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Accessibility
Mutation of kri1l causes definitive hematopoiesis failure via PERK-dependent excessive autophagy induction

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Dysregulation of ribosome biogenesis causes human diseases, such as Diamond-Blackfan anemia, del (5q-) syndrome and bone marrow failure. However, the mechanisms of blood disorders in these diseases remain elusive. Through genetic mapping, molecular cloning and mechanism characterization of the zebrafish mutant cas002, we reveal a novel connection between ribosomal dysfunction and excessive autophagy in the regulation of hematopoietic stem/progenitor cells (HSPCs). cas002 carries a recessive lethal mutation in kri1l gene that encodes an essential component of rRNA small subunit processome. We show that Kri1l is required for normal ribosome biogenesis, expansion of definitive HSPCs and subsequent lineage differentiation. Through live imaging and biochemical studies, we find that loss of Kri1l causes the accumulation of misfolded proteins and excessive PERK activation-dependent autophagy in HSPCs. Blocking autophagy but not inhibiting apoptosis by Bcl2 overexpression can fully rescue hematopoietic defects, but not the lethality of kri1l cas002 embryos. Treatment with autophagy inhibitors (3-MA and Baf A1) or PERK inhibitor (GSK2656157), or knockdown of beclin1 or perk can markedly restore HSPC proliferation and definitive hematopoietic cell differentiation. These results may provide leads for effective therapeutics that benefit patients with anemia or bone marrow failure caused by ribosome disorders.

Keywords: Hematopoietic stem cells; zebrafish; kri1l; ribosome biogenesis; autophagy; PERK; misfolded/unfolded protein

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Introduction

Vertebrate hematopoiesis is an evolutionarily conserved and highly regulated process involving the production of differentiated blood cell lineages from hematopoietic stem cells (HSCs) [1-3]. The zebrafish (Danio rerio) is a powerful genetic and developmental model to study the development of vertebrate circulatory system,
especially hematopoiesis [4, 5]. Definitive hematopoiesis in zebrafish is highly conserved with mammals [6]. It occurs at the ventral wall of dorsal aorta in a structure called aorta-gonad-mesonephros (AGM) around 28 hour post-fertilization (hpf) [7]. By 2 days post-fertilization (dpf), the AGM-derived HSCs migrate to caudal hematopoietic tissue (CHT), an embryonic structure analogous to the mammalian fetal liver [8-10], for rapid pool expansion and lineage differentiation.

Normal ribosome biogenesis is critical for cell survival, proliferation and function. In eukaryotic cells the small subunit (SSU) processome, a ribonucleoprotein complex, plays essential roles in 18S rRNA maturation, small ribosomal subunit assembly and subsequent ribosome biogenesis [11]. The SSU processome is composed of pre-rRNA, small nucleolar RNAs and more than 70 different associated proteins. Dysfunction of ribosome biogenesis is associated with human diseases, including Diamond–Blackfan anemia (DBA), 5q minus (del (5q−)) syndrome, dyskeratosis congenita and Bowen-Conradi syndrome [12-14]. In addition to carrying genetic mutations affecting ribosome biogenesis, these human diseases share same clinical features, and are thus termed as “ribosomopathies” [13].

Previous works have shown that upregulation of p53 by ribosome dysfunction contributes to bone marrow failure syndromes [15] that have been genetically mapped to loss-of-function mutations in rps14 [16], rps19 [17, 18], rpl11 [19] and rps29 [15]. The apoptotic phenotype of hematopoietic cells in these diseases can be partially restored by p53 downregulation [16, 17]. However, some studies have reported that p53 pathway is not involved in some ribosome biogenesis disorders [20-22]. Furthermore, not all bone marrow samples from patients with del (5q−) syndrome or DBA show p53 activation, suggesting that other mechanisms may contribute to ribosomopathies [23]. For example, loss of pwhp2h causes gut degeneration and hyperactivated autophagy in a p53- and mTOR-independent manner [21]. Knockdown of Rpl22 in zebrafish embryos blocks T-lineage progenitor development, while knockdown of the Rpl22 paralog Rpl22l impairs the emergence of HSC in AGM by abrogating Smad1 expression and Runx1 induction [24].

Autophagy and apoptosis are two major stress-response pathways. Dysregulation of autophagy has been linked to many human diseases such as neurodegeneration [25, 26], autoimmunity and cancer [27, 28]. Multiple upstream signaling mechanisms, including mTOR pathway, unfolded protein response (UPR), ER stress and nutrition stress regulate autophagy, with Beclin1-VPS34 complex playing an important role in autophagy initiation [29, 30]. Autophagy is a critical mechanism that protects HSCs from stress damages [31]. In mice, a conditional deletion of atg7 in HSCs renders the loss of HSC’s self-renewal property and severe myeloproliferation due to a failure of HSPCs to respond normally to stress from reactive oxygen species (ROS) [32]. Appropriate autophagy level is also important for lymphocyte survival [33, 34] and erythroid cell maturation [35-37]. Patients with certain ribosomopathies have elevated levels of autophagy in peripheral blood cells resulted from S6K-induced inhibition on insulin pathway activation [38]. However, the potential cures for these disorders have not been found.

In the present study, we report that kri1l gene is essential for definitive hematopoiesis. Loss of Kri1l, a critical component of SSU complex, causes ribosomal biogenesis defects, accumulation of misfolded proteins and activation of PERK-eif2a signaling. These deficiencies subsequently hyperactivate autophagy and ultimately lead to the inhibition of HSPC proliferation. Treatment with autophagy or PERK inhibitors, or knockdown of beclin1 or perk by morpholino (MO), can successfully rescue HSPC proliferation and lineage differentiation in kri1l mutant.

