The NOTCH1-dependent HIF1α/VGLL4/IRF2BP2 oxygen sensing pathway triggers erythropoiesis terminal differentiation

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Abstract

Hypoxia is widely considered as a limiting factor in vertebrate embryonic development, which requires adequate oxygen delivery for efficient energy metabolism, while nowadays some researches have revealed that hypoxia can induce stem cells so as to improve embryonic development. Erythroid differentiation is the oxygen delivery method employed by vertebrates at the very early step of embryo development, however, the mechanism how erythroid progenitor cell was triggered into mature erythrocyte is still not clear. In this study, after detecting the upregulation of VGLL4 in response to oxygen levels, we generated vgl4b mutant zebrafish using CRISPR/Cas9, and verified the resulting impaired heme and dysfunctional erythroid terminal differentiation phenotype. Neither the vgl4b-deficient nor the γ-secretase inhibitor IX (DAPT)-adapted zebrafish were able to mediate HIF1α-induced heme generation. In addition, we showed that vgl4b mutant zebrafish were associated with an impaired erythroid phenotype, induced by the downregulation of alas2, which could be rescued by ifr2bp2 deletion. Further mechanistic studies revealed that zebrafish VGLL4 sequesters IRF2BP2, thereby inhibiting its repression of alas2 expression and heme biosynthesis. These processes occur primarily via the VGLL4 TDU1 and IRF2BP2 ring finger domains. Our study also indicates that VGLL4 is a key player in the mediation of NOTCH1-dependent HIF1α-regulated erythropoiesis and can be sensitively regulated by oxygen concentrations. On the other hand, VGLL4 is a pivotal regulator of heme biosynthesis and erythroid terminal differentiation, which collectively improve oxygen metabolism.

1. Introduction

Aerobions, organisms that require oxygen to survive, have evolved various mechanisms for absorbing oxygen from their environment in order to avoid continuous hypoxia, erythropoiesis is one of the most indispensable mechanisms widespread in vertebrates [1,2]. Hemoglobinogenesis, which occurs during terminal erythroid differentiation, involves the acquisition of heme, iron and globin chains via the upregulation of alas2, tfr1 and other related genes [3]. This process is regulated by a complex of repressive proteins that bind to the promoters of developmentally-regulated erythroid genes [4]. However, the signals required to trigger the dissociation of this regulatory complex remain unknown.

During development, the embryo is subjected to low oxygen concentrations, before peripheral blood circulation is established [5,6]. As a member of the hypoxia inducible factors (HIF) family, HIF1α responds to cellular oxygen concentrations with high levels of sensitivity [7]. Under hypoxic conditions (1–5% oxygen), HIF1α is stabilized in the cytoplasm and translocated to the nucleus to bind the promoter regions of various genes, triggering downstream transcriptional events [8–11]. HIF1α has been reported to be involved in hematopoietic stem cells (HSCs) formation of the aorta-gonad-mesonephros (AGM) region [7,12], as well as the expansion of hematopoietic stem and progenitor cells (HSPCs) via the hypothalamic-pituitary-adrenal (HPA)-central
2.3. Morpholinos and mRNA microinjection

Supplementary Tables 1

mers that cover upstream and downstream regions of the deleted sites. containing corresponding wild type (wt) genes as templates and pri-
the depletion that less than 40bp, we conducted a PCR using plasmids

As an excellent model [15], zebrafish erythropoiesis comprises two sequential waves [16]: Primitive erythropoiesis begins in the posterior lateral mesoderm (PLM) and subsequently the intermediate cell mass (ICM), which contains gata1a proerythroblasts and erythroblasts [17] and generates all erythrocytes in the circulatory system until 4 days post-fertilization (dpf) [18]. From 24 h post-fertilization (hpf), gata1a primary erythroid cells differentiate into mature erythrocytes, which express alas2 [19]. During definitive erythropoiesis in zebrafish, HSCs from the ventral arterial wall (VDA) at approximately 26 hpf [20], then moved into caudal hematopoietic tissue (CHT), thymus, and finally the pronephros from 4 dpf to adulthood [21], which can differentiate into erythrocytes.

Vestigial Like Family Member 4 (VGLL4) is a transcription cofactor which have no DNA binding domain and exert their biological functions through interaction with various transcription factors via their TONDU (TDU) domains [22–24]. VGLL4 contains two TDU domains, which exhibit different preferences for interaction with proteins on certain occasions [25]. For example, VGLL4 antagonizes TEA domain transcription factor/Yes associated protein (TEAD/YAP) signaling during cardiac growth largely via TDU1 [26], while delays malignant progression in breast cancer by interacting with TEA Domain Transcription Factor 1 (TEAD1) predominantly via TDU2 [27]. Among three paralogs of zebrafish vgl4, vgl4b showed highest similarity with human vgl4 [28].

