The *Firre* locus produces a trans-acting RNA molecule that functions in hematopoiesis

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RNA has been classically known to play central roles in biology, including maintaining telomeres, protein synthesis, and in sex chromosome compensation. While thousands of long noncoding RNAs (lncRNAs) have been identified, attributing RNA-based roles to lncRNA loci requires assessing whether phenotype(s) could be due to DNA regulatory elements, transcription, or the lncRNA. Here, we use the conserved X chromosome lncRNA locus *Firre*, as a model to discriminate between DNA- and RNA-mediated effects in vivo. We demonstrate that (i) *Firre* mutant mice have cell-specific hematopoietic phenotypes, and (ii) upon exposure to lipopolysaccharide, mice overexpressing *Firre* exhibit increased levels of pro-inflammatory cytokines and impaired survival. (iii) Deletion of *Firre* does not result in changes in local gene expression, but rather in changes on autosomes that can be rescued by expression of transgenic *Firre* RNA. Together, our results provide genetic evidence that the *Firre* locus produces a trans-acting lncRNA that has physiological roles in hematopoiesis.
transcription occurs at thousands of sites throughout the mammalian genome. Many of these sites are devoid of protein-coding genes and instead contain long noncoding RNAs (lncRNAs). While lncRNA loci have been implicated in a variety of biological functions, comparatively few lncRNA loci have been genetically defined to have RNA-based roles. Indeed, deletions of entire lncRNA loci have uncovered a number of in vivo phenotypes1–5; however, this approach alone is confounded because in addition to the lncRNA transcript, lncRNA loci can also exert function through DNA regulatory elements6–8, the promoter region9, as well as by the act of transcription10,11. Thus, attributing RNA-based role(s) to lncRNA loci requires testing whether other regulatory modes potentially present at the locus have molecular activity that could contribute to phenotypic effects12,13.

In this study, we use the functional intergenic repeating RNA element, (Firre) locus as a model to discriminate between DNA- and RNA-mediated effects in vivo. We selected this locus for our study because it is syntenically conserved in a number of mammals, including human14–17, and because studies have reported diverse biological and molecular roles. Early characterization of the FIRRE locus in human cell lines identified it as a region that interacts with the X-linked macrosatellite region, DXZ4, in a CTCF-dependent manner18–21. Further analyses of the Firre locus demonstrated that it produces an lncRNA that escapes X-inactivation15,22–24, although it is predominately expressed from the active X chromosome25,26. Studies using cell culture models suggest that the Firre locus has biological roles in multiple processes, including adipogenesis26, nuclear architecture15,19,21, and in the regulation of gene expression programs15,27. In addition, there is some evidence for roles of the FIRRE locus in human development and disease28–31. Collectively, these studies demonstrate the diverse cellular and biological functions for the Firre locus. However, the biological roles of Firre as well as disentangling DNA- and RNA-mediated function(s) for the Firre locus have not been explored in vivo.

Using multiple genetic approaches, we describe an in vivo role for the Firre locus during hematopoiesis. We report that Firre mutant mice have cell-specific defects in hematopoietic populations. Deletion of Firre is accompanied by significant changes in gene expression in a hematopoietic progenitor cell type, which can be rescued by induction of Firre RNA from an autosomal transgene within the Firre knockout background. Mice overexpressing Firre have increased levels of pro-inflammatory cytokines and significantly impaired survival upon exposure to lipopolysaccharide (LPS). Finally, the Firre locus does not contain cis-acting RNA or DNA elements (including the promoter) that regulate neighboring gene expression on the X chromosome (nine biological contexts examined), suggesting that Firre does not function in cis. Collectively, our study provides evidence for a trans-acting RNA-based role for the Firre locus that, thus far, has physiological importance for hematopoiesis.

Results
The Firre locus produces an abundant lncRNA. We first sought to investigate the gene expression properties for Firre RNA in vivo. To determine potential spatial and temporal aspects of Firre RNA expression during development, we performed in situ hybridization in wild-type (WT) mouse embryos (E8.0–E12.5). Notably, we detected Firre RNA in many embryonic tissues, including the forebrain, midbrain, pre-somatic mesoderm, lung, forelimb, hindlimb, liver, and heart (Fig. 1a). Since noncoding RNAs have been described to be generally expressed at lower levels compared with protein-coding genes32–35, we determined the relative abundance of Firre RNA in vivo. We performed RNA-seq on eight different WT embryonic tissues and plotted the expression of noncoding and coding transcripts. Consistent with previous reports32–35, we observed that noncoding transcripts were generally less abundant than protein-coding transcripts (Fig. 1b). Despite most lncRNAs being expressed at low levels, we found that Firre, like Malat1 (refs.36–38), is an abundant transcript (Fig. 1b). Next, since Firre is located on the X chromosome and escapes X-inactivation15,22–24, we investigated whether Firre has different expression levels in male and female WT tissues. While levels of Firre RNA varied across embryonic tissue types, within individual tissues, male and female samples exhibited similar expression levels of Firre, despite escaping X-inactivation (Fig. 1c).

Firre knockout and overexpression mice are viable and fertile. To investigate the in vivo role of Firre and assess DNA- and RNA-mediated effects, we generated both Firre loss-of-function and Firre overexpression mice. To delete the Firre locus in vivo, we generated a mouse line containing a floxed allele (Firseboxed) from a previously targeted mouse embryonic stem cell line15 and mated to a CMV-Cre deleter mouse39. This produced a genomic deletion (81.8 kb) that removed the entire Firre gene body and promoter (henceforth called ΔFirre) (Fig. 1d). We confirmed the deletion of the Firre locus by genotyping (Supplementary Fig. 1) and examined Firre RNA expression. As expected, we did not detect Firre RNA in ΔFirre embryos by whole-mount in situ hybridization or by RNA-seq (Fig. 1a, d).

Since Firre is found on the X chromosome, we first sought to determine if deletion of the locus had an effect on the expected sex ratio of the progeny. Matings between ΔFirre mice produced viable progeny and had a normal frequency of male and female pups (Supplementary Table 1) that did not exhibit overt morphological, skeletal, or weight defects (Supplementary Fig. 2). Moreover, deletion of Firre did not impact expression levels of Xist RNA in embryonic tissues or perturb Xist RNA localization during random X chromosome inactivation (XCI) in mouse embryonic fibroblasts (MEFs) (Supplementary Fig. 3A–C).

