of the heart region (Fig. 7A) shows that *bty* expression was present in the diencephalon of forebrain but not in the chondrocranium, the pharyngeal cartilage or the posterior commissure. Within the layers of the eye, *bty* mRNA was present in ganglion cells, amacrine cells, bipolar cells and photoreceptors but not the inner plexiform layer or lens. The erythrocytes in the heart did not express *bty* but the chamber wall showed the expression. Fig. 7B shows the transverse section cutting from the midbrain to the yolk. The expression of *bty* was showed in the mesencephalon, the diencephalon but not in the chondrocranium, the pharyngeal cartilage or the tract of posterior commissure. Fig. 7C shows the transverse section from the midbrain to the yolk and Fig. 7D shows the transverse section cutting from the hindbrain to the yolk. *bty* expression was present in the midbrain, hindbrain, pronephric ducts, pectoral fin and gut. The erythrocytes in the artery did not show *bty* expression. Figs. 7E-H show the transverse sections cutting from the trunk to the yolk and yolk extension. *bty* expression was present in the gray matter of spinal cord, liver, pancreas, esophagus, gut and partial pronephric ducts. *bty* mRNA was not expressed in the white matter of the spinal cord, the notochord or the erythrocytes in common cardinal vein or dorsal aorta.
Figure 6 bty mRNA expression in zebrafish at high-pec stage (48 hpf). (A) Transverse section cutting the anterior head region. (B) Transverse section from the hindbrain to the heart. (C) Transverse section from the hindbrain to the yolk through the otic vesicle. (D) Transverse section from the caudal hindbrain to the yolk. (E) Transverse section from the anterior spinal cord to the yolk through pectoral fin bud. (F) Transverse section from the spinal cord to the yolk extension.
Figure 7 bty mRNA expression in zebrafish at protruding-mouth stage (72 hpf). (A) Transverse section cutting from the head to the anterior of the heart region. (B) Transverse section cutting from the midbrain to the yolk. (C) Transverse section passes through the midbrain to the yolk. (D) Transverse section cutting from the hindbrain to the yolk. (E-H) Transverse sections cut from the trunk to the yolk and yolk extension.
Figure 8 shows *bty* mRNA expression pattern at 96 hpf stage. The transverse sections cutting from the head region to the heart (Fig. 8A-B) show that *bty* expression was present in diencephalon and mesencephalon but not in commissure, chondrocranium or pharyngeal cartilage. In the eyes, *bty* mRNA was present in ganglion cells and photoreceptors but not the inner plexiform layer or the lens. Consistent with results from 72 hpf, *bty* mRNA is expressed in the chamber wall of heart but not in the erythrocytes. Figs. 8C-E show the transverse sections cutting from the head to the yolk. *bty* transcript was present in the metencephalon, the mesencephalon, the diencephalon in the brain. *bty* expression was also present in esophagus, kidney, liver and pronephric ducts. *bty* expression was not present in chondrocranium, pharyngeal cartilage or erythrocytes in the atrium or artery. Figs. 8F-H show the transverse sections cutting from the trunk to the yolk extension. *bty* expression was present in spinal cord, pancreas, liver, gut and pronephric ducts. Consistent with the sections of earlier stages, the expression of *bty* was not present in the notochord or the most parts of somites. *bty* mRNA did not appear to be expressed in the erythrocytes in the dorsal aorta or the axial vein (Fig. 8H).

1.2 The expression of *bty* in development of different tissues

1.2.1 The expression of *bty* in gut tissue

Among the three germ layers, the endoderm generates the gut tissue including the pharynx, the esophagus, the stomach, the pancreas, the liver and the intestine
Figure 8 *bty* mRNA expression pattern at 96 hpf stage. (A-B) Transverse sections cutting from the head region to the heart. (C-E) Transverse sections cutting from the head to the yolk. (F-H) Transverse sections cutting from the trunk to the yolk extension.
(Warga et al. 1999). The intense staining of the endoderm layer from *in situ* hybridization using *bty* probe in the early stage (12-18 hpf) is in concert with the intense staining in the differentiated gut tissue later (24hpf-96hpf). The continuous expression of *bty* mRNA indicated that Bty might be involved in the differentiation and development of gut tissue.

