Zebrafish Models for Dyskeratosis Congenita Reveal Critical Roles of p53 Activation Contributing to Hematopoietic Defects through RNA Processing

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Abstract

Dyskeratosis congenita (DC) is a rare bone marrow failure syndrome in which hematopoietic defects are the main cause of mortality. The most studied gene responsible for DC pathogenesis is DKC1 while mutations in several other genes encoding components of the H/ACA RNP telomerase complex, which is involved in ribosomal RNA (rRNA) processing and telomere maintenance, have also been implicated. GAR1/nola1 is one of the four core proteins of the H/ACA RNP complex. Through comparative analysis of morpholino oligonucleotide induced knockdown of dkc1 and a retrovirus insertion induced mutation of GAR1/nola1 in zebrafish, we demonstrate that hematopoietic defects are specifically recapitulated in these models and that these defects are significantly reduced in a p53 null mutant background. We further show that changes in telomerase activity are undetectable at the early stages of DC pathogenesis but rRNA processing is clearly defective. Our data therefore support a model that deficiency in dkc1 and nola1 in the H/ACA RNP complex likely contributes to the hematopoietic phenotype through p53 activation associated with rRNA processing defects rather than telomerase deficiency during the initial stage of DC pathogenesis.

Introduction

Dyskeratosis congenita (DC) is a rare bone marrow failure syndrome associated with abnormal skin pigmentation, nail dystrophy, mucosal leukoplakia, pulmonary fibrosis, and an increased susceptibility to both hematopoietic and solid cancers [1]. 85% of DC patients experience bone marrow failure that accounts for 80% of all DC-related mortality [2]. Discovery that the telomerase complex gene DKC1 was mutated in a subset of DC patients provided the first insight into a potential mechanism [3,4].

DKC1 encodes dyskerin, a pseudouridine synthase that complexes with box H/ACA small nuclear RNAs involved in posttranscriptional modification of ribosomal RNA (rRNA) through conversion of uridine (U) to pseudouridine (Y). Mutations in the catalytic domain of dyskerin lead to Hoyeraal-Hreidarsson syndrome resulting in a severe form of DC including immunodeficiency, growth retardation, and microcephaly. Dyskerin is also associated with the RNA component of telomerase that contains an H/ACA RNA motif.

Telomerase is a multimeric ribonucleoprotein complex responsible for maintaining telomere length in cells whose incomplete lagging strand synthesis and oxidative DNA damage result in progressive shortening of replicated DNA. Telomere shortening is associated with aging and genomic instability whose impact is widespread-healthy individuals with shorter telomeres possess a higher lifetime incidence of cancers [5] and shortened telomeres are associated with diverse pathologies including psychiatric disease [6], cardiovascular disease [7], idiopathic pulmonary fibrosis [8], and diabetes [9].

The telomerase complex consists of the transcriptase subunit hTERT, the rRNA pseudouridylation dyskerin subunit adjoined to NOPI0, NHP2, and GAR1, and the hTR rRNA (encoded by TERC) providing the template for reverse transcription. The telomerase elongation and replication process is then continued by the shelterin complex.

At present, approximately 50% of DC patients have an identified mutation in one of eight genes involved in the telomerase complex [DKC1, TERC (encoding hTR), TERT, NHP2, NOP10], the Cajal body localizing co-factor TANG1 [10,11], the relatively unknown gene C16orf57 [12], or the shelterin complex (TINF2) [13].

NOLA1 encodes GAR1p, a small nucleolar ribonucleic protein (snRNP) that is critical for yeast 18 S rRNA maturation [14] and pseudouridylation of other precursor rRNAs [15]. It forms a complex with DKC1, NHP2, and NOP10; however, there are no reported NOLA1 mutations in any human patients to date. Whereas knockdown of telomerase complex genes DKC1 and NOP10 result in a subsequent decrease in TERC expression,
knockdown of GAR1p does not reduce TERC expression [16] suggesting that its critical role in rRNA maturation may involve non-telomerase complex associations.

