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# Genomic conservation of erythropoietic microRNAs (erythromiRs) in white-blooded Antarctic icefish

**Dr. Thomas Desvignes**[a], **Prof. H. William Detrich III**[b], and **Prof. John H. Postlethwait**[a,*]

[a]Institute of Neuroscience, University of Oregon, Eugene, OR, 97403, USA

[b]Department of Marine and Environmental Sciences, Northeastern University Marine Science Center, Nahant, MA, 01908

## Abstract

White-blooded Antarctic crocodile icefish are the only vertebrates known to lack functional hemoglobin genes and red blood cells throughout their lives. We do not yet know, however, whether extinction of hemoglobin genes preceded loss of red blood cells or vice versa, nor whether erythropoiesis regulators disappeared along with hemoglobin genes in this erythrocyte-null clade. Several microRNAs, which we here call erythromiRs, are expressed primarily in developing red blood cells in zebrafish, mouse, and humans. Abrogating some erythromiRs, like *Mir144* and *Mir451a*, leads to profound anemia, demonstrating a functional role in erythropoiesis. Here, we tested two not mutually exclusive hypotheses: 1) that the loss of one or more erythromiR genes extinguished the erythropoietic program of icefish and/or led to the loss of globin gene expression through pseudogenization; and 2) that some erythromiR genes were secondarily lost after the loss of functional hemoglobin and red blood cells in icefish. We explored smallRNA transcriptomes generated from the hematopoietic kidney marrow of four Antarctic notothenioids: two red-blooded species (bullhead notothen *Notothenia coriiceps* and emerald notothen *Trematomus bernacchii*) and two white-blooded icefish (blackfin icefish *Chaenocephalus aceratus* and hooknose icefish *Chionodraco hamatus*). The *N. coriiceps* genome assembly anchored analyses. Results showed that, like the two red-blooded species, the blackfin icefish genome possessed and the marrow expressed all known erythromiRs. This result indicates that loss of hemoglobin and red blood cells in icefish was not caused by loss of known erythromiR genes. Furthermore, expression of only one erythromiR, *mir96,* appears to have been lost after the loss of red blood cells and hemoglobin – expression was not detected in the erythropoietic organ of hooknose icefish but was present in blackfin icefish. All other erythromiRs investigated, including *mir144* and *mir451a,* were expressed by all four species and thus are present in the genomes of at least the two white-blooded icefish. Our results rule out the hypothesis that genomic loss of any known erythromiRs extinguished erythropoiesis in icefish, and suggest that after the loss of red blood cells, few erythromiRs experienced secondary loss. Results suggest that functions

*Corresponding author: Prof. Postlethwait, John H.: jpostle@uoneuro.uoregon.edu.

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independent of erythropoiesis maintained erythromiRs, thereby highlighting the evolutionary resilience of miRNA genes in vertebrate genomes.

**Keywords**

miRNA; Notothenioidei; hematopoiesis; Channichthyidae; *mirc144*

## 1. Introduction

Since the first report by the Norwegian biologist Ditlef Rustad of a fish with “colorless blood” that he caught near the sub-Antarctic Bouvet Island (Bouvetøya) in December 1927 and the confirmation in 1954 by Johan Ruud that this species, the blackfin icefish *Chaenocephalus aceratus*, lacks both red blood cells and hemoglobin (Hb) (Ruud, 1954), the “crocodile icefish” of the Southern Ocean have puzzled physiologists. Icefish comprise the only vertebrate clade whose members lack red blood cells and the oxygen transport protein hemoglobin throughout their life cycles. Spurred by these seminal observations, contemporary molecular biologists and physiologists have sought to understand the evolutionary mechanism(s) that led to the loss of mature red blood cells and hemoglobin in icefish and the physiological traits that enable these unique vertebrates to survive without oxygen-binding proteins in their blood (Braasch et al., 2015; Cheng and Detrich, 2007; Holeton, 1970; Kock, 2005a, 2005b; Near et al., 2006; Sidell and O’Brien, 2006).

Among the approximately 130 notothenioid species currently recognized, only the 16 species of the notothenioid crown group, the icefish family Channichthyidae, have lost functional hemoglobin genes (Cheng and Detrich, 2007; Giordano et al., 2015; Near et al., 2006; Sidell and O’Brien, 2006). The loss of functional hemoglobin genes is a shared, derived feature (synapomorphy) that left 15 of the 16 icefish species with a pseudogenized α-globin gene and no β-globin gene, and one species, Jonah’s icefish (*Neopagetopsis ionah*), with an inactive αβ-globin pseudogene complex, a “genomic fossil” derived by gene introgression (Cocca et al., 1995; di Prisco et al., 2002; Near et al., 2006). Furthermore, all notothenioids lost myoglobin (Mb) expression in their skeletal muscles (Sidell et al., 1997), and six species of icefish have lost expression of myoglobin in addition in their heart muscle by multiple independent mutational events (Borley and Sidell, 2010; Grove et al., 2004; Sidell et al., 1997; Sidell and O’Brien, 2006; Small et al., 2003). Several other teleost lineages have also lost cardiac myoglobin expression, including the circum-Arctic three-spined stickleback (*Gasterosteus aculeatus*), which possesses a pseudogenized myoglobin gene (Hoffmann et al., 2011; Macqueen et al., 2014).

