

## RED CELLS, IRON, AND ERYTHROPOIESIS

Elavl1a regulates zebrafish erythropoiesis via posttranscriptional control of *gata1*Xi Li,<sup>1,2</sup> Yi-Chien Lu,<sup>1</sup> Kezhi Dai,<sup>1</sup> Ingrid Torregroza,<sup>2</sup> Timothy Hla,<sup>1</sup> and Todd Evans<sup>2</sup><sup>1</sup>Center for Vascular Biology, Department of Pathology and Laboratory Medicine, and <sup>2</sup>Department of Surgery, Weill Cornell Medical College, Cornell University, New York, NY

## Key Points

- Elavl1 is a protein that binds to the 3'-UTR of RNA encoding *Gata1*.
- Elavl1 controls embryonic erythropoiesis by enhancing expression of *Gata1*.

The RNA-binding protein Elavl1 (also known as HuR) regulates gene expression at the posttranscriptional level. Early embryonic lethality of the mouse knockout challenges investigation into hematopoietic functions for Elavl1. We identified 2 zebrafish *elavl1* genes, designated *elavl1a* (the predominant isoform during embryogenesis) and *elavl1b*. Knockdown of Elavl1a using specific morpholinos resulted in a striking loss of primitive embryonic erythropoiesis. Transcript levels for early hematopoietic regulatory genes including *lmo2* and *scl* are unaltered, but levels of *gata1* transcripts, encoding a key erythroid transcription factor, are significantly reduced in *elavl1a* morphants. Other mesoderm markers are mostly unchanged by depletion of Elavl1a. The 3'-untranslated

region (UTR) of *gata1* contains putative Elavl1a-binding sites that support robust expression levels when fused to a transfected luciferase reporter gene, and Elavl1a binds the *gata1* 3'-UTR sequences in a manner dependent on these sites. Moreover, expression of a transgenic reporter specifically in developing embryonic erythroid cells is enhanced by addition of the *gata1* 3'UTR with intact Elavl1-binding sites. Injection of *gata1* messenger RNA partially rescues the erythropoiesis defect caused by Elavl1 knockdown. Our study reveals a posttranscriptional regulatory mechanism by which RNA-binding protein Elavl1a regulates embryonic erythropoiesis by maintaining appropriate levels of *gata1* expression. (*Blood*. 2014;123(9):1384-1392)

## Introduction

During vertebrate embryogenesis, 2 sequential waves of hematopoiesis occur; they are termed primitive and definitive. In zebrafish, primitive erythropoiesis initiates in the intermediate cell mass in posterior lateral mesoderm, giving rise to erythrocytes and macrophages before 1 day postfertilization (dpf), followed by definitive hematopoiesis when stem/progenitors are produced from hemogenic endothelium in the ventral wall of the dorsal aorta. At the time of primitive erythroid cell generation, lymphocytes and granulocytes arise from an anterior lateral mesoderm domain. After 2 dpf, a major niche for hematopoiesis is present in caudal hematopoietic tissue in the tail. Eventually, the kidney serves as the major site of hematopoiesis through adulthood in the zebrafish.<sup>1</sup>

*Gata1* is a key transcription factor that is essential for blood cell development. In addition to controlling cell survival and proliferation, *Gata1* directly binds to and activates expression of erythroid-specific globin genes, the erythropoietin receptor, and its own promoter.<sup>2-4</sup> Knockout of *Gata1* in the mouse leads to embryonic lethality due to severe yolk sac anemia, consistent with a loss of primitive erythropoiesis.<sup>5</sup> A zebrafish *gata1* mutant has normal expression levels for markers of hematopoietic stem/progenitor, myeloid, and lymphoid cells, but a complete loss of erythroid gene expression through 24 hours postfertilization (hpf).<sup>6</sup>

RNA-binding proteins (RBPs) are recognized to play key regulatory roles in development, including hematopoiesis. Several RBPs bind AU-rich elements (AREs) in the 3'-untranslated region

(UTR), which are present in ~5% to 8% of mammalian transcripts.<sup>7</sup> Several distinct RBPs have been identified, including ARE-binding factor-1 (AUF1), embryonic lethal and abnormal vision (ELAV) family members, ZFP36 family members, and KH-type splicing regulatory protein (KSRP). AUF1 has 4 distinct isoforms (p37, p40, p42, and p45) and regulates many genes that are involved in hematopoiesis, for example, *Bcl2* and *Il8*.<sup>8,9</sup> Knockout of all 4 AUF1 isoforms in mice leads to reduced numbers of splenic T and B cells, suggesting that AUF1 has an important role in lymphopoiesis.<sup>10</sup> KSRP can also regulate many hematopoietic target genes, including *Cxcl2*, *Cxcl3*, *Il6*, and *Tlr4*.<sup>11</sup> Recently, analysis of *Zfp36L2* knockout mice showed the critical role of this RBP in self-renewal of erythroid progenitors.<sup>12</sup> These studies indicate that posttranscriptional gene regulatory mechanisms are important in hematopoiesis.

The ELAV family contains 4 members, the ubiquitously expressed *Elavl1* (HuR, HuA), and neuronal *Elavl2* (HuB), *Elavl3* (HuC), and *Elavl4* (HuD). Recently, ELAVL4 was also shown to be expressed in pancreatic  $\beta$  cells and to regulate the translation of insulin.<sup>13</sup> *Elavl1* has been shown to play an essential role in embryonic development and stem/progenitor cell survival in adult mice.<sup>14,15</sup> Hematopoietic and intestinal progenitors were significantly depleted in *Elavl1*<sup>-/-</sup> conditionally deleted mice due to a p53-dependent cell death program.<sup>14</sup> During embryonic development, *Elavl1*<sup>-/-</sup> embryos appeared normal at day 8.5; however, embryos could not survive beyond embryonic day 14.5 (E14.5).<sup>15</sup> At E10.5 to E12.5,

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knockout embryos exhibited defects in placental development. We showed recently that knockdown of *elavl1* during zebrafish embryogenesis generates defects in angiogenesis of the subintestinal vein plexus, consistent with its role in regulation of vascular endothelial growth factor-A expression.<sup>16</sup> However, the function of *elavl1* in embryonic hematopoietic development has not been characterized, in part due to the early embryonic lethality of the mouse knockout. Here, we used the zebrafish model to evaluate functional roles of *elavl1* during embryonic blood development. We report that *elavl1* regulates *gata1* expression at the posttranscriptional level and thereby promotes embryonic erythropoiesis.