In this study, we observed the rapid upregulation of vgl4b by mild low oxygen concentrations at the hemoglobin accumulation phase of zebrafish embryonic development, and showed that this can be mimicked by increased hif1a expression. Furthermore, VGLL4 regulates Interferon Regulatory Factor 2 Binding Protein 2 (IRF2BP2) transcriptional activity by protein sequestration, thus ensuring effective oxygen delivery in erythroid terminal differentiation.

2. Materials and methods

2.1. Zebrafish maintenance and the generation of mutants

The line of Tg(gata1a: dsRed) [29] was used in this study. After the generation of vgl4b mutant zebrafish was achieved using CRISPR/Cas9, the genotyping PCR primers were designed as follows: forward 5′-GGTGAACCAGCTGAAAGCTGTAACC-3′; reverse 5′-CATGGCAATGGATGTGATCTCAGA-3′. The vgl4b-/- Tg(gata1a: dsRed) zebrafish was achieved using CRISPR/Cas9; normal Tg(gata1a: dsRed), following anesthesia with a solution of 0.1% tricaine (Sigma). Whole embryos were collected as well, then carefully dissected and maintained in 0.9 × phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS) on ice before being placed on a Falcon nylon cell strainer (Beckton Dickinson); gravity was then used to obtain a single-cell suspension. For gata1a+ erythrocyte sorting, at least 60 embryos per group were needed to separate 105 erythrocytes for further analysis, including cell staining, quantitative PCR and RNA-sequencing.

2.2. Plasmid construction

Zebrafish genes, and mutants, were amplified from reverse transcription products and inserted into the tagged PCS2+ vector with their respective primers. In order to generate the luciferase reporter, several 1–2 kb upstream frames of zebrafish vgl4 and alas2 were amplified by PCR and ligated into the Kpn 1 and Sac 1 sites of the PGL3 basic vector (Promega). In order to construct key mutants and mutant sequence with the deletion that less than 40bp, we conducted a PCR using plasmids containing corresponding wild type (wt) genes as templates and primers that cover upstream and downstream regions of the deleted sites. The construction of mutants in which larger segments were deleted was generated using the same method as that for wild-type plasmid construction. All the primers used for plasmid construction are given in the Supplementary Tables 1–5.

2.3. Morpholinos and mRNA microinjection

Morpholinos (MOs) and mRNA microinjection are extremely valuable research tools used to study embryo development in vivo. While MOs mimic gene knock-down, mRNA microinjection simulates gene expression. The MOs were designed using GeneTools (www.genetools.com) [30] and the sequences are as follows: Zebrafish hf2bp2a MO: 5′-AGCAATCGCTTCTCTTGCCGCAA-3′; zebrafish tad1a MO: 5′-CATGGCAATGGATGTGATCTCAGA-3′; zebrafish hf1 MO: 5′-GGAACGATCTAAGCAGAGACAAG-3′.

2.4. Histology

Histological samples were disposed for Hematoxylin and Eosin (H & E) staining, and Prussian blue staining as described previously [32].

2.5. Flow cytometry and cell sorting

Peripheral blood was collected by cardiac puncture from two adult zebrafish lines, normal Tg(gata1a: dsRed) and vgl4b+/− Tg(gata1a: dsRed), following anesthesia with a solution of 0.1% tricaine (Sigma). Whole embryos were collected as well, then carefully dissected and maintained in 0.9 × phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS) on ice before being placed on a Falcon nylon cell strainer (Beckton Dickinson); gravity was then used to obtain a single-cell suspension. For gata1a+ erythrocyte sorting, at least 60 embryos per group were needed to separate 105 erythrocytes for further analysis, including cell staining, quantitative PCR and RNA-sequencing.

2.6. Cell staining

Sorted cells, or collected blood samples, were concentrated onto slides by centrifugation at a speed of 500 rpm for 3 min using a Cytospin column. These were then stained with Wright–Giemsa solution to observe erythrocyte modality.

2.7. Embryo staining

Embryos were fixed overnight before Sudan Black staining [33]. Embryos were stained with O-dianisidine according to a previous study [34]. In brief, the working concentration of o-dianisidine solution was 0.6 mg/mL and contained 0.01 M sodium acetate (pH 5), 40% ethanol and 0.8% hydrogen peroxide. Finally, fresh embryos were stained and gently shaken in the dark for 30 min at room temperature.

2.8. Embryo staining transmission electron microscopy (TEM)

Zebrafish embryos at 3dpf were collected and conducted using a previous method [35].

2.9. Extraction of DNA and RNA and quantitative PCR

DsRed+ cells were sorted from Tg(gata1a: DsRed) controls and Tg (gata1a: DsRed) vgl4b mutants at 72hpf. Total RNA was then extracted using TRIzol reagent (Life Technologies) and then reverse transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo). Amplification products were synthesized using a SYBR Green Real-time PCR Master Mix (TOYOBO).