Whether the loss of functional hemoglobin genes in icefish evolution preceded the loss of red blood cells or extinction of erythropoiesis came first is as yet an unanswered question. To address this issue, we must understand the cascade of events that led to the disruption of globin genes in icefish genomes and the disappearance of red blood cells, which are produced in the pronephric (head) kidney marrow of teleost fish (Fänge, 1994; Witeska, 2013). Did the fixation of deleterious mutations in hemoglobin genes lead to the loss of red blood cells, despite their near-universal additional role in carbon dioxide/bicarbonate

metabolism and transport (Maffia et al., 2001; Tufts et al., 2002)? Alternatively, did red blood cells disappear first, followed by mutations that rendered hemoglobin genes nonfunctional? If erythropoiesis disappeared first, what mechanism led to the suppression of red blood cell development? Did positive regulators of erythropoiesis become non-functional? Or did ancestral icefish evolve mechanisms that actively suppress erythroid development?

Although many protein-coding genes regulate erythropoiesis, such as *gata1* (Galloway et al., 2005; Welch et al., 2004), *myb* (Vegiopoulos et al., 2006), *spi1b* (also known as *pu.1* (Rhodes et al., 2005)) and *bty* (discovered by subtractive hybridization using the hematopoietic transcriptomes of red-blooded and white-blooded notothenioids (Yergeau et al., 2005)), microRNAs also play a key role. miRNAs are small endogenous non-coding RNAs that regulate gene expression post-transcriptionally by binding to specific mRNAs and, together with the RNA-Induced Silencing Complex, either mediate transcript decay or repress transcript translation (See Carthew and Sontheimer, 2009; Christodoulou et al., 2010; Desvignes et al., 2015; Kosik, 2010 for reviews on biogenesis and function). In vertebrates, including teleost fish, several miRNAs are necessary for the formation of red blood cells and functional hemoglobin, and we refer to them here as “erythromiRs” in analogy to the well-described muscle-specific microRNAs referred to as “myomiRs” (McCarthy, 2008). For example, the erythromiR *MIR155* is expressed strongly in early stages of erythropoiesis but dramatically weaker at later stages. In contrast, *MIR451A* displays the opposite expression pattern, increasing in expression more than 200-fold between progenitor stages and late stages of erythropoiesis (Masaki et al., 2007). Furthermore, knockdown and knockout experiments showed that *mir451a* and its clustered companion *mir144* produce microRNAs that are major erythropoietic factors required for erythroid precursor maturation in zebrafish (Dore et al., 2008; Du et al., 2009; Fu et al., 2009; Pase et al., 2009; Yu et al., 2010), mouse (Patrick et al., 2010; Rasmussen et al., 2010; Yu et al., 2010; Zhan et al., 2007) and human (Kim et al., 2015, 2013). In addition, the mature products of both *Mir144* and *Mir451a* protect red blood cells from oxidative stress in zebrafish, mouse, and human (Sangokoya et al., 2010; Yu et al., 2010). Other miRNA genes are also important erythropoietic factors, including *mir23a*, *mir126*, and *mir223* (see Azzouzi et al., 2012; Bhagavathi and Czader, 2010; Havelange and Garzon, 2010; Lawrie, 2010; Listowski et al., 2012; Mohammdai-asl et al., 2015; Sayed and Abdellatif, 2011; Undi et al., 2013; Zhang et al., 2012 for reviews).

In addition to a role in erythropoiesis, miRNAs participate more generally in hematopoiesis. Some miRNAs, such as *mir150*, *mir155,* and *mir223*, also play roles in the development of megakaryocytes/platelets and the T cell and B cell lineages (see Bhagavathi and Czader, 2010; Havelange and Garzon, 2010; Sayed and Abdellatif, 2011; Undi et al., 2013; Zhang et al., 2012 for review), while some other miRNAs, such as *mir142* or *mir181*, regulate development of blood cell lineages other than erythrocytes (Chen et al., 2004; Fan et al., 2014; Kramer et al., 2015). These data show that miRNAs are key regulators of hematopoiesis, including erythropoiesis, and suggest the hypothesis that loss of one or more miRNAs could have been important in the evolution of the erythrocyte-null, hemoglobin-null phenotypes of white-blooded icefish.