Despite the clear association between DC patients and shortened telomere lengths, it remains unclear if shortened telomeres are the sole driver behind the disease phenotype. DC patients with DCK1 and TEN2 mutations typically present at younger ages and with more physical exam abnormalities than patients with TERC or TERT mutations yet there is no difference in telomere lengths between these subgroups [17]. Recently a subset of six DC patients harboring mutations in C6orf57 all had normal telomere lengths despite severe disease penetrance at a young age [12]. It is therefore possible that other pathways aside from telomere maintenance are responsible for the disease phenotype.

Other bone marrow failure syndromes such as Diamond Blackfan Anemia (DBA) have been associated with p53 pathway activation [18]. Up-regulation of p53 has been reported in Dck1-deficient mouse hepatocytes [19], suggesting that p53 may be involved in the pathogenesis of DC as well.

We utilized a morpholino oligonucleotide (MO) knockdown approach to study the mechanism(s) by which the DC-associated gene DCK1 results in hematopoietic stem cell failure. To further understand H/ACA RNPs complex interactions and its role in DC-related hematopoietic failure we also took advantage of a retroviral insertion mutational model of nola1. We report that both models result in reduced hematopoiesis, increased p53 expression, and defective ribosomal biogenesis all without detectable changes in telomerase function. These data suggest involvement of a telomerase-independent mechanism by which hematopoietic failure manifests in dyskeratosis congenita patients.

**Results**

Dck1 morphant and nola1 mutant show similar morphological abnormalities

To study the function of dck1 during embryonic zebrafish development we conducted a knockdown experiment with a splicing morpholino targeting the border between exon 4 and intron 4. This morpholino is predicted to cause inclusion of the 1.5 kb fourth intron into the mRNA transcript resulting in a truncated protein due to a pre-mature stop codon. Using primers spanning exons 4 and 6, morphants demonstrated a 1.5 kb larger dck1 transcript with correctly spliced dck1 transcript reduced to 10% of wild type levels (Fig. 1A b and c). These data demonstrate efficient knockdown of dck1. From 3 days post-fertilization (dpf), dck1 morphants started to exhibit smaller heads and eyes, fail to develop the swim bladder, and develop edema (Fig. 1A e and g). Morphants died around 7 dpf.

We identified a zebrafish line (PKU #13771) carrying a proviral DNA integration in the first exon of nola1 through a retrovirus insertion screen [20]. Protein sequence homology alignment revealed that GAR1/nola1 is highly conserved among different species and human GAR1 and zebrafish nola1 protein sequences share 81.1% similarity and 79.0% identity. Genomic synteny relationship analysis also suggested that zebrafish nola1 is the ortholog of the human GAR1 (Fig.S2A and B). Zebrafish nola1 is ubiquitously expressed in most cells during early stages of development starting at 2 hours post-fertilization (hpf). Afterwards, nola1 is mainly expressed in the brain and intestinal organs (Fig.S1). Homozygous nola1 mutants shared similar morphologic changes with dck1 morphants (Fig. 1B b and d) and mutants died at 7 to 10 days post-fertilization (dpf). Genotyping confirmed that all embryos with the mutant phenotype were homozygous for the retroviral insertion (data not shown). Real time quantitative PCR (qPCR) demonstrated greater than 90% reduction in nola1 mRNA expression in homozygous mutants (Fig. 1B g). The mutant phenotype was rescued by injection of nola1 mRNA or GAR1 mRNA (Fig. 1B I and Fig.S2C c), demonstrating the functional conservation of GAR1/nola1. Overall, functional studies of dck1 and nola1, two components of the H/ACA RNP complex, established that their functions were essential for zebrafish larval development and their deficiencies caused similar morphologic abnormalities.

Both dck1 morphants and nola1 mutants have hematopoietic defects

To determine if dck1 and nola1 deficiencies in zebrafish would cause hematopoietic defects similar to DC patients, we analyzed multiple markers of primitive and definitive hematopoiesis by RNA whole-mount in situ hybridization. Prior to 3 dpf, no difference in circulation and hemoglobin staining was detected between dck1 morphants, nola1 mutants and their wild type siblings (data not shown). Additionally, only minimal reduction in expression of the hematopoietic markers spl and bmy2 was seen in nola1 mutants at 24 hpf (data not shown). These data suggest that primitive hematopoiesis is, at most, minimally affected by dck1 or nola1 deficiency.

