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A point mutation of zebrafish *c-cbl* gene in the ring finger domain produces a phenotype mimicking human myeloproliferative disease

Running title: Zebrafish *c-cbl* mutant induces HSPC proliferation

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Abstract
Controlled self-renewal and differentiation of hematopoietic stem/progenitor cells (HSPCs) are critical for vertebrate development and survival. These processes are tightly regulated by the transcription factors, signaling molecules, and epigenetic factors. Impaired regulations of their function could result in hematological malignancies. Using a large-scale zebrafish N-ethyl-N-nitrosourea (ENU) mutagenesis screening, we identified a line named *LDD731*, which presented significantly increased HSPCs in hematopoietic organs. Further analysis revealed that the erythoid/myeloid lineages in definitive hematopoiesis were increased while the primitive hematopoiesis was not affected. The homozygous mutation was lethal with a median survival time around 14–15 days post-fertilization. The causal mutation was located by positional cloning in the *c-cbl* gene, of which the human ortholog, *c-CBL*, is found frequently mutated in myeloproliferative neoplasms (MPN) or acute leukemia. Sequence analysis showed the mutation in *LDD731* caused a histidine-to-tyrosine substitution of the amino acid (aa) codon 382 within the RING finger domain of c-Cbl. Moreover, the myeloproliferative phenotype in zebrafish seemed dependent on the Flt3 (fms-like tyrosine kinase 3) signaling, consistent with that observed in both mice and humans. Our study may shed new lights on the pathogenesis of MPN and provide a useful *in vivo* vertebrate model for drug screenings for this syndrome.

Key words: hematopoietic stem/progenitor cells; *c-cbl*; myeloproliferative neoplasms (MPN); *flt3*; N-ethyl-N-nitrosourea (ENU) mutagenesis screening

Introduction

Myeloproliferative neoplasms (MPN) are clonal disorders of multi-lineage hematopoietic cells, at a stage between hematopoietic stem cells (HSCs) and common myeloid progenitor cells (CMPs). The disease phenotypes are characterized by over-proliferation of one or more myeloid cell lineages in the bone marrow (BM) and increased numbers of mature and immature myeloid cells in the peripheral blood.[1-4] MPN cases have the propensity to evolve into acute myeloid leukemia (AML). Certain signals or pathways involved in the normal development and regulation of HSPCs may be deregulated in the affected tumor cells of MPN. Hence, decoding the molecular mechanisms by which HSPCs are transformed in MPN will shed lights on the pathogenesis of this disease syndrome, as well as on the biology of normal hematopoiesis.

In vertebrates, the long-term self-renewal HSCs are formed during definitive hematopoiesis. They originate from aorta-gonad-mesonephros (AGM), expand, migrate and seed into different hematopoietic organs, and finally localize to the marrow. HSCs are the original resource of progenitors of blood lineages and terminally differentiated cells throughout a vertebrate life. The proliferation and differentiation states of HSCs and progenitors are tightly regulated by a number of networks including those of transcription factors, epigenetics modifiers, post-transcriptional regulators, signaling molecules and cell-cell interaction molecules.[5] Any abnormal regulations of the self-renewal and differentiation of HSPCs could result in hematological disease, such as MPN and leukemia.[6-8]

The zebrafish (Danio rerio) is an excellent organism for the study of hematopoiesis and hematologic disorders[9, 10] owing to its unique characters such as *en utero* and rapid development, and an approximately 80% genetic conservation between this species and the human being.[11] Similar to that in mammals, hematopoiesis in zebrafish can be divided into two successive waves: primitive and definitive ones. The primitive wave originates from Intermediate Cell Mass (ICM) and Rostral Blood Island (RBI), analogous to the yolk sac hematopoiesis in

mammals and only gives rise to erythroid and myeloid cells. Definitive hematopoiesis initiates from the ventral wall of the dorsal aorta (VDA), which is analogous to mammalian AGM and starts at around 28 hours post fertilization (hpf).[12] The VDA-derived HSCs then migrate to the caudal hematopoietic tissue (CHT), an intermediate hematopoietic site analogous to the fetal liver in mammals, where they expand and mainly differentiate into erythrocytes and myeloid cells.[13] The VDA-derived HSCs also migrate to the thymus, where lymphocytes are produced. By 5 days post-fertilization (dpf), these HSCs ultimately colonize the kidney, the definitive hematopoietic organ in adult fish, analogous to mammalian BM.[14, 15] Although the temporal and spatial development paths of HSPCs in vertebrates have been elucidated, the molecular mechanisms that regulate the origination, migration and maintenance of HSPCs are largely unknown. Over the past decade, efforts in genetic screening and functional studies identified dozens of mutants with defective hematopoiesis in zebrafish. Although many of these mutants have now been cloned and used as animal models for investigating similar human diseases, only a few mutants are found to have abnormal phenotypes of HSPCs.[11]

To gain further insights into the regulation of zebrafish definitive hematopoiesis, we performed a large-scale forward genetic screening and identified a line named *LDD731*, in which a hematopoietic phenotype screening showed significantly increased HSPCs. Positional cloning and sequence analysis revealed a mutation of *c-cbl* gene in zebrafish, which could produce a phenotype mimicking human MPN. We also analyzed potential pathways downstream of c-Cbl. Our results showed that the homozygous mutants definitely represent a vertebrate genetic model of human MPN and could be used for further genetic and chemical genetic studies of the disease.

