Evolution of hemoglobin loci and their regulatory elements

Sjaak Philipsen[1] and Ross C. Hardison[2]

[1] Department of Cell Biology Ee1071b, Erasmus MC, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. Email j.philipsen@erasmusmc.nl Tel. +31-10-7044282
[2] Department of Biochemistry and Molecular Biology, Huck Institute for Comparative Genomics and Bioinformatics, The Pennsylvania State University, University Park, PA 16802, USA. Email rch8@psu.edu Tel. +1-814-8630113

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Abstract

Across the expanse of vertebrate evolution, each species produces multiple forms of hemoglobin in erythroid cells at appropriate times and in the proper amounts. The multiple hemoglobins are encoded in two globin gene clusters in almost all species. One globin gene cluster, linked to the gene *NPRL3*, is preserved in all vertebrates, including a gene cluster encoding the highly divergent globins from jawless vertebrates. This preservation of synteny may reflect the presence of a powerful enhancer of globin gene expression in the *NPRL3* gene. Despite substantial divergence in noncoding DNA sequences among mammals, several epigenetic features of the globin gene regulatory regions are preserved across vertebrates. The preserved features include multiple DNase hypersensitive sites, at least one of which is an enhancer, and binding by key lineage-restricted transcription factors such as GATA1 and TAL1, which in turn recruit coactivators such as P300 that catalyze acetylation of histones. The maps of epigenetic features are strongly correlated with activity in gene regulation, and resources for accessing and visualizing such maps are readily available to the community of researchers and students.

Globin genes: a model system for developmental regulation of high-level, tissue-specific gene expression

Hemoglobins play a central role in the physiology of species with multiple organs by carrying oxygen from a source, such as lungs or gills, to peripheral organs, such as muscles, that use the oxygen for aerobic metabolism. Hemoglobins also help carry the product of aerobic metabolism, carbon dioxide, back to the organ from which the carbon dioxide is expired, and they can modulate the effects of nitrogen oxides. The hemoglobins transport these gases within cells called erythrocytes (or red blood cells); indeed the hemoglobins are highly abundant in these cells – and only these cells.

These critical functions of hemoglobins can be understood as an adaptation of multi-organ species to the opportunities of an oxygen-rich environment. Globins and the genes encoding them are ancient, being found in all three major kingdoms of life [1]. The ancestral heme-globin complex likely had catalytic oxidation-reduction activity in nitrogen oxide metabolism [2-4]. However, this catalytic activity is suppressed in some hemoglobins, thereby allowing the hemoglobins to function in gas transport without catalyzing chemical reactions. This is the case for vertebrate and invertebrate hemoglobins. The familiar $\alpha_2\beta_2$ tetrameric structure predominates among vertebrate hemoglobins, but a variety of hemoglobin tertiary structures have been described in invertebrates [5]. Furthermore, several globins in addition to the classic

tetrameric erythroid hemoglobins have been discovered in vertebrates. These include myoglobins, cytoglobins, and neuroglobins [6]. Thus the globin superfamily is large and pervasive across the biosphere, and members of the superfamily are responsible for a wide range of activities [4]. In this review, we will focus on the vertebrate hemoglobins (and their genes) responsible for gas transport in the blood.

A remarkable feature of vertebrate hemoglobins is that multiple forms of this protein are used for oxygen transport at different stages of development. In placental mammals (eutherians), one form of hemoglobin is dominant in erythrocytes circulating in embryos (primitive erythrocytes) while a different form is used in adult erythrocytes, and in some cases a distinct fetal form is also produced. The different hemoglobins may be adaptive for the differences in oxygen tension at the source organs, e.g. needing a higher oxygen affinity hemoglobin at the fetal placenta than at the adult lung. However, this production of different hemoglobins at progressive developmental stages is not limited to eutherians. To our knowledge, every vertebrate organism examined makes different forms of hemoglobin, and when they have been studied in a developmental context, distinct forms are made at different stages of development. While the full physiological significance of the developmental diversity of hemoglobins is not yet understood, it is clear that the multiplicity of hemoglobins produced in a developmentally controlled manner is a strongly conserved feature across vertebrates, including the jawless vertebrates (agnathans), which are the most distantly related extant vertebrate relatives to humans.

The production of different hemoglobins at progressive stages of development has particular importance for human health. Hemoglobinopathies such as sickle cell disease and thalassemias are the most common forms of inherited disease world-wide [7]. The pathophysiology of each of these diseases almost always involves the hemoglobins produced during adult life. Thus an enduring hope for potential therapies has been the strategy of re-activating the production of hemoglobins that were previously made in fetal life. Recent progress in this strategy is based on our understanding of the mechanisms of gene regulation in the families of genes encoding the globins.

This review will cover the general themes emerging about regulation of globin gene families from an evolutionary and mechanistic perspective. The evolutionary studies are revealing common features of hemoglobin gene regulation, which can be understood best by combining the DNA sequence comparisons of evolutionary approaches with comparisons of additional biochemical features, such as chromatin accessibility, histone modifications, and transcription factor (TF) occupancy. These latter features are referred to as epigenomic,

meaning that they are proteins (e.g. TFs) or biochemical modifications (e.g. DNA methylation or histone acetylation) that lie on top of (epi-) the genetic material (DNA), but do not alter the DNA sequence as such. This review will illustrate how comparisons of genomes and epigenomes lead to insights about regulation and human disease. We also will point readers to resources for examination of epigenomic data for any gene in human or mouse erythroid or related cell types, so that the approaches discussed here can be applied to other genes and gene families.

Globin genes are located in multi-gene loci containing embryonic/fetal and adult genes

Vertebrates have diversified remarkably since they arose about 550 million years ago (MYA) in the early Paleozoic era (Figure 1). An early divergence separated the jawless vertebrates (agnathans such as lamprey and hagfish) from those with jaws (gnathostomes). The latter large group contains many of the animals familiar to us, including fish (cartilaginous and ray-finned), amphibians, reptiles and birds, and mammals. Each major group had separated from the others by around 250 MYA, in the early Mesozoic era. Diversification of the eutherian mammals is thought to have occurred primarily in the past 65 MY.

Despite the long evolutionary time and striking differences between species, some aspects of the arrangements of globin genes have been preserved. Such strong conservation of gene arrangement is indicative of a function, such as regulation, that requires the observed arrangement. When we observe less change than expected, either in DNA sequence or gene arrangement, we infer that changes are disadvantageous and therefore removed from the relevant populations; i.e. we infer that the feature was under purifying or negative selection. Thus mapping the arrangements of globin genes and their neighbors has been of considerable interest for decades. As we will see, the inference that gene arrangement is important has been borne out by the discovery that major regulatory elements are located in genes or intergenic regions distal to the globin genes.