Recently, Xu et al. (2015) looked for potential erythropoietic suppressor miRNAs in white-blooded icefish and reported that *mir16b*, *mir152*, and *mir1388* were over-expressed in the pronephric kidney of hooknose icefish *Chionodraco hamatus* (See Appendix for etymology) compared to their expression in the red-blooded emerald notothen *Trematomus bernacchii*. Injection of each of these three miRNAs into zebrafish embryos reduced the production of red blood cells (Xu et al., 2015), but other developmental defects also occurred that can appear due to non-specific deleterious effects of over-expression experiments (Jin et al., 2015; Zhang et al., 2013). Despite their interesting findings and important dataset, these authors did not examine the global conservation of erythromiRs in icefish, leaving open the possibility that icefish genomes may have lost some key erythromiR genes that are required for normal erythropoiesis or erythroid maturation.

We took advantage of the recently published reference genome of the red-blooded bullhead notothen, *Notothenia coriiceps* (Shin et al., 2014), smallRNA sequencing data from the red-blooded emerald notothen *T. bernacchii* and the white-blooded hooknose icefish *C. hamatus* (Xu et al., 2015), and smallRNA sequencing data that we generated from *N. coriiceps* and the white-blooded blackfin icefish *Chaenocephalus aceratus*, to test two hypotheses regarding the role of erythromiRs in the novel erythroid phenotypes of icefish. In Hypothesis 1), the loss of known erythromiR genes in Antarctic icefish correlates with the loss of functional red blood cells and/or the disruption of their globin genes; and in Hypothesis 2), the loss of hemoglobin and red blood cells by icefish secondarily allowed the loss of some or all erythromiR genes in some icefish lineages.

## 2. Materials and Methods

### 2.1. Fish samples

Specimens of *C. aceratus* and *N. coriiceps* were collected by bottom trawls or baited fish traps deployed from the *ARSV Laurence M. Gould* south of Low Island or west of Brabant Island in the Palmer Archipelago (April–May, 2014). Fish were transported alive to Palmer Station, Antarctica, where they were maintained in flow-through seawater aquaria at −1.5 to 1°C. Samples of pronephric (head) kidney, the major site of erythropoiesis in teleost fish (Fänge, 1994; Witeska, 2013), were dissected and stored in RNAlater until further use at the University of Oregon. Procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Oregon (#10-26) and of Northeastern University (#12-0306 R).

### 2.2. SmallRNA-sequencing and analysis

Total RNAs were extracted from the pronephric kidney of one male *C. aceratus* and one male *N. coriiceps* using the Zymo Research Direct-zol™ RNA MiniPrep kit according to the manufacturer's instructions. Two species-specific smallRNA libraries were then prepared and barcoded using the BiooScientifc NEXTflex™ small RNA sequencing kit with 15 PCR cycles and sequenced by Illumina HiSeq2500 at the University of Oregon Genomics Core Facility. Raw single-end 50-nt long reads were deposited in the NCBI Short Read Archive under accession numbers SRP069031 and SRP069032 for *C. aceratus* and *N. coriiceps* respectively. Pronephric kidney smallRNA reads from *T. bernacchii* and *C.*

*hamatus* were retrieved from (Xu et al., 2015). The method used to generate the libraries for the latter two species was not reported in detail (Xu et al., 2015).

Reads from the four pronephric kidney libraries were processed identically using a new bioinformatic tool, *Prost!*, which is available online at https://github.com/uoregon-postlethwait/prost (Batzel et al., 2015). Briefly, raw reads were trimmed from adapter sequences, filtered for quality using the FASTX-Toolkit, size-selected for lengths between 17 and 25 nucleotides, filtered for a minimum of five identical reads, and grouped by genomic location using the published *N. coriiceps* genome assembly as a reference genome (Shin et al., 2014). Groups of sequences were then annotated against mature and hairpin sequences present in miRBase Release 21 (Kozomara and Griffiths-Jones, 2013), the extended zebrafish miRNA annotation (Desvignes et al., 2014), and the spotted gar annotation (Braasch et al., 2016). Gene nomenclature follows recent conventions (Desvignes et al., 2015), including those for zebrafish (Bradford et al., 2011).