Materials and Methods

Zebrafish maintenance, mutagenesis and positional cloning

Zebrafish strains were managed under standard conditions as described in the Zebrafish Book (http: //zfin.org/zf_info/zfbook/zfbk.html). For all experiments, zebrafish embryos were

cultured in E3 embryo water consisting of 0.045% sea salt and 0.002% methylene blue as a fungicide. To inhibit pigment formation and facilitate *in situ* hybridization, the embryos were incubated with 0.0045% 1-phenyl-2-thiourea (Sigma). Zebrafish strain Tuebingen (Tu) was mutagenized; Wild Indian Karyotype (WIK) was used as the mapping strain. N-ethyl-N-nitrosourea (ENU) mutagenesis was carried out as previously described.[16] The mutant *LDD731* (Tu background) allele was mapped by out-crossing heterozygous Tu fish into the polymorphic wild-type (WT) strain WIK. We scanned the genome for linked simple sequence-length polymorphism (SSLP) markers by bulk segregation analysis (BSA) using standard methods. The SSLP markers used for BSA were selected from the Massachusetts General Hospital Zebrafish Server website (MGH) (http://zebrafish.mgh.harvard.edu).[17, 18] Fine mapping using SSLP markers was carried out to narrow down the genetic interval. The cDNAs of the candidate genes in the interval were sequenced from the total cDNAs of pooled homozygous mutants; candidate mutation was confirmed by sequencing the genomic DNA of individual mutant. All primers used for the mapping are provided in supplemental data (Supplementary Table).

In this work, all animal experimental procedures were in compliance with local and international regulations. All protocols were approved by State Key Laboratory for Medical Genomics, Shanghai Institute of Hematology, RuiJin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Forward genetic screening

To obtain mutants that potentially affect the definitive HSPCs, we performed the ENU treatment and the forward genetic screening. The gene marker used was *c-myb*, of which the transcriptional expression is considered to be specific for the HSPCs. The gene expression was detected by using the technology of whole-mount *in situ* hybridization (WISH).

Single color WISH

Antisense RNA probes were transcribed using linearized constructs with T3 or T7 polymerase (Ambion) in the presence of digoxigenin (DIG, Roche)-labeled UTP using the DIG-RNA Labeling Kit (Roche). The DIG-labelled antisense probes used for the WISH analysis included *flk1*, *c-myb*, *runx1*, *scl*, *pu.1*, *l-plastin*, *mpo*, *gata1*, and *alpha-embryonic globin1* (*αe1*) genes. DIG-labelled antisense probes for these genes were synthesized with T3 or T7 polymerase (Ambion). WISH was performed as described previously, using 5-nitro-blue tetrazoliumchloride/ bromo-4-chloro-3'-indolyl phosphate p-toluidine salt (NBT/ BCIP, Vector Laboratories） as substrates.[19] A clutch of embryos (approximately 30-180 eggs/clutch) were mounted in 4% methylcellulose and captured under a Nikon SMZ1500 microscope equipped with a Nikon DXM1200F digital camera and ACT-1 software.

Survival curve

The Kaplan-Meier survival curve was generated using Graphpad Prism 5 software. The survivals of the embryos in three independent experiments were evaluated between mutants (n=57) and their siblings (n=156) . Given that all homozygous *LDD731* mutant embryos exhibited an increase in cell numbers at the CHT, which could be easily observed under a bright-light dissecting scope after 5 dpf, the genotypes and the phenotypes were tightly linked. The homozygous mutant embryos could be distinguished from the heterozygous mutant siblings and WT siblings at 5 dpf, according to the phenotypes described previously, and then raised separately under identical conditions. The survival of each group was monitored and recorded daily from 5 dpf to 33 dpf.

Plasmids construction and morpholinos microinjection

The zebrafish WT and mutant cDNA of the *c-cbl* gene were amplified from reverse transcription products and cloned into pCS2+ vector. Primer sequences are provided in the supplementary data (Supplemental Table). Zebrafish *c-cbl* probe was synthesized from the plasmid. Human *FLT3* cDNA was cloned into pFlag-CMV4. Human *c-CBL* cDNA was cloned into

pEGFP-C1 (pEGFP-wtCBL), and then the c-CBL-H398Y mutant (pEGFP-mutCBL) was engineered. The sequences of the primers used for cloning are provided in the Supplementary Table. Morpholino oligonucleotides (MOs) were designed by and purchased from Gene Tools. The MO sequences (the control mismatch MO, *c-cbl*-splicing MO and *flt3* MO) and the primer sequences for identifying alternative splicing products of *c-cbl* are also provided in the Supplementary Table.

Immunostaining of phosphorylated histone H3 protein (pH3) and fluorescent *in situ* hybridization analysis of zebrafish embryos

To detect both *c-myb* RNA and mitosis marker pH3 simultaneously, the embryos were hybridized in advance with the DIG-labeled antisense *c-myb* RNA probe, as described for single color WISH. After washing with MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20, pH7.5) and treatment with a block solution (in PBS, 0.3% Triton X-100, 1% DMSO, 10 mg/mL BSA, 10% normal goat serum), the embryos were incubated at 4 °C overnight with a peroxidase (POD)-conjugated anti-DIG antibody (1:500; 11207733910, Roche, SWISS) and stained with Alexa Fluorcy3-conjugated tyramide as substrate (NEL75200, PerkinElmer, USA) according to the instructions of the manufacturer. After an incubation with blocking solution for 1 hour at room temperature, the embryos were incubated with primary antibody anti-pH3 (ser10) (1:500; sc-8656-R, Santa Cruz Biotechnology, USA) at 4 °C overnight. Afterward, the primary antibody was removed and the embryos were washed with incubating buffer (in PBS, 0.3% Triton X-100, 1% DMSO, 10 mg/mL BSA, 2% normal goat serum) for four times and incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (1:500; A11034, Invitrogen, USA) for 4 hours. Images were taken using Olympus Fluoview1000 confocal microscope (Institute of Health Sciences, Shanghai Institutes for Biological Sciences and Graduate University) with a 40× objective.