## Materials and methods

### Zebrafish maintenance

Wild-type and *p53*<sup>-/-</sup> mutant zebrafish<sup>17</sup> were maintained at 28.5°C. Embryos were collected from adult fish after natural matings and staged as described.<sup>18</sup> All animal studies were approved by the Weill Cornell Medical College Animal Care and Use Committee. Morpholinos were injected at the 1-2 cell stage and were either translation-blocking (Mo1: 5'-TGTGGTCTT CGTAACCGTTCGACAT) or splice-blocking (Mo2: 5'-AGAGCACCTTA TGTCACATTACCTT).

### Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using single-stranded RNA probes labeled with digoxigenin-uridine 5-triphosphate (Roche) essentially as described.<sup>19</sup> The *gata1*, *lmo2*, *scl*, *myod*, *nkx2.5*, and *pax2a* riboprobes were synthesized and hybridized as described.<sup>20</sup> Briefly, fixed and staged embryos were washed in phosphate-buffered saline with Tween 20 for 5 minutes and prehybridized for 3 hours, followed by hybridization with the indicated probe overnight. After a series of stringent washes, embryos were blocked in 2% bovine serum albumin, 1% fetal bovine serum saline buffer, incubated with alkaline phosphatase-conjugated antibody overnight, and stained with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) substrate (Roche).

### Cell culture

The mouse erythroleukemia (MEL), HEK293, and HEK293T cell lines were maintained in Dulbecco modified Eagle growth medium containing high glucose (4.5 g/L) (Sigma-Aldrich), L-glutamine, 10% FBS, and 1× antibiotics (Invitrogen). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### RT-PCR and quantitative real-time PCR

First-strand complementary DNA (cDNA) synthesis was performed using the Omniscript Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Qiagen). Primers (forward: 5'-GAGTCTTTCAGCA GCATTGG; reverse: 5'-CCTGATGCCTGATCAAC) were used for amplification of total *elavl1a* and *elavl1b* cDNA. For quantitative RT-PCR (qPCR), the cDNA was mixed with SYBR Green Mix (Quanta) and product measured using an ABI 7500 real-time PCR machine (Applied Biosystems). Each sample was prepared in triplicate, and all experiments were repeated 3 times. The cycle threshold values were determined using the 2<sup>ΔΔT</sup> method.<sup>21</sup>

### Whole-mount immunohistochemistry

Zebrafish embryos were fixed overnight in 4% paraformaldehyde and processed for whole-mount immunohistochemistry with anti-ELAVL1 antibody (Clontech), and then incubated with horseradish peroxidase-conjugated secondary antibody (KPL). Bright-field images were captured using a Zeiss AxioScope2 microscope with an AxioCam digital camera or with a dissecting microscope (Olympus).

### Western blotting

Zebrafish embryos were deyolked or directly lysed in radioimmunoprecipitation assay buffer on ice for at least 30 minutes. Protein concentrations of

each sample were measured using a BCA kit (Thermo Scientific). Protein (15 μg) was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Protein was transferred to polyvinylidene difluoride membranes (Millipore) and immunoblotted with anti-human ELAVL1 monoclonal antibody (1:5000; Clontech or Santa Cruz Biotechnology), anti-β-Actin monoclonal antibody (1:5000; Sigma-Aldrich), or anti-human GATA1 polyclonal antibody (1:5000; Abcam) for at least 1 hour at room temperature or 4°C overnight. Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (KPL) was added and signal developed in enhanced chemiluminescence solution (Millipore).

### Immunoprecipitation

Cell lysates were precleared with Sepharose beads (GE Healthcare) and incubated with anti-ELAVL1 antibody (Clontech or Santa Cruz Biotechnology)-conjugated agarose beads overnight. The beads were washed twice with 0.1% Triton X-100 in phosphate-buffered saline, then boiled with sample loading buffer for western blotting. The binding of Elavl1 with *gata1* messenger RNA (mRNA) was measured by immunoprecipitation followed by qPCR as described.<sup>22</sup> Transfected HEK293 cells or MEL cells were lysed with polysome lysis buffer (100 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.5% Nonidet P-40, 10 μM dithiothreitol) supplemented with RNase and protease inhibitors. The supernatant was incubated overnight at 4°C with no antibody, control immunoglobulin G (IgG), or anti-Elavl1 monoclonal antibody (Clontech or Santa Cruz Biotechnology) and then incubated with protein G Dynabeads (25 μL) for 4 hours at 4°C. After washing 5 times, the pellet was treated with 10 units of DNase I (Promega) in 100 μL of buffer for 10 minutes at 37°C, and then treated with 5 μg of proteinase K (Roche) in 100 μL of buffer for 30 minutes at 55°C. The supernatant was collected and RNA extracted using acid phenol chloroform (Ambion). Extracted RNA was subjected to qPCR analysis.

### Luciferase reporter assays

PCR products of mouse *Gata1* or zebrafish *gata1* 3'-UTR fragments were amplified from full-length cDNA constructs (OpenBiosystems) and then subcloned into the pmir-glo vector (Promega), a dual luciferase (Firefly and Renilla) reporter. Mutations were introduced in the predicted Elavl1-binding sites using the QuickChange II kit (Agilent) using the following primers: site I, 5'-GTAAATTATGTTGTACACACGCGCCGCGCCCTCAATA AATGCATCC and 5'-GGATGCATTTATTGAGGGCGCCGGCGCGTG TGTACAACATAATTTAC; site II, 5'-CTCAATAAATGCATCCCGTC CTGCGCGCTTAAAAGAGATGCATTAAGC and 5'-GCTTTAATG CATCTCTTTCAACGCGCGCAGGACGGGATGCATTTATTGAG; site III, 5'-GAAACTGTGAGCTATATCGCGCGAATGTAAATATTGATG and 5'-CATCAATTTACATTCGCGGATATAGCTCACAGTTTC. Vectors, with or without *elavl1a* morpholino (MO), were injected into 1-cell stage embryos. For some experiments, the reporter was placed under regulation of the 5-kb upstream *gata1* promoter sequence. After 24 hours, 10 to 20 embryos were collected as 1 sample and luciferase activity was measured in lysates using a luminometer (Promega). Firefly luciferase values were normalized with *Renilla* luciferase values and for each experiment "n" was at least 3 samples. At least 3 replicates were tested per experiment.