Because the ΔFirre allele removes the entire gene body, this model does not allow us to distinguish between DNA- and RNA-mediated effects. Therefore, in order to be able to investigate the role of Firre RNA, we generated a doxycycline (dox)-inducible Firre overexpression mouse. This mouse model was engineered to contain a Firre cDNA downstream of a tet-responsive element (henceforth called tg(Firre)), and was mated to mice that constitutively express the reverse tetracycline transcriptional activator (rtTA) gene (combined alleles henceforth called FirreOE) (Fig. 1e). This approach enabled systemic induction of Firre RNA in a temporally controllable manner by the administration of dox. Moreover, by combining the FirreOE and ΔFirre alleles (henceforth called Firre rescue), we could test whether Firre RNA expression alone is sufficient to rescue phenotypes arising in the ΔFirre mice, thereby distinguishing DNA- and RNA-based effects.

To confirm expression of transgenic Firre RNA, tg(Firre) females were mated with rtTA males and placed on a dox diet the day a copulatory plug was detected, and embryos were collected at E11.5 for analyses. Compared with sibling control embryos, we detected increased Firre RNA in FirreOE embryos by whole-mount in situ hybridization (Fig. 1f) and by quantitative reverse transcription-PCR (qRT-PCR) (heart, 16-fold; forebrain, 26.6-fold; and forelimb, 11.5-fold) (Fig. 1g). Moreover, matings between tg(Firre) and rtTA mice fed a dox diet produce viable progeny that overexpress Firre at expected male and female frequencies (Supplementary Table 1).

Firre RNA has been reported to be largely enriched in the nucleus of mouse embryonic stem cells (mESCs)15,40, neuronal
precursor cells, and HEK293 cells, and also has been reported in the cytoplasm of a human colon cell line. Thus, we investigated the subcellular localization of Firre in the genetic models using RNA fluorescent in situ hybridization (RNA FISH). In contrast to ΔFirre MEFs, we detected pronounced localization of Firre RNA in the nucleus of WT MEFs (Fig. 1h). In dox-treated Firre rescue MEFs, which only produce Firre RNA from the transgene, we detected Firre RNA in both the nucleus and cytoplasm (Fig. 1h), which corresponded to approximately a 2.7-fold increase in Firre RNA relative to WT (Fig. 1i). Notably, the Firre rescue transgenic model showed both nuclear and cytoplasmic localization of Firre, suggesting a threshold level control for nuclear localized Firre.

Tissue-specific gene dysregulation in ΔFirre tissues. Given the broad expression profile of Firre RNA (Fig. 1a), we took an initial unbiased approach to explore the potential biological roles for the
**Fig. 1** Mouse models to interrogate the in vivo function of Firre. a Whole-mount in situ hybridization for Firre RNA in WT mouse embryos at E8.0 (n = 4), E9.5 (n = 4), E10.5 (n = 5), E11.5 (n = 6), E12.5 (n = 4), and ΔFirre E11.5 embryos (n = 3). Scale bar equals 1 mm. b Distribution of transcript abundances for protein-coding (light gray) and noncoding (dark gray) genes in WT E11.5 heart tissue (representative tissue shown from seven additional tissues). Vertical lines indicate Firre (red) and Malat1 (blue). c Expression of Firre shown as fragments per kilobase of transcript per million mapped reads (FPKM) from RNA-seq in E11.5 WT male (n = 3) and female (n = 3) forebrain (FB), midbrain (MB), pre-somatic mesoderm (PSM), lung (LU), forelimb (FL), hindlimb (HL), liver (LIV), and heart (HRT). The data shown as mean ± standard error of the mean (SEM). d Firm knockout mouse (red). Schematic of mouse X chromosome ideogram showing the Firre locus relative to Xist. UCSC genome browser diagram of the Firre locus (shown in opposite orientation). Dashed lines indicate the genomic region that is deleted in ΔFirre mice; single isoX scar upon deletion (gray triangle). Histone modifications and transcription factor binding sites in mouse embryonic stem cells (mESC-Brue4, ENCODE/LICR, mm9). RNA-seq tracks for the Firre locus in WT and ΔFirre E11.5 forelimbs. e Schematic of doxycyline (dox)-inducible Firre overexpression mouse (FirreOE). Tet-responsive element (TRE), minimal CMV promoter (mCMV), reverse tetracycline transcriptional activator (rtTA), and β-globin polyA terminator (pA). f In situ hybridization for Firre at E11.5 in control (WT or tgtFirre) + dox (n = 4) and FirreOE + dox (n = 3) embryos. g qRT-PCR for Firre expression shown as fold change (FC) in dox-treated E11.5 control and FirreOE hrt, fb, and fl. h RNA FISH for Firre in male WT, ΔFirre, and Firre rescue MEFS. DAPI (blue) marks the nucleus and Firre RNA is shown in green. Scale bar equals 10 μm. i qRT-PCR for Firre expression shown as FC in male WT, ΔFirre, Firre rescue + dox, and Firre rescue no dox MEFS. Expression normalized to β-actin in the control or WT sample. The data plotted as mean ± CI at 98%. Source data are provided in the Source Data file.

Firre locus, and performed poly(A)+ RNA-seq on eight E11.5 tissues from WT and ΔFirre embryos (forebrain, midbrain, heart, lung, liver, forelimb, hindlimb, and pre-somatic mesoderm). As expected, Firre expression was not detected in any of the ΔFirre tissues (Supplementary Data 1–8). Deletion of Firre was accompanied by significant changes in gene expression in all tissues examined (>1 FPKM, FDR<0.05) (Fig. 2a, b; Supplementary Data 1–8). Across these eight tissues, we identified a total of 3910 significantly differentially expressed genes, of which 271 genes were differentially expressed in two or more tissues (Supplementary Data 1–8). Interestingly, gene ontology (GO) analysis of the commonly dysregulated genes showed that deletion of the Firre locus affected genes involved in hemoglobin regulation and commonly dysregulated genes showed that deletion of the Firre locus (shown in opposite orientation). Dashed lines indicate the genomic region that is deleted in ΔFirre mice; single isoX scar upon deletion (gray triangle). Histone modifications and transcription factor binding sites in mouse embryonic stem cells (mESC-Brue4, ENCODE/LICR, mm9). RNA-seq tracks for the Firre locus in WT and ΔFirre E11.5 forelimbs. e Schematic of doxycyline (dox)-inducible Firre overexpression mouse (FirreOE). Tet-responsive element (TRE), minimal CMV promoter (mCMV), reverse tetracycline transcriptional activator (rtTA), and β-globin polyA terminator (pA). f In situ hybridization for Firre at E11.5 in control (WT or tgtFirre) + dox (n = 4) and FirreOE + dox (n = 3) embryos. g qRT-PCR for Firre expression shown as fold change (FC) in dox-treated E11.5 control and FirreOE hrt, fb, and fl. h RNA FISH for Firre in male WT, ΔFirre, and Firre rescue MEFS. DAPI (blue) marks the nucleus and Firre RNA is shown in green. Scale bar equals 10 μm. i qRT-PCR for Firre expression shown as FC in male WT, ΔFirre, Firre rescue + dox, and Firre rescue no dox MEFS. Expression normalized to β-actin in the control or WT sample. The data plotted as mean ± CI at 98%. Source data are provided in the Source Data file.