### 1.2.2 The expression of *bty* in bone related tissue

During gastrulating, the converging movement of hypoblast cells forms the chordamesoderm, which is the precursor of notochord (Trinkaus et al. 1992). Although *bty* expression was present in the region of chordamesoderm, it was not appeared in the notochord. During segmentation period, the notochord differentiates in an anterior-posterior sequence and some of its central cells acquire a large vacuole and become the structural elements (Kimmel et al. 1995). In addition, *bty* expression was not present in chondrocranium or the pharyngeal cartilage, which were both primitive skeletal structures. This implies that Bty may be involved in the early differentiation of chordamesoderm but was not expressed in the structural bone tissue thereafter.

### 1.2.3 The expression of *bty* in nervous system

The central nervous system of zebrafish begins to form during gastrulation. With the completion of gastrulation, the neural plate is then derived from neuroectoderm. Folding and convergence of cells in neural plate further forms the neural tube which differentiates to advanced neural structure later (Strahle et al. 1994). Fig. 1 shows a low
level of bty expression in the ectoderm at 6-somite stage (12hpf) while Fig. 2 shows intense levels of bty expression in the advanced brain structures such as the telecephalon and the diencephalon of the forebrain, the midbrain (mesencephalon) and the hindbrain (rhombencephalon) at 20-somite stage (18hpf) (Kimmel 1993). Figs. 3-8 show consistent bty expression pattern in the brain and the spinal cord of zebrafish embryos from prim-5 stage (24hpf) to 4dpf. In general, bty exhibits a widespread expression in the brain spatially and temporally except for some highly specified regions such as inner plexiform layer of the eye, several commissure regions within the brain and the white matter along the spinal cord. All regions that show no expression of bty are regions highly specified in structure and function, many of which are fibrous or conductive. For instance, the commissure consists of axon bundles that connect adjacent subdivisions in the brain; the inner plexiform layers consist of neurofibrils; the white matter consists mostly of myelinated axons. In these tissues, transcriptional activity is very limited.

1.2.4 The expression of bty decrease in somites with development

Paraxial mesoderm, adjacent to the chordamesoderm, is the origin of somites (Kimmel et al. 1995). bty was expressed in paraxial mesoderm during gastrulation and the expression was still visible within the somites at prim-15 stage (30hpf) but decreased shortly after. At prim-25 stage (36 hpf), the expression of bty could barely be detected in somites. bty expression in muscle cells was diminished with muscle maturation. Mature muscle cells contain more than one nucleus due to single muscle cell (myocyte)
During muscle maturation, many single myocytes will fuse together into a structure called syncytium, which then developed into a large bundle of contractile muscle fiber. Once muscle fibers are formed, unless damaged, it will maintain a stable structure with no further fusion of myocytes. Such stability results in a very low level of proliferation, differentiation and apoptosis activity after maturation in muscle tissues. This implies that Bty may be involved in the early development of muscle cells, but not in mature muscle fibers.