2.10. Whole mount in situ hybridization (WISH)

Whole-mount mRNA in situ hybridization was conducted as described previously [36]. Digoxigenin (DIG)-labeled RNA probes were
transcribed with T7, T3 or SP6 polymerase (Ambion). Probes labeled with DIG (Roche) were detected using anti-digoxigenin Fab fragment antibody (Roche) with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) staining (Vector Laboratories).

2.11. RNA-Seq of zebrafish kidney marrow

Zebrafish kidney marrow were dissected from 6-month-old fish after anesthetized in ice, at least 15 fishes per group, then the kidney marrow were made into single-cell suspensions in 20 ml of 4°C PBS and were resolved in TRIZol reagent (Life Technologies) after centrifuged and removed the supernatant. Total RNA was extracted for RNA sequencing. RNA libraries were constructed using MGI Easy RNA Library Prep Set according to the manufacturer’s protocol. Libraries were sequenced using BGISEQ500 platforms.

2.12. Cell transfection and luciferase reporter assay

HEK293T cells was cultured in DMEM (Gibco) with 10% FBS (Gibco). Plasmid samples were transfected into cells using Effectene Transfection Reagent (QIAGEN), and were then harvested after 48–48 h before protein extraction, or luciferase reporter assays conducted with the Dual Luciferase Reporter Assay Kit (Promega), as previously described [37].

2.13. Fluorescent analysis of heme levels [38]

The fluorescent heme assay was conducted using a protocol described previously [39].

2.14. Western blotting and co-immunoprecipitation assay (Co-IP)

Cells were prepared as described previously [40]. Western blotting and co-immunoprecipitation assay were conducted using a previous methods [41]. Cells and tissues were degraded by RIPA lysis buffer (Beyotime) containing proteinase inhibitors (Roche). This mixture was then shaken on ice for 30 min and centrifuged at 15,000 × g for 30–45 min until a clear precipitate appeared. The supernatant was then removed and a rabbit anti-HA antibody (Sigma) and protein G agarose beads (Sigma, 30 μl per sample) were added and shaken overnight at 4°C. The next day, samples were washed three times in RIPA buffer and centrifuged at 6000 × g for 1 min. The supernatant was then removed and mixed with sample buffer (Laemmli 2 × concentrate, Sigma). Proteins were then analyzed by immunoblotting with mouse anti-FLAG (Sigma).

2.15. Western blotting of wt and mutant VGLL4b proteins

VGll4b sequences were amplified by PCR using cDNA from wt and vgll4b−/+ zebrafish, respectively, and then inserted into FLAG-tagged PCS2+ vector to construct flag-tagged vgll4b plasmids. These plasmids were subsequently transfected into H293T cells and protein bands were visualized using an anti-FLAG antibody.

2.16. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) [37]. Statistical significance was determined by a two-tailed unpaired t-test. For survival analysis, Kaplan–Meier survival curves were analyzed, statistical significances were calculated using GraphPad Prism 5.0. Quantitative data were collected from at least three independent experiments. For WISH and other embryo staining images, analysis involved at least 20–30 embryos. Positive areas of gene expression in every embryo involved in WISH analysis were selected and calculated as a proportion of the total captured embryo area using Photoshop CS6.

3. Results

3.1. VGll4b is upregulated in mild hypoxia during zebrafish embryonic differentiation

First, we conducted two experimental groups of zebrafish embryos, comprising normoxic condition as control and hypoxic condition (generated by MGC Anaero Pack incubation: 2.5 L, 5%–6% O2), result showed that vgll4b was up-regulated obviously in mild hypoxic condition (Supplementary Fig. 1A).

3.2. VGll4b-deficient zebrafish model displayed an increased mortality rate

The zebrafish model of mutant vgll4b (vgll4b−/−) was successfully established in our laboratory using CRISPR/Cas9 (Supplementary Fig. 1B); eight nucleotides were deleted leading to a non-functional Sma1 restriction site (Supplementary Fig. 1D) and resulting in a truncated VGLL4b protein of 22.4kD without any TDU domain (Supplementary Figs. 1C and D).

Survival analysis indicated a higher mortality rate in this vgll4b mutant line at 48 hpf, implying embryo dysplasia, followed by sustained and consistently higher mortality rates during adulthood, which is different from the survival curve of a premature-aging phenotype [42] (Supplementary Fig. 1E).

3.3. Mutation of vgll4b led to abnormal erythrocyte in zebrafish

First, splenomegaly and increased irregular erythrocytes accumulation in vgll4b mutants implied increased clearance of abnormal erythrocytes; Kidney paramorphia and increased erythroid precursors implied compensatory generation of erythrocytes (Fig. 1A, red arrow). Flow cytometry analysis of both adult fish peripheral blood and embryonic single cell suspensions showed normal erythrocyte counts (Supplementary Figs. 2A and B and Fig. 1B) while reduced heme levels in vgll4b−/− zebrafish (Fig. 1B).