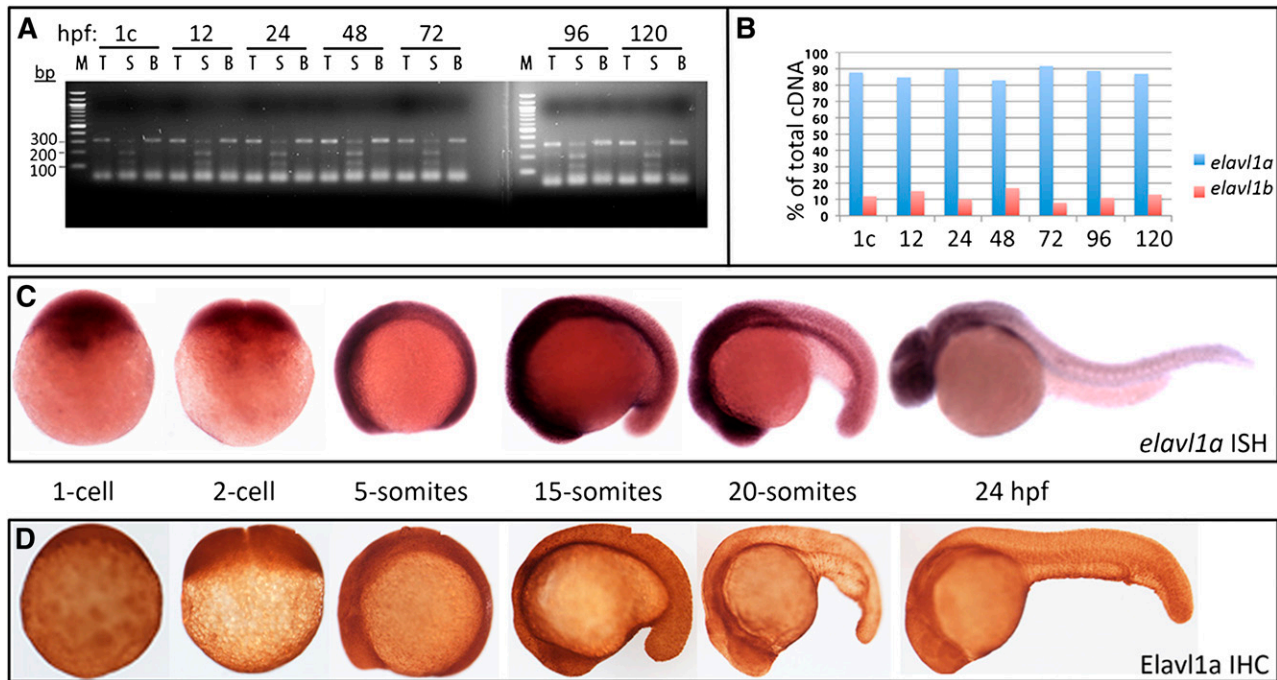
### Statistics

Data are expressed as means ± SEM. Statistical significance was determined by the 2-tailed unpaired Student *t* test or  $\chi^2$  test with Prism software. For the rescue experiment in Figure 2, the statistical analysis used each of the 4 criteria as a degree of freedom in  $\chi^2$  analysis. Thus, we did not require an embryo to be fully rescued along all 4 characteristics, and an embryo rescued for even 1 of the characteristics was scored as a rescue.

## Results

### Elavl1a regulates zebrafish embryonic development

ELAVL1 is highly conserved in vertebrates, and by protein-homology comparison in the ENSEMBL database, 2 zebrafish Elavl1 orthologs



**Figure 1. *Elavl1a* is the major isoform expressed during zebrafish embryogenesis.** (A) A 322-bp fragment of cDNA derived from both *elavl1a* and *elavl1b* mRNA (total, T) was amplified from embryos harvested from the 1-cell (1c) stage to 120 hpf embryos, as indicated, and digested by Sca1 (S) or Bfa1 (B) restriction enzymes, which recognize polymorphisms specific to each gene. The *elavl1a* cDNA is cut into 120- and 200-bp fragments by Sca1, whereas *elavl1b* cDNA is cut into 100-bp and 220-bp fragments by Bfa1. (B) Quantification of *elavl1a* and *elavl1b* relative transcript abundance according to the data in panel A. (C) Representative embryos are shown at different embryonic stages as indicated, following in situ hybridization with the *elavl1a* antisense probe. No signal was detected using a control sense probe (not shown). (D) Representative embryos are shown following immunohistochemistry of Elavl1 protein at approximately the same stages. All are lateral views, other than 1- to 2-cell stages with anterior to the left. For both ISH and IHC,  $n > 20$  embryos per sample. IHC, immunohistochemistry; ISH, in situ hybridization.

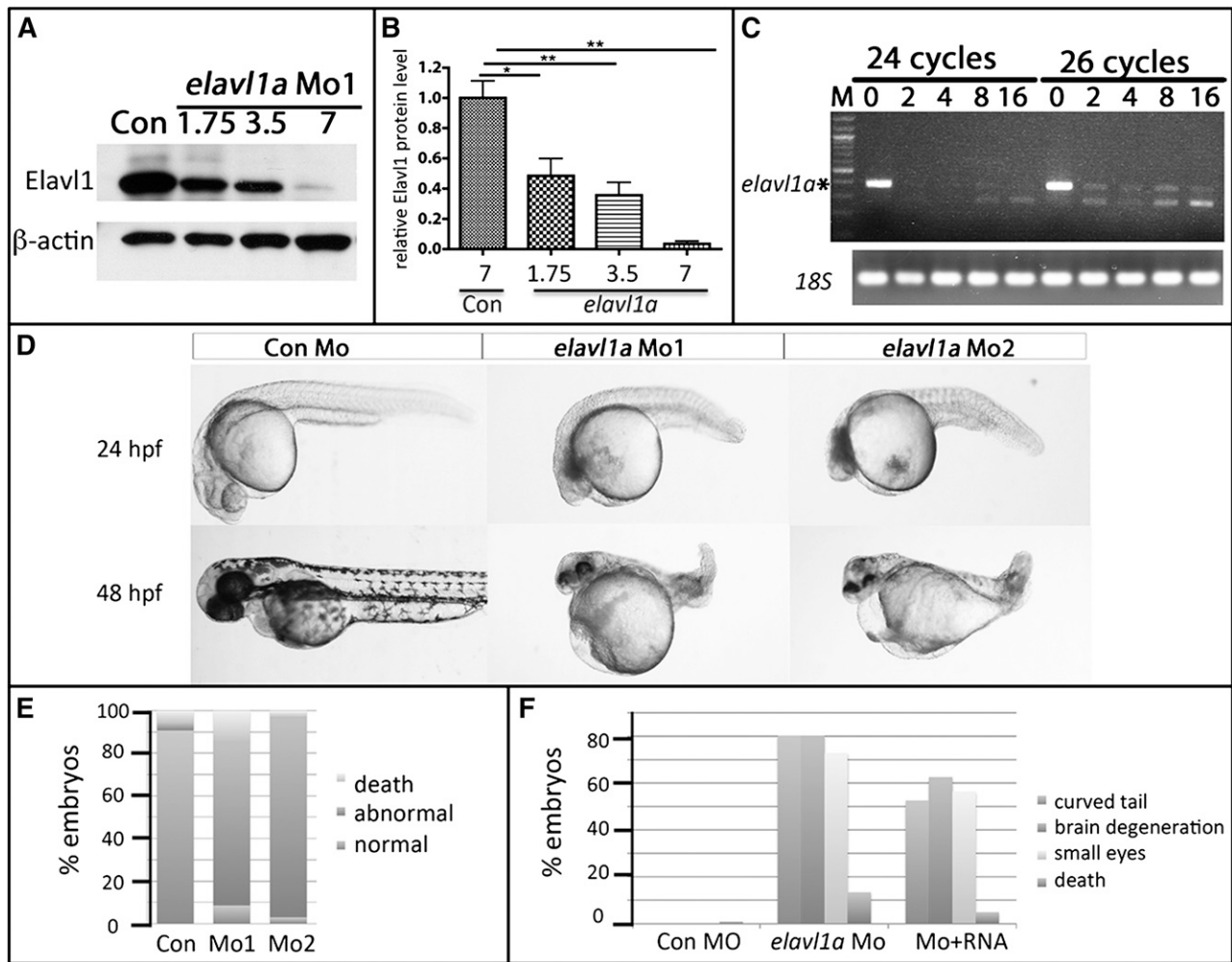
were clearly identified (supplemental Figure 1, available on the *Blood* Web site), encoded by genes we designated *elavl1a* and *elavl1b*. Predicted protein sequence analysis showed that both orthologs are highly related, with Elavl1a and Elavl1b 85% and 82% similar to the human protein, respectively. Genomic structures of the zebrafish *elavl1a* and human ELAVL1 genes also show high conservation (supplemental Figure 2). Like the human gene, zebrafish *elavl1a* has 6 exons, with the coding region starting within exon 2, and lengths of exons 3 to 5 are identical (exon 3: 104 bp, exon 4: 154 bp, exon 5: 226 bp). In contrast, zebrafish *elavl1b* is encoded by 8 exons, with the open reading frame initiating from exon 4. Therefore, zebrafish *elavl1a* is more similar to human ELAVL1. The zebrafish genome contains distinct (single copy) genes for *elavl2*, *elavl3*, and *elavl4* that are also highly conserved among vertebrates.