However, expression of definitive hematopoietic stem cell (HSC) markers runx1 and c-myb were significantly reduced in both dck1 and nola1 deficient fish at 30 hpf and 3 dpf, respectively (Fig. 2A, Fig. 2B a, b, c and d). Nola1 mutants also had greatly decreased hemoglobin as shown by o-dianisidine staining after 4 dpf (Fig. 2B i and j). This reduction persisted until 7 dpf (data not shown), indicating that the decrease in red blood cells was not caused by a delay of development. The decreased number of red blood cells may be due to the decreased production and/or decreased cell survival in definitive HSCs.

To explore whether nola1 mutation resulted in reduced development in all definitive blood lineages, we examined granulocyte and lymphocyte development using mpx and rag1 expression, respectively. Compared with wild type siblings, mutants’ lymphoid cells were absent at day 4 (Fig. 2Be-2Bf). In contrast, mutants had minimal change in the granulocyte marker mpx at 3 dpf (Fig. 2Bg-2Bi). These data indicate that definitive hematopoiesis is compromised to varying degrees by decreased nola1 expression.

In conclusion, similar to what is observed in human DC patients, the deficiencies of dck1 and nola1 in zebrafish result in defective hematopoiesis. We therefore view these zebrafish as potential models for DC.

Hematopoietic reduction is mediated by p53

The p53 pathway is activated in some animal models of congenital hematopoietic diseases, including zebrafish model of Diamond Blackfan Anemia (DBA) [18]. The p53 network is also activated in telomerase deficient zebrafish [21]. However, the mechanism responsible for regulation of hematopoiesis by the p53 pathway is not clear. Our qPCR results showed p53 expression was increased greater than 4 fold in dck1 morphants and nola1 mutants. P53 downstream genes such as the pro-apoptotic gene bax and the cell cycle regulator gene cyclin G1 also had significantly increased expression (Fig. 3A, C) suggesting that the p53 pathway is activated with the H/ACA RNPs complex deficiency.

To determine if p53 inhibition could rescue the hematopoietic defects seen in our models, we microinjected p53 morpholino (blocking ATG translation) into dck1 morphants. Although the inhibition of p53 did not rescue the morphological abnormalities...
seen in the dkc1 morphants, down-regulation of p53 did rescue c-myb and runx1 expression in dkc1 morphants (Fig. 3B). In a different experiment, we crossed p53 null fish (tp53M13K) with nola1 mutant fish and tested interaction of these two genes in a stable genetic mutant background. Detection of hemoglobin by the o-dianisidine staining on embryos of p53+/+ nola1+/+; p53−/− nola1+/-; p53−/− nola1−/− showed that the anemia observed in nola1 mutants was rescued in the p53 null background (Fig. 3D, arrows). Similarly, genetic mutation of p53 partially rescued c-myb expression (Fig.S3).

Overall, our data suggest that the hematopoietic defects in dkc1 morphants and nola1 mutants are p53-dependent. The p53 pathway may function in hematopoiesis by regulating the apoptosis and cell cycle of HSC and the differentiation of blood cell lineages.