The first preserved feature of vertebrate hemoglobin genes is their presence in multi-gene clusters. The genes encoding hemoglobins have now been mapped across the wide diversity of vertebrates [8-19]. In all cases, multiple hemoglobin genes are found together (Figure 2). For reptiles, birds, and mammals, the genes encoding α-like globins are clustered together on one chromosome, while the genes encoding the β-like globins are clustered on a different chromosome. We refer to the former locus as the α-like globin gene cluster or *HBA* cluster, and the latter as the β-like globin gene cluster or *HBB* cluster. In all cases that have been investigated thoroughly, genes at the left side of *HBA* and *HBB* clusters (as diagrammed in Figure 2) are expressed in embryonic erythrocytes, while genes on the right side of the

clusters display a broader developmental expression pattern and include the globins expressed in adult erythrocytes. While many of the globin genes encode polypeptide components of hemoglobin, no protein product has been discovered from the genes labeled μ and θ in Figure 2, despite the presence of orthologous genes in multiple species [6]. Thus a role for these genes, if any, remains to be determined.

In fish and amphibians, the genes encoding α-like globins are clustered together with the β-like globin genes, frequently appearing as pairs of an α-like and a β-like globin gene. In zebrafish, the set of α-βgene pairs expressed in early developmental stages is at one end of the gene cluster, while those expressed at later stages are at the other end [10]. A similar pattern is observed in frogs; the globin genes expressed in tadpoles are separated from those expressed in adult frogs [12, 14, 20]. The genome assembly of the frog *Xenopus tropicalis* is not sufficiently complete to address the gene arrangements unambiguously. One possibility inferred from the current assembly is shown in the model in Figure 2. The current genome assembly of the genome of the elephant shark, representing cartilaginous fish, shows at least one cluster with genes encoding α-globin, β-globin, and cytoglobin.

The most distant vertebrate with a genome sequence assembly is the lamprey, an agnathan. The globins of lampreys are monomeric and appear to be more closely related to vertebrate cytoglobins than to vertebrate hemoglobins, leading to the inference that the gas-transporting activity of erythrocyte hemoglobins has arisen twice by convergent evolution [11]. Notably, the agnathan hemoglobin genes are arranged as clusters in two different loci (Figure 2, [18]).

The second preserved feature of vertebrate globin gene clusters is the presence of more than one multi-gene cluster. In almost all species, two multi-gene clusters have been identified. For shark and frog, no clear evidence is available for a second multi-gene cluster, but this could reflect the incompleteness of the genome assemblies and correlated work.

The genes flanking the globin multi-gene clusters are conserved across vertebrates

Examination of the genes flanking the globin gene clusters reveals three distinct loci, two of which are used in each species. In almost all mammals and birds, the *HBA* cluster is located between the *NPRL3* and *LUC7L* genes. The mouse *HBA* cluster no longer has *LUC7L* downstream of the globin genes (to the right in Figure 2) because of a chromosomal rearrangement, but *NPRL3* has been retained upstream [8, 13]. The *HBB* cluster in birds and mammals is embedded in a large cluster of *OR* genes encoding olfactory receptors. Single-copy genes can be far away from the *HBB* cluster, but in all cases with sufficient contiguity to the

genome assembly, the *DCHS1* gene is located upstream and the *RRM1* and *STIM1* genes are located downstream of the *HBB* cluster in birds and mammals. A similar arrangement appears to be present for the *HBA* and *HBB* clusters in turtle, except for an inversion downstream of the *HBA* cluster that may obscure the presence of *LUC7L.*

The linkage to *NPRL3* is also observed for an amphibian (frog) and every species of ray-finned fish examined (three are shown in Figure 2). These cases can be viewed as a combined cluster of *HBA* and *HBB* genes adjacent to *NPRL3*. A second globin gene cluster is found on a different chromosome in the ray-finned fish. This cluster is smaller, sometimes with three genes, but both α-like and β-like globin genes are found in the smaller cluster [10, 21]. This second cluster is adjacent to the genes *ARHGAP17* and *LCMT1* on one side, and frequently the gene *RHBDF1* is on the other side. A paralogous copy of *RHBDF1* (i.e. a related gene generated by duplication) is also frequently close to the first gene cluster containing *NPRL3*. This second locus is clearly distinct from the *OR* cluster harboring the *HBB* complex in mammals. The annotation and assembly of the shark genome is less complete than for the genomes of ray-finned fish, and while *NPRL3* has not been mapped unequivocally to the globin gene cluster, the genes *RHBDF1* and *LUC7L* flank the cluster. Thus it is possible that the shark globin gene cluster has an arrangement of flanking genes similar to that in other vertebrates.

Remarkably, one cluster of agnathan globin genes is also flanked by *NPRL3*, and the other is flanked by *RHBDF1* [18]. Therefore even in these distantly related species, two different loci harboring globin genes are present, and those loci show similar flanking genes to those in the jawed vertebrates.

Examination of globin gene clusters across the full span of vertebrates consistently shows that the *NPRL3* gene is adjacent to one of the hemoglobin multi-gene clusters. In almost every species examined, *NPRL3* is located adjacent to the *HBA* complex or to a combined *HBA-HBB* complex. Current apparent exceptions such as the shark may simply reflect incomplete assembly and annotation. Furthermore, *NPRL3* is adjacent to a globin gene cluster in agnathans [18], despite the substantial divergence of these monomeric hemoglobins from the tetrameric hemoglobins in gnathostomes [11].

In summary, a striking picture is emerging of conservation of synteny, clustering, and gene order around globin gene loci. Two or more genes present on the same chromosome are syntenic, and when such genes are retained in the same order (and often the same orientation) in different species, we can be confident in concluding that an ancestral arrangement of genes has been preserved over evolution. However, over a large enough span of evolutionary distance, chromosomal rearrangements will break synteny. In fact, for comparisons across

major groups of vertebrates (e.g. mammals and birds), the conservation of synteny does not extend much further than the regions shown in Figure 2 [8, 13]. Importantly, the phylogenetic span over which synteny and gene order surrounding globin gene clusters is conserved appears to be greater than for many other loci. We infer from this strong conservation that the surrounding genes are important, and as we will examine later, one important function attributed to this arrangement is regulation of globin gene expression.

The *HBB* cluster in birds and mammals arose by a transposition

The linkage of a globin gene cluster to *NPRL3* is found in all vertebrates, and thus we infer that this is a characteristic derived from the same arrangement in the ancestral vertebrate. The evolutionary history of the other globin gene clusters is more complex. The *HBB* cluster in birds and mammals is in a different locus from the non-*NPRL3*-linked cluster in fish, and thus they do not share a common ancestral arrangement. It is therefore likely that the *HBB* cluster transposed into the *DCHS1-OR-RRM1-STIM1* locus in the last common ancestor to reptiles, birds, and mammals (Figures 1 and 2, [6, 17]). The source of those globin genes is unclear. It could be the genes that were linked to *LCMT1*. In mammals, no globin genes are present around *ARHGAP17-LCMT1*, and thus one could propose movement from that locus to the *OR* locus. However, it is also possible that one or more *HBB* genes from the *HBA-HBB* combined locus, linked to *NPRL3*, was the source. In either scenario, genes were lost from one or more of the globin gene loci now seen in contemporary fish, and the transposed *HBB* genes underwent a series of duplications and divergences to form the contemporary *HBB* gene cluster with developmentally regulated genes.