Results

cas002 mutant displays a hematopoietic failure phenotype

In a large-scale ENU mutagenesis screen for definitive hematopoietic mutations, we obtained cas002, a novel mutant with severe hematopoietic defects and recessive lethality. cas002 embryos are morphologically indistinguishable from wild-type siblings before 3 dpf, with normal blood flow and heart beats (Figure 1A-1B). However, whole-mount in situ hybridization (WISH) of cmyb reveals a markedly reduced HSPC population in caudal hematopoietic tissue (CHT) of mutant embryos at 3 dpf (Figure 1C-1D), and in CHT, thymus and kidney at 5 dpf (Figure 1E-1F). cas002 mutant embryos eventually die at 6-10 dpf with abnormal head shape, cardiac edema and smaller eyes.

To examine hematopoiesis phenotype in detail, we performed WISH analysis of different cell lineage markers: gata1 (erythrocyte progenitors), ael-globin (embryonic erythrocytes), l-plastin (pan-myeloid cells), lyz and mpo (neutrophils). The expression of these markers is the same as wild-type siblings at 3 dpf (Supplementary information, Figure S1), but becomes significantly reduced in cas002 mutant embryos at 5 dpf (Supplementary information, Figure S2A-S2J). The expression of rag1 (T lymphocyte), is substantially reduced at 4 dpf in cas002 (Supplementary information, Figure S2K-S2L),
Figure 1 Hematopoietic defects and positional cloning of cas002 mutant. (A-B) Light microscope images of zebrafish wild-type (WT) and cas002 embryos at 3 dpf. (C-F) WISH analysis of cmyb expression in WT and cas002 embryos at indicated development stages. Black arrows indicate thymus, kidney marrow and CHT. (C'-D') enlarged CHT regions in C and D. (G) Genetic mapping of the cas002 region on chromosome 3. Bulk segregation analysis locates cas002 mutation to Chr. 3. Fine mapping using SSLPs narrows down to a region between markers 219-BX-5 and 220-CU-6, containing kri1l and four other genes as indicated. (H) The sequencing results of kri1l cDNA from mutant embryos show a 38 bp deletion (MU) compared with kri1l cDNA from WT embryos. (I, J) The sequencing result of kri1l genomic DNA shows a T-G transversion at the kri1l exon 1-intron 1 consensus splicing donor site (I), which causes a frame shift (H) and a premature stop codon leading to the production of a truncated Kri1l protein (J). (K) Synteny between zebrafish kri1l I and human KRI1 loci. (Left) Six genes, including KRI1, are located within a genomic region on human chromosome 19. (Right) Six zebrafish homologs are listed according to their map positions on chromosome 3 (Ensembl website). CHT, caudal hematopoietic tissue; KM, kidney marrow; Mb, mega base; T, thymus; het, heterozygote.
indicating that lymphoid development is also impaired. In contrast, primitive hematopoiesis and vascular morphogenesis are intact in cas002 mutant embryos (Supplementary information, Figure S3). To identify the onset of hematopoiesis failure in cas002 mutant embryos, we traced expression of cmyb and runx1, another HSC marker, at earlier developmental time points (Supplementary information, Figure S4). Both cmyb and runx1 are expressed normally in the AGM from 36 hpf to 40 hpf (Supplementary information, Figures S4A-S4D and S4C’-S4D’) and at 36 hpf (Supplementary information, Figure S5A-S5B), respectively, suggesting a normal hematopoiesis in the AGM at these stages. A marginal decrease of cmyb expression is detectable in the CHT region at 40 hpf (Supplementary information, Figure S4C’’-S4D’’). By 4 dpf, the expression of cmyb is almost undetectable in the CHT, kidney and thymus of cas002 mutant embryos (Supplementary information, Figure S4K-S4L). Another HSPC marker scl is also markedly reduced from 3 dpf (Supplementary information, Figure S5C-S5H). In addition, cas002 mutant embryos have significantly fewer EGFP+ cells (marked by eGFP under cmyb promoter) than the wild type (Supplementary information, Figure S6A-S6G). Taken together, these results suggest that definitive HSPCcs in the CHT are severely disrupted in cas002 mutant embryos.

cas002 mutant carries a defective kri1l gene

To understand the mechanism of hematopoietic failure in cas002 mutant, we carried out positional cloning [39]. The mutation was first mapped on chromosome 3 by bulk segregation analysis (BSA). Simple sequence length polymorphism (SSLP)-based fine mapping established that the mutation lies within a 300 kb region between two markers: 219-BX-5 and 220-CU-6 (Figure 1G). We sequenced all five candidate genes in this region, and found a 38 bp deletion in kri1l cDNA in cas002 mutant (Figure 1H). Genomic DNA sequencing of kri1l gene revealed that a consensus splicing donor site at the boundary between exon1 and intron1 is disrupted by a T-to-G transversion in cas002 mutant (Figure 1I). This mutation yields an abnormally spliced transcript with a frame shift and a premature stop codon in exon2. The altered transcript is predicted to encode a highly truncated Kri1l peptide 32 amino acid in length (Figure 1J). This point mutation is not found among five commonly used laboratory zebrafish strains (Tu, AB, WIK, Longfin and Shanghai; Supplementary information, Figure S6H), excluding the possibility of single-nucleotide polymorphism [40]. The zebrafish kri1l gene is 62% identical with the human KRI1. The kri1l locus on zebrafish chromosome 3 is syntenic to a region of human chromosome 19 that contains the KRI1 gene, based on the conserved locations of neighboring orthologous gene pairs (SIPR5, SMARCA4 and LDLR; Figure 1K). These findings suggest that zebrafish kri1l gene is an ortholog of human KRI1.

Mutated kri1l causes cas002 phenotypes

To confirm that the mutation in kri1l gene is responsible for cas002 phenotypes, we microinjected kri1l ATG morpholino (MO) and splicing MO to suppress translation and maturation of kri1l mRNA, respectively, into wild-type zebrafish embryos. A construct containing a 60-bp fragment including kri1l ATG MO-binding site fused to the N-terminus of EGFP (Supplementary information, Figure S7E) was co-injected with kri1l ATG MO; the expression of EGFP was successfully blocked (Supplementary information, Figure S7A-S7D). The kri1l splicing MO caused splicing defect in the endogenous kri1l transcripts (Supplementary information, Figure S7F). cmyb expression and EGFP+ cell numbers in both morphants in Tg(cmyb:egfp) background were significantly reduced, indicating that kri1l knockdown reproduces hematopoietic defects found in cas002 mutant (Figure 2A-2C and Supplementary information, Figure S7G-S7H).