Firre mice have cell-specific hematopoietic phenotypes. Having observed an effect of Firre in regulating gene expression and accentuating the inflammatory response, we further investigated the role of Firre in hematopoiesis (Fig. 3a). We first examined cell populations in the peripheral blood in ΔFirre mice and observed a modest but significant reduction in the frequencies of CD4 and CD8 T cells, whereas the frequencies of B and NK cells were unaffected compared with WT (Fig. 3b; Supplementary Fig. 5A). To investigate the cause of this reduction, we examined the thymus (to assess for a defect in T-cell development) and the bone marrow (to assess for a defect in hematopoietic progenitor cells). There was no block in thymic development in ΔFirre mice, as normal frequencies of cells were observed at each developmental stage (Supplementary Fig. 5B, upper panels). However, we noticed that the absolute number of cells was generally lower in ΔFirre mice at every developmental stage, suggestive of a pre-thymic defect in progenitor development (Supplementary Fig. 5B, lower panels). Consistent with this, in the bone marrow compartment, we observed a significant reduction in both the

LPS potentiates the innate immune response in FirreOE mice. Firre is expressed in many innate immune cell types (Fig. 2d). Cell-based approaches have shown that Firre can be transcriptionally upregulated upon exposure to lipopolysaccharide (LPS) and can modulate the levels of inflammatory genes in a human colorectal adenocarcinoma cell line (SW480)27, mouse macrophage cell line (RAW264.7)27, as well as in an injury model using cultured primary rat microglial cells30. Thus, we hypothesized that dysregulation of Firre might alter the inflammatory response in vivo and reasoned that investigating the inflammatory response in Firre loss- and gain-of-function mice could provide insight into the DNA- and RNA-mediated effects of Firre. To test this, we employed a commonly used endotoxic shock model by administering LPS intraperitoneally to cohorts of WT, ΔFirre, FirreOE no dox, and dox-fed FirreOE mice in order to stimulate signaling pathways that regulate inflammatory mediators43 (Fig. 2e).

We administered two different LPS preparations, one which broadly stimulates the pattern recognition receptors toll-like receptors (TLR) 2, 4, and nitric oxide synthase, and an ultrapure LPS preparation that specifically stimulates TLR4 (refs44–46). At 5 h post LPS injection, we measured serum cytokine levels. Notably, we observed that FirreOE dox-fed mice administered broad-acting LPS had significantly higher levels of inflammatory cytokines, including TNFa, IL12-p40, and MIP-2 compared with WT (Fig. 2f). In contrast, we did not observe a significant difference for these cytokines in LPS-treated ΔFirre mice (Fig. 2f).

Consistent with the increased cytokine response using broad-acting LPS, dox-fed FirreOE mice administered TLR4-specific-acting LPS also had significantly higher levels of TNFa, IL12-p40, and MIP-2 compared with WT (Fig. 2g), albeit at lower serum concentrations compared with the broad-acting LPS (Fig. 2f, g).

In addition, we confirmed that overexpressing Firre RNA alone (without LPS) does not result in increased serum levels of TNFa, IL12-p40, and MIP-2 (Supplementary Fig. 4).

Because increased levels of TNFa is a hallmark of endotoxic shock47–49, we next tested whether the levels of Firre RNA had an impact on survival following LPS treatment. We administered 5 mg/kg of TLR4-specific-acting LPS to WT (n = 30), ΔFirre (n = 18), FirreOE no dox (n = 13), and FirreOE dox-fed (n = 17) mice, as well as a saline control group and monitored for 6 days. At this dose, across two independent cohorts, dox-treated FirreOE mice showed a significantly higher susceptibility to LPS compared with WT mice (P<0.0001, Mantel–Cox) and uninduced FirreOE animals (P = 0.0063, Mantel–Cox) (Fig. 2h). Whereas ΔFirre mice did not show a significant difference in the level of susceptibility to LPS (P = 0.1967, Mantel–Cox test) (Fig. 2h). Collectively, these results indicate that endogenous Firre does not appear to be necessary for a physiological inflammatory response to LPS, but that ectopic levels of Firre RNA can modulate this inflammatory response in vivo independent of genomic context, consistent with an RNA-based role for Firre.
Fig. 2 Modulation of Firre impacts genes with roles in the blood. a Schematized E11.5 tissues used for RNA-seq. WT (n = 6) shown in black, and ΔFirre (n = 6) shown in red. The number of differentially expressed genes shown below each tissue. b Heatmap of replicate embryonic tissues. c GO analysis for genes found dysregulated in four or more tissues. d Firre expression across multiple mouse blood cell lineages (RNA-seq data from bloodspot.eu, GSE60101). e Experimental approach for cytokine and survival experiments. f Cytokine measurements in serum at 5 h post intraperitoneal (i.p.) injection of 5 mg/kg LPS (broad acting) in WT (n = 8), ΔFirre (n = 8), FirreOE control diet (n = 6), FirreOE dox diet (n = 5–6), and saline-injected WT (n = 2) and ΔFirre (n = 2) (two independent experiments shown). g Cytokine measurements in serum at 5 h post i.p. injection of 5 mg/kg LPS (specific acting) in WT (n = 6), FirreOE control diet (n = 5), FirreOE dox diet (n = 4). Cytokine data are plotted as mean ± SEM, and significance determined by an unpaired two-tailed t test. h 6-day survival plot of mice injected with 5 mg/kg LPS (specific acting) in WT (n = 30), ΔFirre (n = 18), FirreOE control diet (n = 13), and FirreOE dox diet (n = 17) or saline control group (n = 10) consisting of WT, ΔFirre, and FirreOE mice over two independent experiments. Statistical difference determined by a by a Mantel-Cox test with P < 0.05 deemed significant. Source data are provided in the Source Data file.
To assess whether the observed defect in hematopoiesis could be due to a progenitor-intrinsic effect of Firre deficiency, we performed a competitive chimera transplant assay using an HSC-enriched population. We isolated an HSC-enriched population (lin−Sca1+c-Kit+CD34+CD135−) from WT (CD45.2), ΔFirre (CD45.2), and congenic WT (CD45.1), and then separately mixed WT (CD45.2) or ΔFirre (CD45.2) cells at an equal ratio with congenic WT (CD45.1). We then transplanted this cell mixture into lethally irradiated CD45.1 recipient mice (Supplementary Fig. 6A, B) and assessed the long-term reconstitution ability of WT and ΔFirre HSCs to repopulate blood cell lineages in vivo. We observed that ΔFirre/CD45.2 donors had significant reductions in the frequencies of CD4 and CD8 T cells, B cells, and NK cells in the peripheral blood of recipient mice compared with WT/CD45.2 donors (P = 0.0028, P = 0.0051, P = 0.0114, P = 0.0068, respectively, two-tailed Mann–Whitney U), indicating that ΔFirre-donors were markedly outcompeted at repopulating the blood (Supplementary Fig. 6C). Together, these data are consistent with a progenitor-intrinsic role for Firre in hematopoiesis.