The evolutionary rationale for having separate *HBA* and *HBB* gene clusters is thought to be that this precludes gene conversion events between *HBA* and *HBB* genes, thus promoting fine-tuning of the developmental expression patterns and protein sequences of the α-like and β-like globins. An instructive example is provided by the two γ-globin genes, encoding fetal β-like globins, which have been recently acquired through duplication events in Old World monkeys including humans.

Proximal and distal regulation in the multi-gene clusters

The globin genes are expressed exclusively in erythroid cells, they are expressed at extremely high abundance when activated, and different genes are expressed at different developmental stages. All three aspects of regulation, *viz*. tissue-specificity, high abundance, and developmental control, have been studied intensively, often using groundbreaking

biochemical, genetic, and genomic approaches. For this review, we will discuss some aspects of globin gene regulation that correlate with the evolutionary analyses.

Globin genes have a canonical promoter structure that directs transcription to start at the appropriate location. The sequences conferring this promoter-proximal regulation are found in common for many globin genes, and include a TATAA box at -30bp from the transcription initiation site [22], a CCAAT box at -50bp [23], and a CACCC box at -80bp [24]. The TATAA box is a landing platform for the general transcription factor complex TFIID, and is considered a hallmark of strong tissue-specific promoters. The CCAAT box is a potential binding site for an array of transcription factors, such as the ubiquitously expressed hetero-trimeric NF-Y transcription factor [25] and α-CP1 [26]. The CACCC box element is recognized by members of the **s**pecificity **p**rotein / **K**rüppel-**l**ike **f**actor (SP/KLF) transcription factor family [27]. This element confers tissue-specificity as it is bound by KLF1, the only erythroid-specific member of the 26-strong mammalian SP/KLF family [28, 29]. The importance of these motifs for high-level globin expression is illustrated by promoter variants leading to thalassemic phenotypes in patients [24, 30, 31], and by systematic analysis of transgene expression in cultured cells and transgenic mice [9].

By themselves the promoters are insufficient to drive high-level transcription of the globin genes in all erythroid cells [32-34]. To achieve this, a series of erythroid-specific enhancer elements is typically required. These enhancer elements are also clustered and located distally from the globin genes. The globin gene loci provided the earliest evidence for long-distance regulation in addition to proximal control [35-37]. A series of DNaseI hypersensitive sites (DHS) upstream of the human embryonic ε-globin gene (*HBE*, Figure 3) [37] was shown to confer position-independent, high-level expression to a linked globin gene in transgenic mice [35]. This region was termed the Locus Control Region (LCR) and is a defining feature of all mammalian *HBB* clusters studied to date (orange dots in Figure 2). For the human *HBA* locus, a major regulatory element was identified located in intron 5 of the *NPRL3* gene [36], which is now referred to as Multispecies Conserved Sequence R2 (MCS-R2) [13, 38]. Similar to the LCR, the MCS-R2 is also part of a series of erythroid-specific DHS (Figure 3) [8, 39]. Genetic dissection of the distal regulatory elements has shown that both MCS-R1 and MCS-R2 are major regulatory elements [39, 40]. This arrangement of multiple DHS is typical for mammalian *HBA* clusters (Figure 3).

Interestingly, it was found that the importance of the *NPRL3* intron 5 MCS-R2 element for high-level activation of the globin genes differs between species. Deletion of the homologous element in mice resulted in only a modest reduction in α-globin expression [40]. We now know

that the clustered DHS work together in an additive fashion [39, 41, 42]; the *NPRL3* intron 5 MCS-R2 element appears to have a more dominant role in α-globin gene activation in humans than it does in mice.

The origin of the *HBB* LCR remains obscure. It could have been derived from the *HBA* MCS-R2, if that part of *NPRL3* were included in the transposition. Regulation of the fish globin clusters flanked by the *ARHGAP* and *RHBDF* genes has not been studied in detail yet [10, 21]. One or more DHS have been mapped in this globin gene locus in both *Fugu* and in zebrafish [10, 21], and it is possible that this could be an important regulatory element (orange dots in Figure 2). Thus the LCR could also have been be derived from this element. Alternatively, the *HBB* LCR could have arisen *de novo*, in which case it acquired binding sites for an array of transcription factors very similar to those seen in the globin loci linked to *NPRL3* (Figure 3).

Epigenomic features across mammalian *HBA* and *HBB* loci

Despite the different evolutionary paths of the *HBA* and *HBB* loci, many aspects of their regulation are conserved. Indeed, not only are the genes in both clusters subject to control of tissue-specificity, high abundance, and developmental switches, but the production of proteins from each locus must be balanced to make the globin polypeptides for the $\alpha_2\beta_2$ hemoglobin tetramer. Maps of epigenetic features associated with gene regulation have been produced across the genomes of erythroid-related cell types in mouse and human, both from individual labs and from large consortia. Examination of these maps reveals substantial similarities between the *HBB* and *HBA* loci, and strong conservation between mouse and human (Figure 3). These similarities in maps suggest similarities in regulatory mechanisms.

A cluster of regulatory elements, marked by DHS, is distal to the globin genes in both loci (Figure 3). This cluster is referred to as the locus control region, or LCR, for the *HBB* locus. At least one of the DHS in each distal regulatory region is a strong enhancer as assayed by gain-of-function reporter gene assays or by deletional analysis. In humans, such strong enhancer activity is associated with MCS-R1 and MCS-R2 in the *HBA* locus and 5'HS2 in the *HBB* LCR. Activated globin genes are marked by a DHS at the promoter and often broader, proximal nuclease cleavage sensitivity.

The regulatory elements marked by distal and proximal DHS are occupied by the co-activator P300 (Figure 3). This enzyme catalyzes the acetylation of lysine 27 of histone H3, leading to a strong signal for H3K27ac, spreading from the positions occupied by P300. Co-activators are recruited by transcription factors bound to specific sequences. The maps show binding by key transcription factors such as GATA2 and GATA1 (at different stages of erythroid

differentiation) and TAL1 to many of the regulatory elements. Indeed, co-binding by GATA factors and TAL1 is strongly predictive of induced expression of target genes [43]. Furthermore, more selective binding of NFE2 is observed for strong enhancers, in keeping with previous observations that the DNA binding motifs for NFE2 were critical for erythroid-specific transcriptional enhancement [8, 36, 44]. Selective binding by the erythroid transcription factor KLF1 is also observed at regulatory elements [45-47]. The protein CTCF is bound at the extremities of the *HBA* and *HBB* loci. Some CTCF-bound sites are also bound by components of cohesin, suggesting that they are involved in forming distinct structures within the chromatin, which may in turn play roles in demarcating domains of regulation [48-50]. Overall, the epigenomic maps at the *HBA* and *HBB* loci are strikingly similar.