To provide further evidence that kri1l is defective in cas002 mutant, we performed a rescue experiment by microinjection of synthetic wild-type kri1l mRNA into cas002 mutant embryos. As monitored by WISH analysis of cmyb expression, we found that the definitive hematopoiesis was fully rescued by wild-type Kri1l overexpression (Figure 2D-2F). In summary, results from positional cloning, MO phenocopy and mRNA rescue experiments strongly suggest that the T-to-G mutation in zebrafish kri1l gene is responsible for the defective hematopoiesis in cas002 mutant embryos. We thus rename the mutant as kri1l<sup>ca002</sup>.

To understand the role of kri1l in embryogenesis, especially in definitive hematopoiesis, we examined the temporal and spatial expression of kri1l by WISH analysis. The kri1l transcript is expressed as maternal mRNA. During early development, kri1l is expressed ubiquitously throughout the embryo and enriched in somites at 18 hpf and eyes at 22 hpf (Supplementary information, Figure S8).

In yeast, KRI1 is a component of the SSU complex, and plays an essential role in 40S ribosome subunit formation and ribosomal polysome assembly. Mechanistically, loss of KRI1 results in instability of 18S rRNA precursor and dramatic reduction of mature 18S rRNA [41]. We asked whether kri1l<sup>ca002</sup> mutant embryos have similar defects in ribosome biogenesis. E-bioanalyser analysis of total RNA revealed a dramatic reduction in
Figure 2 Mutation in kri1 gene is responsible for cas002 phenotypes. (A-C) Morpholino knockdown of kri1 phenocopies cas002 mutant. kri1 ATG MO and splicing MO were injected into wild-type embryos at one-cell stage. At 3 dpf, the injected embryos were fixed and analyzed for cmyb expression by WISH. The percentage of morphants with the reduced cmyb expression phenotype is listed at the bottom of B and C. (D-F) Transient expression of wild-type kri1 mRNA rescues cas002 mutant. cas002 mutant embryos at one-cell stage were injected with synthesized wild type kri1 mRNA. The injected embryos were fixed for analysis of cmyb gene expression using WISH at 4 dpf. After WISH and photographing, all embryos were genotyped by sequencing of genomic DNA; the percentage of the rescue was then evaluated. The percentage of fully rescued mutant embryos is about 54% (50/92), while the rest are partially rescued. (A'-F') Details of cmyb expression in CHT regions in A-F. (G-H) E-Bioanalyser analysis of total RNA isolated from WT and kri1/cas002 embryo pools (each pool of 12 embryos) at 3 dpf. A significant reduction in the 18S peak but unchanged 28S peak in kri1/cas002 results in an elevated 28S/18S rRNA ratio. (I) The 18S rRNA reduction can be restored in mutant embryos by injection of kri1 WT mRNA.

mature 18S rRNA in kri1/cas002 embryos, while the amount of 28S rRNA was normal (Figure 2G-2H), which is consistent with previous observations in KRI1-deficient yeast cells. Reduced 18S rRNA could be restored by microinjection of wild-type kri1 mRNA into kri1/cas002 mutants (Figure 2I). We further examined the total protein level by bicinchoninic acid (BCA) protein quantitation and confirmed impaired protein synthesis in kri1/cas002 mutant embryos (Supplementary information, Figure S9A-S9C) ($P = 0.0003$). Sequencing of 18S rRNA in wild-type and kri1/cas002 mutant embryos showed no difference; however, 80S ribosome and polysome formation were impaired in kri1/cas002 mutant embryos with excessive 60S ribosome subunits (Supplementary information, Figure S9D).

Bcl2 rescues hematopoiesis in kri1/cas002 mutant

To elucidate the mechanism of defective HSPCs in kri1 mutant, we examined HSPC proliferation by calculating the proportion of phospho-histone 3 (pH3) immunostaining-positive cells in total HSPC (cmyb WISH positive) during hematopoietic development (Figure 3A, 3F, 3G-3R and Supplementary information, Figure S10). At 40 hpf, the percentage of proliferative HSPCs (pH3$^+$ cmyb$^+$) in kri1/cas002 mutant embryos was nearly the same as that in wild-type siblings in the AGM (Figure 3A and Supplementary information, Figure S10A-S10H; $P = 0.0873$), but was significantly reduced in the CHT (Figure 3A and Supplementary information, Figure S10I-S10P; $P < 0.0001$), and was further decreased in the CHT at 2
Figure 3 Transient expression of Bcl2 rescues the defective hematopoiesis in kri1l cas002 mutant embryos. (A) Statistical analysis of the proportion of proliferative HSPCs (cmyb and PH3 double positive) in total HSPCs in kri1l cas002 mutant embryos and wild-type siblings during hematopoietic development (40 hpf-2 dpf). N ≥ 10 for each group. 40 hpf AGM, p = 0.0873; 40 hpf CHT, p < 0.0001; 2 dpf CHT, p < 0.0001. Error bars represent SEM. **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test). (B) Proportion of kri1l cas002 mutant embryos with classified hematopoiesis phenotype (indicated by cmyb staining) with or without microinjection of p53 MO, treatment with caspase inhibitor (Z-VAD-FMK) or Bcl2 expression, respectively. (C-E) The CHT hematopoiesis is recovered by Bcl2 overexpression in kri1l cas002 mutant embryos. Embryos from two heterozygous parents were injected with bcl2 mRNA at one-cell stage and harvested for WISH analysis. After WISH and photographing, all embryos were genotyped by genomic DNA sequencing; percentage of rescue was then calculated. 26 out of 33 mutant embryos injected at one-cell stage with bcl2 mRNA are fully rescued, while the rest of embryos are partially rescued. (C’-E’) The enlarged CHT regions. (F) Percentage of the proliferative HSPCs in the CHT of kri1l cas002 mutant embryos and wild-type siblings with or without Bcl2 overexpression at 3 dpf. N = 12 for each group. Without Bcl2 overexpression, p < 0.0001; With Bcl2 overexpression, p = 0.3136. Error bars represent SEM. **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test). (G-R) Representative confocal images of double staining of cmyb RNA (red) and PH3 protein (green) in the CHT at 3 dpf. G, K and O are bright-field images overlaid with fluorescent staining. Scale bars, 50 µm.
Autophagy is hyperactivated in kri1f<sup>pan002</sup> mutant