Figure 1. Molecular and phenotype analysis of dkc1 morphant and nola1 retroviral insertion mutantant of zebrafish. (A) Splicing MO targeting dkc1 sequence caused the inclusion of intron 4 into mRNA (a). Semi-quantitive PCR data showed an increase of 1.5 kb in the PCR product in dkc1 morphants (b). According to Real Time PCR result, expression of dkc1 was diminished by more than 90% when high dose (15 ng) of MO was injected (c). Pictures of embryos at 3 dpf (d and e) and 5 dpf (f and g) showed smaller eyes and smaller head (red arrows in d, e, f and g) in dkc1 morphants. Compared to wild type embryos, dkc1 morphants developed edema, and had fewer red blood cells (red arrowhead in d, e, f and g). d and f: wild type controls; e and g: dkc1 morphants. (B) A retroviral insertion in exon1 of the nola1 gene led to the phenotype of smaller eyes, smaller head, and cardiac edema in nola1 homozygous mutant. Red arrow indicated the smaller eyes and smaller head, and red arrowhead showed edema in nola1 homozygous mutant at 3 dpf (a and b) and 5 dpf (c and d). Microinjection of nola1 mRNA (f), but not eGFP mRNA (e) can rescue the mutant phenotype of nola1 homozygous mutants at 5 dpf (red arrow and arrowhead in e and f). a and c: wild type siblings; b, d, e and f: nola1 homozygous mutants. Expression of nola1 was reduced more than 90% in nola1 homozygous mutants (g). All the pictures of embryos are lateral view with anterior to the left. doi:10.1371/journal.pone.0030188.g001

No telomerase activity defects are observed in dkc1 and nola1 deficient embryos but rRNA processing is inhibited

H/ACA RNP complex functions in both rRNA pseudouridylation and telomere maintenance [22]. To determine which function of H/ACA RNP complex plays a more important role in the initiation of hematopoietic failure in DC, we measured the telomerase activity and rRNA processing in both dkc1 and nola1 deficient fish.
There was no significant difference in whole embryo lysate telomerase activity between morphants, mutants, and wild-type siblings at 3 dpf (Fig. 4Aa, b). To exclude the possibility of HSC-specific telomerase defects, we injected dkc1 MO into one-cell stage of Tg(c-myb:GFP) fish embryos, FACS sorted the GFP positive cells, and measured their telomerase activity. No difference in telomerase activity was detected in sorted cells (Fig. 4Ac), suggesting that the hematopoietic failure seen in mutants/morphants is independent of telomerase function.

However, production of 18S rRNA was defective in dkc1 morphants at 48 hpf (Fig. 4Ba, b, arrow in a) prior to observation of the morphologic phenotype. In addition, nola1 mutants showed...
approximately 25% less 18 S rRNA relative to total RNA at 3 dpf (Fig. 4B-c, d, arrow in c). There was no statistically significant change in production of 28 S rRNA. We obtained the same results from Northern blotting analysis using probes that were specifically designed to detect the internally transcribed sequence 1 (ITS1), internally transcribed sequence 2 (ITS2), and 18 S rRNA. Decrease of the rRNA precursor generating 18 S rRNA was observed in the lanes probed with ITS1 in both of dkc1 morphant at 48 hpf and nola1 mutant at 3 dpf compared with the wild type controls (arrows in Fig. S4B). Importantly, a significant reduction of total amount of 18 S rRNA was detected in the lanes probed with 18 S rRNA in both models. However, no significant difference was revealed in the lanes probed with ITS2, indicating that the processing of 28 S rRNA and 5.8 S rRNA appears to be normal or much less affected. Taken together, our data suggest that defects in rRNA processing, and not telomerase activity, may play the central role in DC patients’ bone marrow failure.

Discussion

The goal of this study is to reveal the cause of bone marrow failure in DC. The prevailing view is that DC is caused by telomere maintenance defects [23]. Indeed, mutations of several components of telomerase and shelterin complexes have been found in DC patients and in many cases telomere shortening is reported. However, not all data fit this notion. DKC1, NOP10, and NHP2 are involved not only in telomere maintenance but are also components of the H/ACA RNP complex. This conserved complex also performs site-specific pseudouridylation of ribosomal RNAs [24]. In a Dkc1-deficient mouse model, deregulation of ribosome function and hematopoietic defects have been observed in early development while telomere shortening has been detected only in later generations [25]. Defects of rRNA maturation in a Drosophila mutant for an ortholog of this gene have also been reported [26]. In mouse Dkc1-deficient hepatocytes, accumulation of rRNA precursors and induction of p53 pathway have been found [19]. P53-independent cell cycle arrest of cells depleted with dyskerin was also noted [27]. Based on findings described here, we believe that defects of ribosome biogenesis constitute an early event in the pathogenesis of DC and may be the major cause of bone marrow failure in this disease, although we cannot exclude the possibility that telomere shortening may contribute to DC at later stages. Our results agree with studies of Dkc1 mutant mice, which suggested that deregulated ribosome function is important for the initiation of DC [25]. These data may be relevant to some clinical cases of DC characterized by normal telomeres [28].
We found that the effect of deficiency of H/ACA RNP complex genes on hematopoiesis is mediated by p53. Defects in HSC development in dkc1 and nola1-deficient zebrafish can be lessened by p53 inhibition. Zebrafish mutant for nola1 has a phenotype similar to dkc1 morphant including hematopoietic defects. Deficiency of both proteins results in p53 up-regulation and the hematopoietic defects are rescued by p53 inhibition. While this manuscript was in preparation, Pereboom et al reported that zebrafish mutation of nop10, another component of H/ACA RNP complex, caused similar p53 dependent phenotype due to RNA processing defect [29]. Therefore, our data together with previous reports reveal an important role for p53 activation in mediating blood specific defects in DC as well as related diseases such as DBA [18]. Therapeutics targeting p53 activation may have beneficial effects in treating these types of diseases.