1.2.5 The expression of bty in blood related lineage

a) Blood and blood vasculature

The lateral plate mesoderm gives rise to the heart and blood of the circulatory system including the hemangioblast cells, the precursors of blood vessels and blood cells (Moody 1999; Gilbert 2003; Orkin et al. 2008). In zebrafish, the intermediate mass of mesoderm is the early forerunner of the hematopoietic blood island (Kimmel et al. 1995). The cardiac progenitors derive from the anterior of lateral plate mesoderm during gastrulation. The angioblast progenitors, which give rise to the blood vessels during vasculogenesis and angiogenesis, are derived from dorsal lateral plate mesoderm. The dorsal aorta and the cardinal vein are formed when the angioblast cells migrate to the midline from the lateral plate mesoderm (Vogel et al. 2000). The presence of bty expression in the lateral plate mesoderm at 12 hpf (Fig. 1), the ICM region at 18 hpf (Fig. 2) and the differentiated tissue including dorsal aorta, cardinal vein and pronephric ducts subsequently at 24hpf
(Fig. 3C), 30hpf (Fig. 4E), 36hpf (Fig. 5D) and 48hpf (Fig. 6D), indicates that Bty is involved in erythropoietic program and might also function in the formation of the vascular lineage. The erythrocytes in the heart (Fig. 7A and Fig. 8B), the artery (Fig. 7C) and the cardinal vein (Figs. 7E-F, Figs. 8D-F) did not show bty expression. The whole mount in situ hybridization of MOs treated zebrafish embryos suggests Bty is required in terminal erythroid differentiation (Yergeau et al. 2005) while the erythrocytes lack bty expression. This suggests that Bty plays a role in erythroid differentiation but it is not required for the maintenance of erythrocytes. Therefore the expression of bty vanished during erythrocyte maturation.

b) Pronephros

The intermediate mesoderm generates the urogenital system including kidney and gonads. The pronephric progenitor cells emerge in the intermediate mesoderm as a stripe of tissue, which is adjacent to the ventro-lateral paraxial mesoderm (Drummond et al. 2010). During somitogenesis, the pronephric primordium lies under the second and third somites, under which pronephric ducts grow caudally (Kimmel et al. 1995). The HSCs in the AGM migrate to the pronephros either through CHT or directly in definitive hematopoiesis. From 4dpf, the HSCs appear in the pronephros which is functionally equivalent to mammalian bone marrow (Murayama et al. 2006; Bertrand et al. 2008; Paik et al. 2010). bty was expressed in the intermediate mesoderm region and its expression was also shown in the pronephric tubes (Fig. 6) and pronephric ducts from 24 hpf (Fig.
3C). The expression of bty in the pronephros indicates its involvement in development of pronephros and/or the hematopoiesis taking place there.

2. Rescue of MO knockdowns with mouse TRIMs

Bioinformatics analysis indicated that TRIM27 and TRIM39 were plausible candidates for mammalian BTY gene. To determine whether these TRIMs play the same role in the erythroid genetic pathway as Bty, mouse TRIM27 and TRIM39 mRNA were co-injected with MO Zebb302a (5ng) in embryos separately. Figure 9 shows that the mouse TRIM27 mRNA was able to rescue the knockdown phenotype of embryos (32-hpf) at doses of 10 and 100pg. Higher doses caused more erythrocytes to recover. With 100pg of rescue mRNA, the erythrocytes were restored to nearly wild type level. However, Figure 10 shows that both 10 and 100pg of mouse TRIM39 mRNA were not able to rescue the knockdown phenotype of embryos. These results suggest that TRIM27 gene is the most possible mammalian ortholog for zebrafish bty.
**Figure 9** Rescue the zebrafish *bty* morphant phenotype by co-injection of an antisense MO and mouse *Trim27* mRNA. Embryos were stained with *o*-diansidine at 32-hpf to detect the hemoglobin-expressing red blood cells. (A) Embryo injected with Zebb302a antisense MO, 5ng. (B) Wild-type embryo, uninjected. (C) Embryo co-injected with Zebb302a MO (5ng) and mouse *Trim27* mRNA (10pg). (D) Embryo co-injected with Zebb302a MO (5ng) and mouse *Trim27* mRNA (100pg).
**Figure 10** Rescue the zebrafish *bty* morphant phenotype by co-injection of an antisense MO and mouse *Trim39* mRNA. Embryos were stained with o-diansidine at 32-hpf to detect the hemoglobin-expressing red blood cells. (A) Embryo injected with Zebb302a antisense MO, 5ng. (B) Wild-type embryo, uninjected. (C) Embryo co-injected with Zebb302a MO (5ng) and mouse *Trim39* mRNA (10pg). (D) Embryo co-injected with Zebb302a MO (5ng) and mouse *Trim39* mRNA (100pg).
Discussion