Phosphorylated histone H3 protein (pH3) staining in

zebrafish embryos

The procedure was similar to the immunostaining of pH3 and fluorescent *in situ* hybridization, but without the *c-myb* single color WISH. The treated embryos (n=50) were incubated with rabbit anti-phosphohistone H3 antibody (1:500; sc-8656-R, Santa Cruz Biotechnology, USA) at 4 °C overnight, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:500; 31460, Invitrogen, USA) in the second day. The DAB chromogenic reagent kit was used for developing color. The embryos were mounted in 4% methylcellulose and images captured under a Nikon SMZ1500 microscope equipped with a Nikon DXM1200F digital camera and ACT-1 software.

Monitoring of blood flow

Homozygous *LDD731* mutants (n=6) and their siblings (n=6) were anesthetized with 0.03% tricaine (Sigma-Aldrich), mounted in 1% low-melt agarose in a 35-mm coverslip-bottomed dish at 5 dpf. Movies were captured by a Zeiss Axio Zoom V16 microscope equipped with a Zeiss AxioCam MRm digital camera with 80× magnification.

Vascular phenotype analysis

c-cbl-splicing MO was injected into the one-cell embryos of the transgenic zebrafish (*flk1*: mCherry),[20] whose vascular endothelial cells are specifically labelled with mCherry fluorescence. At 5 dpf, the embryos were anesthetized with 0.03% tricaine (Sigma-Aldrich), mounted in 1% low-melt agarose in a 35-mm coverslip-bottomed dish and captured by Olympus FV1000 scanning confocal microscope with a UPLSAPO 20× objective.

Sudan Black staining and *o*-dianisidine staining

Sudan Black staining was used to detect the granules of the granulocytes. After treatment with 4% formaldehyde, the embryos (n=55) were incubated in Sudan Black liquid for 20 min at 5 dpf. *o*-Dianisidine staining was used to check the hemoglobin; a brown color indicated the presence of

hemoglobin in zebrafish embryos. The embryos (n=92) were dechorionated at 36 hpf and stained for 15 min in the dark in an *o*-dianisidine dye solution at 5 dpf.

Analyzing cell composition in peripheral blood by flow cytometry and Giemsa staining

The blood cells collection procedure is the same as that in flow cytometry anslysis and Giemsa staining. The Zebrafish larvae were anesthetized at 5 dpf. One embryo was pipetted onto a glass slide at each time. The E3 containing 5% tricaine was replaced with 0.9× PBS containing heparin at 50 units/mL and 5% FBS; a couple of buffer exchanges were performed. The tail of the larval was cut by a small blade and the embryo was allowed to bleed. The blood cells of the homozygous *LDD731* mutants (n=20) and their siblings (n=50) were collected separately into two test tubes (compatible with flow cytometry machines) on ice. Flow cytometry analysis was then performed as previously described.[24] Briefly, the samples were treated with Hoechst 33342 for 10 min in dark in advance to label the DNA and exclude the impurity interference. Approximately 1×10^4 blood cells were sufficient according to the preliminary experiment. The machine model of flow cytometry was BD LSR Fortessa™ X-20. The cells of different lineages were recognized primarily according to the scatter parameters, because of the lack of the antibodies specific for hematopoietic lineages of zebrafish. Wright Giemsa staining was performed as following. Centrifugation at 500 rpm for 5 min was carried out to concentrate the samples, and the blood cells were then re-suspended to allow the blood to smear by cytofuge. Approximately 20 μL of Giemsa stain A was added to the blood smears for 1 min; subsequently, 50 μL of Giemsa stain B was added for 10 min. The blood smears were washed out, and the photos were taken under oil immersion lens using the ACT-1 software.

Real-time Quantitative PCR

Total RNAs were extracted from 20 zebrafish embryos using Trizol reagent (Invitrogen). The RNA was reverse transcribed

using random hexamers and SuperScript III Reverse Transcriptase (Invitrogen). 2× PCR Mix (TaKaRa, Premix Ex Taq™) containing SYBR Green I was used for the real-time quantitative PCR analysis with the Applied Biosystems 7900HT Fast Real-Time PCR System. The relative expression values were normalized against the internal control *gapdh*; qPCR primer sequences are listed in supplementary data (Supplementary Table).

Analysis of c-CBL ubiquitin ligase activity

The ubiquitin ligase activity of the mutant c-CBL protein, compared with the WT c-CBL, was determined by transiently transfecting 293T cells (purchased from ATCC) with 2 μg of pEGFP-wtCBL or of pEGFP-mutCBL, together with 8 μg of pFlag-FLT3. The cells were washed and collected 48 hours after transfection, and then lysed in ice-cold radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS). After centrifugation at 13,000g for 10 min at 4 °C, the supernatant of the lysates were incubated with a FLT3-specific polyclonal antibody (3462S, Cell Signaling Technology, USA) overnight at 4 °C. Approximately 20 μL of Protein A Sepharose beads (17528001, GE Healthcare Life Sciences, Sweden) were then added and incubated at 4 °C for 4 hours. Afterward, the beads were washed three times in ice-cold RIPA buffer, mixed with 80 μL sodium dodecyl sulfate (SDS) sample loading buffer, and denatured at 95 °C before immunoblot analysis with antibodies against ubiquitinylated proteins (clone FK2) (PW8810, Affiniti Research Products Ltd., UK), c-CBL (610442, BD Transduction Laboratories, USA) or FLT3.