In contrast to the ΔFirre model, mice overexpressing Firre RNA in the WT background (FirreOE) had normal frequencies of CD4, CD8, and B cells, but had a significant reduction in the frequency of NK cells in the peripheral blood compared with control mice (Fig. 3c; Supplementary Fig. 5D). A decrease in the frequency of NK cells in dox-fed Firre− rescue mice, where only Firre RNA from the transgene is expressed, was also observed (Supplementary Fig. 7). In the bone marrow of dox-fed FirreOE mice, we did not observe significant changes in the frequencies of HSC, multipotent progenitor (MPP), common myeloid progenitor (CMP), or CLPs compared with control samples (Fig. 3c; Supplementary Fig. 5E). Taken together, these results identify cell type-specific defects during hematopoiesis, whereby alterations of Firre impact the ratios and numbers of particular blood cells produced during hematopoiesis.

**Firre RNA has a trans-acting role in vivo.** Next, we wanted to further investigate the DNA- and RNA-mediated effects of the Firre locus using a cell type that was dysregulated in the ΔFirre immunophenotyping analysis. We selected to use the CLP as a model, because this was the earliest hematopoietic defect identified and because Firre is highly expressed in this progenitor cell
type. Therefore, further investigation could provide insight into the physiological effects of modulating Firre in a progenitor cell population. Because the ∆Firre mouse contains a deletion that removes all potential DNA regulatory elements, the IncRNA, and the promoter (thus removing the act of transcription), this mouse model does not allow us to distinguish between DNA- and RNA-mediated effects.

To directly test whether the decrease in the frequencies of CLPs observed in the ∆Firre mice is mediated by an RNA-based mechanism, we reasoned that overexpressing Firre RNA in the ∆Firre background would enable to test for a RNA-mediated role. To this end, we generated multiple cohorts of mice that contained the FirreOE alleles in the ∆Firre background (Firreescue) and induced transgenic Firre RNA expression by placing mice on a dox-diet. From multiple cohorts of WT, ∆Firre, and dox-fed Firreescue mice, we assessed CLP frequency by flow cytometry in total bone marrow and lineage-depleted bone marrow (to enrich for hematopoietic non-lineage committed cells).

Consistent with the previous data (Fig. 3b), we also observed a significant increase in the frequency of CLPs in total bone marrow from ∆Firre mice (n = 17, mean CLP frequency = 0.0532) compared with WT mice (n = 16, mean CLP frequency = 0.0829) (Fig. 4b) and in lineage-depleted bone marrow from ∆Firre mice (n = 9, mean CLP frequency = 0.3011) compared with WT (n = 9, mean CLP frequency = 0.2167) (Supplementary Fig. 8A, B). In contrast, dox-fed Firreescue mice, which only expressed transgenic Firre RNA, we observed that the frequency of CLPs was significantly increased (n = 15, mean CLP frequency = 0.0810) compared with ∆Firre mice, and restored to approximately that of WT in total bone marrow (Fig. 4b). Consistent with this data, in lineage-depleted bone marrow, we also observed a significant increase in the frequency of CLPs in dox-fed Firreescue mice (n = 11, mean CLP frequency = 0.2809) compared with ∆Firre mice (Supplementary Fig. 8). Thus, induction of transgenic Firre RNA alone is sufficient to rescue the reduction in frequency of CLPs observed in ∆Firre bone marrow. These data suggest that Firre RNA, rather than DNA, exerts a biological function during hematopoiesis.

**Transgenic Firre RNA restores gene-expression programs in vivo.** To gain further insight into the molecular roles of Firre in the CLPs, we took a gene expression approach, because alterations of the Firre locus and RNA have previously been shown to impact gene expression. Moreover, we reasoned that we could test if changes in gene expression in the loss-of-function model could be rescued by expressing only transgenic Firre RNA. To this end, we isolated CLPs by fluorescence-activated cell sorting (FACS) from the bone marrow of age- and sex-matched WT, ∆Firre, and dox-fed Firreescue mice, and performed poly(A) + RNA-seq. As expected, Firre RNA was not detected in the ∆Firre samples, and expression of transgenic Firre RNA in the Firreescue samples was detected at levels above WT (Fig. 4c). Differential gene expression analysis between WT and ∆Firre CLPs identified 89 significantly differentially expressed genes (FDR<0.1) (Fig. 4d; Supplementary Data 9). GO analysis of the differentially expressed genes showed that deletion of Firre in CLPs affected genes involved in lymphocyte activation, cell adhesion, and B-cell activation (Fig. 4e).

Next, we determined if induction of Firre RNA in the Firreescue model could rescue expression of the 89 significantly dysregulated genes found in ∆Firre CLPs. We compared the CLP RNA-seq from ∆Firre and Firreescue mice and identified 4656 genes with significant changes in gene expression (FDR<0.1) (Supplementary Data 10). Notably, 78 of the 89 genes that were significantly differentially expressed in ∆Firre CLPs were found to be significantly and reciprocally regulated in Firreescue CLPs (P = 2.2e-16, Fisher exact test) (Fig. 4d). For example, Ccnd3, Ly11, and Cblp1 are significantly downregulated in ∆Firre CLPs, but are found significantly upregulated in Firreescue CLPs to (Fig. 4f). Further, genes such as Maa, Fam46c, and Icos were found significantly upregulated in ∆Firre CLPs, but their expression was significantly reduced in Firreescue CLPs (Fig. 4f). We also noted that several immunoglobin heavy and light-chain variable region genes were reciprocally regulated in our analyses (Fig. 4d). Taken together, these data suggest that ectopic expression of Firre is sufficient to restore a gene expression program in an RNA-based manner in vivo.