1. Bty is a possible TRIM/RBCC E3 ubiquitin ligase

   Like most studied TRIM/RBCC proteins, bty exhibits a broad expression pattern including gut tissue, brain, spinal cord, pronephric ducts and early blood vessels including dorsal aorta and vein. The loss-of-function study suggested that bty was essential in red blood cells formation in zebrafish embryos (Yergeau et al. 2005). Consistent with its crucial role in erythropoiesis, bty expression has been found in multiple tissues including but not limited to the specific hematopoietic tissues such as the intermediate cell mass (ICM) and the aorta-gonad-mesonephros (AGM). The disruption of Bty synthesis does not cause abnormalities or defects of development other than the deficiencies in the production of erythrocytes. Therefore, although bty mRNA was found to be expressed in the brain, the gut tissue, and some other tissues, it was not required for their development and maintenance, at least before 48 hpf when the presumably degradation and/or dilution of morpholino oligonucleotides took place.

   Compared with the two E1 activating enzymes and the approximate 40 E2 conjugating enzymes, numerous predicted E3 ubiquitin ligases (around 600 in human) are suggested to be responsible for the specificity and regulation of ubiquitination of appropriate substrates (Deshaies et al. 2009; Schulman et al. 2009). Although some members of TRIM/RBCC protein family have been shown to be expressed in a restricted pattern from single tissue to multiple organs, around half of the TRIM/RBCC proteins are
widely expressed in adult tissues (Ozato et al. 2008). The ubiquitous expression of TRIM/RBCC proteins, in concert with the broad expression of E3 ligases, may imply an involvement in fundamental cell activities and crucial pathways which are common in different types of cells. An example is the study of the expression profile of the E3 ligase Axotrophin. Although the axotrophin null mice only showed the premature neural degeneration and corpus callosum agenesis, axotrophin mRNA was expressed in many tissues including brain, muscle, kidney, pancreas, etc (Metcalf et al. 2005; Szigyarto et al. 2010). Another example is HLS5, a member of the TRIM/RBCC protein family. HLS5 (hemopoietic lineage switch 5), also known as TRIM35, was reported to suppress globin synthesis by modulating GATA-1 activity (Endersby et al. 2008). Although it was reported to be involved in the specific hematopoietic program, the expression of HLS5 was detected in branchial arches, spinal cord, dorsal root ganglia, limb buds and brain during embryogenesis and in an even wider range in adult tissues (Lalonde et al. 2004).

Most E3 ligases in the TRIM family interacted with more than one ubiquitin conjugating E2 enzyme (Meroni et al. 2011). E2 enzymes were demonstrated to mediate the initiation and elongation of ubiquitin chains, which is important to the fate of the modified substrate (Ye et al. 2009). One E3 ligase may bind to different E2s due to the nature of different tasks, thus complete different types of ubiquitination to the substrate. Different ubiquitination patterns will eventually determine the fate of the substrate. In addition, although E3 ligase is concerned with high specificity of substrate,
in some cases; it still may have more than one substrate. The capacity of interacting with variable E2s and different substrates might be a crucial factor that enables one single E3 ligase to be involved in different cell activities and to exhibit a broad expression pattern (Sato et al. 2011).

Ubiquitination has an essential role in regulating numerous cellular pathways and maintaining protein homeostasis (Ciechanover 1998; Sun et al. 2004). Several E3 ligases are important regulators of hematopoiesis. The E3 ligase c-Cbl (casitas B-cell lymphoma) mediates degradation of STAT5, which negatively regulate hematopoietic stem cell development (Rathinam et al. 2008). Another E3 ligase, SCFSKP2 is required in human erythroid progenitor cell proliferation for downregulation of p27Kip via proteasomal degradation (Carrano et al. 1999; Mamillapalli et al. 2001; Bouscary et al. 2003). Other published E3 ligases involved in differentiation of the hematopoietic progenitors and regulation of the HSC activities include Itch, FLRF, PcG Complex 1 and Fbw7 (Jing et al. 2008; Ohtsubo et al. 2008; Thompson et al. 2008; Rathinam et al. 2011). We propose Bty could be another potential regulator for erythroid differentiation as an E3 ubiquitin ligase. Bty is located in cells involved in erythropoiesis but is also distributed in many tissues. The specific functions of Bty will be determined by its E2 and substrate partners in different cell types.