Notably, mutations of GAR1/nola1 have not been reported in DC patients [16]. Earlier studies showed that H/ACA snRNA accumulation did not require GAR1 [14,30,31]. However, recent data revealed a crucial role of Gar1/nola1 in the formation of functional H/ACA RNPs. First, Dyskerin forms a complex with

**Figure 4. Analysis of telomere maintenance and rRNA processing in zebrafish dkc1 and nola1 deficiency.** (A) TRAP Assay results showed no difference between dkc1 morphants, nola1 mutants and control embryos at 3 dpf when the mutant phenotype onset (a and b). We injected dkc1 MO into Tg(c-myb:GFP) fish. GFP-positive HSC were isolated by cell sorting (FACS) at 3 dpf. GFP positive (GFP+) and negative (GFP−) cells from control embryos (0 ng MO) and dkc1 morphants (15 ng MO) were subjected to TRAP Assay. IC: Internal control; -ctrl: only lysis buffer but no embryo extracts added group. (B) Analysis of RNA processing showed that reduction of 18 S rRNA in dkc1 morphant (a and b) at 48 hpf and in nola1 mutant (c and d) at 3 dpf, but slight change or no change of 28 S rRNA. The average fold changes of percentage of 18 S rRNA and 28 S rRNA are represented by the bar graphs. WT: wild type control; MO: dkc1 morphant; nola1−/−: nola1 mutant. ** indicates very significant changes at p<0.01, and * indicates significant changes at p<0.05 on the basis of independent Student t tests.

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Nop10, Nhp2, and Naf1, and then this complex binds the nascent precursor of H/ACA RNA to form inactive pre-H/ACA RNP, which still lacks Gar1 [24]. The last step of H/ACA RNP assembly is the replacement of Naf1 with Gar1, which controls the transition from inactive pre-RNP to functional H/ACA RNP. Gar1/nola1 is highly conserved among species as illustrated by a transition from inactive pre-RNP to functional H/ACA RNP. Assembly is the replacement of Naf1 with Gar1, which controls the last step of H/ACA RNP precursor of H/ACA RNA to form inactive pre-H/ACA RNPs, Nop10, Nhp2, and Naf1, and then this complex binds the nascent precursor of H/ACA RNA to form inactive pre-H/ACA RNP, which still lacks Gar1 [24]. The last step of H/ACA RNP assembly is the replacement of Naf1 with Gar1, which controls the transition from inactive pre-RNP to functional H/ACA RNP. Gar1/nola1 is highly conserved among species as illustrated by a transition from inactive pre-RNP to functional H/ACA RNP.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described [38] using c-myb, runx1, mpx, rag1 [36] and nola1 riboprobes. Sequences of primers used for nola1 probe synthesis were: nola1/RTR: 5'-AGGAGGAGTTGATATCAGAGTCA-3' and nola1/RTF1: 5'-AGGAGGAGTTGATATCAGAGTCA-3' and nola1 RTR1: 5'-CCCATGCGGCTGACTATTTCCACAT-3'. Runx1 expression analysis in nola1 mutation was performed at 30 hpf. At this stage, the mutants were not distinguishable from wild type according to the morphology. After in situ hybridization, ~25% (12/50) embryos exhibited the reduction of runx1 shown as in figure 2 Bd, and ~75% (38/50) showed the normal pattern as seen in figure 2 Bc. We confirmed those having expression shown in figure 2 Bc as nola1 mutants and those in figure 2 Bc as wild type or heterozygous by PCR genotyping.