Small molecular chemical compound treatment

FLT3 (fms-like tyrosine kinase 3) inhibitor PKC412[21] (sc200691) and Lestaurtinib[22] (sc 218657) with stock concentrations of 10 mM, were purchased from Santa Cruz Biotechnology (USA) and stored at −80 °C. We carried out the dosage determination by treating the zebrafish larvae (n=40) in E3 embryo water with PKC412 at concentrations of 50 nM, 100

nM, 300 nM, 500 nM, 1 μM, or with Lestaurtinib at concentrations of 100 nM, 300 nM, 500 nM, 1 μM, 10 μM, 20 μM and 50 μM. The working concentration of 100 nM was determined for PKC412, based on our own study of toxic/lethal effects of the drug and taking reference of a previous report[23]. To reduce the side effects, the use of the drug at early embryonic life (0-24 hpf) was avoided. With regard to Lestaurtinib, since there was no drug dosage information available for zebrafish, the working concentration of 500 nM was chosen in this study according to our own observation of the toxic/lethal effects versus therapeutic activities of the drug. The embryos were treated with the above mentioned dosages of the two agents from 2 dpf to 5 dpf, and were fixed for *c-myb* WISH staining at 5 dpf accompanied by morphologic observation under a bright-light dissecting scope.

Assay of inhibition of FLT3 phosphorylation in 293T cells

The 293T cells were transiently transfected with 8 μg of pFlag-FLT3 and 2 μg of pEGFP-wtCBL or pEGFP-mutCBL; PKC412 or Lestaurtinib was then added into the medium 24 hours after transfection. The cells were washed and collected 48 hours after transfection, and then lysed in ice-cold RIPA buffer. The supernatant was mixed with SDS sample loading buffer and denatured before the immunoblot analysis with antibodies against FLT3, c-CBL, and phosphorylated FLT3 (pFLT3) (pTyr591, 3466S, Cell Signaling Technology, USA).

Statistical Analysis

Data were analyzed with the Graphpad Prism 5 software using the two-tailed Student's *t*-test. The plot error values were calculated by standard error of the mean (SEM). Significance was defined as $p<0.05$. Data conforms to normal distribution. All data in this study were repeated for at least twice.

Results

A zebrafish mutant with abnormally increased definitive hematopoiesis in the CHT and kidney

Since impaired regulations of self-renewal and differentiation of HSPCs can result in hematological diseases including malignancies, and *c-myb*+ cells in the definitive hematopoietic tissues have been generally accepted as HSPCs, we performed a large-scale forward ENU-mutagenesis screening with WISH at 5 dpf. The first step in our screening strategy was to rule out individuals exhibiting apparently abnormal phenotypes of morphologic development, especially those with abnormal blood flow, heart and brain. The screening of aberrant hematopoietic phenotypes in our zebrafish model was then carried out using *c-myb* expression as a read-out by WISH. All the identified mutants should fall into three categories: increased, decreased or ectopic expression of *c-myb*. The *LDD731* line was thus selected and established from zebrafish with a phenotype of increased *c-myb* expression.[13, 24] In the *LDD731* mutants, *c-myb* expression exhibited an obviously increased level in the pronephros region and the CHT compared with siblings at 5 dpf (Figures 1a and 1b). We further analyzed other lineage specific hematopoietic markers to evaluate whether the more differentiated cells were affected. Both *gata1*, a marker of erythroid progenitors, and *alpha-embryonic-globin1 (αe1)*, a marker of mature erythrocytes, were significantly elevated in the CHT region of *LDD731* mutants, suggesting that erythropoiesis at 5 dpf was significantly increased (Figures 1c–1f). The expressions of myeloid cell marker *mpo* and the monocyte marker *l-plastin* were also aberrantly increased in pronephros as well as the CHT in *LDD731* mutants, compared with that of siblings at 5 dpf (Figures 1g–1j). Given that definitive hematopoiesis originates from AGM at about 28–60 hpf and that cells expressing *runx1* or *c-myb* in AGM at 36 hpf are considered HSPCs, we further examined whether the AGM HSPC population was increased by WISH analysis of *c-myb* gene expression. The results showed no differences in terms of the numbers of cells positive for *c-myb* between the mutants and their siblings, suggesting that the HSPCs at this stage were not

yet affected in the *LDD731* mutants (Supplementary Figures 1A and 1B). The expression of different lineage markers was tested at 3 dpf, and an increased level was presented in all markers (Supplementary Figures 1C–1H).

We also examined the primitive hematopoiesis at 22 hpf, which is a representative time point for the stage of primitive hematopoiesis. When WISH was performed using *scl* (for HSCs), *gata1* (for erythroid progenitors), *pu.1* (for myeloid progenitors) and *mpo* (for myeloid cells) probes at 22 hpf, the expression levels of these genes were all normal in *LDD731* mutants, indicating that the primitive hematopoiesis was not influenced by the mutation (Supplementary Figure 2).

Abnormal hematopoietic phenotype was attributed to intrinsic defects of hematopoietic system in *LDD731* mutants

In *LDD731* mutants, the *c-myb*+ cells in the kidney marrow (KM) (Supplementary Figures 3A and 3B) and CHT (Supplementary Figures 3G and 3H) were significantly increased, whereas the histological examination of pronephric ducts showed no obvious defect (Supplementary Figures 3C and 3E). This result suggested that the increased number of *c-myb*-expressing cells was not attributed to the abnormal structure/ function of pronephric ducts, but should be ascribed to the intrinsic abnormalities of the HSPCs of definitive hematopoiesis. In support to this result, WISH analysis of *scl* expression as the marker of HSPCs showed increased number of *scl*+ cells (Supplementary Figures 3I and 3J). We also verified the expression of *rag1*, a marker of the thymus-dependent lymphocytes, but did not find obvious abnormality of lymphoid development (Supplementary Figures 3D and 3F).