Maps such as those shown in Figure 3 are available genome-wide for a large number features, including chromatin accessibility, multiple histone modifications, and many transcription factors [51-62]. Expression data for protein-coding and noncoding genes, largely from RNA-seq approaches, are also available from a large number of cell types. These data can be powerful resources to generate hypotheses about regulation that can be tested experimentally by individual investigators. Thus it is important to provide easy access to the data. A list of some of these resources, along with URLs, is provided in Table 1. A multi-investigator project, called VISION (for **V**al**I**dated **S**ystematic **I**ntegrati**ON** of epigenomic data in hematopoiesis) is an ongoing effort to compile, integrate and model the effects of candidate regulatory elements on expression, to validate those models experimentally, and provide the results freely to the community. Figure 3 was generated by using a subset of the data compiled and displayed by VISION.

Common epigenomic features across fish and mammalian *HBA* and *HBB* loci

Similar regulatory landscapes for globin loci are observed across large phylogenetic distances. In the time since mammals and ray-finned fish diverged, the genome sequences have become quite different. Only a very small subset of the human genome aligns to any fish genome, and the alignments are largely confined to protein-coding exons. The exceptions of noncoding regions conserved between mammals and fish have proven to be dramatic examples of conserved regulatory regions, but these are rare. Not even all protein-coding exons are conserved between human and fish.

This sparse conservation makes the conserved synteny of *HBA* loci in mammals with the *NPRL3*-linked globin genes in fish (Figure 2) even more striking. However, when the intron in *Fugu* or zebrafish *NPRL3* that should be orthologous to the mammalian intron harboring MCS-

R2 was searched for alignments with human *NPRL3*, no meaningful matches were detected above the background of random matches [8]. Reasoning that short matches such as the binding site motifs for transcription factors may not be detected by large-scale alignments, the relevant introns from *Fugu* and zebrafish *NPRL3* were tested experimentally and shown to be active erythroid enhancers [8, 10] (Figure 4). Furthermore, these introns have matches to binding sites for the battery of erythroid transcription factors found in mammalian globin gene regulatory elements, and ChIP-seq experiments confirm binding. This inability to find regulatory elements by interspecies sequence alignments even applies within the clade of ray-finned fish. The *Fugu Nprl3* intron 5 harboring MCS-R2 aligns with the orthologous sequence from several fish, but not zebrafish. Despite the lack of alignment, both the *Fugu* and zebrafish introns have enhancer activity and appear to bind a similar set of transcription factors [8, 10, 21]. These examples illustrate the power of epigenomic analysis for deep interrogation of regulatory mechanisms. Indeed, conservation of epigenomic features may be a particularly effective means of finding active regulatory elements.

The collection of transcription factors binding to MCS-R2 in ray-finned fish is strikingly similar to those observed at distal regulatory elements in mammals (Figure 4, C and D). High resolution DNase sensitivity maps show footprints, i.e. regions of protection (presumably from transcription factor binding) separated by regions of higher cutting [63, 64]. Those footprints correspond well to the binding site motifs for transcription factors, each of which has been shown by ChIP-seq or other assays to be bound. While the exact number and pattern of binding sites differs among the regulatory elements, the transcription factors bound at the active elements tend to be the same.

Distal regulatory elements are required for high-level expression of the globin genes

The mechanism by which the promoters of the globin genes are activated by the distal regulatory elements was the subject of fierce scientific debates during the 90s of the previous century. A fairly bewildering variety of models were proposed, and while some models were more credible than others, most were not mutually exclusive. Even to date, a detailed time-resolved description of the molecular mechanism is lacking; this will require further development of advanced high-resolution microscopy to follow the dynamic changes in three-dimensional organization of the globin loci in living cells. One model proposed release of RNA polymerase II from the LCR, which would then track along the DNA and start transcription at the first available promoter it encountered [65]. For the chicken *HBA* locus, a full locus transcript including all the globin genes was reported [66]. Linking of the LCR to the globin promoters via extended protein

bridges was another model for transcriptional activation [67]; local repression of embryonic/fetal promoters would prevent activation of these genes in adult erythroid cells. An alternative model, not necessarily excluding a role for most of the other proposed mechanisms, was derived from observations made in bacterial systems in which the formation of DNA loops was observed to accommodate protein-protein interactions between DNA-bound transcriptional regulators [68]. Indirect support for the looping model was obtained from transgenesis experiments in which the position and/or order of genes and regulatory elements was changed [69-72]. The first direct support was obtained using RNA-fishtrap, a method that tags and recovers chromatin in the immediate vicinity of an actively transcribed gene. This revealed that 5'HS2 of the LCR is in close physical proximity to the actively transcribed β-globin gene [73]. Development of Chromosome Conformation Capture (3C) enabled investigation of the three-dimensional structure of loci inside the nucleus independent of transcriptional status [74]. Initially developed *in vitro* [75], then in yeast [74], adaptation of 3C to analysis of mammalian cells showed that, in adult erythroid cells, the DHS of the LCR come in close spatial proximity with the promoter of the β-globin gene, with the intervening DNA looping out [76]. No such interactions were observed in non-erythroid cells. The LCR-promoter interactions were developmentally regulated, leading to the proposal that the DHS form a holo-complex which facilitates activating interactions preferentially with the nearest accessible globin promoter [77]. Binding of repressors to embryonic/fetal globin promoters in adult erythroid cells would exclude participation in this structure, which was termed the active chromatin hub (ACH). Key erythroid transcription factors KLF1 [78] and GATA1 [79] were found to be required for ACH formation in adult erythroid cells. Remarkably, tethering the self-association domain of the GATA1 cofactor LDB1 to the γ-globin promoter forced LCR looping to the γ-globin promoter and resulted in significant reactivation of the fetal gene in adult erythroid cells [80]. For the *HBA* locus, similar mechanisms are operational [39]. Of note, a single nucleotide polymorphism (SNP), located between the upstream DHS and the α-globin genes, was shown to create a decoy promoter interfering with normal activation of the downstream α-globin genes. This single SNP is the cause of α-thalassemia in individuals from Melanesia, illustrating that altered chromatin loop formation can be the underlying cause of human disease [81].

How loop formation is achieved remains to be elucidated. Looping requires that regulatory elements sample the nuclear space in order to come in close proximity to each other. We know that the likelihood of *in cis* enhancer – promoter interactions decreases with distance [69, 70, 82]. Recent investigations of the three-dimensional organization of the genome have revealed that chromatin is compartmentalized by several mechanisms, such as association with

the nuclear lamina [83, 84] and division in topologically associated domains [85]. In addition, the high local densities of proteins and nucleic acids at enhancers and promoters may result in the formation of membraneless organelles, called cellular bodies, which are formed by a process termed phase separation. It has been proposed that the formation of such phase-separated multi-molecular assemblies are an essential feature for the function of super-enhancers [86], clusters of DHS such as those found in the *HBA* and *HBB* loci.