**The Firre locus does not function in cis.** Many IncRNA loci exert function to control the expression of neighboring genes, a biological function called cis-regulation. This occurs through a variety of mechanisms including, cis-acting DNA-regulatory elements, the promoter region, the act of transcription, and the IncRNA. The ∆Firre mouse model enables to test for potential cis-regulatory roles for Firre on the X chromosome, because the knockout removes the entire Firre locus and promoter region (Fig. 1d). To investigate local (cis) effects on local gene expression, we generated a 2 Mb windows centered on the Firre locus and examined whether the neighboring genes were significantly dysregulated across nine biological contexts.

Differential gene expression analysis for WT and ∆Firre CLPs showed that of the 12 genes within a 2 Mb window (excluding Firre), none were differentially expressed (Fig. 4g). Consistent with this finding, we did not observe significant changes in local gene expression (2 Mb windows centered on the Firre locus) in seven of the eight embryonic tissues (Fig. 4g; Supplementary Fig. 9A–F). Indeed, we observed one instance of differential expression in one embryonic tissue (Hs6st2 was slightly but significantly downregulated in the embryonic forebrain, −0.38 log2 fold change, FDR<0.05) (Supplementary Fig. 9A–F). These data demonstrate that the Firre locus does not exert a local effect on gene expression in vivo, and suggest that the Firre IncRNA regulates gene expression in a trans-based manner. Collectively, our study investigates the roles of DNA and RNA at the Firre locus in vivo and genetically defines that the Firre locus produces a trans-acting IncRNA molecule in vivo.

**Discussion**

Classic models used to study noncoding RNAs—ribosomal RNAs, small nucleolar RNAs, tRNAs, and the telomerase RNA component (TERC)—have demonstrated that RNAs serve important cellular functions. This core of possible RNA biology has been greatly expanded by studies that have identified tens of thousands of IncRNAs. Indeed, subsequent molecular and genetic interrogation of IncRNA loci have identified diverse molecular roles and biological phenotypes. However, IncRNA loci can potentially function through a variety of molecular modes: DNA-regulatory elements (including the promoter), the act of transcription itself, and the IncRNA gene product. Therefore, attributing an RNA-based role to a IncRNA locus requires the development of multiple genetic models to determine the activities and contributions of potential regulatory modalities.

In this study, we developed three genetic models in mice for the syntenically conserved IncRNA Firre: loss-of-function, overexpression, and rescue. Notably, we report that deletion of the Firre locus does not impact survival in mice, or despite escaping XCI, skew the sex ratio of progeny. We leveraged the genetic models to discriminate between DNA- and RNA-mediated effects in vivo. We demonstrated that modulating Firre directs cell-specific
defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during hematopoiesis, potentiates the innate immune response upon exposure to LPS, and can restore gene expression defects during 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with our findings, independent studies have found that overexpression of Firre RNA in different cellular contexts can modulate genes in the innate immune response. For example, the levels of select inflammatory genes, including IL12p40, are increased upon overexpression of Firre RNA followed by LPS stimulation in human SW480 cells. In addition, upon overexpression of Firre RNA in an injury model using cultured primary microglial cells, TNF-α levels are increased. The different, yet synergistic approaches (cell-based, ex vivo, and in vivo) provide supporting evidence for an RNA-based role for Firre. In the overexpression approach in this study, we note that the phenotypes could be due to increased levels of Firre in a cell type where it is normally expressed and/or from de novo expression in a cellular context where Firre is not endogenously found. Yet, this strategy may perhaps mimic disease contexts where Firre is found overexpressed. We speculate that Firre could be important in setting functional thresholds for cells. For example, Firre is not only significantly increased in certain cancers, including kidney renal cell carcinoma, lung squamous cell carcinoma, and colon adenocarcinoma, but high levels of Firre significantly associated with poor survival in patients with large B-cell lymphoma.

Finally, our study suggests that Firre does not have a cis-regulatory role on gene expression in vivo. Upon deletion of the Firre locus and its promoter region, we observed global changes in gene expression. Yet, we did not find changes in local gene expression (2 Mb window) at the Firre locus in most embryonic tissues and in CLPs. Thus, potential DNA regulatory elements, the lncRNA, the promoter, and the act of transcription appear to not have regulatory roles on neighboring gene expression in the nine biological contexts (eight embryonic and one cell type) analyzed in this study. Moreover, we observed that deletion of Firre in vivo does not perturb Xist RNA expression in eight embryonic tissues and does not affect random XCI in MEFs, which is consistent with a previous study using cell culture models. Together, these data are notable because cis-acting mechanisms are speculated to be common feature at lncRNA loci. While we did not find any evidence that neighboring genes were significantly dysregulated upon deletion of the Firre locus.

In summary, we have developed genetic models to test the DNA- and RNA-mediated effects of the syntenically conserved lncRNA locus, Firre, in vivo. Our findings provide evidence that the Firre lncRNA locus has a role in hematopoiesis that is mediated by a trans-acting RNA, and further highlights the biological importance of lncRNA-based machines in vivo. While this study defines a role for Firre in a hematopoietic context, it is important to note that deletion of Firre perturbed gene expression in a number of tissues, including the brain, where Firre has been implicated in a rare human disease. Therefore, going forward it will be important to use genetic models to investigate the potential role(s) of Firre in a number of different biological and disease contexts.

**Methods**

**Mouse care and ethics statement.** Mice used in this study were maintained in a pathogen-specific free facility that is under the supervision of Harvard University's Institutional Animal Care Committee. We have compiled with the ethical regulations for animal testing and research in accordance with Harvard University's Institutional Animal Care and Use Committee. Mice used in this study were housed at a density of 2–5 mice per cage containing: Anderson's Bed (The Andersons, Inc), Enviro-Dri (Shepherd Specialty Papers), compressed 2” x 2” cotton nestlet (Ancare), a red mouse hut (BioServ), automatic watering of reverse osmosis deionized water was chlorinated at 2 ppm, and were fed a regular Chow diet (Probio IsoPro RMH 3000 3P75/76).