2. A proposed mechanism of Bty/TRIM27 in erythroid differentiation
Bioinformatic analysis suggested that TRIM27 is a probable mammalian BTY ortholog. Our rescue experiments strongly support this hypothesis. In MO treated zebrafish embryos, the expression of klfd mRNA is down regulated by the suppression of Bty synthesis (Yergeau et al. 2005). klfd is an important transcription factor mediating primitive erythropoiesis (Fu et al. 2009) and is expressed in both primitive and definitive hematopoietic sites such as the ICM and pronephros (Oates et al. 2001). Both TRIM27 and EKLF, the mammalian klfd homolog, also participate in hematopoiesis. The conservation of hematopoietic program among vertebrates suggests TRIM27 and Bty may play the same role in erythroid differentiation.

TRIM27 has been identified via a gain-of-function screening as one of the factors enhancing HSC activity (Deneault et al. 2009). In this study, overexpression of TRIM27 led to increased proliferative output of LT-HSC (Deneault et al. 2009). TRIM27 interacts with the basic helix-loop-helix (bHLH) region of SCL via its B-box and first coiled-coil domain (Bloor et al. 2005). TRIM27 has also been reported to associate with various proteins that mediate diverse functions such as transcriptional repression and proteasomal degradation (Bloor et al. 2005; Fukushima et al. 2006; Gillot et al. 2009). TRIM27 has been shown to be widely expressed and distributes to nucleus or cytoplasm depending on the cell types (Tezel et al. 1999). Immunohistochemistry showed that TRIM27 is also highly expressed in normal male germ cells and also in tumor cell lines (Takahashi et al. 1988; Cao et al. 1996). In addition, central and peripheral neurons, hepatocytes and adrenal chromaffin cells have nuclear expression of
TRIM27 while cytoplasmic expression was detected in plasma cells as well as multiple myeloma cells (Tezel et al. 1999). The broad expression of TRIM27 indicates its potential function in regulation of differentiation and/or growth of different cell types (Tezel et al. 1999).

Histone deacetylase 1 (HDAC1) is associated with both TRIM27 (Kato et al. 2009) and EKLF (Chen et al. 2004). HDAC1 is an enzyme that removes acetyl groups from histones, resulting in chromatin compaction and transcriptional repression (Leipe et al. 1997). HDAC1 regulates variable cellular activities such as cell differentiation and cell cycle through direct interaction or multiprotein complexes (Kato et al. 2009). The importance of HDAC1 in hematopoiesis was demonstrated by the zebrafish hdac1 mutant, which failed to specify HSCs and was not able to express c-myb or runx1 (Burns et al. 2009). HDAC1, together with the transcription factor Sin3A, form a corepressor complex to regulate EKLF in erythroid cell lines (Chen et al. 2004). HDAC1-Sin3A repression of EKLF activity requires lysine 302 (K302), which is acetylated by p300/CBP and is responsible for the interaction between EKLF/KLF1 and the HDAC1-Sin3A protein complex (Chen et al. 2004). In addition, HDAC1 is also involved in SUMO-dependent repression of EKLF via binding to the sumoylated K74 with Mi-2β (Siatecka et al. 2007).

The importance of the HDAC1 and TRIM27 interaction in TBP-2 gene regulation was first studied in cancer (Kato et al. 2009). Results of coimmunoprecipitation experiments demonstrated that both the coiled-coil and Rfp domains of TRIM27 interact
with the N-terminal region of HDAC1 (Kato et al. 2009). In addition, TRIM27 forms a homooligomers via the RING finger, B-box and coiled-coil domains. One of the Rfp domains in TRIM27 also binds NF-YC, another negative regulator of TBP-2. The oligomerized TRIM27 proteins mediated the repression of TBP-2 through interaction with HDAC1 and NF-YC via their Rfp domains (Kato et al. 2009).