## 3. Results and Discussion

### 3.1. Sequencing statistics

After sequence filtering and read grouping as described in section 2.2, the four Antarctic species *N. coriiceps*, *T. bernacchii*, *C. hamatus* and *C. aceratus* provided about 200,000, 6.3 million, 7.8 million, and 4.8 million reads, respectively. Because variations in library preparation protocols precluded statistically sound differential expression analysis among samples (e.g. see Baran-Gale et al., 2013; Hafner et al., 2011; Pritchard et al., 2012; Raabe et al., 2014; Tian et al., 2010), we were not able to perform reliable differential expression analysis with this dataset. The data are, however, robust for qualitative analysis, such as identifying specific miRNAs in each species' smallRNA transcriptome and, therefore providing positive proof for the presence of the encoding gene in each species' genome.

### 3.2. Antarctic fish genomes possess erythropoietic miRNAs

The hypothesis that the loss of erythropoietic miRNAs led to the loss of red blood cells and hemoglobin in white-blooded icefish predicts that red-blooded notothenioids would possess erythromiRs known from other vertebrates but white-blooded icefish would lack one or more of them. To test this prediction, we first examined whether miRNAs currently known to be important in erythropoiesis in vertebrates were expressed in erythropoietic organs of red-blooded and white-blooded notothenioids.

Output of smallRNA transcriptomic reads using the software *Prost!* for the *N. coriiceps* pronephric kidney identified sequences for all miRNAs known to be involved in erythropoiesis in vertebrates (Table 1) and we mapped them onto the *N. coriiceps* genome assembly (Additional File 1). Similar analyses identified all known erythromiRs in the *T. bernacchii* smallRNA dataset (Table 1). The presence of these miRNAs in these two red-blooded Antarctic notothenioids and their expression in the pronephric kidney is consistent with the hypothesis that these miRNAs play a conserved role in erythropoiesis in Antarctic red-blooded notothenioids as they do in other fish and in tetrapods. While some species had sequencing reads from both arms of nearly all erythromiR hairpins, in other species, reads

appeared from only one arm, the 5' or 3' arm; for example, *mir23a* in *N. coriiceps* and *C. hamatus*, or *mir155* in *N. coriiceps, T. bernacchii*, and *C. hamatus*. This situation can occur due to either unequal sequencing levels or asymmetries in arm degradation. The identification of sequencing reads from only one of the two strands, especially when they have perfect sequence conservation, nevertheless definitively demonstrates that the gene is 1) present in the genome, 2) expressed, and 3) has at least one strand processed into a mature form at a significant level. For example, the most highly expressed strand for *mir155* is the 5p strand (MiR155-5p), which is present in libraries from all four species; the complementary strand, however, was only found in *C. aceratus*, likely due to deeper sequencing in this species.

Analysis of smallRNA sequencing data from the white-blooded *C. aceratus* pronephric kidney also revealed expression of all known erythropoietic miRNAs. This finding demonstrates that the blackfin icefish genome possesses the known set of erythomiR genes (Table 1). The *C. hamatus* pronephric kidney sequencing data, in contrast, contained reads for all known erythromiRs with the exception of *mir96,* which was undetected (Table 1). Because MiR96-5p was present in the smallRNA transcriptome of *C. aceratus* hematopoietic marrow, and its sequence is identical to the MiR96-5p of red-blooded notothenioids (Additional File 1), we reject the possibility that the loss of detectable expression of the *mir96* erythromiR gene in an ancestor of all extant icefish caused the loss of red blood cells and hemoglobin.

In sum, the presence and expression of known erythromiRs in the hematopoietic marrow of at least one white-blooded icefish rules out the hypothesis that the loss of one or more known erythromiRs by the most recent common ancestor of icefish triggered the loss of red blood cells and/or functional hemoglobin because such gene losses should be shared by the clade.

### 3.3. Evolution of erythropoietic miRNAs following the loss of erythropoiesis

Given datasets for two white and two red-blooded notothenioids, we then asked whether icefish lost any miRNA genes implicated in vertebrate erythropoiesis secondarily after the clade lost red blood cells and hemoglobin. Because expression of neither the 5' nor the 3' strand of *mir96* was detected in *C. hamatus* but was readily detected in *C. aceratus*, *mir96* expression loss in *C. hamatus* is likely a secondary event that followed red blood cell and globin gene losses.

Assuming that the function of *mir96* in humans – the regulation of embryonic globin expression (Azzouzi et al., 2011) – is conserved in red-blooded teleost fish, then the absence of *mir96* expression in the pronephric kidney of *C. hamatus* may be due to prior evolutionary loss of globin genes, which relaxed selective pressures for conservation of this erythromiR and its pronephric kidney expression. Alternatively, failure to detect *mir96* expression in *C. hamatus* kidney marrow could reflect either: 1) insufficient sequencing depth of its pronephric kidney transcriptome; or 2) loss of expression of *mir96* in pronephric kidney but maybe not in other tissues. These possibilities can be evaluated by deeper sequencing of pronephric kidney libraries, sequencing of a larger variety of tissues, generation of whole genome sequences, and wider phylogenetic sampling with species related to hooknose

icefish, such as the ocellated icefish *Chionodraco rastrospinosus*, Myer's icefish *Chionodraco myersi*, or spiny icefish *Chaenodraco wilsoni* (Near et al., 2012).