Recent reports have shown that Bcl2 functions not only as an anti-apoptosis protein, but also as an anti-autophagy protein by interacting with Beclin1 and disrupting VPS34-Beclin1 complex [44, 45]. To check whether the regulatory role of Bcl2 in autophagy is responsible for the rescue of HSPC in kri1<sup>pan002</sup> mutant, we carried out immunoblotting on the whole embryo lysates using an antibody against an autophagy marker, microtubule-associated protein light chain 3 (Lc3) [46, 47]. We found the level of Lc3-II in kri1<sup>pan002</sup> mutant was significantly higher than that in wild-type embryos (Figure 4A), and it could not be rescued in p53<sup>M214K</sup> mutant background [48] (Supplementary information, Figure S13E). In addition, we found that the level of p62 protein [49], a well-characterized autophagy substrate, was decreased in kri1<sup>pan002</sup> mutant embryos, while the level of autophagy initiation factor Beclin1 was unchanged in the mutant embryos (Figure 4A and Supplementary information, Figure S14A).

In order to directly observe the autophagy level in live kri1<sup>pan002</sup> mutant [46, 50], we injected a mCherry-lc3 fusion RNA (in vitro transcribed from a previously described construct [51]) into wild-type or kri1<sup>pan002</sup> embryos transgenic for cmab:egfp at one-cell stage. At 3 dpf, abundant Lc3-II puncta (indicating autophagosomes) were present in kri1<sup>pan002</sup> EGFP cells in the CHT (Figure 4B-4H; P = 0.004), and this phenomenon became more obvious after chloroquine treatment, which is known to block the degradation of autophagosome [52, 53] (Figure 4B, 4I-4N; P = 0.0008). The increased Lc3-II puncta in HSPC could be found as early as 40 hpf in the CHT of kri1<sup>pan002</sup> mutant (Supplementary information, Figure S16). Analysis of electron micrographs further confirmed a significant increase of autophagosome-like structures above the wild-type level in the CHT region of kri1<sup>pan002</sup> mutant (Supplementary information, Figure S17). These results suggest that the autophagy level is significantly elevated in kri1<sup>pan002</sup> mutant, and this change may correlate with the reduction of HSPCs.

To test whether Bcl2 overexpression rescues the hematopoietic failure in kri1<sup>pan002</sup> mutant embryo through inhibiting autophagy, we evaluated the Lc3-II level in kri1<sup>pan002</sup> mutant with or without overexpression of Bcl2. Consistent with our hypothesis, Bcl2 overexpression significantly reduced the Lc3-II level in kri1<sup>pan002</sup> mutant (Figure 4O). mCherry-Lc3-labeled autophagosomes in EGFP<sup>+</sup> HSPCs were also markedly decreased in Bcl2-overexpressed kri1<sup>pan002</sup> mutant (Figure 4P-4S; P = 0.0021), suggesting Bcl2 rescues kri1<sup>pan002</sup> hematopoietic phenotype through its anti-autophagy function.

Inhibition of autophagy restores hematopoiesis in kri1<sup>pan002</sup> mutant

Autophagy inhibitors have been successfully developed to target different stages of autophagic flux. We hypothesized that the treatment with these inhibitors, or knockdown of the autophagy initiation factor Beclin1, might mimic the effect of Bcl2 overexpression in restoring hematopoiesis in kri1<sup>pan002</sup> mutant. Indeed, kri1<sup>pan002</sup> mutant embryos treated with 3-MA [54] at 10 mM or Bafilomycin A1 (Baf A1) [55] at 25 nM for 36 h, or injected with beclin1 MO at one-cell stage, showed normal
Figure 4 Ectopic expression of Bcl2 inhibits excessive autophagic flux in kri1f<sup>cas002</sup> HSPCs. (A) Representative immunoblotting images of Lc3-I, Lc3-II, Beclin1 and p62 in whole embryo lysates of wild-type (WT) and kri1<sup>cas002</sup> embryos at 3 dpf, treated with or without chloroquine (5 µM) for 6 h before harvest. Tubulin serves as the loading control. (B) Statistics of the average number of autophagosome (indicated by mCherry-Lc3 puncta) in kri1f<sup>cas002</sup> mutant embryos and WT siblings with or without chloroquine treatment at 3 dpf. mCherry-Lc3 puncta are counted in over 20 EGFP<sup>+</sup> cells (in cmyb:egfp background) per embryo, total 6-8 embryos under each condition. Without chloroquine treatment, \( P = 0.0040 \); with chloroquine treatment, \( P = 0.0008 \). Error bars represent SEM. **\( P \leq 0.01 \); ***\( P \leq 0.001 \) (Student’s t-test). (C-N) Representative confocal images of mCherry-Lc3 puncta (autophagosomes) in HSPCs of live zebrafish embryos. Wild type siblings or kri1<sup>cas002</sup> mutant embryos in the Tg (cmyb:egfp) transgenic background, were injected with mcherry-lc3 mRNA at one-cell stage and imaged at 3 dpf with or without pre-treatment with chloroquine (5 µM) for 6 h. Scale bars: 5 µm. (O) Immunoblotting analysis of whole embryo lysates from WT and kri1<sup>cas002</sup> mutant embryos with or without bcl2 mRNA injection. (P) Statistics of the average number of autophagosome for kri1f<sup>cas002</sup> mutant embryos and wild type siblings with Bcl2 overexpression at 3 dpf. mCherry-Lc3 puncta are counted in over 20 EGFP<sup>+</sup> (cmyb: egfp) cells per embryo, total 8-11 individual embryos. \( P = 0.0021 \). Error bars represent SEM. **\( P \leq 0.01 \); ***\( P \leq 0.001 \) (Student’s t-test). (Q-S) Representative confocal images of mCherry-Lc3 puncta (autophagosomes) in HSPCs of live zebrafish embryos with Bcl2 overexpression. Scale bars, 5 µm.
**Excessive autophagy limits HSPC proliferation**

**dpf**, the proportion of pH3 cells in *kri1* mutant embryos treated with BafA1 was almost the same as that in wild type (Figure 5U; *P* = 0.2344). Therefore, inhibition of autophagic flux at different stages is able to rescue HSPC proliferation defects in *kri1* mutant.

