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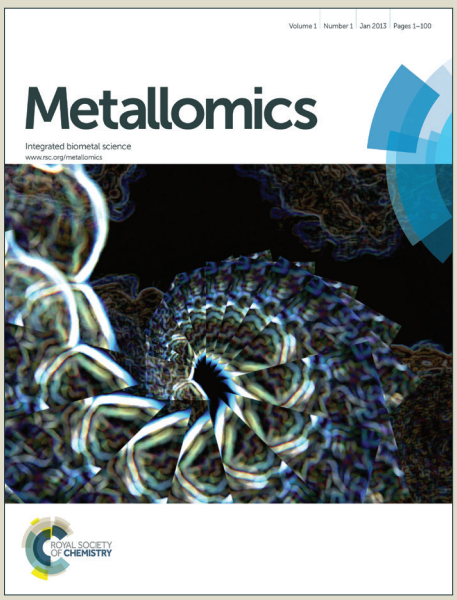

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Iron and oxygen sensing: a tale of 2 interacting elements?

Robert J Simpson and Andrew T McKie

Diabetes and Nutritional Sciences, School of Medicine, Kings College London, 150 Stamford Street,

LONDON SE1 9NH Email: andrew.t.mckie@kcl.ac.uk

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1. Introduction

Iron and oxygen are 2 elements that are indispensable for life in mammals but are toxic in excess. Both are found together in essential proteins such as haemoglobin and interact with each other in many key enzymes including haemoglobin and cytochrome c oxygenase. They also interact to produce toxic compounds (including reactive oxygen species) when in excess. Levels of cellular oxygen and iron can change rapidly and cells must be able to respond in order to survive. Therefore cells have developed a number of regulatory mechanisms which rapidly alter key proteins involved in regulation of cellular oxygen and iron levels. Sensing changes in cellular oxygen and iron levels is a key component of these regulatory pathways and in recent years much progress has been made in understanding these mechanisms. In several cases the proteins involved require both O_2 and iron for optimal activity (e.g. FBLX5, PHDs, IRPs). Prolyl hydroxylases (PHDs) are involved in oxygen sensing and require both iron and oxygen. A reduction in either substrate leads to inactivation of the enzyme and activation of the effectors Hypoxia Inducible transcription Factors (HIFs). HIFs bind to hypoxic response elements (HREs) within promoters leading to increased gene expression. Therefore exposure of cells to low oxygen tension or low iron will result in activation of a similar set of genes. Thus iron deficiency and hypoxia can be considered as similar physiological stimuli and this explains why changing iron status has effects on oxygen dependent processes and vice versa. Thus the primary interaction of iron and oxygen at the active site of proteins is not only significant in enzymes and globins but also in signalling proteins. In recent years, improved understanding of the molecular biology of iron and oxygen metabolising proteins has uncovered new mechanisms for interaction of iron and oxygen. The recent discovery of HREs which bind HIF2 alpha in the promoters of iron metabolism genes such as Dyctb, FPN1 has provided insight into the regulation of iron genes by hypoxia. On the other hand the discovery of an iron responsive element (IRE) within the 5' region of the HIF2 mRNA has added another layer of complexity to this intriguing regulatory network. Thus the regulation of iron and oxygen metabolizing proteins are intimately connected.

In this review we will discuss the implications of this recent work on understanding how iron and oxygen levels regulate iron metabolism both in cells and in the organism and how iron metabolism regulation is linked to oxygen supply via regulation of erythropoiesis.

2. HIF2α and iron sensing in the gut.

Five years ago, the discovery that hypoxia inducible factor 2 alpha (HIF2α) was a key factor in the transcriptional control mechanism for major intestinal iron absorption proteins such as DMT1, ferroportin (Fpn) and Dcytb was established [1, 2]. This finding opened up possibilities to explain previously mysterious details of the regulation of iron absorption, particularly at the intestinal level [3]. HIfs combine with ARNT to form a transcriptional complex for genes with hypoxia response elements (HREs) in their promoters[4] , and the levels of HIFS are controlled by both oxygen and iron. HIF2α protein levels are regulated by degradation through an iron and oxygen sensitive mechanism whereby prolyl hydroxylases (PHDs) are activated by oxygen and/or iron to hydroxylate Hifs. Hydroxylation initiates degradation via ubiquitinylation that depends on the protein von-Hippel Landau tumour suppressor (VHL) [5]. Thus transcription of HRE containing genes such DMT1, Dcytb and Fpn repreasetning the main iron transport proteins in the body can be increased in conditions of low oxygen (hypoxia) or iron deficiency.

Since that major advance further work has supported the importance of intestinal HIF2α in regulation of intestinal iron absorption. Mastrognianaki et al [6] showed that intestinal knockout of HIF2α reduces iron loading in genetically iron loaded mice (Hepcidin knockout mice) while Anderson et al [7] showed that intestinal expression of HIF2α was essential for the intestinal absorption of iron to support erythropoiesis.