Super-enhancers for robust regulation

Recently, clusters of hypersensitive sites such as those found in the *HBA* and *HBB* loci have been re-branded as super-enhancers [87]. The multiple DHS and extensive histone modifications in the super-enhancers have been interpreted as indicating a large, interacting complex of regulatory elements that together produce a stronger regulatory effect than the individual elements acting separately. Such a model predicts that the regulatory elements in a super-enhancer would act synergistically. However, a recent study examining the effects of deleting each of the five DHS of the *HBA* locus super-enhancer, singly and in combination, demonstrated that individual DHS act independently of the other four elements. The DHS operated in an additive fashion with respect to hematological phenotype, gene expression, chromatin structure, and chromosome conformation [39]. These results are entirely consistent with earlier studies on the *HBB* LCR [41, 42, 88-94]. The magnitude of the effects of the deletions of individual elements differed widely. In the *HBA* complex, deletion of each of two of the candidate regulatory elements with all the hallmarks of enhancers (DNase hypersensitivity, histone modifications indicative of active chromatin, binding by key transcription factors, enhancer RNAs, interactions with promoters, interspecies sequence conservation) had almost no impact on expression [39]. This result shows that these candidate regulatory elements are dispensable for globin gene expression in a laboratory setting, but it does not preclude a role under other conditions.

We suggest that, rather than facilitating synergistic interactions or higher-order effects on the 3D structure of the hemoglobin loci, the super-enhancer architecture in the *HBA* and *HBB* loci provides robustness to the system. Such robustness may be the main force driving evolutionary selection on the complex enhancers of the globin loci. Multiple regulatory elements acting independently ensure that expression of the globin genes is fully activated in the vast majority of red cells being produced [95]. Given that an adult human needs to produce over 2 million new erythrocytes every second to replenish worn-out erythrocytes, this is not a trivial consideration.

Developmental regulation of globin gene expression: hemoglobin switching

The recent insights in molecular control of hemoglobin switching elegantly combine the concept of activation of the individual globin genes by the distal regulatory elements via interactions with the globin promoters. The appearance of specific repressor proteins during development renders the promoters of the embryonic/fetal globin genes inaccessible for activation, shifting the DHS-promoter interactions to the adult globin genes. Notably, in the vast majority of cases the embryonic/fetal globin genes are located closer to the DHS along the genomic DNA, with the adult genes located more distally in the locus. Experimentally, the importance of gene order, direction, and distance to the DHS has also been demonstrated using a variety of transgenic approaches in mice [69-72]. The essential role of repressor proteins in orchestrating the switch from embryonic/fetal to adult globin gene expression has now been firmly established with the identification of a regulatory circuit involving MYB [96], KLF1 [97, 98], BCL11A [99] and LRF (also called Pokemon or ZBTB7A) [100]. In adult erythroid cells, MYB activates expression of KLF1, a major activator of terminal erythroid differentiation [101]. KLF1 is a positive regulator of BCL11A [97, 98] and LRF [102] expression, two transcription factors which act as direct repressors of the embryonic/fetal globin genes. Since KLF1 preferentially activates adult globin genes [103, 104], this MYB-KLF1-BCL11A-LRF regulatory circuit results in high-level expression of adult globin genes and very efficient repression of the embryonic/fetal genes in adult erythroid cells. Clinically, this regulatory circuit provides rational targets for directed genome editing in somatic cells or development of novel drugs aimed at reactivation of the fetal β-like globin genes in patients with β-thalassemia and sickle cell disease. A promising recent study showed that removal of a repressor binding site upstream of the γ-globin genes led to substantial increase in fetal hemoglobin and reduced sickling in cells derived from sickle cell patients [105]. While classical transcription factors lack domains with catalytic activity and are therefore as such not very attractive drug targets, they are known to require a host of co-factors in order to exert their functions. These co-factors include histone- and DNA modification enzymes and chromatin remodelers, for which an arsenal of pharmacologic inhibitors is available. Thus, targeting (a combination of) these co-factors is currently a very active area of research. An early example is provided by treatment of a β-thalassemia patient with the DNA methyltransferase inhibitor 5-azacytidine, which resulted in increased fetal hemoglobin expression [106, 107]. This experimental treatment was stopped because of concerns about toxicity, and it remains controversial whether the effects of 5-azacytidine are directly related to inhibition of DNA methylation or due to other metabolic changes in the erythroid cells [108].

Mixed results have been reported on the fetal hemoglobin inducing activities of inhibitors of histone deacetylases [109, 110] and the histone demethylase LSD1 [111, 112]. It is nevertheless encouraging that the increasingly detailed knowledge of the developmental regulation of globin gene expression provides guidance to the development of desperately needed novel pharmacological regimes for the treatment of β-hemoglobinopathy patients. In addition, successful gene therapy of β-hemoglobinopathy patients has been reported for a small number of cases [113, 114]. The gene therapy vectors are based on what could be viewed as an ultra-condensed version of the *HBB* locus, depending entirely on the inclusion of core regulatory elements of the LCR and the β-globin gene to drive high-level erythroid-specific expression of the therapeutic globin gene [115].

In conclusion, the study of globin loci across the vertebrate kingdom has yielded a wealth of information about developmental regulation of multigene loci and provided a paradigm for understanding spatio-temporal transcriptional control of more complex gene clusters such as the *HOX* loci [116]. Furthermore, the detailed studies on evolution of the globin gene clusters have helped to reveal the molecular mechanisms underlying gene regulation in higher eukaryotes. This has profoundly contributed to our understanding of human genetic disease in general, and paved the way for development of novel treatments of the hemoglobinopathies, the most common monogenic disorders in the human population.

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Figure Legends

Figure 1. Major events in globin gene clusters during vertebrate evolution.
The branching pattern for major vertebrate groups is shown along with a time scale for divergences (**m**illions of **y**ears **a**go, MYA). The labels indicate the inferred times and phylogenetic spans of the presence or movement of specific globin gene clusters.

Figure 2. Globin gene clusters across a wide span of vertebrate species.

The arrangement of globin genes and their flanking genes are shown for contemporary species ranging from the jawless vertebrate lamprey to humans. Each gene is shown as a rectangle; those positioned above the lines are transcribed from left to right, those positioned below the lines are transcribed from right to left. Orthologous genes are indicated by rectangles of the same color; boxes for α-like globin genes are yellow and those for β-like globin genes are red. Genes flanking the globin genes are named at their first appearance from the top of the diagram, and names of genes diagnostic for the types of clusters are repeated at the bottom. Almost all species have two of three major clusters. The cluster containing *NPRL3* is found in all species, and is indicated by flanking genes in shades of purple and orange. The cluster containing *LCMT1* is found in ray-finned fish, and is indicated by flanking genes in shades of green. The cluster containing *DCHS1* and *RRM1* is found in reptiles, birds, and mammals, and it is indicated by flanking genes in shades of blue. The latter clusters contain what can be a large number of olfactory receptor (*OR*) genes, and thus only representative *OR* genes are shown in the figure. An orange dot indicates the major distal enhancer for globin gene clusters in species for which experimental evidence has been obtained for such activity. The figure summarizes maps presented in publications (see references in text) or gleaned from annotations of genome sequences.

Figure 3. Epigenetic features in *HBA* and *HBB* clusters in mouse and human.