We next performed the live observation of the bright field of the CHT region at 5 dpf embryos. No obvious differences were found between the *LDD731* mutants and siblings at 5 dpf in terms of blood flow, indicating that the angiogenesis was normal (Supplementary Movies 1 and 2).

To rule out the involvement of other tissue stem/progenitor cell populations in the *LDD731* mutant line, we carried out WISH analysis of the neural and germ stem cells labeled by *nr4a2b*

and *vasa*, respectively. No obvious abnormalities were found between these two cell populations (Supplementary Figures 3K–3N).

LDD731 **mutants harbored homozygous point mutation in the RING Finger domain of *c-cbl* gene**

We explored the molecular basis of the MPN-like phenotype in *LDD731* mutants by using a positional cloning approach. Bulk segregation analysis mapped the *LDD731* allele to chromosome (Chr.) 15. Fine mapping using SSLPs narrowed the mutation to the critical region between the 197-08 and z23655 markers. We sequenced the cDNA of the candidate genes (*ccdc153, c-cbl, mcamb, rnf26, c1qtnf5*) in this region (Figure 2a). A point mutation (C, 1144 C>T, in exon8) was detected in *c-cbl* (Figures 2c-2e), which led to the critical 382nd amino acid (aa) transition from histidine to tyrosine (H382Y) in the only RING finger domain that is evolutionarily conserved (Figure 2b). We examined the sequences at this position in the genomes of the grandfather, grandmother, father, mother and the offsprings of *LDD731*. A pattern accorded with the Mendel's law of autosomal recessive inheritance was noted (Figures 2l-n, Supplementary Figures 4A-4D), with the MPN-like phenotype observed only in homozygotes (9 out of 36 individuals tested) for *c-cbl* mutation, while siblings with heterozygous mutation (19 out of 36 individuals) and WT *c-cbl* gene (8 out of 36 individuals) all bore normal phenotype, as judged by the amount of HSPCs with the expression of the *c-myb* marker. To exclude the possibility of the detected sequence change as a genetic polymorphism, we also verified the sequences at the same positions from five WT zebrafish strains and found that the nucleotide transition change was unique to *LDD731* mutant (Supplementary Figures 4E–4I). The 382nd aa of zebrafish c-Cbl protein corresponds to the 398th aa of c-CBL protein in humans. Synteny analysis shows that the genomic region harboring the *c-cbl* gene is well conserved between zebrafish and humans (Figure 2f). According to the WISH results, the *c-cbl* gene was maternally expressed and its expression in early development was ubiquitous. Noticeably, the gene showed a higher

expression in the lateral mesoderm at 12 hpf and AGM at 36 hpf (Supplementary Figure 4J).

To clarify whether or not *c-cbl* mutation could affect survival, we performed the Kaplan–Meier survival analysis between the homozygous mutants (n=57) and their siblings (n=156). The fish harboring *c-cbl* homozygous mutation showed a significantly low survival rate, with a median survival time of approximately 14–15 dpf on the basis of statistical analysis ($P<0.0001$, Figure 2g). These results suggested that c-Cbl-H382Y mutation induced the phenotype of homozygous *LDD731* mutants and affected the survival of the zebrafish.

The human *c-CBL* reportedly functions as a tumor suppressor gene, and its mutations in hematopoietic malignancies cause a loss of function of the protein. We performed a MO phenocopy experiment to further address whether the phenotype in homozygous *LDD731* mutants was caused by the mutation and resultant loss of function of the *c-cbl* gene. A *c-cbl*-splicing MO against the site at the junction of *c-cbl* exon2 and intron2 was designed to induce an abnormal mRNA splicing pattern with the deletion of *c-cbl* exon2. We checked the MO efficiency in embryos injected with *c-cbl* or control (mismatched) morpholino by using RT-PCR with primers that corresponded to the sequences in exon1 and exon3. The electrophoretogram clearly demonstrated an expected pattern in band sizes (Figures 2h and 2i) in the embryos injected with *c-cbl* or control morpholino. Sequencing results confirmed the loss of exon2 sequence in the *c-cbl* transcripts only in morphants treated with *c-cbl* MO (Supplementary Figures 4K and 4L).

We then microinjected the *c-cbl*-splicing MO into the embryos with WT genotype and found that it could induce the *c-myb* overexpression phenotype similar to that in the homozygous *LDD731* mutants at 5 dpf (Figures 2j and 2k). We then tried to inject this MO into the *LDD731* embryos, and the results demonstrated that in fish heterozygous for *c-cbl* mutation, which could retain a normal hematopoiesis most likely due to the residual activity of c-Cbl produced by the WT allele (Figures 2l and 2m), an increased hematopoiesis was observed in response to the *c-cbl*-splicing MO (Figure 2p). In WT individuals, slightly

increased HSPCs with *c-myb* expression was also observed (Figure 2o). By contrast, in those homozygous mutants for c-Cbl-H382Y, the degree of MPN-like phenotype was not affected by the knockdown of *c-cbl* (Figures 2n and 2q), suggesting the mutation was sufficient to abrogate the activity of c-Cbl.
In addition, we injected *c-cbl*-splicing MO into transgenic zebrafish with *flk1* labeled by mCherry (*flk1*: mCherry) and examined the vascular system at 5 dpf (Supplementary Figure 5). The results confirmed that the angiogenesis at both the dorsal body and the CHT was normal, providing further evidence to suggest that the MPN-like phenotype in *LDD731* line should be ascribed to the genetically determined abnormalities of the HSPCs.