**Accumulation of misfolded proteins trigger PERK-dependent autophagy**

To understand how *Kri1* deficiency-caused ribosome defects trigger excessive autophagy via a p53-independent manner, we hypothesized that impaired ribosome biogenesis might cause dysfunction in protein synthesis to trigger misfolded protein accumulation, which is known to activate autophagy [56, 57].

Protein remodeling factor Hsp110 is known to cooperate with Hsp70 and Hsp40 to dissolve and reactivate aggregated proteins. It is used as a molecular probe to detect puncta foci where misfolded proteins accumulate [58]. We injected an *in vitro* transcribed mRNA coding for *Hspa4a* (zebrafish homolog of human Hsp110) [59] fused to mCherry into one-cell stage wild-type or *Hspa4a-mCherry* embryos stably transgenic for *cmyb:egfp*. Abundant *Hspa4a*-mCherry puncta were observed in *kri1* mutant embryos in the CHT region (Figure 6A-6G; *P* = 0.0002; Supplementary information, Figure S9A-S9C) and cause misfolded proteins to aggregate (Figure 6A-6G). Accumulation of misfolded proteins and inefficient protein synthesis trigger PERK activation (Figure 6H), which subsequently upregulates autophagy [56, 57] (Figure 4A, 4C-4N and Supplementary information, Figure S19A) and LC3-II (Supplementary information, Figure S16). Inhibition of autophagy or PERK signaling using drug treatments (Figure 5, 3-MA and Baf A1 targeting autophagy; Figure 6, GSK2656157 targeting PERK signaling) or MOs (Figure 5, 3-MA and Baf A1 targeting autophagy; Figure 6I and 6L, targeting *beclin1*; Figure 6I and 6L, targeting *perk*/*eif2ak3*) successfully rescue hematopoiesis defects in *kri1* mutant embryos.

The finding is distinct from the known regulatory role of apoptosis in bone marrow failure or anemia caused by ribosome disorders [15-18]. Apoptotic signals are normal in HSPCs in the CHT of *kri1* embryos (Supplementary information, Figure S11). Neither *p53* MO nor caspase 3 inhibitor, nor *p53* null allele, rescued the HSPC defects (Supplementary information, Figure S12, S13A-S13D). Results from our biochemical analyses of Lc3-II level and live imaging of Lc3 puncta in HSPCs, however, attribute hematopoiesis defects in *kri1* embryos to a hyperactivation of autophagy, which can be inhibited by Bcl2 overexpression that is known to disrupt the formation of VPS34-Beclin1 complex during auto-

**Discussion**

Through the characterization of a recessive zebrafish mutant *kri1* with hematopoietic defects we have uncovered a novel connection between ribosome biogenesis and autophagy in HSPCs. Loss of *kri1* blocks HSPC proliferation in the CHT region and depletes most downstream hematopoietic lineages during definitive hematopoiesis. Due to the dysfunction of SSU complex, in which *Kri1* functions as a critical component [41], the level of 18S rRNA is dramatically reduced, although 28S rRNA is spared (Figure 2G-2I). The ribosomal biogenesis defects reduce the rate of protein synthesis (Supplementary information, Figure S9A-S9C) and cause misfolded proteins to aggregate (Figure 6A-6G). Accumulation of misfolded proteins and inefficient protein synthesis trigger PERK activation (Figure 6H), which subsequently upregulates autophagy [56, 57] (Figure 4A, 4C-4N and Supplementary information, Figure S16). Inhibition of autophagy or PERK signaling using drug treatments (Figure 5, 3-MA and Baf A1 targeting autophagy; Figure 6I-6R, GSK2656157 targeting PERK signaling) or MOs (Figure 5D and 5E, targeting *beclin1*; Figure 6I and 6L, targeting *perk*/*eif2ak3*) successfully rescue hematopoiesis defects in *kri1* mutant embryos. 

This finding is distinct from the known regulatory role of apoptosis in bone marrow failure or anemia caused by ribosome disorders [15-18]. Apoptotic signals are normal in HSPCs in the CHT of *kri1* embryos (Supplementary information, Figure S11). Neither *p53* MO, nor caspase 3 inhibitor, nor *p53* null allele, rescued the HSPC defects (Supplementary information, Figure S12, S13A-S13D). Results from our biochemical analyses of Lc3-II level and live imaging of Lc3 puncta in HSPCs, however, attribute hematopoiesis defects in *kri1* embryos to a hyperactivation of autophagy, which can be inhibited by Bcl2 overexpression that is known to disrupt the formation of VPS34-Beclin1 complex during auto-
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Figure S10A-S10H and Figure S16A-S16F). In addition, their departure for the CHT (Supplementary information, Figure S18A-S18K), further supporting the idea that the hematopoietic phenotype in kri1l kas02 is caused by autophagy that is negatively regulated by BeII.

Previous reports have shown that autophagy is essential for HSCs to balance their quiescence, self-renewal and expansion [31]. Activated autophagy driven by FOXO3A-mediated program protects HSCs from starvation-induced apoptosis and maintains HSCs functions [63]. atg7−/− HSCs show ROS accumulation and a loss of colony formation capacity in replying [32]. Similar observations are obtained in Atg5 knockdown and in FIP2000-null HSCs [64-66]. In contrast, kri1l kas02 HSCs accumulate excessive autophagosomes that are harmful to HSPC proliferation. Our observation and previous reports together suggest that autophagy functions as a double-edged sword [67] in HSPCs, and an appropriate and fine-tuned autophagy level is important for HSPC proliferation and maintenance.