In vgll4b−/− embryos, Wright–Giemsa staining revealed significantly paler cytoplasm, methylene blue staining revealed that the cytoplasm of abnormal erythrocytes contained hyperchromatic cytoplasmic DNA thus indicating an immature morphology and dysplasia erythroid terminal differentiation (Fig. 1C); Prussian Blue staining of the spleen showed few iron deposits (Fig. 1D).

3.4. Mutation of vgll4b resulted in ruined mitochondria and stimulated mitophagy in zebrafish erythrocyte

Erythrocyte terminal differentiation involves the elimination of mitochondria [35], which in zebrafish, begins at 3dpf, and is completed by 4 dpf [43,44]. In this study, transmission electron microscopy analysis showed that at 3 dpf, vgll4b mutants presented an immature erythrocyte phenotype with swollen membrane-ruptured mitochondria (Fig. 1E). Quantitative PCR analysis of the mitophagy-related gene, gata1α+ erythrocytes sorted at 3 dpf, revealed a slight increase in bnip3 expression in the mutant animals (Supplementary Fig. 2C), hinting at upregulated mitophagy [45]. Collectively, the data suggested that vgll4b−/− zebrafish generated abnormal and immature erythrocytes, leading to increased levels of compensatory erythroid cell turnover in an attempt to maintain erythroid cell numbers, albeit characterized by lower heme levels and abnormal mitochondria.

3.5. Expression of mutant vgll4b led to heme biosynthesis-related gene inhibition and alas2 downregulation in zebrafish erythropoiesis

Firstly, HSC and lineage-specific markers in vgll4b mutants were examined by whole-mount in situ hybridization (WISH) to detect other hematopoietic lineage changes. We found that with the expression of
the HSC marker c-myb, which was slightly elevated, the levels of all other assayed markers were unchanged (Supplementary Fig. 3).

We next investigated the effect of vgll4b depletion on erythropoiesis, globin chain expression, heme synthesis and hemoglobin maturation. Although we observed a significant reduction in o’dianisidine staining ($P=0.008$; Fig. 2C and L), the expression levels of the erythrocyte progenitor gene marker, gata1a [46], and the globin production-inducing gene, klf1 [47], remained unchanged (Fig. 2A and B). Expression levels of the major embryonic hemoglobin chain genes [48] varied little until 5 dpf, when $hbae1.1$ was slightly downregulated (Fig. 2D–H, Supplementary Figs. 4A–C), suggesting a secondary phenomenon resulting from impaired heme levels [49]. However, we detected the clear downregulation of the genes involved in mitochondrial heme synthesis: $slc25a38$[50], $slc25a37$[51] and $alas2$[52] (Fig. 2I–L).

3.6. Expression of mutant vgll4b led to heme biosynthesis-related gene inhibition and alas2 downregulation in zebrafish erythropoiesis

To determine whether other potential erythropoiesis-related genes were altered, we conducted RNA-sequence analysis of adult zebrafish kidney marrow. The gene ontology (GO) analysis tool that detect vgll4b mutation in zebrafish was significantly associated with tetrapyrrole
binding, heme binding and the redox reaction (Figure 2M). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of categories enriched for differentially-expressed genes (DEGs) indicated that the porphyrin pathway was strongly enriched (Figure 2N). Furthermore, various genes related to heme biosynthesis were indeed downregulated, with *alas2* exhibiting the greatest expression change (Figure 2O and Supplementary Figure 4J).

We next examined the expression of *alas2* every 4 h from 16 to 32 hpf in *vgll4b*/*vgll4b* mutant zebrafish (skipped 28hpf as tested in Figure 2K). As early as 20 hpf, *alas2* levels in the ICM region were markedly reduced, and declined further until 24 hpf (Supplementary Figures 4D and E). From 28 to 32 hpf, *alas2* expression became detectable in the peripheral blood circulation, the VDA, and the yolk sac, while total expression levels remained significantly lower in *vgll4b* mutants (Figure 2K and Supplementary Figure 4G). *Alas2* expression is primarily regulated by the erythroid-specific transcription factors, GATA1 [52,53] and EPO/EPOR [54]. Since these factors were not decreased in the mutant fish (Figure 2A and Supplementary Figure 4H), the molecular mechanisms underlying *alas2* downregulation remain to be determined. As zebrafish embryonic-to-adult globin chain switching occurs at around 10 dpf [48], qPCR analysis of the peripheral blood cells sorted at 18 dpf, showed a significant downregulation of adult hemoglobin and transferrin genes (Figure 2P and Q, respectively).

Taken together, our results demonstrate that the presence of a dysfunctional *vgll4b* gene leads to impaired heme synthesis and hemoglobin generation, primarily via the suppression of *alas2* and other related genes.