HSPCs in homozygous *LDD731* mutants displayed aberrantly increased proliferation ability without major blockage of differentiation

We investigated the mechanism by which *c-cbl* mutation affected hematopoiesis in the CHT and KM. The proliferation of HSPCs in *LDD731* was examined using anti-pH3 staining, a classical method used to evaluate cellular proliferation. The results of anti-pH3 staining showed that cell proliferation at the KM and CHT stages significantly increased in homozygous *LDD731* mutants (Figures 3a–3c). Considering that the pattern was similar to that of *c-myb* expression, we performed double staining of both *c-myb* and anti-pH3. The proliferation of HSPCs (Figures 3d–3k) and pH3/*c-myb* double-positive cells (Figures 3l and 3m) significantly increased at 5 dpf. The increase in *c-myb*-positive cells started from 3 dpf (Supplementary Figures 1A-1D). Hence, we also performed *c-myb*/anti-pH3 double staining at 3 dpf. Indeed, the proliferation of HSPCs significantly enhanced at this early developmental stage (Supplementary Figure 6).
We also determined whether or not the increased proliferation of *c-myb*+ cells results from a differentiation block of the HSPCs in homozygous *LDD731* mutants. We employed *o*-dianisidine and Sudan Black staining to evaluate the maturation statuses of erythrocytes and granulocytes, respectively. In the homozygous

LDD731 mutants, Sudan Black staining at 5 dpf demonstrated that the granulocyte differentiation was not blocked and the cell population significantly increased, especially at the KM stage (Figures 4a and 4b). Similarly, *o*-dianisidine staining showed that homozygous *LDD731* mutants had greater cell population than their siblings (Figures 4c and 4d). Wright–Giemsa staining of peripheral blood of *LDD731* was performed, and the elements of distinct lineages were analyzed. We calculated the ratio of different lineages on the blood smears and the results showed that the percentage of immature red cells was 35.5% in the homozygous *LDD731* mutants as compared to 6% in the WT or heterozygous siblings and the percentage of mature erythrocytes was lower in the homozygous mutants than in the siblings (Figure 4h). In flow cytometry analysis of peripheral blood, the percentage of immature cells also increased, consistent with that in Wright Giemsa staining (Figures 4i–4k). These morphological, histochemical staining and flow cytometry data were also supported by real-time PCR analysis of HSPC markers (*c-myb*, *runx1*, *scl*), myeloid cell markers (*mpo*) and erythroid cell markers (*gata1*, *αe1*) (Figure 4e). The above analyses indicated that the increased percentage of multi-lineage HSPCs in homozygous *LDD731* mutants was likely caused by the increase in HSPC proliferation ability, and their differentiation was not obviously affected.

Increased HSPC proliferation in *LDD731* mutant was dependent on the Flt3 pathway

We then addressed the possible molecules downstream of c-Cbl-H382Y by focusing on c-Cbl substrates in the hematopoietic system. The *flt3* gene is interesting because it encodes the Flt3 receptor tyrosine kinase for the protein Flt3 ligand (FL). Two forms of human FLT3 have been described: a 160 kD membrane-bound protein that is glycosylated at N-linked glycosylation sites in the extracellular domain (mature form) and an unglycosylated 130 kD protein (immature form) that is not membrane-bound. Only the mature form of FLT3 can be stimulated by its ligand, which leads to FLT3 phosphorylation. It is well known that c-CBL binds to the pTyr site in FLT3 via its

tyrosine-kinase-binding (TKB) domain and ubiquitinates FLT3, resulting in a degradation of FLT3 in proteasomes. As a molecule controlling important cellular processes such as the growth, proliferation and survival of HSPCs, FLT3 is involved in the pathogenesis of AML.[25-28] We therefore investigated the human CBL-FLT3 pathway in the 293T cell line since no appropriate agents were available for similar studies in zebrafish. 293T cells were transiently transfected to express FLT3 with WT c-CBL or c-CBL-H398Y. The results showed that the protein level of FLT3 was lower in the WT c-CBL +FLT3 group than in the c-CBL-H398Y+FLT3 group or FLT3 alone group (Figure 5i), suggesting that WT c-CBL could induce FLT3 down-regulation. The higher immunoprecipitated FLT3 protein level in the c-CBL-H398Y+FLT3 group corresponded to a lower ubiquitination level (Figure 5i), indicating that the c-CBL-H398Y lost its E3 ubiquitin ligase activity. All these results suggested that the c-CBL-H398Y homozygous mutant phenotype correlated with the regulation of FLT3.

We subsequently investigated whether or not the pathway was also conserved in zebrafish *in vivo* by using MO injection to down-regulate the fish Flt3 protein in homozygous mutant embryos. We then examined the expression level of *c-myb* in *flt3* morphants in *LDD731*. The results showed that *flt3* MO could partially attenuate the proliferation of HSPC phenotype (Figures 5a–5d) in embryos bearing the homozygous c-Cbl-H382Y mutation. We further explored the effect of the FLT3 pathway inhibitor PKC412 and the FLT3/JAK2 inhibitor Lestaurtinib on the homozygous mutant phenotype. The doses of drugs used were experimentally determined by achieving the lowest lethality and the least effects on normal development as determined by morphological abnormalities. After treatment with PKC412 or Lestaurtinib, the embryos were fixed and stained using the *c-myb* probe. These two drugs seemed to subtly reduce *c-myb* expression in WT (Supplementary Figures 7A-7D) fish while both agents caused a drastic reduction of *c-myb* expression in homozygous *LDD731* mutants (Figures 5e-5h). This finding indicated that PKC412 and Lestaurtinib could partly decrease the proliferation of HSPCs. To further

look at the effects of the two drugs on FLT3 expression, we treated FLT3+CBL-transfected 293T cells with Lestaurtinib and PKC412 at the same concentrations as those used in the zebrafish embryonic experiments. Both drugs efficiently down-regulated pFLT3 levels (Figure 5j) without altering the mRNA level of *FLT3*[29]. Overall, these data suggested that the proliferation of HSPCs partially depended on the Flt3 pathway.