We quantified the relative transcript levels derived from each of the 2 zebrafish *elavl1* genes during embryogenesis, by designing a single pair of PCR primers that amplify cDNA derived from both genes. Restriction enzyme polymorphisms were then used to distinguish the relative contribution of each gene to the total cDNA pool.<sup>23</sup> Semiquantitative RT-PCR experiments demonstrated that *elavl1a* represents the major expressed isoform, constituting 80% to 90% of total *elavl1* transcripts throughout early embryogenesis, from the 1-cell stage until 120 hpf (Figure 1A-B). Given that *elavl1a* has a more conserved genomic structure and predominates for expression during embryonic development, we focused our functional studies on this gene. In situ hybridization experiments confirmed that *elavl1a* transcripts are deposited maternally and expressed widely during early stages of embryogenesis (Figure 1C).

When the zebrafish protein was expressed in HEK293 cells, monoclonal anti-ELAVL1 antibodies generated against the human

protein specifically cross-reacted with the zebrafish Elavl1a protein, according to western blotting analysis (supplemental Figure 3A). Using this antibody, whole-mount immunostaining was performed with zebrafish embryos, which confirmed that Elavl1 is expressed maternally and then ubiquitously through 120 hpf of development (Figure 1D). There was general concordance between RNA and protein expression patterns.

To determine whether *elavl1a* plays a role in zebrafish embryonic development, 2 MOs were designed and validated to specifically target the *elavl1a* gene including a translational blocking MO (Mo1) and a splice-blocking MO (Mo2), as indicated in supplemental Figure 2A. Using the cross-reacting antibody, we confirmed the efficacy of both Mo1 and Mo2, finding that 7 to 8 ng effectively eliminates the targeted protein expression, while 3.5 ng reduces expression levels to around 40% of controls (Figure 2A-B). Mo2 effectively blocked the generation of properly spliced RNA product (Figure 2C). To avoid potential off-targeting effects using higher concentrations, we used 4 ng of MO for subsequent experiments, which reproducibly achieves a knockdown of >50%. Our previous study<sup>16</sup> reported angiogenesis phenotypes at 72 hpf. Here, we show that at 24 hpf, embryos injected with 4 ng of *elavl1* Mo1 (373 of 408 embryos, 91%) or Mo2 (130 of 134 embryos, 97%) showed a general developmental delay, curved tails, neural defects including small eyes, and pericardial edema; the fish failed to survive beyond 5 dpf (Figure 2D-E). As a further test for whether these phenotypes were specifically induced by loss of Elavl1, mRNA encoding mouse ELAVL1, which does not contain the MO-binding site (75 pg per embryo), was coinjected with 4 ng of *elavl1a* Mo1, and this partially rescued defective development ( $\chi^2$ ,  $P < .001$ , Figure 2F). The embryos were never rescued for all aspects of development, although



**Figure 2. Knockdown of Elavl1a disrupts normal embryonic development.** (A) Representative western blotting analysis of lysates derived from zebrafish embryos injected with 7 ng of control MO (Con) or those injected with increasing doses of *elavl1a* Mo1. Cell lysates were prepared at 24 hpf. (B) Quantification of *elavl1a* Mo1 knockdown efficiency based on western blotting data; results were averaged from 3 independent experiments. Error bars represent SD. \* $P < .05$ ; \*\* $P < .001$ . (C) Semiquantitative RT-PCR demonstrates efficacy of *elavl1a* splicing MO (Mo2) knockdown. Amplifications were for 24 or 26 cycles. 18S RNA was measured as control. (D) Embryos that had been injected at the 1-cell stage with 4 ng of control MO are normal, whereas those injected with either Mo1 or Mo2 are defective and grossly similar at 24 hpf and 48 hpf. (E) Quantification of embryos showing characteristic developmental defects (as in panel D) caused by either of the 2 MOs. (F) Quantification of embryonic defects in embryos injected with control (Con) MO, *elavl1a* MO, or coinjected with *elavl1a* MO and murine *Elavl1* mRNA. (B-F) The results are compiled from several independent experiments with  $n > 150$ .