For each gene cluster, the first row shows the positions of genes, followed by tracks for known ***c****is*-**r**egulatory **m**odules (CRMs, red boxes), accessible chromatin measured by ATAC-seq or DNase-seq, occupancy by the co-activator P300, modification of chromatin at histone H3 acetylated on lysine 27 (H3K27ac), and occupancy by GATA2, GATA1, TAL1, NFE2, KLF1, and CTCF. Numbers to the left of each signal track give the maximum value for the signal shown; only peak positions are shown for KLF1 binding in human HUDEP-2 cells [102]. The values were obtained from different programs and thus are not comparable between tracks, but the signal values along each track are meaningful. The orange dots indicate major distal enhancers. The transcriptional orientation of globin genes is from left to right. Maintaining consistency in orientation of the globin genes required a reversal of orientation from the reference genome sequence for the *HBB* clusters; this is indicated by showing the direction to the nearest telomere as TEL>. The epigenetic features were determined either in primary erythroid cells and tissues or in cell lines with erythroid character. Abbreviations for cell types are: ERY=erythroblast from mouse bone marrow, FL=fetal liver (mouse or human), G1E=immortalized erythroid-

differentiated mouse ES cells with a knockout of the *Gata1* gene, K1ER=immortalized erythroid-differentiated mouse ES cells with a knockout of the *Klf1* gene that have KLF1 restored as a fusion with an estrogen receptor domain, K562 or K56=transformed human cell line that has some erythroid properties, HUDEP-2=immortalized human erythroid progenitor cell line, and PBDE=peripheral blood derived erythroblasts from humans. The data are from many sources (see references in text), and can be viewed and downloaded from resources of the VISION project (URL is in Table 1).

Figure 4. Conservation of epigenetic signals *versus* genome sequences in distal regulatory regions of globin gene clusters.

(A) Binding of GATA1 and DNase accessibility at the major regulatory element (MCS-R2) for the globin gene cluster on chromosome 3 of zebrafish. The signal tracks are from Figure 4 of Ganis et al. [10]; they are aligned with a gene map from the genome assembly. The proteins inferred to be bound at MCS-R2 are shown as colored icons in the zoomed-in view. (B) Genome sequence conservation and divergence at MCS-R2 in other fish. The *HBA* cluster from *Fugu* on chromosome 5 is shown, highlighting the MCS-R2 by showing the inferred proteins bound. Underneath the gene map is a track showing the likelihood that DNA segments are under purifying selection. That PhyloP Cons score is estimated from sequence alignments of multiple species, which are shown as dark rectangles indicating aligned sequences. Note that the intron containing the *Fugu* MCS-R2 aligns with sequences from three other fish, but not to zebrafish. (C) DNase footprints for the *HBA* MCS-R2 in humans. The high density DNase-seq analysis [63, 64] was done on highly erythroid human fetal liver (FL) tissues and in K562 cells. Regions of frequent cleavage (greater accessibility in chromatin) have a high signal on the tracks. Positions of bound transcription factors, determined from other studies, are shown as colored icons. (D) DNase footprints for the *HBB* LCR 5'HS2 in humans.

Table 1. Resources for obtaining and visualizing epigenetic data

Project	Description or goal	URL
VISION	Generate and compile epigenomic data: integrate, model, and validate. Focus on erythro-myeloid lineages	http://www.bx.psu.edu/~giardine/vision/
CODEX	Curated collection of epigenomic data in hematopoietic cells and stem cells	http://codex.stemcells.cam.ac.uk
ENCODE	Generate and integrate epigenomic data across a wide variety of cell types	https://www.encodeproject.org
BLUEPRINT	Generate epigenomic in human hematopoietic cells	http://www.blueprint-epigenome.eu/

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Figure 1.