### 3.4. A case study: the conservation of erythropoietic *mir144* and *mir451a* genes in white-blooded icefish

In vertebrates, the *mir144/451a* cluster (alias *mirc144*), plays a central role in the developmental progression of erythroid precursors to mature red blood cells (Dore et al., 2008; Du et al., 2009; Fu et al., 2009; Kim et al., 2015, 2013; Pase et al., 2009; Patrick et al., 2010; Rasmussen et al., 2010; Yu et al., 2010; Zhan et al., 2007). In mammals, in addition to the erythropoietic function of *mirc144*, recent work suggests a role of *Mir144* and *Mir451a* in cardiomyocyte function and development (Kuwabara et al., 2015; Song et al., 2014; Wang et al., 2012). In teleosts, some erythropoietic miRNAs (e.g., *mir150*, *mir155* and *mir223*, Table1) are known to participate in the formation of other blood cell types in addition to erythrocytes as well as in the development of other tissues. In contrast, in teleosts, *mir144* and *mir451a* are the only erythromiRs we are aware of that have been shown by functional experiments to play a role exclusively in erythropoiesis and not in the development of other blood cell lineages or other tissue and organs. When these genes are knocked-down or knocked-out in zebrafish (Dore et al., 2008; Du et al., 2009; Fu et al., 2009; Pase et al., 2009; Yu et al., 2010), erythropoiesis is impaired but no other embryonic defects, including heart defects, appear. Thus, disruption of the *mirc144* cluster would be an attractive candidate for causing loss of erythropoiesis in icefish, without disrupting the development of other blood cell types, including myeloid and lymphoid lineages, because if the *mirc144* cluster was indeed erythropoiesis-specific in teleosts, then its loss could have hypothetically impaired red blood cell maturation in icefish ancestors as loss of the cluster loss does in zebrafish, mouse and human today. And because the function of the cluster in teleost fish appears to relate only to erythropoiesis, the loss of red blood cells might have occurred first, followed by the loss of this *mirc144* cluster in disuse analogous to pseudogenization of hemoglobin genes. The detection of *mir144* and *mir451a* expression in the erythropoietic tissues of two species of erythrocyte-null icefish, however, shows that icefish genomes conserve the clustered *mir144* and *mir451a* genes.

Conservation of the *mir144* and *mir451a* genes in icefish genomes and their expression in the icefish hematopoietic organ, however, doesn't necessarily mean that the function of these miRNAs is also conserved. Indeed, rearrangements of genes within genomes are sometimes associated with changes in gene regulation. Therefore, we analyzed whether syntenies around *mirc144* were conserved between the bullhead notothen and other ray-finned fish and humans. Conservation of synteny and expression for the *mirc144* cluster in *N. coriiceps* would suggest a conserved function of the cluster at least in red-blooded notothens. Our results showed that the genomic environment of the *mirc144* cluster was well conserved between *N. coriiceps* and several vertebrates including human (Figure 1A–B). Conserved synteny analyses in the white-blooded icefish *C. aceratus* and *C. hamatus* are not yet possible due to the lack of a reference genome assembly for any icefish species. Consequently, whether the icefish *mirc144* cluster experienced rearrangements that may have altered *mir144* and *mir451a* function remains an open question.

Another hypothesis for a role of the *mirc144* cluster in the white-blood phenotype would be that icefish process these miRNAs differently from red-blooded vertebrates, so that these miRNAs are non-functional in the erythropoietic context. Our analysis of the miRNA-seq data showed that the nucleotide sequences of MiR144-3p, the mature miRNA originating from the 3' side of the precursor hairpin, was perfectly conserved across all investigated ray-finned fish (Figure 1C), consistent with the observation that MiR144-3p is the active mature product originating from the *mir144* gene in erythropoiesis (Dore et al., 2008; Fu et al., 2009; Kim et al., 2013; Rasmussen et al., 2010). In contrast, the nucleotide sequence of MiR144-5p, the mature miRNA originating from the 5' side of the precursor hairpin, showed evolved nucleotide differences in vertebrates, including a lineage-specific one-nucleotide change (A to T) in perciformes, represented here by the three-spined stickleback and notothenioids (Near et al., 2015, 2013) (Figure 1C). MiR144-5p additionally displays a seed-shift of one nucleotide at the 5' end in Antarctic fish, which is a change in the position of the seed region of the miRNA by one nucleotide (Figure 1C), and which can potentially have major functional repercussions (Desvignes et al., 2015). Given that the reference MiR144-5p sequences for human, spotted gar and zebrafish were obtained from tissues other than pronephric kidney (Braasch et al., 2016; Desvignes et al., 2014) and/or miRBase (Kozomara and Griffiths-Jones, 2013), we cannot, however, rule out the possibility that the most expressed MiR144-5p isomiR in some tissue other than pronephric kidney has a 5' start similar to the one observed in human, gar and zebrafish, and that, in the pronephric-kidney specifically, the most expressed MiR144-5p isomiR has a 5' start similar to the one observed in notothenioid fish. SmallRNA sequencing of pronephric-kidney from other teleost species, especially those closely related to notothenioids should provide answers to this issue.