Isolation of hematopoietic stem cells and FACS analysis

300 transgenic zebrafish embryos expressing GFP under c-myb promoter were dechorionated with protease at 3 days post fertilization (dpf) and washed with Ca2+-free Kinger’s solution for 15 min. Embryos were incubated with 0.25% Trypsin-EDTA at 20 degrees Celsius for 30–50 min and then centrifuged at 300 g for 5 min. Cells were washed once with suspension solution [Lebovitz medium L-15, 0.8 M CaCl2, 1% PBS] and then resuspended in suspension solution. Suspension was filtered through a mesh capped tube. FACS sorting was performed with a flow cytometer sorter (BD AriaII). GFP positive and negative cells were sorted out.

In vitro measurement of telomerase activity

The TRAPEze® telomerase detection kit (Millipore) was used according to the manufacturer’s instructions. The PCR products were separated with 10% polyacrylamide gels and visualized by ethidium bromide staining.

Analysis of RNA processing

RNA was isolated from 30 embryos at different stages (24 hpf, 48 hpf and 3 dpf) using Trizol (Invitrogen, Carlsbad, CA, USA).

Materials and Methods

Ethics Statement

All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Peking University. The reference from IACUC of Peking University is LSC-ZhangB-1.

Zebrafish Lines

Zebrafish (Danio rerio) were raised and maintained under standard laboratory conditions [32,33]. Wild type fish lines were AB or Tübingen (TU). Nola1 mutant fish line was identified from an insertional retrovirus mutagenesis screen [20].

Microinjection of morpholino oligonucleotides and mRNAs

Sequence of splicing dclk1 MO (Gene Tools, Corvallis, OR, USA): 5'-GGTAAATCGACCTACGGTGCA-3'. P35 ATG MO was described previously [51]. Sequences of primers used for detecting the knockdown of dclk1 in morphants were: dclk1/RTF1: 5'-GAACATCAGGACGGCTCATT-3' and dclk1/RTR1: 5'-ATGCCCACTATCTTGTGC-3'.

To make mRNA, coding sequences of GARI and nola1 were PCR amplified and cloned into the pCS2+ vector. mRNA were synthesized by in vitro transcription using mMESSAGE mMACHINE® SP6 Kit (Ambion) according to the manufacturer’s instructions. Sequences of primers for cloning the coding sequences of GARI and nola1 were: GARI/RTF1: 5'-GGGATCCCGAGGGTTCAGCCGAGGAA-3' and GARI/RTR: 5'-CCGTCGAGGAGAGGACCCACCCAGTTTGGTGGC-3'; nola1/RTF2: 5'-GGGATCCAGGGTTCAGCCGAGGAA-3' and nola1 RTTR2: 5'-CGGCGTCGAGTCCATGGTGGAGGATC-3'.

DNA isolation, reverse transcription and Real Time PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from 30–40 embryos according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription using 2 ug RNA with Oligo(dT)12-18 (Invitrogen, Carlsbad, CA, USA). Real Time PCR was conducted using FastStart Universal SYBR Green Master (Roche, Diagnostics, Indianapolis, IN, USA) and a MyiQ Single-Color PCR thermal cycler (BioRad, Hercules, CA, USA). Each experiment was performed in triplicate and was repeated three times. Real Time PCR primers used for p53 signaling pathway were described previously [36]. Sequences of Real Time PCR primers for dclk1 and nola1 were: dclk1/RTF1: 5'-GAACATCAGGACGGCTCATT-3' and dclk1/RTR1: 5'-ATGCCCACTATCTTGTGGC-3'; nola1 RTF1: 5'-AGGAGGAGTTGATATCAGAGTCA-3' and nola1 RTR1: 5'-CCCATGCGGCTGACTATTTCCACAT-3'. Dclk1 and nola1 mRNA expression were normalized to β-actin using the method described previously [37] and compared to wild-type siblings.