Morrison group has reported that the rate of protein synthesis in HSCs is highly regulated; either increased or decreased protein synthesis impairs HSC function [68]. Naive HSPCs undergo massive expansion upon arrival in CHT. It is conceivable that HSPCs is more sensitive to ribosome disorders due to the proliferative stress associated with the expansion. Indeed, both autophagy level and proliferation status of HSPCs in AGM are normal before their departure for the CHT (Supplementary information, Figure S10A-S10H and Figure S16A-S16F). In addition, cells in other proliferative tissues, such as cranial region and gut, carry more Lc3-mCherry puncta-positive autophagosomes (data not shown). These observations together suggest proliferative state of stem cells may render them particularly sensitive to Kri1l deficiency.

How SSU defects cause the accumulation of misfolded proteins remains elusive. Kri1l deficiency causes defective polysome formation, which includes reduction of functional polysomes or even disassembled polysomes accompanied with excessive 60S subunits. Reduced polysomes or excessive 60S subunits may lead to disorder in protein synthesis, and cause de novo synthesis of unproductively folded protein. Especially for larger proteins, the well-coupled translation and folding kinetics are vital for the correct production of functional proteins [69]. Unfolded protein response (UPR) was recently reported to regulate HSCs [70]. kri1l kas02 HSCs have a dramatic increase of Hspa4a and ubiquitin-modified proteins, indicating an accumulation of misfolded/unfolded proteins. This leads to the activation of PERK signaling, reflected by an increased level of phospho-eif2a. Importantly, blocking of PERK signaling is sufficient to inhibit excessive autophagy and to rescue definitive hematopoiesis in kri1l kas02 embryos. However, only the transcription level of ATF-4 (downstream of PERK-eif2a arm of UPR), but not BIP, ATF6, chop, xbp1 and xbp1ls (markers for other signaling arms of UPR), is significantly increased in kri1l kas02 mutant (Supplementary information, Figure S2D), implying that either only PERK-eif2a signaling cascade is strongly activated in Kri1l deficiency-induced UPR, or excessive autophagy in kri1l kas02 mutant is triggered by a PERK-dependent, but UPR-independent signaling mechanism.

A model of S6K-mediated inhibition of insulin pathway has been proposed to explain how RPS19 or RPS7 deficiencies induce the upregulation of autophagy level in peripheral blood cells of patients and zebrafish mor-

Figure 5 Autophagy inhibitors prevent hematopoiesis failure in kri1l kas02 mutant embryos. (A-D) Representative images of cmyb expression pattern in kri1l kas02 embryos with or without treatment of autophagy inhibitors, or with microinjection of beclin 1 MO. kri1l kas02 embryos were treated with 3-MA (10 mM) or Baf A1 (25 nM) from 36 hpf to 72 hpf. After cmyb WISH and imaging, all embryos were genotyped, the percentage of the rescue was calculated. 18 out of 40 mutant embryos treated with 3-MA are fully rescued, while 17 out of 40 mutant embryos are rescued partially. 12 out of 26 mutant embryos treated with Baf A1 are fully rescued, while 11 out of 26 mutant embryos are rescued partially. 7 out of 26 mutant embryos injected beclin 1 MO are fully rescued, while 11 out of 26 mutant embryos are rescued partially. (E) Percentage of kri1l kas02 mutant embryos with classified hematopoiesis phenotype with or without treatment of autophagy inhibitors, or with microinjection of beclin 1 MO. (F-K) 3-MA inhibits excessive autophagy in HSPCs in kri1l kas02 mutant embryos. Representative confocal images of mCherry-Lc3 puncta (autophagosomes) in kri1l kas02 mutant embryos in Tg (cmyb:egfp) transgenic background are shown. Scale bars, 5 µm. (L) Quantitative analysis of F-K. The numbers of autophagosomes are decreased in kri1l kas02 embryos with 3-MA treatment, p = 0.0037. (M-T) Baf A1 treatment restores HSPCs proliferation in kri1l kas02 mutant embryos. Double staining of cmyb RNA (red) and phospho-histone 3 (pH3) protein (green) in the CHT region of embryos at 3 dpf. M and Q show bright-field images overlaid with fluorescent staining. Scale bars, 50 µm. (U) Percentage of proliferative HSPCs in the CHT of kri1l kas02 mutant embryos and wild type siblings with or without Baf A1 treatment at 3 dpf N = 10. Without Baf A1 treatment, p = 0.0014; with Baf A1 treatment, P = 0.2344. Error bars represent SEM. **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test).
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We also show an upregulation of S6K and its downstream effector-RPS6 in kri1l<sup>cas002</sup> mutant embryos (Supplementary information, Figure S22A). However, phospho-RPS6 signaling is dominant in peripheral blood cells, but not in EGFP<sup>+</sup> HSPCs in either wild-type or kri1l<sup>cas002</sup> mutant (Supplementary information, Figure S22C-S22K), suggesting S6K was not the major regulator to induce excessive autophagy in kri1l<sup>cas002</sup> HSPCs. Taken together, we propose that the deficiency of Kri1l causes an impaired ribosome biogenesis, resulting in the accumulation of misfolded proteins. Aggregation of misfolded proteins activates PERK signaling, which in turn triggers a significant increase of autophagy. Excessive autophagy finally leads to an inhibition of definitive hematopoiesis. The S6K-RPS6 axis might be involved in the regulation of Kri1l-deficient peripheral blood cells rather than HSPCs (Supplementary information, Figure S22B). In addition, we found that rapamycin treatment could not rescue hematopoietic failure phenotype in kri1l mutant (Supplementary information, Figure S20A-S20C).