Sequencing data originating from the *mir451a* gene in all four species clearly demonstrate that the primary miRNA *pri-miR451a* and the mature MiR451a are well conserved across evolution (Figure 1D–E). MiR451a is the only known miRNA to be processed in a non-canonical, Dicer-independent pathway involving Argonaute2 cleavage followed by exonuclease nibbling of the 3' tail of the miRNA, which results in the formation of multiple isomiRs displaying a tiling pattern (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). Our finding that all four notothenioid species exhibit this characteristic tiling pattern (Figure 1E) demonstrates that the maturation process for MiR451a is also conserved in Antarctic notothenioids.

Together, the conservation of the *mir144* and *mir451* genes in the genomes of the two icefish species studied here and the shared pattern of isomiR processing between red-blooded and white-blooded notothenioids in hematopoietic tissues demonstrate that neither the loss of these miRNA genes nor the modification of their processing are directly responsible for the erythrocyte-null phenotype of icefish. A role for the cluster in the white-blooded phenotype, however, can't be entirely ruled out because the paucity of samples available for this study prevented us from conducting a statistically robust differential expression analysis among species and because reference genome assemblies for icefish species are not yet in hand.

Nevertheless, the conservation of the *mirc144* cluster, its expression in kidney marrow, and its conserved processing in two species of icefish despite the lack of erythropoiesis suggest

that the cluster may perform functions other than erythropoiesis in the pronephric kidney and/or in other tissues (e.g. heart ventricle) of notothenioids and perhaps of other teleosts.

## 4. Conclusions

Study of erythromiRs in erythropoietic organs of red-blooded and white-blooded Antarctic fish revealed that the loss of red blood cells and hemoglobin was caused neither by the loss of miRNA genes known to be necessary for erythropoiesis in red-blooded vertebrates nor by the loss of their expression in hematopoietic marrow. Furthermore, our results showed that expression of only one erythromiR (*mir96*) appears to have reduced below detection in one of the two icefish species, the hooknose icefish, consistent with secondary loss after the extinction of hemoglobin genes and erythropoiesis. The conservation of erythromiRs in sequence and expression despite the loss of erythropoiesis could be explained if these non-coding RNAs play roles in the development of non-erythropoietic blood cell lineages, perhaps other myeloid lineages, like megakaryocytes, mast cells, or myeloblasts, or in the lymphoid lineage leading to B-cells and T-cells, lineages that apparently develop normally in icefish (Bhagavathi and Czader, 2010; Havelange and Garzon, 2010; Sayed and Abdellatif, 2011; Undi et al., 2013; Zhang et al., 2012). In addition, erythromiRs may persist in icefish because they perform additional functions in non-hematopoietic tissues or functions other than erythropoiesis in blood-producing organs. For example they might help protect cells from oxidative stress (Sangokoya et al., 2010; Yu et al., 2010), which is harsher in Antarctic fish due to the 1.6 fold increase in oxygen content of the frigid Southern Ocean (Giordano et al., 2015). The persistence of these miRNAs in icefish genomes may also highlight the evolutionary resilience of miRNA genes once they have become embedded as fine regulators in several genetic pathways (Lee et al., 2007; Peterson et al., 2009; Wheeler et al., 2009).,

Our finding of erythromiRs in white-blooded icefish does not, however, rule out the possibility that evolution of the miRNA system participated in the evolution of the white-blooded icefish phenotype. One hypothesis is that erythromiR binding sites on targeted messenger RNAs were lost or greatly modified or that new targets evolved. Precise annotation of potential targets of erythromiRs in the 3’UTRs of Antarctic fish mRNAs and the sequencing of both the miRNAs and their mRNA target sites will be necessary to unravel the full role of miRNAs in the loss of red blood cells and hemoglobin and the dramatic adaptive evolution that followed in the Channichthyidae family.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Appendix. The hooknose icefish Chionodraco hamatus