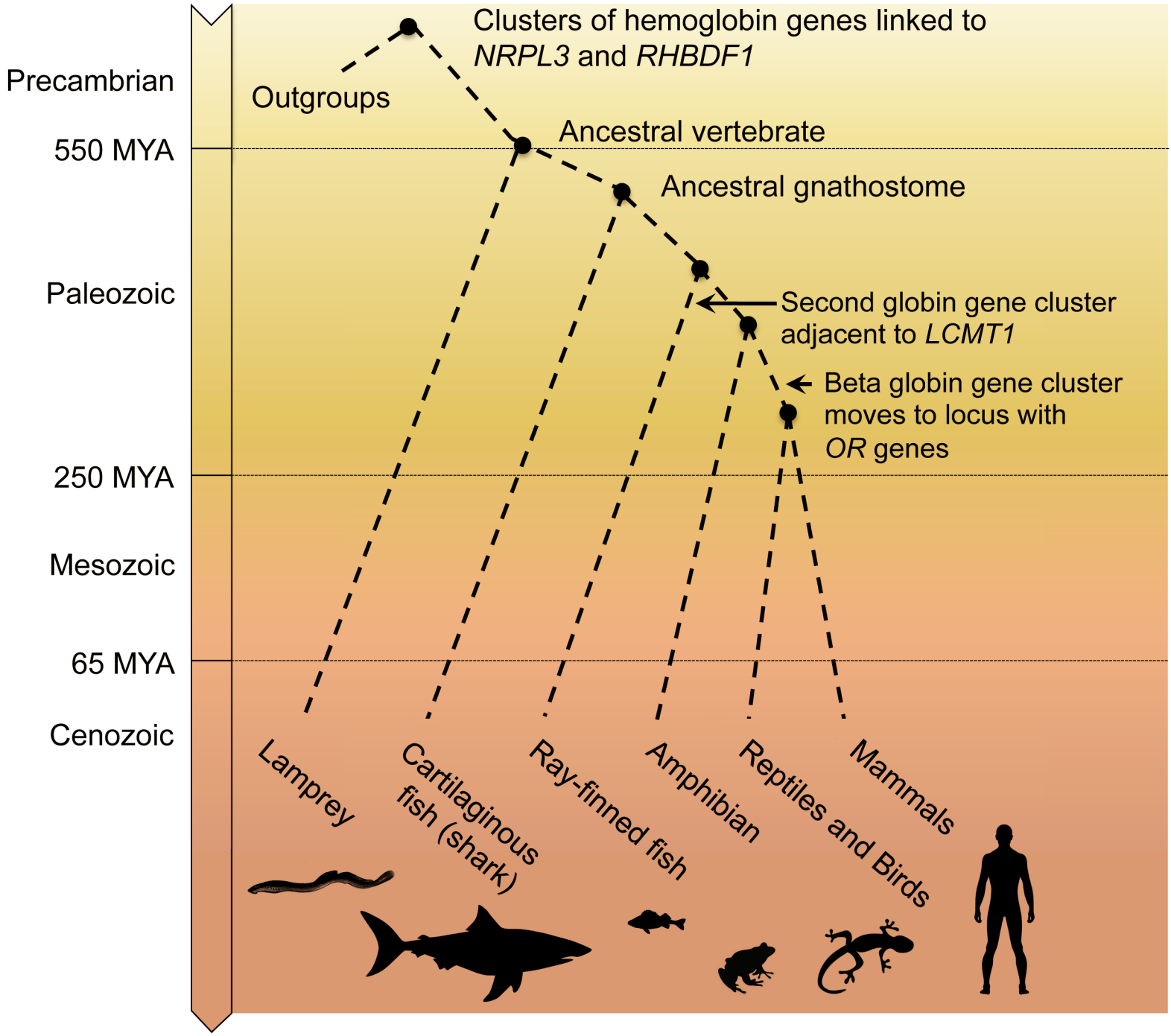
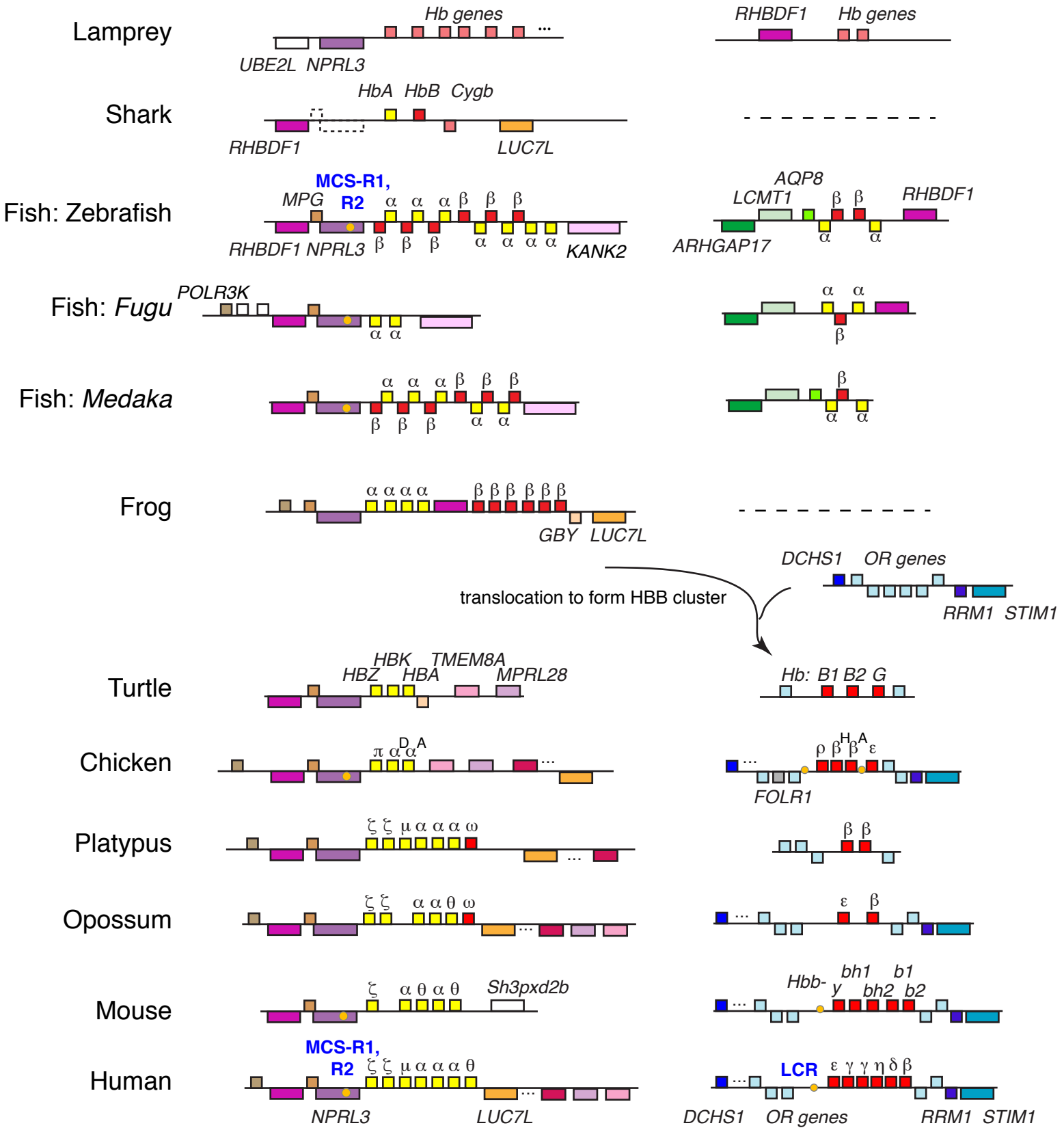

Clusters of hemoglobin genes linked to
NRPL3 and RHBDF1
Outgroups
Ancestral vertebrate
Ancestral gnathostome
Second globin gene cluster
adjacent to LCMT1
Beta globin gene cluster
moves to locus with
OR genes
Precambrian
550 MYA
Paleozoic
250 MYA
Mesozoic
65 MYA
Cenozoic
Lamprey
Cartilaginous
fish (shark)
Ray-finned fish
Amphibian
Reptiles and Birds
Mammals

Figure 2.