**Firre mouse strains and genotyping.** A previously targeted allele for Firre in JEM embryonic stem cells (mESC) (Jax 023253) was inserted into blastocysts (Harvard Genome Modification Facility), and subsequent progeny were screened for the FirreΔ allele (Jackson Laboratory, 029931). To generate a deletion of the Firre locus in all tissues, female FirreΔ mice were mated to male B6.C-Tgf(CMV-Cre)1Cgn/j mice (CMV-Cre) (Jackson Laboratory, 00654). We screened the progeny for the ΔFirre allele by PCR genotyping (described below) for Firre Δk allele, knockout (ΔFirre), neomycin, hygromycin, and cre alleles (Supplementary Fig. 1A, B). A female containing the ΔFirre and CMV-Cre alleles was subsequently mated to C57BL/6 J mice in order to remove CMV-Cre and to propagate the ΔFirre allele. Progeny were PCR genotyped for Firre wild-type, ΔFirre, and cre alleles. Progeny containing only the ΔFirre allele were backcrossed to C57BL/6 J mice for three generations (N3) (Jackson Laboratory, 03008). Mice were inbred by intercrosses through heterozygous and homozygous breeding strategies.

To generate an inducible Firre-overexpressing allele (tg(Firre)) in mice, we cloned a mouse Firre cDNA into a Tet-On vector (pTRE2), where the β-globin promoter was removed to remove potential disease-causing and disease-promoting genetic variation. Next, we used EcoR and Not restriction enzymes to digest the Firre cDNA to the Firre loci, and β-globin poly(A) terminator. The digested DNA fragment was purified according to Harvard Genome Modification Facility protocol, and the purified DNA fragment was injected into the C57BL/6 J mouse (Jackson Laboratory) blastocysts. Progeny were screened for the tg(Firre) cassette by PCR genotyping for the tg(Firre) allele, and male founder mice were identified and individually mated to female C57BL/6 J mice (Jackson Laboratory, 000664). To overexpress tg(Firre) N2 and N3 generation, females were mated to male B6:NFBV female mice (Jackson Laboratory, 016532) and at the plug date females, and the subsequent progeny were either placed on a normal chow diet or 625 mg/kg doxycycline-containing chow (Envigo, TD.01306) until the experimental end points. A colony of male rats were mated with female to breed to C57BL/6 J females for up to four generations.

Cytokine analysis and in vivo endotoxin challenge. To investigate the cytokine response in vivo, we used two different preparations of LPS from Escherichia coli (InvivoGen, lrr-134. Sigma, L2630) and Ultrapure LPS (from InvivoGen, tlr-3ples) and dissolved in 0.9% saline solution (Teknova, S5825). We administered either 0.9% saline or 5 mg/kg broad-acting LPS (Sigma, L2630) by i.p. injection using 30-G insulin syringes (BD, 324811) to two female mice cohorts 8–10 weeks old (WT, ΔFirreOE) no dox, and dox fed (ΔFirreOE). We also administered a different preparation of LPS at 5 mg/kg that is TLR4-specific (InvivoGen, tlr-3ples) by i.p. injection in mice 5–10 weeks old (WT, ΔFirreOE no dox, and dox fed (ΔFirreOE). At 5 h post i.p. injection, mice were euthanized, and peripheral blood was collected by cardiac puncture and allowed to clot for 30 min at room temperature with gentle rotation. After clotting, samples were centrifuged at 1000 rpm for 10 min at 4 °C, and serum was collected. Cytokine analysis was performed on serum diluted twofold in PBS pH 7.4 (Eve Technologies, Chemokine Array 31-Plex). Measurements within the linear range of the assay are reported. Endotoxin survival experiments were performed over two independent experiments using mice 9–16 weeks old. WT (mean age = 12.9 weeks), ΔFirreOE no dox (mean age = 13.6 weeks), and dox fed ΔFirreOE (mean age = 13.7 weeks). Survival rate were reported.
prepared as described above and administered by i.p. injection, and mice were monitored for moribund survival over 6 days. Mice were housed at a density of 3–5 mice per cage by Anderson’s Bed (The Andersons, Inc), Enviro-Dri (The Andersons, Inc), compressed 2" x 2" cotton nestlet (Ancare), and a red mouse hut (BioServ). The following supportive care was provided during the duration of the experiments: hydrogel, a small cup containing powdered diet mixed with water, and a heating pad (5” x 8.6” x 6”) was placed externally on the bottom of the cage (Kobayashi).

Whole-mount in situ hybridization. We generated a digoxigenin-labeled anti-sense riboprobe by in vitro transcription (Roche, 1127763910) against Fire from a 428 bp sequence (Supplementary Fig. 1C) that corresponds to the 5’ end of the Fire transcript. In situ hybridization was performed on a minimum of three embryos per stage and/or genotype. For whole-mount staining, we fixed embryos in 4% paraformaldehyde for 18 h at 4 °C, followed by three washes in 1× PBS for 10 min at room temperature. We then dehydrated the embryos for 5 min at room temperature in a series of graded methanol solutions (25%, 50%, 75%, methanol containing 0.85% NaCl, and 100% methanol). Embryos were stored in 100% methanol at -20 °C. Next, we rehydrated embryos through a graded series of 75%, 50%, 25% methanol/0.85% NaCl solutions for 5 min at room temperature. Embryos were next washed twice in 1× PBS with 0.1% Tween-20 (PBST) and treated with 10 mg/mL proteinase K in 1× PBST for 10 min (E0, E9.5, E10.5, and E11.5). Samples were fixed again in 4% paraformaldehyde/0.25% glutaraldehyde in PBST for 20 min at room temperature, and washed twice in 1× PBST. We then incubated samples in pre-hybridization solution (50% formamide, 2× saline–sodium citrate (SSC), 0.1% SDS, 500 ng/mL RNase A) for 1 h at 68 °C, and then incubated overnight in 500 ng/mL of Fire antisense riboprobe at 68 °C for 16 h. Post hybridization, samples were washed in stringency buffers and incubated in 100 ng/mL RNase A at 37 °C for 1 h. Next, samples were washed in 1× maleic acid buffer with 0.1% Tween-20 (MBST) and incubated in Roche Blocking Reagent (Roche, 1091676) with 10% heat-inactivated sheep serum (Sigma, S2263) for 4 h at room temperature. We used an anti-digoxigenin antibody (Roche, No.11093274910) at 1:5000 and incubated the samples for 18 h at 4 °C. Samples were washed eight times with MBST for 15 min, five times in MBST for 1 h, and then once in MBST for 16 h at 4 °C. Samples were washed 3x for 5 min at room temperature with NTMT solution (100 mM NaCl, 100 mM Tris–HCl (pH 9.5), 50 mM MgCl2, 0.1% Tween-20, 0.1% levamisole). The in situ hybridization signal was developed by adding BM Purple (Roche, 1144270401). After the colorimetric development, samples were fixed in 4% paraformaldehyde and cleared through a graded series of glycerol/1× PBS and stored in 80% glycerol. Embryos were imaged with a Leica M216FA stereo-microscope (Leica Microsystems) equipped with a DFC300 FX digital imaging camera in Leica Application Suite v2.3.4 R2.