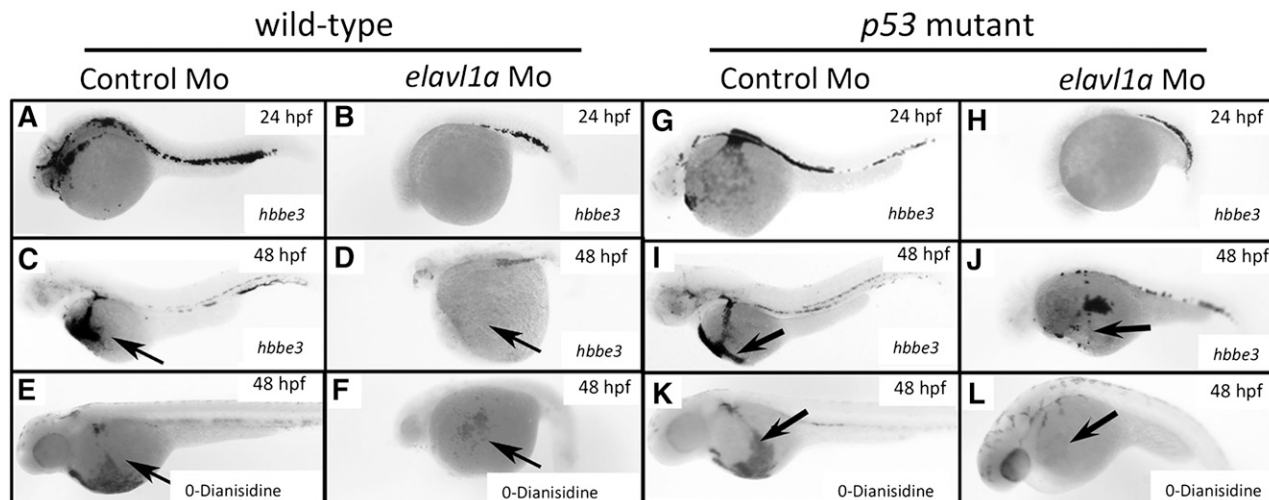
this could be due to technical challenges in the rescue assay. These experiments indicate that *elavl1a* is essential for early zebrafish development.

#### Elavl1a regulates erythropoiesis

Visual inspection suggested that blood cells were markedly reduced or absent in the circulation of *elavl1a* morphants. We therefore evaluated hematopoiesis in these embryos. Indeed, the morphants had significantly decreased levels of globin transcripts and hemoglobin as shown by in situ hybridization for *hbbe3* at 24 or 48 hpf and o-Dianisidine staining for hemoglobin at 48 hpf (Figure 3A-F). The difference was more pronounced at 48 hpf in the morphant, indicating that the defect in erythropoiesis was not due simply to developmental delay. Morphants coinjected with *elavl1a* mRNA were also stained for globin transcripts and hemoglobin at 48 hpf, as shown by representative samples in supplemental Figure 4. While few of the MO-injected embryos showed substantial staining, this was increased to 55% or 83% of the embryos (for hemoglobin or globin RNA, respectively) when *elavl1a* mRNA was coinjected.

We used a *gatal:dsRed* transgenic line to purify erythroid cells by flow cytometry from batches of wild-type or morphant embryos, followed by cytopsin to evaluate blood morphology. Erythroid cells, which are far fewer in the morphant embryos, appear relatively large and immature compared with wild type, as shown by representative images in supplemental Figure 5.

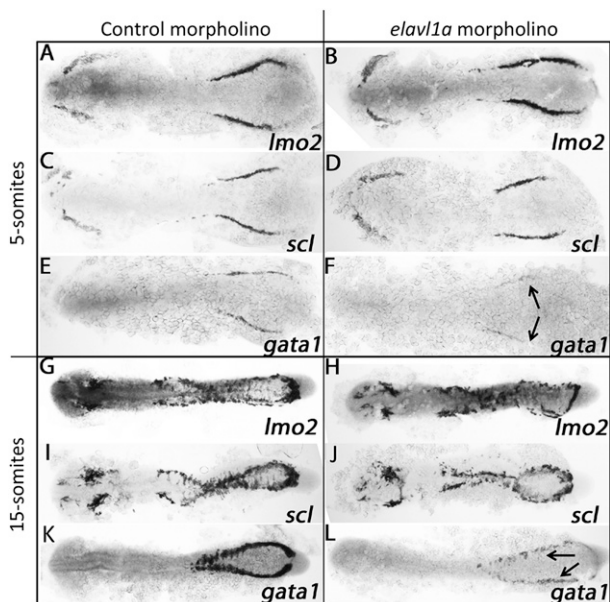
ELAVL1 can regulate p53 expression following UV irradiation by direct association with the p53 3'-UTR region, by regulating p-phosphorylated von Hippel-Lindau tumor suppressor protein, or indirectly by stabilizing MDM2, a p53 protein E3 ligase.<sup>14,24,25</sup> Therefore, we tested whether the loss of *hbbe3* signal was caused by p53-induced apoptosis. A similar loss in *hbbe3* transcripts at 24 or 48 hpf, as well as the absence of o-Dianisidine staining at 48 hpf, was found following *elavl1a* knockdown in p53<sup>-/-</sup> mutant embryos, although brain degeneration and eye phenotypes were improved (Figure 3G-L). In addition to the erythroid deficit, the pericardial edema phenotype was not rescued in p53<sup>-/-</sup> mutants. Thus, the erythropoiesis defect occurs independently of p53, suggesting that *elavl1a* specifically regulates embryonic erythropoiesis during zebrafish development.



**Figure 3. Erythropoiesis is defective in *elavl1a* morphants, regardless of *p53* status.** Whole-mount in situ hybridization was performed detecting *hbbe3* transcripts at 24 hpf (A-B) or 48 hpf (C-D). Shown are representative embryos injected with *elavl1a* MO that displayed reduced *hbbe3* transcript levels (27 of 33), compared with normal controls (38 of 40). (E-F) o-Dianisidine staining was used to detect hemoglobinized cells at 48 hpf. The representative *elavl1a* morphants show strikingly reduced hemoglobin staining (40 of 46), compared with normal embryos injected with control MO (60 of 60). (G-L) The same analyses were carried out using *p53*<sup>-/-</sup> embryos. All embryos are oriented lateral with anterior to the left.

### Elavl1a regulates *gata1* expression

To investigate the molecular basis for the erythropoiesis defect following Elavl1a depletion, the expression of various hematopoietic regulatory genes was evaluated by in situ hybridization. At the 5-somite stage, expression of the stem/progenitor markers *lmo2* and *scl* appear normal in control and *elavl1a* morphants (Figure 4A-D), whereas levels of the erythroid *gata1* transcript are decreased (Figure 4E-F). This loss of *gata1* transcript levels was still evident at the 15-somite stage, while transcripts for *lmo2* and *scl* are similar in