Although lmo2 promoter-induced expression of wild-type Kri1l rescued defective hematopoiesis in kri1l<sup>cas002</sup> embryos (Supplementary information, Figure S21), suggesting a cell autonomous role of Kri1l in HSPCs, it is also worth to note the presence of other cell types in the CHT. These include niche cells forming the hematopoietic microenvironment and differentiated hematopoietic lineage cells. They may also undergo an upregulation of autophagic flux in kri1l<sup>cas002</sup> mutant. Since appropriate cell-cell interactions in hematopoietic niche are vital for HSPC self-renewal, non-cell autonomous effects may also contribute to the defective hematopoiesis in kri1l<sup>cas002</sup> mutant.

In conclusion, an intact ribosome biogenesis, optimal protein synthesis and an appropriate level of autophagy are critical for HSPC maintenance and proliferation. During the first wave of HSPC expansion in the CHT region, a higher level of protein synthesis is needed, which makes HSPCs more sensitive to dysfunctional ribosome biogenesis [68, 71]. In kri1l<sup>cas002</sup> HSPCs, impaired ribosome biogenesis causes ribosomal stress and an upregulation of autophagy, which subsequently results in impaired proliferation of HSPCs. Decreased HSPCs can be restored by the treatment of autophagy inhibitors, PERK inhibitor and PERK/Beclin1 knockdown (Figure 7). This study suggests that autophagy level may be useful for the clinical diagnosis of anemia or bone marrow failure caused by ribosomopathies. Our finding also suggests patients with certain types of ribosomopathy may benefit from treatments with autophagy inhibitors, such as lys05 or spautin-1 [71]. In addition, the mechanism revealed in this study may also play an important role in the progression of other human diseases caused by ribosome abnormalities.

Materials and Methods

Zebrafish husbandry and MO/mRNA microinjection

Zebrafish stock maintenance, ENU mutagenesis and positional cloning were performed as previously described [39, 72, 73]. Zebrafish facility and study were approved by Institutional Animal Use Review Board of Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences. Morpholino oligonucleotides (MOs) were ordered from Gene Tools, LLC. Capped mRNAs were transcribed from linearized pCS2<sup>+</sup> plasmids (mMessage Machine; Ambion), purified and diluted to 150 ng/µl for microinjection into zebrafish embryos at 1-cell stage.

ENU mutagenesis and positional cloning

ENU mutagenesis and positional cloning were performed as previously described [39, 72]. The cas002 (Tu background) allele

Figure 6 Accumulation of unfolded proteins and PERK activation leads to excessive autophagy and hematopoiesis failure. (A-G) Representative confocal images (A-F) and Quantitation (G) of Hspa4a-mCherry puncta (aggregation of misfolded protein) in HSPCs of live zebrafish embryos. Wild-type siblings or kri1l<sup>cas002</sup> mutant embryos in the Tg (cmyb:egfp) transgenic background were injected with hspa4a-mcherry mRNA at one-cell stage and imaged at 3 dpf. The numbers of misfolded protein puncta are increased in kri1l<sup>cas002</sup> embryos compared to that in wild-type siblings, P ≤ 0.0002. Hspa4a-mCherry puncta were counted in over 30 EGFP<sup>+</sup> cells (in the Tg (cmyb: egfp) background) per embryo, total 10-12 embryos. Error bars represent SEM. **P ≤ 0.01; ***P ≤ 0.001 (Student’s t-test). Scale bars, 5 µm. (H) Representative immunoblotting analysis of the level of protein ubiquitination and phospho-eif2α in whole embryo lysates of WT and kri1l<sup>cas002</sup> embryos at 3 dpf injected with Flag-tagged ubiquitin plasmid at one-cell stage. Tubulin serves as the loading control. (I) Treatment with PERK inhibitor GSK2656157 or injection of perk MO rescues cmyb expression in HSPCs. Kri1l<sup>cas002</sup> mutant embryos were treated with GSK2656157 (20 µM) from 36 hpf to 72 hpf, or injected with perk MO at one-cell stage. After WISH and imaging, all embryos were genotyped. 14 out of 35 mutant embryos treated with GSK2656157 are fully rescued, while 17 out of 35 mutant embryos are rescued partially. 9 out of 35 mutant embryos injected with perk MO are fully rescued, while 11 out of 35 mutant embryos are rescued partially. (J-L) Proportion of kri1l<sup>cas002</sup> mutant embryos with classified hematopoiesis phenotype with or without GSK2656157 treatment, or with microinjection of perk MO. (M-R) GSK2656157 inhibits excessive autophagy in kri1l<sup>cas002</sup> HSPCs. Representative confocal images of mCherry-Lc3 puncta (autophagosomes) in kri1l<sup>cas002</sup> mutant embryos in Tg (cmyb:egfp) transgenic background are shown. Scale bars, 5 µm.
Figure 7 Working model for Kri1l dysfunction-induced hematopoietic defects and its treatment. (A) In normal HSPCs, ribosome biogenesis is tightly regulated; autophagy occurs at an appropriate level. HSPCs expand massively in the CHT region and differentiate into multiple hematopoietic lineages, including erythrocytes, myeloid cells (neutrophils and monocytes) and lymphocytes. (B) In kri1l cas002 HSPCs, abnormal ribosome biogenesis leads to the accumulation of misfolded proteins, which triggers PERK activation and excessive autophagy. The excessive autophagy leads to inhibition of HPSC proliferation, depletion of HSPC pool, and a profound hematopoiesis failure. (C) Blocking of PERK signaling with specific inhibitor (GSK2656157), Bcl2 overexpression or treatment with autophagy inhibitors (3-MA, Baf A1) reduces autophagy level and restores HSPC proliferation defects and hematopoietic lineage differentiation in kri1l cas002 mutant embryo.

was mapped by out-crossing Tu background heterozygous fish into polymorphic WIK background wild-type strain. We scanned the genome for linked SSLP markers by BSA. SSLP markers [74, 75] used for BSA were selected from the Massachusetts General Hospital Zebrafish Server website (http://zebrafish.mgh.harvard.edu). Fine mapping using mainly SSLP markers was carried out to narrow down the genetic interval. The cDNAs of candidate genes in the interval were cloned and sequenced from pooled mutants, and candidate mutation was confirmed by sequencing genomic DNA of individual mutant embryo. All primers used for this study are provided in Supplementary Table 1.