The Antarctic icefish *Chionodraco hamatus* (Lönnberg, 1905) lacks a common name. Because the use of common names sometimes facilitates communication, we propose to

apply “hooknose icefish” to the Antarctic icefish *Chionodraco hamatus* (Lönnberg, 1905). The qualifier “hooknose” derives from the etymology of *hamatus*, from the Latin “*h mus,*” meaning “*hook*”, referring to the prominent spine on the nose. The genus *Chionodraco* contains two other species: the ocellated icefish *Chionodraco rastrospinosus*, DeWitt & Hureau, 1979, and Myer’s icefish *Chionodraco myersi*, DeWitt & Tyler, 1960. *C. rastrospinosus* also has a well-developed rostral spine as its scientific name suggests, whereas *C. myersi* has only a small rostral knob.

## Highlights

- Loss of hemoglobin and red blood cells in white-blooded icefish is not due to the extinction of microRNA genes currently known to regulate vertebrate erythropoiesis (erythromiRs).
- Known vertebrate erythromiR genes are conserved and expressed in blackfin icefish, but *mir96* expression in hematopoietic marrow appears to have been secondarily lost in hooknose icefish.
- The major erythromiR genes *mir144* and *mir451a* are conserved in white-blooded Antarctic icefishes despite the lack of red-blood cells.
- Results highlight the resilience of miRNA genes in animal genomes and suggest that some erythromiRs may have functions beyond their roles in red blood cell formation.