3.7. *Vgll4b*-depletion retarded *hif1a*-regulated hemoglobin maturation

The mitochondrial biosynthesis of heme is critical for oxygen transport and resistance to oxidative stress [29]. In order to test the resistance to oxidative stress, we assessed the survival of the four experimental groups; wild-type (WT) and *vgll4b* mutant embryos, each under either normoxic or hypoxic conditions (using the MGC AnaeroPack® System, set to 2.5 L, 5%–6% O2). *Gata1α*+ erythrocytes from each group were collected at 3 dpf and RT-PCR was performed to evaluate the expression of genes associated with heme biosynthesis. The results showed that *vgll4b* mutants exhibited inferior survival relative to WT embryos under normoxic conditions, and that incubation under hypoxic conditions, exacerbated the difference in their progenies (P = 0.002 vs. P < 0.001, respectively; Figure 3A). Furthermore, genes, such as *alas2*, were upregulated in WT embryos in the hypoxia group, consistent with compensatory heme synthesis hypothesis, as observed previously in a mouse model [55]. In contrast, we detected the dramatic downregulation of *alas2*, *uro*, *sle25a37*, and *sle25a38* expression in *vgll4b* mutants, along with normal levels of the *sle25a38* homologs, *sle25a39* [56], and *sle25a39*, which are ubiquitously expressed and not known to be involved in heme biosynthesis. Additionally, these genes were not upregulated during hypoxia (Figure 3B)

Taking into account that: i) elevated *hif1a* levels can induce heme biosynthesis by upregulating *alas2* expression [55]; and ii) *hif1ab* (which shares a high level of similarity with human *hif1a*) is highly expressed in the great vessels of zebrafish between 24 hpf and 28 hpf [57]; we next set out to explore the possibility that HIF1α-induced erythroid compensation is mediated by VGLL4. *Hif1ab* mRNAs were injected into either WT control or *vgll4b* mutant embryos, and the expression levels of *alas2*, *hbae3* and a hemarker were quantified using o’dianisidine staining. In *vgll4b* mutant embryos, a clear drop in expression in *alas2* expression levels and o’dianisidine staining occurred, even following *hif1ab* mRNA injection. In contrast, the expression levels of *hbae3* remained unaltered (Figure 3C and D). Importantly, *vgll4b* mRNA rescued the impaired hemoglobin phenotype triggered by *hif1a* MO knockdown (Figure 3E and F).

Based on our TEM analysis of erythroid morphology, *hif1ab* mRNA injection rendered the 3 dpf erythrocyte more fusiform-shaped and less mitochondria-like, similar as seen in WT erythrocyte at 4 dpf, implying accelerated erythroid terminal differentiation. However, *hif1ab* mRNA injection did not rescue this phenotype in *vgll4b* mutants. Erythrocyte receiving a *Hif1a* MO injection, exhibited a similar, albeit milder phenotype than *vgll4b* mutants (Figure 3G). Taken together, our data suggest that HIF1α-induced heme biosynthesis is partly dependent on VGLL4.

3.8. HIF1α - VGLL4 axis induced erythroid differentiation dependent on NOTCH1

We set out to find potential genes downstream *hif1a*. On condition that genes *notch1* [58], *gata1α* [59], *kfl1* [60,61], *fl1* [62] can all be regulated by *hif1a* and they have all been reported to affect hematopoiesis, we set to inject all the corresponding mRNAs to detect *alas2* expression level, among them we overexpressed *notch1* intracellular domain (NICD) in presentation of NOTCH1 [58]. As a result, only HIF1α and NICD led high expression of *alas2*, which was attenuated by *vgll4b* mutation (Figure 4A and B). Furthermore, HIF1α could not stimulated *alas2* expression any longer when WT zebrafish embryo were exposure to DAPT (GSI-IX, γ-secretase inhibitor IX) at 300 μM, implied an *alas2*-inducing axis of HIF1α-NOTCH1-VGLL4 (Figure 4C).

To verify a potential target site of HIF1α or NOTCH1 to *vgll4b*, two successive 4863 bp segments of the sequences upstream of the *vgll4b* coding region were cloned: *alas2*promoter (−2992 to −523) and *alas2*promoter (−710 to +1871) (Figure 4D). Luciferase reporter gene analysis results reflected that HIF1α did not make significant difference to *vgll4b* expression, while an about 400 bp segment (−1622 to −1210) upstream of the *vgll4b* open reading frame seems to be a binding site of NOTCH1 and can mediated the stimulation of NICD to *vgll4b* express (Figure 4E and G).
3.9. VGLL4b activation of heme biosynthesis is mainly dependent on its TDU1 domain and can be enhanced by mutation of the K225 acetylation site

As VGLL4 is an ancillary transcription factor which can interact with several proteins via its two TDU domains [63], and has an acetylation site at K225 (equivalent to K221 in zebrafish VGLL4b) that is important for its impact on VGLL4-TEAD compound [26], five mutants of VGLL4b were constructed: VGLL4bKM1 (an A to R change at K221), VGLL4bKM2 (an A to R change of K224); VGLL4bDT1 (TDU1 domain deletion), VGLL4bDT2 (TDU2 domain deletion), and VGLL4bDT1DT2 (deletion of both TDU domains) (Fig. 5A).