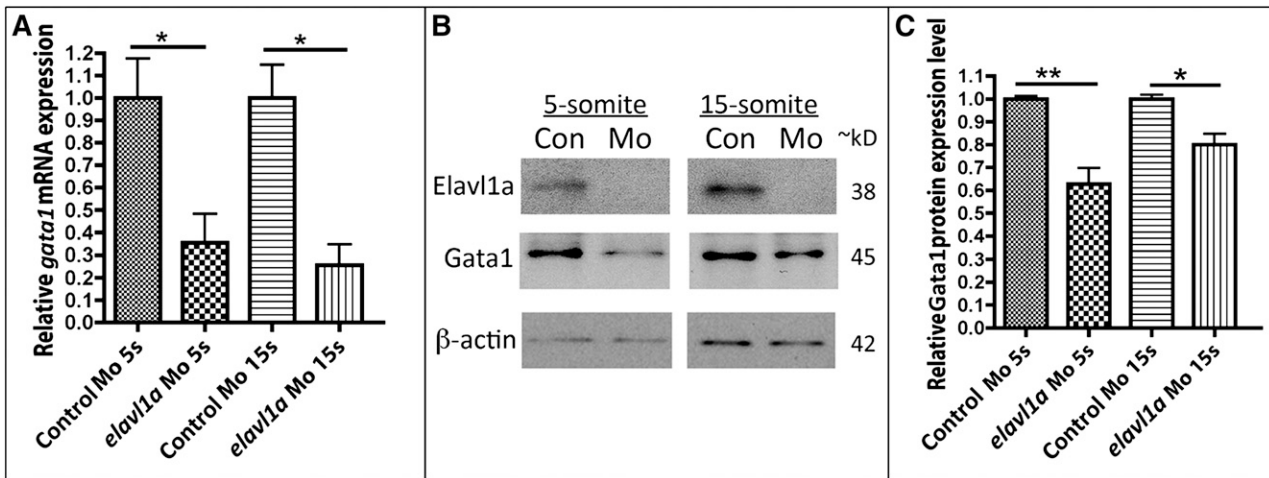


**Figure 4. Elavl1a regulates *gata1* transcript levels.** Shown are representative embryos following in situ hybridization for hematopoietic markers at the 5-somite (A-F) or 15-somite (G-L) stages in embryos that had been injected with 4 ng of control MO (left panels) or *elavl1a* Mo1 (right panels). Transcripts were analyzed for *lmo2*, *scl*, and *gata1* as indicated, and embryos were flat-mounted, shown dorsal with anterior to the left. For each sample,  $n > 20$ , and the loss of *gata1* transcripts (indicated by the arrows) was reproducible in independent experiments.

control and *elavl1a* morphants (Figure 4G-L). qPCR analysis confirmed the overall reduction in *gata1* mRNA levels of *elavl1* morphants at both 5- and 15-somite stages of development (Figure 5A). Therefore, *gata1* mRNA levels are specifically decreased during stages of primitive erythropoiesis following depletion of Elavl1a, while other hematopoietic regulatory genes including *lmo2* and *scl* are relatively unaffected. We also examined the expression levels of Gata1 protein. Anti-human GATA1 polyclonal antibody recognized zebrafish Gata1 protein expressed in HEK293 cells (supplemental Figure 3A). In *elavl1a* morphants, the Gata1 protein levels are reduced at both 5- and 15-somite stages, although less so than for the RNA, compared with control morphants ( $P < .05$ , Figure 5B-C). The western blots are less quantitative compared with qPCR data, and therefore might under-estimate the loss of Gata1 protein. However, if loss of Elavl1 leads primarily to a destabilization and loss of RNA (rather than deregulating translation) the Gata1 protein that is synthesized might be relatively stable up to these stages of development. These data indicate that *elavl1a* regulates the expression of *gata1*, an essential regulator of erythropoiesis during embryonic development.

### Elavl1a function in mesoderm is relatively restricted

We next tested whether the phenotype described in *elavl1a* morphants is specific to blood cells, or represents a more general defect in mesoderm lineage differentiation. For this purpose, whole-mount in situ hybridization experiments evaluated transcript levels for *myod* (somatic mesoderm and muscle), *ntl* (axial mesoderm and notochord), *pax2a* (lateral mesoderm and pronephros), and *nkx2.5* (anterior lateral mesoderm and cardiomyocytes). As shown in Figure 6A-L, *elavl1a* morphant embryos exhibit similar transcript patterns compared with the control embryos at the 5- or 15-somite stages for *myod*, *ntl*, and *pax2a*. Interestingly, *nkx2.5* (the cardiac mesoderm marker) is relatively decreased in the *elavl1a* morphants at the 15-somite stage (Figure 6M-N). The results suggest that knockdown of *elavl1a* does not cause a general defect in mesoderm development, but causes specific defects in expression of *gata1* and *nkx2.5*, which could explain phenotypes in erythropoiesis (*gata1*) and perhaps contribute to pericardial edema (*nkx2.5*).



**Figure 5. *Gata1* transcript and protein levels are reduced in *elavl1a* morphants.** (A) qPCR analysis was performed on embryos at the 5-somite (5s) or 15-somite (15s) stages (N = 3 experiments, \**P* < .05). (B) Representative western blotting experiment. (C) Quantification of *Gata1* protein levels, normalized to  $\beta$ -actin, following western blotting, averaged from n = 6 experiments. \**P* < .05; \*\**P* < .01. Error bars represent SD.

**Elavl1a binds *gata1* mRNA and promotes expression**

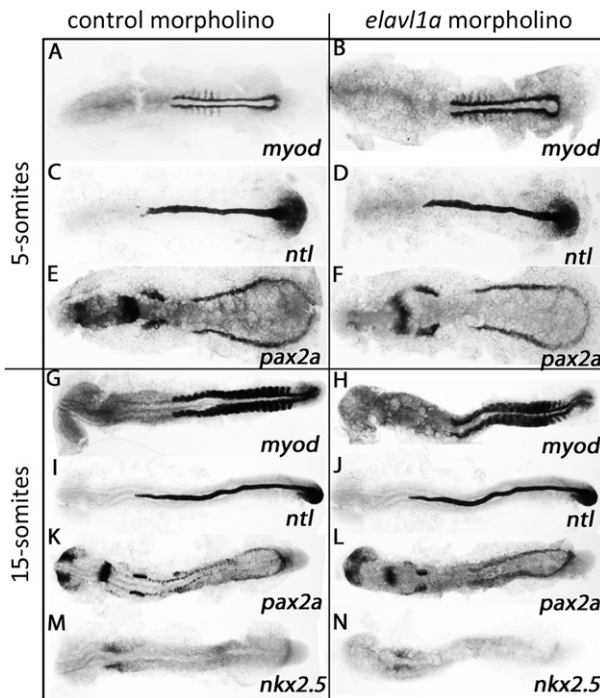
Elavl1 is known to positively regulate gene expression by binding to ARE elements in the 3'-UTR of target mRNAs.<sup>26</sup> Because *gata1* transcripts were significantly depleted in *elavl1a* morphant embryos, we analyzed the zebrafish *gata1* untranslated region and found 3 potential Elavl1-binding sites (supplemental Figure 6). We hypothesized that by binding to these sites Elavl1a could stabilize *gata1* transcript levels. Therefore, we tested whether Elavl1 can bind directly *gata1* 3'-UTR sequences by performing immunoprecipitation assays. Using HEK293 cell lysates as a positive control, monoclonal antibodies were shown to precipitate both human and