RNA-seq in embryonic tissues preparation and analysis. For WT and ΔFire RNA-seq in embryonic tissues, we dissected tissues (forebrain, midbrain, heart, lungs, liver, forelimb, hindlimb, and pre-somite mesoderm) from E11.5 embryos RNA-seq in embryonic tissues, we dissected tissues (forebrain, midbrain, heart, lungs, liver, forelimb, hindlimb, and pre-somite mesoderm) from E11.5 embryos and with a 10-cycle PCR enrichment to minimize PCR artifacts. The indexed libraries were pooled in groups of six, with each pool containing a mix of WT and ΔFire samples and from each tissue and sex, and genes with FDR ≤0.05 were deemed significant (Supplementary Data 1-8). Gene Ontology analysis was performed using the Biological process enrichment tool (GOSlim) with the Gene Ontology database. Genes with an FDR ≤0.01 were deemed significant in a comparison between wild-type and ΔFire (Supplementary Data 9) and genes with an FDR ≤0.1 in the ΔFire and FireΔneo comparison were deemed significant (Supplementary Data 10). Gene Ontology analysis was performed on significantly differentially regulated genes (FDR ≤0.01) in the WT and ΔFire CLP comparison using Panther.

qRT-PCR. Embryonic tissues or cells were homogenized in TriZol (Invitrogen), and total RNA was isolated using RNase mini columns (Qiagen) on a QIAcube (Qiagen). In total, 300 ng of the isolated RNA was used as input to synthesize cDNA (SuperScript IV Vilo Master Mix, Invitrogen, 11756050). Primers used qRT-PCR experiments: F_b-act: GCTGTATTCCCCTCCATCGTG, R_b-act: CACAGTGGCTGGCTGGTTG; F_Fire: AAATCGAGAGCAATCGGACG, R_Fire: CCGTGCGGCGTGCTTTTTTG. Experiments were performed on a ViiA7 (Applied Biosciences). qRT-PCR data were analyzed by the ΔACT method.

Distribution of Fire expression across wild-type tissues. For each of the eight WT embryonic tissues, FPKM estimates of all protein-coding or noncoding genes (biotypes selected: protein coding, lincRNA, and processed transcript) were gathered and filtered for expression > 1 FPKM. Density and expression plots were generated using ggplot2_2.2.1 (geom_density()) in R version 3.3.3.

Flow-cytometry analysis. Zombie Aqua Fixable Viability Kit (Biolegend, 423101) was used as a live-dead stain, and used according to the manufacturer’s protocol. For cell analysis of the peripheral blood, thymus, and bone marrow, age and sex-matched mice were used. Peripheral blood was collected by cardiac puncture or by tail vein collection into 10% by volume 4% citrate solution. The following antibodies were added (1:100) to each sample and incubated for 30 min at room temperature Alexa Fluor 700 anti-mouse CD8a clone 53-6.7 (Biolegend, 100730), PE/Dazzle-594 anti-mouse CD4 clone GK1.5 (Biolegend, 104565), APC anti-mouse CD19 clone 6D5 (Biolegend, 115512), Alexa Fluor 488 anti-mouse NK-1.1 clone PK136 (Biolegend, 108718), PE anti-mouse CD3 clone 17A2 (Biolegend, 100205), and TruStain FcX (anti-mouse CD16/32) clone 93 (1:50) (Biolegend, 101319). For competitive chimera experiments, Pacific Blue anti-mouse CD45.2 clone 104 (Biolegend, 109820) and Pacific Blue anti-mouse CD45.1 clone 104 (Biolegend, 108767) were used. Red blood cells were then lysed for 15 min at room temperature using BD FACS Lysing Solution (BD, 349202). Cells were washed twice in 1× PBS with 1% BSA and then resuspended in 1% paraformaldehyde or 1% PBS with 0.2% BSA.

Thymi were collected and homogenized in ice cold PBS over a 40-micron filter. Thymus and bone-marrow antibodies (1:100) for 30 min at room temperature: Alexa Fluor 488 anti-mouse CD25 clone 9G6 (Biolegend, 102017), PE/Cy7 anti-mouse/human CD44 clone IM7 (Biolegend, 103030), PE anti-mouse TCR β chain clone H57-597 (Biolegend, 109208), APC anti-mouse/human CD45R/B220 clone RA3-6B2 (Biolegend, 103212), iFluor 450 anti-mouse CD69 clone H1.2F3 (Invitrogen/Ebioscience, 4809182), Alexa Fluor 700 anti-mouse CD8a clone 53-6.7 (Biolegend, 100730), and PE/Dazzle-594 anti-mouse CD4 clone GK1.5 (Biolegend, 100456). Cells were washed twice in 1× PBS with 1% BSA and then resuspended in 1% paraformaldehyde.

Bone marrow was collected from both femurs and tibias (four bones total per mouse) following the end-maturation process (post 40–42 days old) containing 5% fetal bovine serum (FBS) ( Gibco, 26140079) and 10 mM EDTA. Cells were then pelleted, resuspended, and passed through a 70-micron filter. The resulting single-cell suspension was then incubated with the following antibodies (1:100) for 60 min on ice: Alexa Fluor 700 anti-mouse CD16/CD32 clone 93 (Biolegend, 108138), PE/Cy7 anti-mouse/human CD44 clone IM7 (Biolegend, 103030), APC anti-mouse CD34 clone HM34 (Biolegend, 128612), PE anti-mouse CD135 clone A2F10 (Biolegend, 105350), and Pacific Blue anti-mouse Lin/CD3/CD19/CD4/CD8/CD16/CD11b clone (20 µL per 1× 10^7 cells) (Biolegend, 114976/119/ M1/70 (Biolegend, 133310). Red blood cells were lysed by 15 min at room temperature using BD FACS Lysing Solution (BD, 349202) or BD Pharm Lyse (BD, 555899). Cells were washed twice in 1× PBS with 1% BSA and then resuspended in 1% paraformaldehyde or 1× PBS with 0.2% BSA.