Interestingly, VGLL4bKM1 had a superior rescue effect relative to the wild type VGLL4b, indicating enhanced protein-protein interaction caused by the K221 mutation, leading to more effective rescue of the erythroid dysfunction. As expected, VGLL4bKM2 achieved a similar rescue effect to the wild type protein. Furthermore, VGLL4bDT2 exhibited a better rescue effect than VGLL4bDT1 and VGLL4bDT1DT2, implying that VGLL4b likely exerts its function in erythropoiesis by...
interacting with a protein that preferentially binds to the TDU1 domain, rather than TDU2 (Fig. 5B and C).

RT-PCR at 3 dpf showed similar results in genes of uros and slc25a38a (Supplementary Fig. 5E).

3.10. VGLL4b induces heme biosynthesis and erythrocyte terminal differentiation via the sequestration of IRF2BP2, thus inhibiting the IRF2BP2-mediated suppression of target genes

The VGLL4 TDU domains form bridges to interact with proteins such as TEAD1(26), IRF2BP2 [64] and Myocyte Enhancer Factor 2B (MEF2) [65]. Therefore, we injected irf2bp2a, irf2bp2b, tead1a, tead3b, mef2ca and mef2cb mRNAs into zebrafish embryos. The results showed that the overexpression of both irf2bp2a and irf2bp2b, suppressed heme
generation (Supplementary Fig. 5A, i, iv and v), while the other four genes had no effect (Supplementary Fig. 5A, B and D). This result was further confirmed by whole-embryo RT-PCR analysis (Supplementary Fig. 5C).

We next confirmed a direct interaction between zebrafish VGLL4b and IRF2BP2a by co-immunoprecipitation (Co-IP) experiments conducted in H293T cells (Fig. 5D). Consistent with previous reports [66], in this study, this interaction appeared to be more dependent on zebrafish VGLL4b TDU1 domain (Fig. 5D).

Interestingly, irf2bp2a MO knockdown rescued the impaired heme

generation in irf2bp2b−/− mutants. More importantly, the effects exerted by irf2bp4a overexpression in the mutants were also abolished by irf2bp2a MO (Fig. 5E and F). This surprising result shows that irf2bp2a knockdown negated irf2bp4a’s influence, meaning that without irf2bp2a, irf2bp4a has little effect on erythroid maturation. Similar results were observed in irf2bp4a−/− embryos (Supplementary Figs. 5F and G), suggesting that VGLL4b function in the context of heme
generation and alas2 expression, is dependent on IRF2BP2.

3.11. VGLL4b induced alas2 expression by reducing the IRF2BP2-mediated transcriptional suppression, and uncoupled the co-suppressive effects of IRF2BP2 and its partner ETO2

To verify whether the inhibition of heme generation in irf2bp4a mutants was mediated by IRF2BP2, we next examined the influence of irf2bp2a on alas2 expression and heme generation. Previous studies had identified an IRF2BP2 binding site in the alas2 gene promoter region using the MEL cell line [4]. To identify the IRF2BP2 target site in the zebrafish alas2 gene, two successive 5361 bp segments upstream of the alas2 coding region were cloned: alas2prom (−679 to +2433) and alas2prom (−2996 to −82) (Supplementary Fig. 6A). A series of constructs were then generated for use in luciferase reporter gene analysis, which showed that a 240 bp segment (−2700 to −2460) upstream of the alas2 open reading frame (ORF), referred to as alas2prom, was a potential target site for IRF2BP2 (Supplementary Figs. 6B–C). This target site also contains several predicted binding sites for other erythroid terminal differentiation regulators [4,67].

CBFA2/RUNX1 Partner Transcriptional Co-Repressor 3 (CBFA2T3, namely ETO2) is a transcriptional co-repressor involved in the 

co-ordination of erythroid cellular proliferation, differentiation, and maturation [4,68], which can interact with IRF2BP2 to co-suppress erythroid terminal differentiation [4]. We performed comprehensive luciferase assays to dissect the roles of IRF2BP2, ETO2 and VGLL4 in the regulation of alas2 expression (Fig. 6A). While VGLL4 or ETO2 alone did not exhibit any repressive effect, IRF2BP2 alone suppressed luciferase activity by approximately 30%, compared with control samples (P = 0.004). Importantly, the co-expression of ETO2 further enhanced IRF2BP2-mediated transcriptional repression (P = 0.001). However, this enhancement was counteracted by VGLL4a in a dose-dependent manner. VGLL4b only lost its ability to counteract IRF2BP2/ETO2 transcriptional repression activity following the deletion of its TDU1 domain (VGLL4bΔTDU), consistent with our previous in vivo rescue data (Fig. 5B and D).