zebrafish versions of the Elavl1 protein (shown in supplemental Figure 3B). Therefore, the interaction between Elavl1 protein and *gata1* transcript was tested by RNA immunoprecipitation. When zebrafish Elavl1a and a transcript containing the *gata1* 3'-UTR sequences were expressed in HEK293 cells, the Elavl1a-specific antibody showed a 20-fold increase in RNA pull-down compared with the controls using IgG or no antibody (Figure 7A), suggesting that Elavl1a binds directly to the 3'-UTR of the *gata1* mRNA. The efficiency of pull-down was substantially decreased when the expressed RNA contained site-specific mutations in the putative Elavl1a-binding sites. These data indicate that Elavl1 binds to specific elements in the 3'-UTR of the *gata1* mRNA. Sequence analysis suggested that the mouse *Gata1* 3'-UTR contains 2 predicted Elavl1 predicted binding sites, and sequence alignment suggests that in zebrafish and mouse *Gata1* at least 1 of these 3'-UTR elements is conserved (supplemental Figure 4). Therefore, we hypothesized that murine *Gata1* RNA is also a direct target of mouse ELAVL1 and performed RNA immunoprecipitation assays using lysates from the MEL cell line. We found a ninefold enhanced precipitation of *Gata1* transcripts using anti-ELAVL1 antibody compared with controls using IgG or no antibody (Figure 7B).

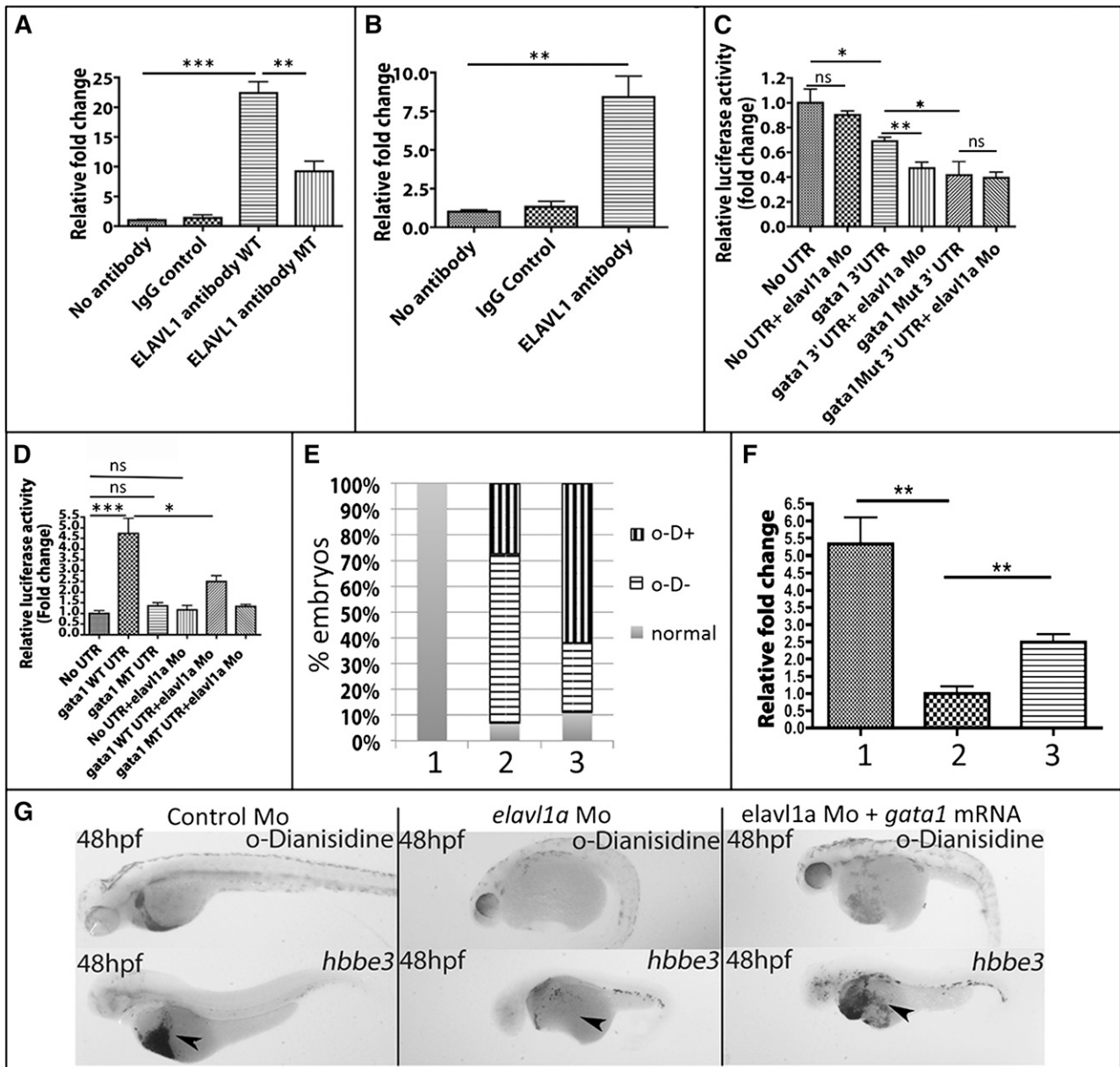
We next tested directly whether *cis* elements that promote Elavl1a binding affect *gata1* transcript function. For this purpose, wild-type or mutated versions of the zebrafish *gata1* 3'-UTR were fused downstream of a luciferase reporter gene and constructs were microinjected into developing embryos. Luciferase activity was quantified (normalized by activity of a control *Renilla* luciferase reporter in the same pmir-Glo vector) comparing control luciferase, the luciferase fused to wild-type *gata1* 3'-UTR, or the luciferase fused to the *gata1* UTR with putative Elavl1-binding sites mutated. As shown in Figure 7C, *elavl1* MOs strongly suppress *gata1* 3'-UTR-linked luciferase expression. Similar low levels of luciferase were found when the Elavl1-binding sites are mutated on the 3'-UTR (and are not further affected by inclusion of the MO). These data suggest that the binding of Elavl1a on the *gata1* 3'-UTR promotes the expression of this erythroid-specific transcription factor.