Discussion

In this study, we isolated the zebrafish *LDD731* line through genetic screening to identify novel factors that regulate definitive hematopoiesis in HSPC development. The definitive hematopoiesis was specifically affected but the primitive hematopoiesis was normal in the homozygous mutants (Figure 1 and Supplementary Figure 2). Further analysis revealed that the HSPC origin was normal in the AGM (Supplementary Figures 1A and 3B), but the number of HSPCs dramatically increased in the CHT and KM because of the hyperproliferation of these cells. The number of both erythroid and myeloid cells also increased. This result implied that the differentiation of HSPCs was apparently not arrested (Figures 3 and 4, Supplementary Figure 6). We injected *c-cbl*-splicing morpholino into *flk1* transgenic lines labeled with mCherry to explore the effects of H382Y mutation on angiogenesis. The vessels were normal even at 5 dpf (Supplementary Movies 1 and 2, Supplementary Figures 5A–5F). Most of the homozygous mutant zebrafish larvae died before 1 month post-fertilization, and the median survival time was approximately 14 and 15 dpf (Figure 2g). The proportion of different lineages in peripheral blood was checked through blood smears and flow cytometry. Our results showed that more immature cells were present in the homozygous mutants than in their siblings. This phenotype mimicked that of human MPN, indicating that *LDD731* could be used as a model for MPN research.

Using positional cloning, we characterized the gene mutation at the RING finger domain of the *c-cbl* gene, which caused H382Y transition (Figure 2). c-Cbl[30] is a member of the Cbl family and

functions as an E3 ubiquitin ligase that participates in the development and functions of HSCs.[31] All Cbl proteins have a highly conserved N-terminal region that contains two domains critical for protein function: the TKB domain that recognizes and binds to phosphorylated tyrosine residues, and the RING finger domain that is important for c-Cbl protein to bind ubiquitin-conjugating enzymes (E2 enzymes) and for the transfer of ubiquitin moieties from E2 enzymes to the target molecules.[32, 33] The ubiquitination of the substrate is followed by the proteasome-dependent proteolysis of substrate proteins. c-Cbl thus functions as negative regulator of receptor and non-receptor tyrosine kinases, which may be required to restrict myeloid proliferation.

Recent studies have shown homozygous mutations of *c-CBL* in human hematopoietic malignancies.[34-39] Sargin et al.[27] reported the first *c-CBL* mutation (R420Q, in exon9) in a patient with AML, whereas Caligiuri et al.[40] identified *c-CBL* mutations in 4 of 12 AML cases and demonstrated a *c-CBL* splice site mutation with an 18 base pair deletion in the AML cell line MOLM-13. In addition, *c-CBL* exon 8/9 deletion mutants were found in 1.1% of 279 patients with AML/MDS.[41, 42] In MPNs, the JAK2-V617F mutation[43] alone cannot explain the phenotypic heterogeneity, whereas a 2%–8% frequency of *c-CBL* mutations has been reported.[35, 37, 39, 44] Grand et al.[45] identified the mutation of c-CBL-H398Y in MPN patients, which corresponds well to the c-Cbl-H382Y mutation in our zebrafish system. Notably, c-CBL-H398Y displayed a loss of ubiquitin ligase activity. The incidence of *c-CBL* mutations in MPNs ranges between 5% and 15%,[42, 46] with the highest rates occurring in chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia.[47-51] *c-CBL* mutations are related to poor-prognosis MPNs.[35]

Advances have been made over the past years to understand the mechanism by which *c-CBL* mutations drive the pathogenesis of MPN.[52] In mice with a mutation in the RF domain of c-Cbl, a MPN was developed, which progressed to leukemia.[53] Importantly, the Flt3 signaling pathway is augmented in hematopoietic progenitors in these animals. FLT3 is a substrate

of c-CBL,[54] and its mutations occur in one-third of AML patients with poor outcome.[55]

In this work we determined whether or not human c-CBL mutation can lead to an attenuation of FLT3 ubiquitination levels (Figure 5i). Our results showed that total FLT3 protein levels increased while the ubiquitination levels decreased in the 293T cells co-transfected with FLT3 and the c-CBL mutant compared with that of WT c-CBL, indicating that the mutated c-CBL lost its function and thus could not ubiquitinate FLT3. Through *flt3* MO injection or treatment with FLT3 inhibitors, we found that the MPN phenotype could be partially reduced in the *LDD731* line (Figures 5a–5h). This result provides new evidence that c-Cbl-H382Y can up-regulate the Flt3 pathway and lead to the hyperproliferation of HSPCs. To date, several FLT3 kinase inhibitors have been investigated in preclinical and clinical studies. In *c-cbl* mutation mice, suppressing Flt3 signaling through mating with Flt3 ligand (FL) knockout mice prevents leukemia development.[53] Another important yet still unanswered issue is the mechanism underlying the progression of MPN to leukemia. Other molecular and/or cytogenetic defects that trigger abnormal proliferation and block the differentiation of HSPCs must be identified. In our future work, we will try to introduce other mutations such as activated JAK2a into *LDD731* to address possible cooperative effects of these mutations in leukemogenesis.[56, 57] Finally, *LDD731* zebrafish line may provide an ideal animal model to screen for therapeutic agents against MPN and even leukemia.[58-60]

Acknowledgement
We thank Profs. Zhu Chen for carefully revising the paper and guiding the work and Qiang Li critical reading of the manuscript, all members of the Laboratory of Development and Disease (LDD) for their technical assistance and helpful discussions, and for Drs. Anskar Leung and Bai-Liang He for sharing the *flt3* morpholino. This work was supported in part by Chinese National Key Basic Research Project (2013CB966800), Special Grant of Ministry of Health (201202003), Mega-projects of Science Research for the 12th Five-Year Plan (2013ZX09303302), and the National Natural Science Foundation of China (81123005)

Conflict of interest
The authors declare no competing financial interests.