Whole-mount o-dianisidine staining

Hemoglobin was detected by whole-mount o-dianisidine staining as described previously [36]. Briefly, 25 embryos under anesthesia were stained in o-dianisidine solution [0.6 mg/ml o-dianisidine, 0.01 M sodium acetate (pH 4.3), 0.65% hydrogen peroxide, 40% ethanol] for 15 min in the dark followed by two washes with PBS.
and cleaned up with Qiagen RNaseasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. RNA samples were analyzed using Agilent RNA 6000 Nano Kit (Agilent, Waldbronn, Germany) according to the manufacturer’s instructions.

Northern blotting analysis

Total RNA was isolated from 50 embryos at 48 hpf for dclk1 or 3 dpf for nola1 using Trizol (Invitrogen, Carlsbad, CA, USA) and cleaned up with Qiagen RNaseasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Northern blots were performed as previously described [39]. Total RNA (1.0 μg per lane) was separated by electrophoresis on a 2% formaldehyde, 1.2% agarose gel and blotted onto a nylon membrane (Hybond-N, Amersham Biosciences). RNA blots were cross linked to the membrane by UV irradiation and probed with DIG labeled ITS1, ITS2 and 18 S rRNA probes using prehybridization and hybridization solution (Roche, Diagnostics, Indianapolis, IN, USA) at 65 °C. The intensity of staining relative to the background was measured using ImageJ program.

Supporting Information

**Figure S1** Temporal and spatial expression pattern of nola1. (A) Semi-quantitative PCR result showed that nola1 was expressed from about 2 hpf. (B) Non-specific expression of nola1 was detected during the early stage of zebrafish development (a, b). Afterward, nola1 expression was mainly detected in the brain and some inner organs. a: nola1 expression at 7 hpf; b: nola1 expression at 10 hpf; c and d: nola1 expression at 4 dpf. a, b: lateral view; c: lateral view with anterior to the left; d: dorsal view with anterior to the left. (TIF)

**Figure S2** Nola1 is highly conserved among different species. (A) Analysis result using Clustal W software showed protein sequences of nola1 from different species have high similarity. (B) Synteny analysis data show the evolutionary conservation of nola1. (C) Microinjection of GAR1 mRNA can partially rescue the mutant phenotype of nola1 homozygous mutants at 5 dpf (red arrow and arrowhead in a, b and c). a: wild type sibling; b: nola1 homozygous mutant; c: nola1 homozygous mutant injected with human GAR1 mRNA. All of the pictures of embryos are lateral view with anterior to the left. (TIF)

**Figure S3** Rescue of hematopoietic defects of nola1 mutant in p53 mutant background. (A) p53+/−/nola1+/−; (B) p53+/−/nola1−/−; (C) p53−/−/nola1−/−. Number of HSC (marked by c-myc, red arrows) was partially rescued in nola1 and p53 double mutant at 3 dpf. (TIF)

**Figure S4** Deficiencies of both dclk1 and nola1 lead to the defects of rRNA processing. (A) Schematic figure modified from previous report [29] shows the overview of rRNA processing. B: A significant decrease of a precursor strand generating ITS1 and 18 S rRNA was detected in the lanes probed with ITS1 with both dclk1 morphant and nola1 mutant compared with wild type controls (arrows in a and b). The total amount of 18 S rRNA was reduced significantly as shown in the lanes probed with 18 S rRNA probe. The intensity of staining of 18 S rRNA relative to the background was measured using ImageJ program. In contrast, no obvious difference was detected in the lanes probed with ITS2 probe. (TIF)

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Author Contributions

Conceived and designed the experiments: YZ BZ SL. Performed the experiments: YZ BZ SL. Analyzed the data: YZ BZ SL. Contributed reagents/materials/analysis tools: YZ ND. Wrote the paper: YZ KM ND BZ SL.

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