Together, the association among TRIM27, HDAC1-Sin3A and EKLF, the repressive effect of HDAC1-Sin3A corepressor complex and E3 ligase activity of TRIM27 (Gillot et al. 2009) imply that TRIM27 may play an important role in erythropoiesis. Since the knockdown of bty suppressed the expression of klfd, I propose that TRIM27 may function upstream of EKLF to mediate the erythroid differentiation. One potential genetic pathway is that TRIM27, as an E3 ligase, mediates a proteasomal degradation of HDAC1-Sin3A and promotes erythroid differentiation through preventing the suppression of EKLF (Figure 11).
Figure 11 Proposed genetic pathway of TRIM27. TRIM27 may act as an E3 ligase and mediate a proteasomal degradation of HDAC1 to promote erythroid differentiation through preventing the suppression of EKLF via HDAC-Sin3A corepressor complex.
Conclusion

The distribution of bty in the zebrafish embryo is consistent with a role in erythropoiesis as well as many functions as yet unknown. My work on the bty gene of zebrafish has led to the identification of TRIM27 as the probable mammalian ortholog. Clearly, the zebrafish provide an outstanding model system for future analysis of bty/TRIM27.
**Future Prospects**

Whether TRIM27 affect erythroid differentiation and the activity of EKLF can be investigated in mammalian hematopoietic cell lines such as MEL (murine erythroleukemia). The expression level of EKLF can be tested by immunoblotting or real-time RT-PCR. If the knockdown of TRIM27 by RNAi results in the decrease of expression level and/or activity of EKLF and further lead to the deficient erythrocytes development while the enforced TRIM27 causes the increase of expression level and/or activity of EKLF and promotes erythroid differentiation, the former hypothesis can be verified. Hence, the results will be in concert with earlier studies of TRIM27 by Deneault and his colleagues since the enforced TRIM27 has been demonstrated to promote HSC activity (Deneault et al. 2009).

The influence of TRIM27 on erythroid differentiation by association with EKLF can be investigated by RNAi knockdown in mammalian hematopoietic cell lines such as MEL (murine erythroleukemia). If the proposed mechanism is true then the knockdown of TRIM27 will result in a decrease of EKLF expression, as shown by immunoblotting or real-time RT-PCR, which would lead to a deficiency of erythrocytes upon DMSO induction. In contrast, enforcing TRIM27 would result in an increased level of EKLF expression and thereby promote erythroid differentiation. These results would be in concert with earlier studies where enforced TRIM27 was shown to promote HSC activity (Deneault et al. 2009).
Former analysis indicates that HDAC1 might be a potential downstream target substrate of TRIM27 mediating proteasomal degradation due to the direct binding between them. To characterize the association level of EKLF with HDAC1 and SIN3A, the lysates of MEL cells with TRIM27 knockdown or overexpression would be collected and examined by immunoprecipitation with EKLF antibodies and the presence of HDAC1 and SIN3A determined by immunoblot analysis.

The E3 ligase activity of TRIM27 on HDAC1 can be studied in vivo via ubiquitination assay using HEK293 cells with overexpression of TRIM27, HDAC1, ubiquitin and specific E2s such as UbcH1, UbcH5A, 5B, 5C, UbcH6, which were identified from former studies (Gillot et al. 2009). The expected mono-ubiquitinated and poly-ubiquitinated forms of HDAC1 can be determined by immunoblotting. The probable interaction of zebrafish Bty and Hdac1 could be further tested if the results of above are positive.

To further elucidate the function of Bty and confirm the specificity of the in situ results, reverse genetic tools TILLING (Targeting Induced Local Lesions in Genomes) and ZFNs (zinc-finger nuclease) could be incorporated to obtain a zebrafish knockout.
References