Supplementary information is available at (*leukemi*a)'s website.

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Figure legends:

Figure 1. Homozygous *LDD731* mutants displayed a dramatic increase in definitive hematopoiesis. (a and b) WISH analysis of *c-myb* expression at 5 dpf. *c-myb*+ cells represent the definitive HSPCs. (c–f) WISH analysis of the expression of *gata1* (marker of erythroid progenitors) and *αe1* (marker of mature erythrocytes) at 5 dpf. (g–h) WISH analysis of *mpo* (marker of myeloid cells) expression. (i and j) In *LDD731* mutants, *l-plastin* expression significantly increased in pronephros compared with their siblings at 5 dpf. Abbreviation: T (thymus), KM (kidney marrow), CHT (caudal hematopoietic tissue). The site marked with asterisks in 1b was amplified and showed in Supplementary Figure 3H, with 3G as a control.

Figure 2. Positional cloning of mutated gene in *LDD731* line. (a) Genetic map of the *LDD731* region on Chr.15. Bulk segregation analysis positioned the *LDD731* allele to Chr.15. Fine mapping using SSLPs narrowed down the region between markers of 197-08 and z23655. The *c-cbl* gene is located in this region. (b) Structure of WT zebrafish c-Cbl protein and location of the mutation within the RING domain. The zebrafish c-Cbl 382nd aa corresponds to the human c-CBL 398th aa. (c–e) Sequencing results of *c-cbl* cDNA from homozygous mutants, heterozygote mutants, and WT siblings. (f) Synteny analysis of the *c-cbl* gene. (g) Kaplan–Meier survival analysis between the homozygous mutants and their siblings. (h) Primer design for *c-cbl*-splicing MO. (i) Detection of splicing MO efficiency. (j–k) *c-cbl*-splicing MO injection into WT embryo could mimic the phenotype of increased *c-myb* expression in *LDD731* as revealed by WISH. (l–n) *c-myb* expression in uninjected *LDD731* embryos. Note that the over-expression of *c-myb* was observed only in homozygotes for *c-cbl* mutation (9 out of 36 individuals tested, l), while siblings with heterozygous mutation (19 out of 36 individuals, m) and WT *c-cbl* gene (8 out of 36 individuals, n) bore normal phenotype. (o–q) *c-myb* expression in *c-cbl*-splicing MO-injected *LDD731* embryos

Figure 3. Proliferation of HSPCs increased at 5 dpf. (a and b) Proliferation of *LDD731* homozygous mutants increased as shown in the pH3 staining. (c) Statistical analysis of pH3+ cells in regions a, b, and c between *LDD731* homozygous mutants and their siblings. (d–k) Confocal imaging of *c-myb*+ and pH3+ cells in CHT between *LDD731* mutants and their siblings. pH3+ cells were labeled with GFP, and *c-myb*+ cells were labeled with RFP. (l) Statistical analysis of pH3+ cells in CHT between *LDD731* homozygous mutants and their siblings. (m) Statistical analysis of pH3+ cells and *c-myb*+ cells in CHT between *LDD731* homozygous mutants (n=6) and their siblings (n=6) (*t*-test, $p < 0.05$).

Figure 4. Proliferation of the definitive HSPCs was not accompanied by obvious differentiation arrest. (a–b) Sudan Black staining of the homozygous *LDD731* mutants at 5 dpf demonstrated that myeloid differentiation was not blocked and that the proliferation of myeloid cells was significantly increased. The black arrows point to the expression sites. (c–d) *o*-Dianisidine staining was performed to detect the hemoglobin expression of mature erythroid cells. The black arrows point to the expression sites. (e) Real-time PCR of HSPC markers (*c-myb*, *runx1*, *scl*), myeloid cell marker (*mpo*) and erythroid cell markers (*gata1*, *αe1*). (f and g) Wright staining of peripheral blood of embryos from homozygous *LDD731* mutants and siblings. Red arrows mark the immature erythrocytes with a high ratio of nucleus to cytoplasm. Blue arrows mark mature erythrocytes. (h) Proportion of different lineages according to blood smears. (i–k) Flow cytometry analysis of homozygous *LDD731* mutants and siblings.

Figure 5. Increase in HSPCs was dependent on the Flt3 pathway. (a–d) *flt3* MO injection attenuated the phenotype of *LDD731*. (e and f) *c-myb* staining of zebrafish embryos after treatment with 500 nM Lestaurtinib. (g and h) *c-myb* staining of embryos after treatment with 100 nM PKC412. (i) CBL mutation decreased FLT3 ubiquitination levels and upregulated mature FLT3. 293T cells were transfected with FLT3 and/or c-

CBL constructs as indicated. After lysis, FLT3 was immunoprecipitated, blotted, and probed with anti-FLT3 and anti-FK2. (j) Inhibitors were used to treat 293T cells. The cells transfected with FLT3 and c-CBL were treated with or without PKC412 or Lestaurtinib for 24 h. After lysis, protein samples were blotted and probed with FLT3, c-CBL and pFLT3 antibodies.