WISH, TUNEL assay and immunostaining
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). WISH was performed as described previously using NBT/BCIP (Sigma) as substrates [76, 77]. TUNEL was performed with In Situ Cell Death Detection Kit and TMR Red Kit (Roche) following manufacturer’s instruction. To detect both cmyb RNA and mitosis marker pH3 simultaneously, embryos were first hybridized with the DIG-labeled antisense cmyb RNA probe, incubated at 4 °C overnight with a peroxidase-conjugated anti-DIG antibody (1:500; Roche), and stained with Alexa Fluor cy3-conjugated tyramide as substrate (PerkinElmer). The embryos were then incubated with primary anti-pH3 (ser10) antibody (1:500; Santa Cruz), and finally incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (1:500; Invitrogen).

Plasmid construction
The zebrafish cDNA of kri1l gene was amplified from reverse transcription products and cloned into pCS2+ vector. For construction of the kri1l (1-60)-gfp reporter plasmid, the zebrafish cDNA (1-60 aa) of kri1l gene was amplified and cloned into pCS2+-egfp vector. For construction of Tg (lmo2:kr1l) transient transgenesis plasmid, the zebrafish lmo2 promoter was obtained by PCR amplification from lmo2-Cre-PBSK-I-SceI plasmid, and then cloned into the Tol2 transposon backbone together with full-length kri1l gene.
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For construction of the mcherry-lc3 plasmid, the PCR was performed to generate mcherry cDNA without the termination codon, and then replace the EGFP coding region of pEGFP-C1, and the resulting plasmid was named mCherry-C1. The fragment of lc3 was inserted into the corresponding sites in the mCherry-C1 plasmid. The zebrafish cDNA of hspa4a gene without the termination codon was amplified and cloned into pCS2* vector. Then mcherry cDNA was generated by PCR and inserted into pCS2*-hspa4a.

Live imaging of autophagy and unfolded protein in HSPCs

mCherry-Lc3 mRNA or hspa4a-mCherry mRNA was transcribed via the mMessage mMachine SP6 kit (Ambion), and then injected into Tg (cmyb:egfp) transgenic zebrafish embryos at one-cell stage. The live embryos were anesthetized with tricaine and mounted in 3% low melting point agarose for imaging with Olympus FX10000 scanning confocal microscope (under a 60×/1.00 NA water-immersion objective) [79].

Immunoblotting analysis

Embryos were deyolked [80], and then homogenized in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% Triton X-100, protease inhibitor cocktail and phosphatase inhibitor (Roche)). Protein lysates were separated on SDS-PAGE, transferred to nitrocellulose membrane (Amersham Biosciences), and incubated with anti-LC3 (Cell Signaling Technology, 1:1 000), phos-S6K (Cell Signaling Technology, 1: 1 000), phos-RPS6 (Cell Signaling Technology, 1:1 000), phos-eIF2α (Cell Signaling Technology, 1: 1 000), p62 (MBL,1:1 000), Beclin1 (abcam, 1:5 000), p53 (a gift from Jinrong Peng, Zhejiang University) or anti-a-tubulin (Sigma, 1:2 000) antibodies, and then HRP-conjugated secondary Antibodies.

Small molecular compound treatment

Final concentrations of chemicals used in this study were 5 μM chloroquine (Fluka Sigma-Aldrich), 10 mM 3-MA (Sigma), 25 nM Baf A1 (Santa Cruz), 10 μM Z36 (Sigma) and 20 μM GSK2656157 (Selleck). Embryos were incubated with small molecular chemicals in embryo medium at 28.5 °C until collection.

Transmission electron microscopy

For electron microscopy analysis, 3 dpf zebrafish embryos were fixed in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer. The embryos were washed in PBS-glycine to quench free aldehydes, then embedded in gelatin and infiltrated in 2.3 M sucrose, and subjected to rapid freezing in liquid N2 and infiltrated in 2.3 M sucrose, and subjected to rapid freezing in liquid N2 and infiltrated in 2.3 M sucrose, and subjected to rapid freezing in liquid N2 and infiltrated in 2.3 M sucrose, and subjected to rapid freezing in liquid N2 and infiltrated in 2.3 M sucrose, and subjected to rapid freezing in liquid N2 and infiltrated in 2.3 M sucrose, and subjected to rapid freezing in liquid N2, 50 mM thick cryosections were cut at ~120 °C using an Ultracut-S ultra microtome (Leica Microsystems). Sections were directly viewed in a FEI Tecnai G2 Spirit Twin electron microscope (FEI).

Quantitative PCR analysis

Total RNAs were extracted from 20 zebrafish embryos using Trizol reagent (Invitrogen). 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 actin (QPCR primer sequences were listed in Supplemen-
tary information, Table S1).

Quantitation of rRNA and polysome level

As previously described [21], total RNA was extracted from WT or kri1f00022 mutant zebrafish and then analyzed on an Agilent 2100 E-Bioanalyzer according to the manufacturer’s instructions. 140 WT or kri1f00022 larvae at 72 hpf were deyolked, washed and resuspended in cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM KCl, 10 mM MgCl2, 1% Triton X-100, 2 mM DTT, 0.5% sodium deoxycholate and 0.1 mg/ml cycloheximide) containing 100 U/ml RNase inhibitor (Promega), Complete Protease Inhibitor Cocktail (Roche), sodium vanadate, sodium fluoride and PMSF. Next, samples were homogenized by small pestles. Lysates were incubated on ice for 15 min and centrifuged (12 000 rpm, 10 min at 4 °C) to pellet the nuclei and cellular debris. The supernatant of each sample was loaded onto a continuous 15% - 45% (w/v) sucrose gradient in high salt resolving buffer (20 mM HEPES (pH7.4), 150 mM KCl, and 10 mM MgCl2) generated by a Biocomp gradient master. The mixture was next centrifuged in a Beckman SW41Ti rotor (Beckman Coulter) at 36 000 rpm for 3.5 h at 4 °C, and the absorbance at 254 nm was determined with an EM-1 UV Monitor (Bio-Rad).

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