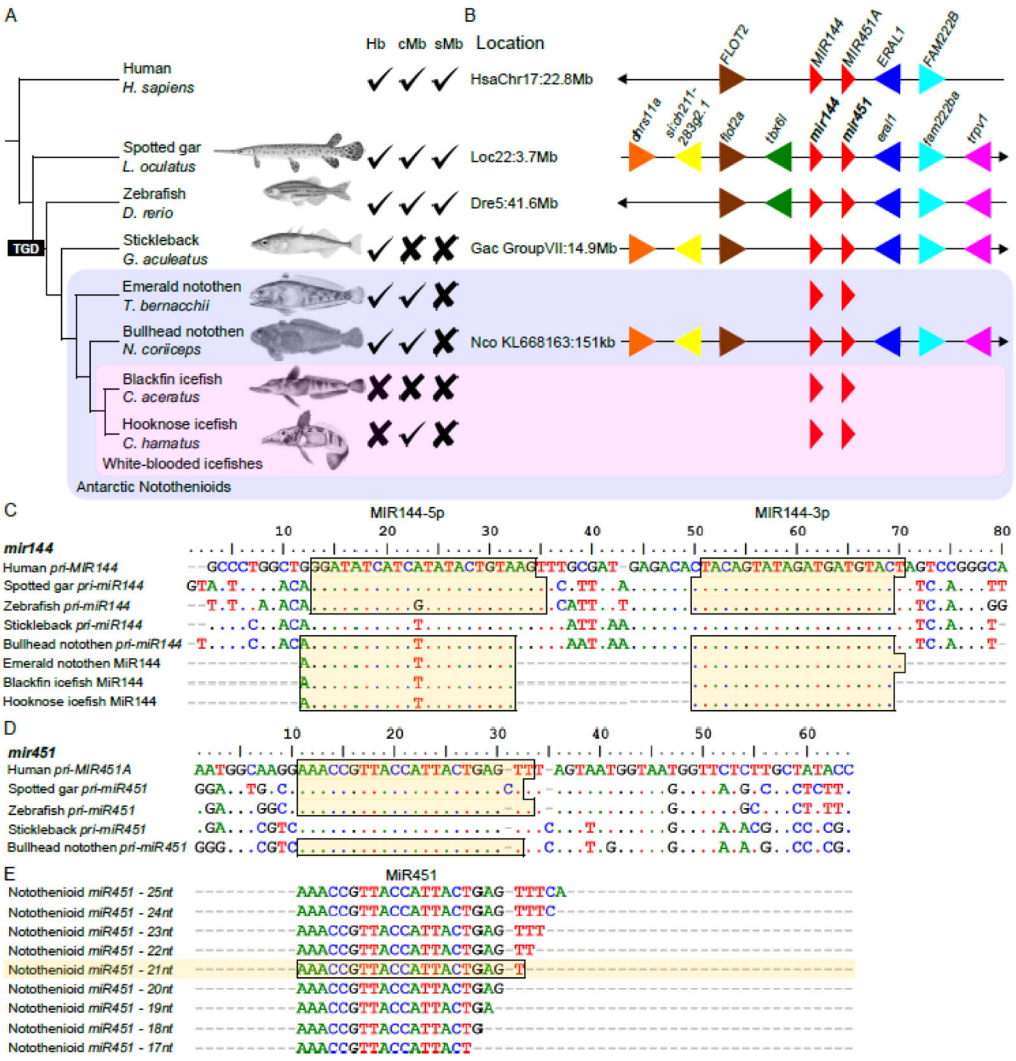


**Figure 1. Evolutionary conservation of the *miR144/451a* miRNA gene cluster**

A) Phylogenetic relationships among vertebrates with human (*Homo sapiens*), spotted gar (*Lepisosteus oculatus*), zebrafish (*Danio rerio*), three-spined stickleback (*Gasterosteus aculeatus*), and four Antarctic notothenioids. The table records for each species the presence/absence of genes encoding hemoglobin (Hb) and cardiac and skeletal muscle myoglobin (cMb and sMb, respectively). B) Synteny conservation of the *mir144/451a* cluster (*mirc144*) among vertebrates. The figure lists the approximate location of the cluster for each species. Small black arrows at the ends of chromosome segments indicate the direction of the chromosome/scaffold in the corresponding genome assemblies deposited in Ensembl as of November 2015. Note that synteny relationships for the mir144 and mir451 genes of the emerald notothen and the two icefish species are unknown due to the absence of reference genomes. C) Alignment of cDNA sequences for human, spotted gar, zebrafish, stickleback, and bullhead notothen partial primary *miR144* RNAs (*pri-miR144*). Dots denote conserved nucleotides, and dashes indicate indels. The most highly expressed isomiRs in the smallRNA sequencing data are highlighted in pale yellow for emerald notothen, blackfin icefish and hooknose icefish; for these mature MiR144 sequences, dashes are introduced to

align the isomiRs with respect to the five *pri-miR144* sequences. Mature MiR144 sequences for stickleback are unknown. Genomic *pri-miR144* sequences are not available for *T. bernacchii*, *C. aceratus*, and *C. hamatus*. D) Sequence alignments for human, spotted gar, zebrafish, stickleback, and bullhead notothen partial primary *miR451a* RNAs (*pri-miR451a*). Dots denote conserved nucleotides; dashes denote indels (insertions or deletions). The most highly expressed isomiRs for MiR451a are highlighted in pale yellow. E) In all four Antarctic notothenioids, isomiRs both smaller and larger than the most highly expressed isomiRs were also found in the sequencing data and reflect post-transcriptional enzymatic trimming. The mature MiR451a sequence of stickleback is unknown.

**Table 1**

Presence of erythromiR genes in Antarctic notothenioid genomes deduced from smallRNA-sequencing data. The list of erythromiRs was compiled from several reviews on miRNA function in erythropoiesis and hematopoiesis (Azzouzi et al., 2012; Bhagavathi and Czader, 2010; Havelange and Garzon, 2010; Lawrie, 2010; Listowski et al., 2012; Mohammdai-asl et al., 2015; Sayed and Abdellatif, 2011; Undi et al., 2013; Zhang et al., 2012). Gene names in boldface indicate those shown to be involved in erythropoiesis in teleost fish, and the supporting reference is given. Superscript “P” and “W” identify miRNAs known to be involved in platelet and white blood cell formation respectively.

| miRNA gene | *Trematomus bernacchii* | *Notothenia coriiceps* | *Chaenocephalus aceratus* | *Chionodraco hamatus* | References in teleosts |
|---|---|---|---|---|---|
| *mir15a* | □ | □ | □ | □ | |
| ***mir16b*** | □ | □ | □ | □ | (Xu et al., 2015) |
| ***mir23a*** | □ | □ | □ | □ | (Zhu et al., 2013) |
| *mir24* [W] | □ | □ | □ | □ | |
| *mir96* | □ | □ | □ | □ | |
| *mir103* [W] | □ | □ | □ | □ | |
| ***mir126*** [P] | □ | □ | □ | □ | (Grabher et al., 2011) |
| ***mir145*** | □ | □ | □ | □ | (Muhseen and Abbood, 2014) |
| ***mir144/451a*** | □ | □ | □ | □ | (Du et al., 2009; Fu et al., 2009; Pase et al., 2009; Yu et al., 2010) |
| *mir150* [P,W] | □ | □ | □ | □ | |
| ***mir152*** | □ | □ | □ | □ | (Xu et al., 2015) |
| *mir155* [P,W] | □ | □ | □ | □ | |
| *mir462* (aka | □ | □ | □ | □ | |
| *mir191*) | □ | □ | □ | □ | |
| *mir210* | □ | □ | □ | □ | |
| *mir221/222* [W] | □ | □ | □ | □ | |
| ***mir223*** [P,W] | □ | □ | □ | □ | (Roberto et al., 2015) |
| ***mir1388*** | □ | □ | □ | □ | (Xu et al., 2015) |