IRF2BP2 contains a potential DNA-binding zinc finger motif and a protein-protein interaction ring finger motif [64,66,69]; Therefore, we constructed two mutants with each of these domains deleted: IRF2BP2aΔZinc and IRF2BP2aΔRing, respectively (Fig. 6B). Co-IP analysis showed that IRF2BP2 interacted with VGLL4a via amino acids 1050–1476, and the deletion of the zinc finger did not interfere with this interaction (Fig. 6C). Furthermore, we found that in the luciferase assays, IRF2BP2aΔZinc could no longer repress alas2 expression, negating both the synergistic effect of ETO2 and the rescue function of VGLL4a simultaneously. Interestingly, while IRF2BP2aΔRing still repressed alas2 expression to some extent, it completely lost either the ability to synergize with ETO2 or be rescued by VGLL4a. These results indicate that, by coordinating zebrafish alas2 expression, both the zinc finger and the ring finger domains of IRF2BP2 are indispensable. For productive interactions with either ETO2 or VGLL4, ring finger domain of IRF2BP2 is indispensable. Thus, the inhibitory function of VGLL4a, mediated via IRF2BP2, depends on both its zinc

generation and alas2 expression. Therefore, the interpretation that high cbga2d3 (the gene encoding ETO2) levels suppressed heme biosynthesis feasibly via its endogenous partners (Fig. 6E and F).

4. Discussion

Low oxygen density is typical during the early stage of embryonic development [5,6]. On sensing hypoxic conditions (1–5% oxygen), HIF1α is a sensitive oxygen responder [8–11] and has been widely reported to be essential for HSC formation in the AGM [7,12] and expand HSPCs [13]. However, persistent hypoxia is detrimental to embryonic
development [70,71] and biological evolution [72,73], and can in some cases result in serious disease or death [74]. Thus, erythropoiesis represents a considerably more effective method for oxygen consumption. To our knowledge, this study is the first to delineate the oxygen sensing pathway used in erythropoiesis terminal differentiation to improve oxygen consumption, and thus serves to expand our knowledge on the relationship between erythroid differentiation and the environment.

Heme synthesis [29] is indispensable for the erythroid differentiation and oxygen delivering, and regulates the expression of the adult α- and β-globin genes to ensure balanced synthesis [3]. Dysfunction of ALAS2 in erythroblasts within the hematopoietic organs of erythroblasts, typically results in impaired heme biosynthesis, excessive iron accumulation and oxidative stress-induced cell damage, as a consequence of inadequate PPIX levels, relative to the available iron. Thus
patients with ALAS2 dysfunction invariably exhibit hypochromic, microcytic anemia [75] and iron deposition in the cytoplasm [16,76], observations which are largely consistent with the vgll4b mutant zebrafish phenotype [77]. However, vgll4b mutant zebrafish exhibit normal erythrocyte counts without iron deposition, which could be potentially explained by impaired iron absorption mechanisms [76,77] (Figure 2Q). As a result, decreased iron absorption reduces oxidative stress-induced cell damage, thereby not altering erythrocyte counts.

The vgll4b mutant embryos exhibited normal β embryonic globin chain expression until 4 dpf, while further qPCR analysis showed that the adult globin genes were significantly down-regulated in vgll4b mutants (Fig. 2P), thus confirming the impaired adult globin levels, reported in vgll4b mutants [4].

It has been reported that the VGLL4-TEAD1 interaction can be enhanced by the mutation of the K225 acetylation site in VGLL4 TDU1 [26]. TDU1 is crucial for the interaction of human VGLL4 with human IRF2BP2, as demonstrated in C2C12 mouse myoblasts [64]. On deletion of both TDUs, VGLL4 is still able to interact with IRF2BP2 in HEK293T cells [66]. Furthermore, the TDU domains were shown not to be required for the interaction of VGLL4 with IRF2BP2 and the regulation of PD-L1 in human lung cancer A549 cells [66]. In this study, however, the interaction between zebrafish VGLL4b and IRF2BP2a is dependent on the TDU1 domain. The mutation at the TDU1 acetylation site, enhanced the rescue effect exerted by VGLL4b (Fig. 5B iv). Consequently, TDU1 deletion significantly weakened: i) the VGLL4b/IRF2BP2a interaction (Fig. 5D); ii) the ability of VGLL4b to rescue ala2 expression (Fig. 6A); and iii) the ability of VGLL4b to rescue zebrafish heme biosynthesis (Fig. 5B vi). Collectively, these results assign an important role to the TDU1 domain of VGLL4 in the context of heme biosynthesis. Interestingly, TDU1 appears to have different roles in different mouse cells and is important in zebrafish in terms of promoting the IRF2BP2/VGLL4b interaction. However, while TDU1 appears critically important for the IRF2BP2/VGLL4b interaction in zebrafish and humanized animal models, its...
function may be redundant in this regard, in human cells. Whether the IRF2BP2/VGLL4 protein interaction is compensated for in more advanced species like humans, or whether this interaction is highly dependent on the species-specific environmental conditions, deserves further explored.