**Elavl1a regulates *gata1* expression and erythropoiesis in vivo**

To confirm that Elavl1a regulates *gata1* in erythroid progenitors, wild-type or mutated zebrafish *gata1* 3'-UTR sequences were fused downstream of the firefly luciferase reporter in transgenic constructs



**Figure 6. Mesoderm is not generally disturbed in *elavl1a* morphants.** (A-N) Shown are representative embryos following in situ hybridization for *myod*, *ntl*, *pax2a*, and *nkx2.5* at 5- or 15-somite stages in control or morphant embryos, as indicated. Embryos are flat mounted, viewed dorsally, anterior to the left. For each representative sample, n > 20.



**Figure 7. Elavl1 binds to the *gata1* transcript and Elavl1a-binding sites in the 3'-UTR affect gene expression.** (A) Association of Elavl1 protein and *gata1* transcript demonstrated by RNA immunoprecipitation. Transcripts including the *gata1* 3'-UTR (WT) or with the putative Elavl1a-binding sites mutated along with Elavl1a expression vectors were transfected into HEK293 cells. IP experiments used no antibody, IgG control antibody, or Elavl1a-specific antibodies, as indicated, to precipitate RNA, and relative levels of the *gata1* 3'-UTR in the precipitate were quantified by qPCR. Fold-change is shown normalized to the no-antibody control group.  $**P < .01$ ;  $***P < .001$ . (B) Lysates from MEL cells were likewise immunoprecipitated with ELAVL1-specific antibody and murine *Gata1* RNA quantified in the pull-down.  $**P < .01$ . (C) Reporter gene assay in zebrafish embryos. Luciferase reporters contained no UTR, zebrafish *gata1* 3'-UTR, or the mutated isoform 3'-UTR. Relative firefly luciferase activity was normalized by *Renilla* luciferase activity. All data are averaged from at least 3 experiments.  $*P < .05$ ;  $**P < .01$ . Error bars represent SD. (D) Erythroid lineage-specific reporter assay in zebrafish embryos. Embryos were injected with transgenes regulated by the *gata1* erythroid-specific promoter. Relative firefly luciferase activity was normalized by *Renilla* mRNA luciferase activity. All data are averaged from at least 3 experiments.  $*P < .05$ ;  $**P < .01$ . Error bars represent SD. (E) Quantification of phenotypes for embryos in each study group, including: lane 1, control embryos; lane 2, morphant embryos; or lane 3, morphant embryos, that were also injected with 12.5 ng of *gata1* mRNA, which can partially rescue the blood cell phenotype. Embryos were scored as relatively normal, strongly positive for o-Dianisidine-stained cells, even though the cells fail to circulate (o-D+) or were negative for o-Dianisidine (o-D-).  $\chi^2$ ,  $P < .001$ . (F) Results from qPCR experiments measuring *hbbe3* transcripts (in lane 1, control Mo-injected; lane 2, *elavl1a* Mo-injected; or lane 3, *elavl1a* Mo-injected embryos) that were coinjected with *gata1* mRNA. Transcript levels in control and RNA-injected morphants are plotted relative to those from *elavl1a* morphants;  $**P < .01$ . (G) Shown are representative 48 hpf embryos stained for hemoglobin (o-Dianisidine, top panels) or processed by in situ hybridization for globin transcripts (*hbbe3*, bottom panels). For each representative sample,  $n > 20$ . MT, mutated; ns, not significant.

with the reporters placed under control of the erythroid-specific *gata1* promoter, which restricts expression to the developing red cells in the embryonic intermediate cell mass.<sup>27-29</sup> Constructs were injected into zebrafish embryos and reporter activity was quantified at 24 hpf. The transgenic embryos containing the wild-type *gata1* 3'-UTR expressed significantly higher levels of luciferase activity compared

with transgenic embryos harboring the mutant construct. Furthermore, this increased luciferase activity was suppressed by knockdown of *elavl1a* by a coinjected MO (Figure 7D). To determine whether *gata1* is a critical gene targeted by Elavl1 affecting erythropoiesis, *gata1* mRNA was injected in *elavl1a* morphants. Indeed, 40% of the embryos showed a substantial increase in hemoglobin staining

at 48 hpf compared with uninjected morphants, ( $\chi^2$ ,  $P < .001$ ), and a partial but significant rescue of embryonic globin RNA expression according to qPCR and in situ hybridization experiments (Figure 7E-G).

## Discussion

Elavl1 is an RNA-binding protein that we find is well conserved between humans and zebrafish. Zebrafish Elavl1 is encoded by 2 genes, presumably duplicated and then retained in the fish genome. The predominant isoform expressed during embryogenesis is encoded by the *elavl1a* gene, with transcripts and protein found as maternal products and then widely throughout the developing embryo. We showed that knockdown of this gene has major effects on embryonic development, such that by 48 hpf, the body axis is much shorter and neural tissues are degenerated. The embryos also develop with a striking deficit of embryonic blood cells. While defects in hematopoiesis may be caused indirectly through early embryonic morphogenetic alterations, our data suggest phenotypes that are caused by direct deregulation of *gata1*, a key regulator of the erythroid lineage. In contrast to markers for other mesodermal derivatives, *gata1* transcript levels are decreased even as early as the 5-somite stage, and coinjection of *gata1* RNA is able to at least partially restore erythropoiesis to the *elavl1a* morphant embryos. The results indicate that altered *gata1* transcript levels caused by knockdown of Elavl1a protein are sufficient to deregulate embryonic erythropoiesis. At least some activity for Elavl1 appears to function through binding sites present in the *gata1* 3'-UTR. We did not find a difference for rescue, comparing the *gata1* mRNAs with wild-type or mutated 3'-UTR, but this might reflect technical challenges of the rescue assay, since overexpression with injected RNA could override the balance of regulatory mechanisms acting through the 3'-UTR.

In summary, the major finding of this study is that the Elavl1 RNA-binding protein can bind and stabilize *gata1* transcripts during embryonic erythropoiesis, as a posttranscriptional mechanism to enhance Gata1 expression and regulate red cell development.

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Embryos depleted of Elavl1a, which is the predominant isoform in zebrafish embryos, had specific defects in *gata1* transcript levels, Gata1 protein levels, and primitive embryonic erythropoiesis. Elavl1 can bind to the *gata1* 3'-UTR, dependent on AU-rich sites that match the Elavl1 consensus-binding sequence, and this feature appears to be conserved in the mouse gene. Our data show that posttranscriptional regulation of *gata1*, a key transcriptional regulatory factor for the erythroid lineage, provides a previously unrecognized post-transcriptional regulatory mechanism for embryonic erythropoiesis.

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## Authorship

Contribution: X.L. conceived the study, carried out experiments, analyzed data, and wrote the manuscript; Y.-C.L. and K.D. performed experiments; I.T. provided expert consultation for experiments and reagents; and T.H. and T.E. conceived the study, analyzed data, and wrote the manuscript.

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