Lamprey
Hb genes
UBE2L NPRL3
RHBDF1 Hb genes
Shark
HbA HbB Cygb
RHBDF1
LUC7L
Fish: Zebrafish
MCS-R1,
R2
MPG
RHBDF1 NPRL3
KANK2
AQP8
LCMT1
RHBDF1
ARHGAP17
Fish: Fugu
POLR3K
Fish: Medaka
Frog
GBY LUC7L
translocation to form HBB cluster
DCHS1 OR genes
RRM1 STIM1
Turtle
HBK TMEM8A
HBZ HBA
MPRL28
Hb: B1 B2 G
Chicken
FOLR1
Platypus
Opossum
Mouse
Sh3pxd2b
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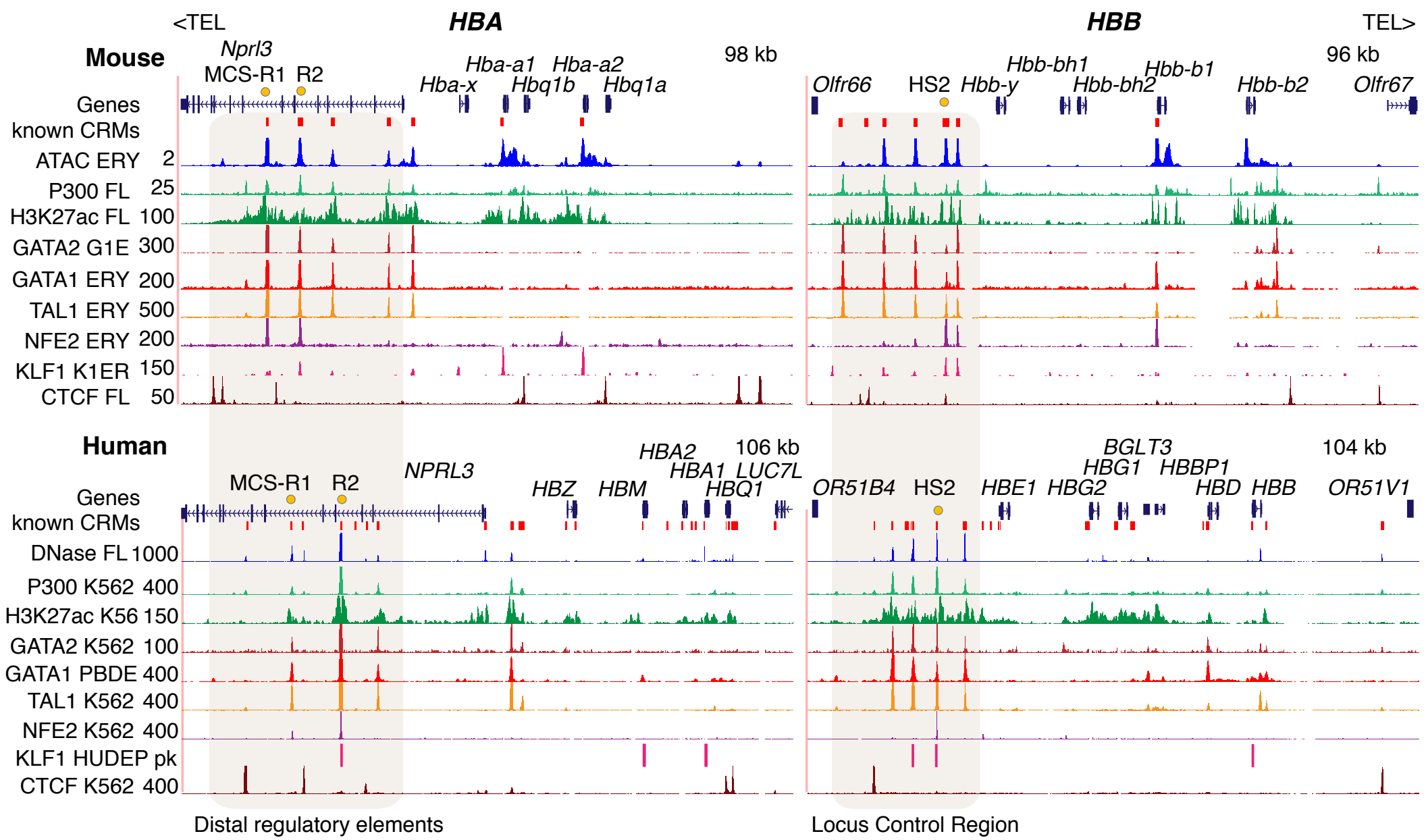
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Hbb-b1
Olfr66
HS2
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Hbb-bh2
Hbb-b2
Olfr67
96 kb
Genes
known CRMs
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P300 FL 25
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GATA1 ERY 200
TAL1 ERY 500
NFE2 ERY 200
KLF1 K1ER 150
CTCF FL 50
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HS2
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HBD
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DNase FL 1000
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H3K27ac K56 150
GATA2 K562 100
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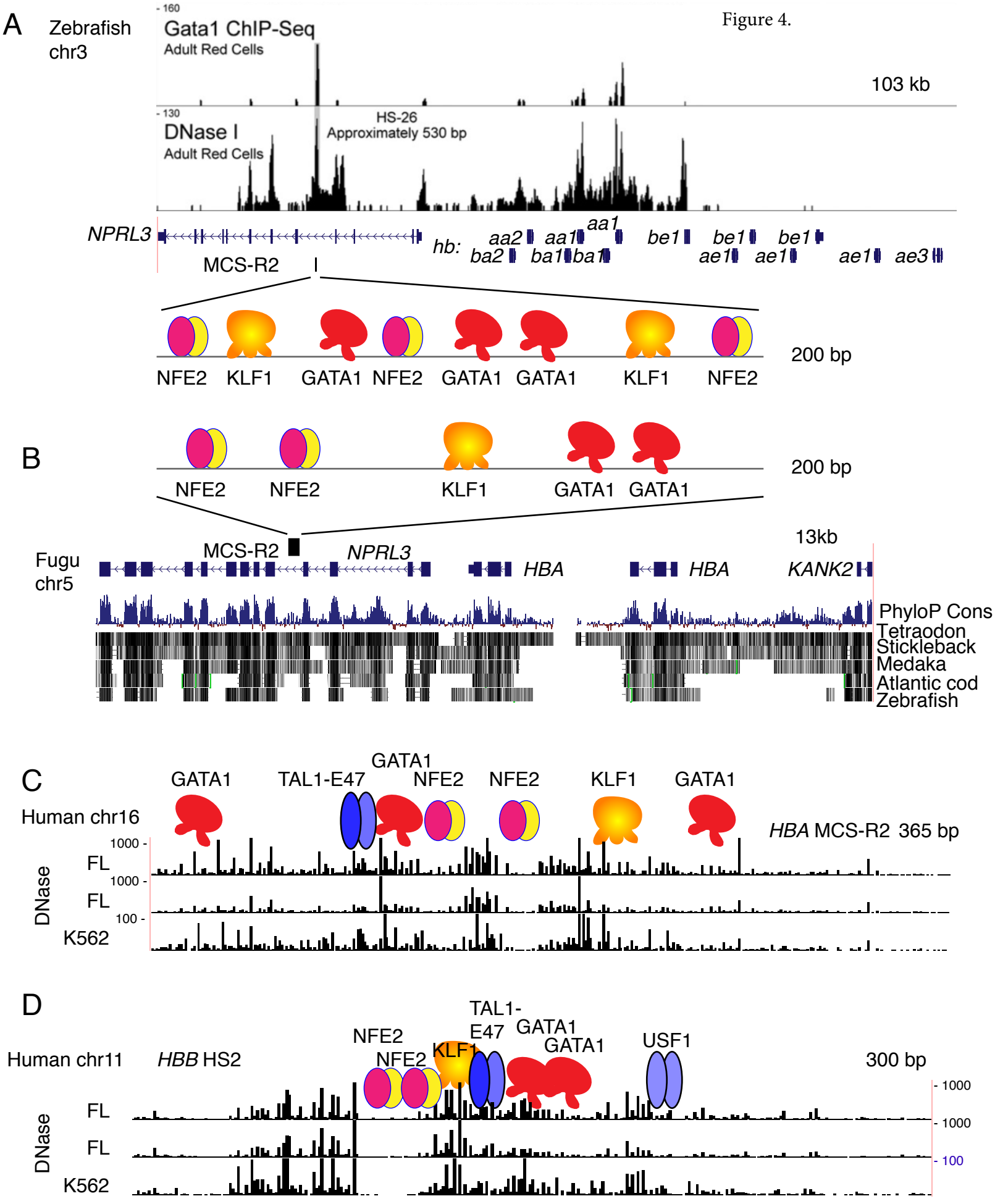
Distal regulatory elements
Locus Control Region

Figure 3.

Figure 4.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DNase I
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HS-26
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NPRL3
hb:
aa2
aa1
aa1
be1
be1
be1
ba2
ba1
ba1
ae1
ae1
ae1
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MCS-R2
NPRL3
HBA
HBA
KANK2
Fugu
chr5
PhyloP Cons
Tetraodon
Stickleback
Medaka
Atlantic cod
Zebrafish
C
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GATA1
TAL1-E47
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NFE2
KLF1
GATA1
Human chr16
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