Bone marrow cytometry was performed on a LSR II (BD). To enumerate cell populations, 50 µL of CountBright Absolute Counting Beads (Invitrogen, C36950) was added to relevant bone marrow and thymus samples. Gating of cell populations was performed using FlowJo 10.4.1 software (Treestar) using the following criteria (applied to live singlets) (Supplementary Fig. 10): CD4 T cells (CD4+ (CD3-); CD8 T cells (CD8+ (CD4-)); NK cells (NK1.1+CD19-CD3-); B cells (CD19+CD3-); double negative (DN) (B220-CD4-CD8-CD25-CD44+); double positive (DP) (CD4+CD8+B207); single positive
(SP) (CD8, CD8+CD4-CD220-); single positive (SP) (CD4, CD4+CD8-CD220-); hematopoietic stem cells (HSC) (LSK [Lin-], Sca1+, c-Kit+, CD45+); multipotent progenitors (MPP) (LSK+CD45+CD135+); common lymphoid progenitors (CLP) (Lin-Sca1+c-Kit+IL7Rα+); and common myeloid progenitors (CMP) (LK[CD34+CD163-]). Negative gates were set using fluorescence-minus one controls (FMO).

Competitive HSC transplant assay. Bone marrow from age- and sex-matched mice was collected and pooled with like genotypes (as described in Flow-cytometry analysis) from that were 8-9 weeks old. PepBoy/CD45.1 (n = 3 females per experiment; mean age = 9 weeks) (Jackson Laboratory, 002144), Firre WT/CD45.2 (n = 3 females per experiment mean age = 8.9 weeks), and ΔFirre/CD45.2 (n = 3 females per experiment; mean age = 8.6 weeks). Bone marrow was lineage-depleted according to the manufacturer's protocol (MiltenyiBiotec, 130-042-401), and cell marker surface staining was performed (as described in Flow-cytometry analysis section) from mice that were 8-9 weeks old and then infected with retro-orbital injection into each experimental PepBoy/CD45.1 male recipient mouse. After transplantation, mice were maintained on antibiotic-containing water (0.4% sulfamethoxazole/0.008% trimethoprim) for 4 weeks, and then switched to automatic watering of reverse osmosis deionized water chlorinated at 2 ppm.

MEF preparations and culture. We generated Firre WT, Firre knockout, and Firre–/– MEFs at E13.5 from intercrosses between male Firre+/+ with female Firre+/– and male Firre–/– with female Firre+/–. Individual embryos were dissected into 1x phosphate-buffered saline (PBS) and were eviscerated, and the head, forelimbs, and hindlimbs were removed. Embryo carcasses were placed into individual 6cm2 tissue culture plates containing 1 ml of pre-warmed 37 °C Tryпле (Thermo Fisher, 12604013) and were incubated at 37 °C for 20 min. Embryos were dissociated by gently pipetting with a P1000 tip and MEF media was added. Cells were cultured for 5–7 days, and cryostacks of individual lines were generated. Subsequent experiments were performed from thaws from the cryostocks up to passage 3. MEFs were genotyped for Firre WT, knockout, rta, (γFirre), and sry alleles. MEF culture media: 1x eagle’s minimum essential medium (MEM) with 2.5% FBS using FACS (BD Aria). Recipient mice, PepBoy/CD45.1 (n = 10 males per experiment; mean age = 8.6 weeks), were lethally irradiated using a split 9.5 μl dose (3 h apart). Firre WT and ΔFirre HSCs were separately mixed at a 1:1 ratio with PepBoy/CD45.1 HSCs. In total, 100 μl containing 4000 mixed cells were transplanted by retro-orbital injection using 30-gauge insulin syringes (BD, 328411) into lethally irradiated recipients. Forty-eight hours post transplant, 100,000 helper marrow cells from male PepBoy/CD45.1 were transplanted by retro-orbital injection into each experimental PepBoy/CD45.1 male recipient mouse. After transplantation, mice were maintained on antibiotic-containing water (0.4% sulfamethoxazole/0.008% trimethoprim) for 4 weeks, and then switched to automatic watering of reverse osmosis deionized water chlorinated at 2 ppm.

Fire Fish probe design and RNA FISH. Fire flg oligo probes were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and synthesized by Integrated DNA Technologies (IDT). After Amino Linking (IDT), E11.5 embryos were added to 2% pooled oligos by terminal transferase (New England Biolabs), oligos were labeled with Alexa647 and labeled with Cy3. After Amine-ddUTP (Kerafast) was added to 2 pmol of pooled oligos Firre RNA FISH was performed using a 60X1.4 NA objective lens, Orca ER camera (Hamamatsu), and Volocity software (Perkin Elmer). All probes were prepared by nick translation using DNA polymerase I (New England Biolab), DNase I (Promega), and Digoxigenin-dUTP (Roche), or Cy3-dUTP (Enzo Life Sciences).

Skeletal preparations. WT and ΔFirre E18.5 embryos were dissected and eviscerated. Samples were fixed in 100% ethanol for 24 h at room temperature. Embryos were then placed in 100% acetone at room temperature and then incubated in staining solution (0.3% alcian blue 8GS (Sigma) and 0.1% Alizarin Red S (Sigma) in 70% ethanol containing 5% acetic acid) for 3 days at 37 °C. Samples were rinsed with distilled water and then placed in 1% potassium hydroxide at room temperature for 24 h. Samples were cleared in a series of incubations with 1% potassium hydroxide in 20%, 50%, and 80% glycerol. Skeletal preparations were placed in 80% glycerol/1x PBS and imaged using a Nikon D7000 camera with a Nikon 28-105 macro lens. Images were captured in the Nikon Electronic Format (NEF), and were processed in the Adobe Camera Raw plug-in where exposure and temperature adjustments were applied to all like images.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. RNA-seq data for Gene Expression Omnibus under the accession numbers GSE125683 and GSE83631. The data that support the findings of this study are available from the corresponding author upon request.

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Data availability. RNA-seq data for Gene Expression Omnibus under the accession numbers GSE125683 and GSE83631. The data that support the findings of this study are available from the corresponding author upon request.
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Competing interests
The authors declare no competing interests.