VGLL4 is reported to stabilize and enhance the suppressive effect of IRF2BP2 on IRF2 expression, in the regulation of PD-L1 expression in malignant cells [66]. In this study, we set out to test vgl4b mutant zebrafish model, and surprisingly detected the potentially IRF2BP2-mediated downregulation of various genes [4], implying that the inhibitory effect of IRF2BP2 was enhanced by vgl4b depletion. This seemingly opposing result observed in zebrafish erythrocytes, compared to tumor cell lines, implies that the VGLL4 and IRF2BP2 relationship may be delicately fine-tuned, depending on the...
environmental setting, with further research required on the relationship between the degradation and bioeffect of IRF2BP2.

Furthermore, since VGLL4 is also essential for heart growth [26] and valve development [80], it makes sense that VGLL4 may be regulated by oxygen level in other tissues. Thus, it is probable that VGLL4 can trigger the oxygen-delivery system development in both the cardiomyocytes and erythrocyte. The involvement of VGLL4 in oxygen sensing, delivery and organ development merits further exploration.

Additionally, other NOTCH family members may be involved in regulating oxygen-dependent organ development processes. For example, NOTCH2 regulates cortical neurogenesis and bone metabolism [81,82], while, NOTCH3 plays a critical role in esophageal squamous cell differentiation and angiogenesis [83,84].

In conclusion, this study shows that vgll4b is up-regulated at low oxygen concentration and can mediate NOTCH1-dependent HIF1α-induced heme biosynthesis. VGLL4b induces alas2 expression by reducing the suppressive effects of IRF2BP2 and its binding partner ETO2. As a result, erythroid maturation is improved through enhanced oxygen metabolism (Fig. 7). Hypoxia is considered as a limiting factor in embryonic development. Our results reveal that oxygen actually acts as a diffusible inducing factor for embryonic erythropoiesis under aerobic conditions. Based on published work, describing how the spatial distribution of oxygen or HIF1α plays a crucial role in local organ development [58,85], we have highlighted a loop that exists between oxygen distribution, molecular modulation and the development of oxygen delivery systems. Thus, the precise regulation of oxygen provision and its sensory downstream receptors are critical factors in embryonic development at spatial and temporal levels.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].
Confl icts of interest
The authors declare that they have no competing financial interests.

Authors’ contributions
JZ contributed to the conception and design of the study. YW, XL and BX performed the experiments and analyzed the data. HY and YZ assisted with the experiments. JZ and YW wrote the paper. All authors read and approved the final manuscript.

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Fig. 7. Bioeffects of IRF2BP2 mutants in synergistic effect with ETO2. (A) Bioeffects of IRF2BP2a mutants on heme biosynthesis suppression in addition to ETO2 detected by o’dianisidine stain. (B) Staining positive area compare among subgroups of IRF2BP2 mutants, depletion of either Zinc Finger or Ring Finger can abolish suppress effect of ETO2. (C) Model for the regulation of heme biosynthesis by alas2 et al., regulated by HIF1α, VGLL4 and IRF2BP2. HIF1α induced alas2 expression dependent on NOTCH1 and VGLL4. VGLL4 induced alas2 expression by reducing the IRF2BP2 transcription suppression and uncoupling the co-suppressor effects of IRF2BP2 and its partner ETO2. Data are shown as the mean ± SEM of at least 15 to 30 embryos in each subgroup. *p < 0.05, **p < 0.01, ***p < 0.001. NS denotes no significant difference.

Conflicts of interest
The authors declare that they have no competing financial interests.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101313.

References
[1] R.S.S. Wu, Hypoxia: from molecular responses to ecosystem responses, Mar. Pollut. Bull. 45 (2002) 35–45.
[2] G.L. Semenza, Regulation of oxygen homeostasis by hypoxia-inducible factor 1, Physiology 24 (2009) 97–106.
[3] D. Chiabrando, S. Mercurio, E. Tolosano, Heme and erythropoiesis: more than a structural role, Haematologica 99 (2014) 973–983.
[4] R. Stadhouders, A. Cico, T. Stephen, S. Thongjuea, P. Kolovos, H.I. Baymaz, X. Yu, J. Demmers, K. Rezstarosti, A. Maas, et al., Control of developmentally primed erythroid genes by combinatorial co-repressor actions, Nat. Commun. 6 (2015).
[5] P.C. Steptoe, R.G. Edwards, J.M. Purdy, Human blastocysts grown in culture, Nature 229 (1971) 132–133.
[6] A.V. Gore, L.M. Pillay, M.V. Galantternik, B.M. Weinstein, The Zebrafish: A Fintastic Model for Hematopoietic Development and Disease vol. 7, Wiley Interdisciplinary Reviews-Developmental Biology, 2018.
[7] P. Imanirad, P. Solaimani Karralaei, M. Gisani, C. Vink, T. Yamada-Inagawa, E. de Pater, D. Kurek, P. Kaimakis, R. van der Linden, N. Speck, et al., HIF1alpha is a regulator of hematopoietic progenitor and stem cell development in hypoxic sites of the mouse embryo, Stem Cell Res. 12 (2014) 24–35.