However an additional complicating factor was that HIF2α mRNA has an iron responsive element (IRE) in its 5' untranslated region and this confers a property of translational repression by active iron regulatory proteins (IRPs) [8]. There are two known IRPs, IRP1 and IRP2 with distinctive regulatory properties- IRP1 is more active in mRNA binding when iron levels are low and oxygen levels are adequate, whereas IRP2 levels increase when iron or oxygen are low [9]. These latter complexities meant that unravelling the details of iron and oxygen regulation of iron absorption required further study and a series of knockout mouse studies, including the use of conditional, tissue specific and multi gene knockouts have been published that help clarify the situation.

3. Iron sensing in other tissues : muscle and bone marrow

Hypoxia is sensed in many tissues locations and cell types including EPO producing kidney cells, stem cell niches, tissues with impaired blood supply due to pathological damage or adipocytes in obesity. This sensing is thus important both in normal physiology and pathology and leads to paracrine and systemic responses. In this section we focus on signalling between primary tissues of iron metabolism, namely muscle and bone marrow and the liver.

It has always been an attractive idea that EPO might coordinate iron supply for enhanced erythopoiesis by stimulating iron supply, especially iron absorption. Several early studies, however, failed to support a direct role for EPO in iron absorption regulation [10-14] as opposed to an enhancement secondary to increased erythropoiesis. The discovery of hepcidin in 2001 was followed by a renewed interest in iron absorption regulation and evidence that EPO might directly regulate hepcidin production by liver via EPO receptor [15] was published. In 2010 Srai et al, [16] reported that a prolonged regime of EPO injection in rats stimulated iron absorption and showed that EPO receptor was present in duodenum. They further showed that the Caco2 cell line, which has features of duodenal cells, also expresses EPO receptor and treatment of this cell line led to enhanced iron transport by the cells. This was in line with a much earlier report of a direct effect of EPO on intestinal iron transport [17]. Many other studies support an indirect effect of EPO on hepcidin

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expression, operating probably via bone marrow [18-22]. More recent studies in man of the effect of EPO injections on iron absorption also support an indirect effect, presumably via bone marrow [23, 24] however a small direct effect of EPO on hepcidin or intestine cannot be excluded.

Erythroid Regulator

Finch initially proposed the 'erythroid regulator' to explain the increases in iron absorption seen in thalasaemia that could be dominant over the 'iron store regulator'[25]. Increases in erythopoeisis induced by EPO or blood loss (phlebotomy) rapidly supress liver hepcidin levels [19, 26]. As discussed above and below there appears to be no direct effect of either EPO or Hifs on hepatic hepcidin levels, however the effect is indirect and is dependent on an intact erythron[19]. Thus hepcidin inhibitory factors secreted from bone marrow cells which would regulate liver hepcidin have long been suspected. The identification of these regulators has been the holy grail of iron metabolism for many years. Several candidates including GDF15 and TWSG1 were initially identified from erythroid precursors[27, 28], however these have been shown to be either not required for erythropoeitic responses (GDF15) or not to be regulated in various physiological conditions where erythropoiesis is altered in the case of TWSG1 [29]. More recently a putative hormone erythroferrone (ERFE also known as fam132b) was identified using gene arrays which focused on genes which changed very early after erythroid stimulus by EPO or phlebotomy in mice. Erythroferrone (ERFE) is a secreted peptide and its mRNA was strongly induced within hours in phlebotomised in mice[30]. Moreover ERFE KO mice develop a deficit in haemoglobin particularly when stressed and injections of ERFE into normal mice result in hepcidin suppression [30]. Thus ERFE appears to be the main erythroid regulator, however the receptor for ERFE and signalling mechanism remain unknown.

Muscle regulator?

After the erythron, skeletal muscle contains the 2nd highest amount iron in the body and therefore it would make some physiological sense if changes in demand for iron in muscle could somehow influence iron supply. A candidate for such coordination of iron supply with demand is hemojuvelin. Hemojuvelin was first discovered as the gene mutated in the severe form of haemochromatosis called juvenile haemochromatosis[31]. It was subsequently found to be a membrane bound BMP co-receptor leading to the finding that BMP signalling is a major regulator of liver hepcidin levels and of iron metabolism [32]. HJV is highly expressed in other tissues such as skeletal muscle and heart and exists as a soluble form (sHJV) which can be detected at high levels in plasma[33]. sHJV acts as an competitive inhibitor of BMP signalling presumably by sequestering BMPs or acting as a 'decoy receptor'. Thus increases in the level of plasma sHJV would reduce hepcidin levels and increase plasma iron levels. Regulation of the serum levels of sHJV either by regulation of tissue HJV levels or regulation of cleavage of the receptor has been an attractive regulatory mechanism. Although some studies have shown that hepatic HJV protein levels appear not to be sensitive to changes in iron metabolism since no changes in liver protein were observed under conditions of iron deficiency or overload or by repeated phlebotomy and EPO treatment [34]. Intriguingly, despite skeletal muscle containing the highest levels of HJV of any tissue, conditional KO of HJV in skeletal muscle in mice had no effect on hepatic hepcidin levels or iron metabolism in contrast to liver KO of HJV which recapitulated the phenotype of juvenile haemochromatosis [35, 36] It should be noted, however,

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that the authors did not stress or provoke increased iron need in muscle and thus HJV may be required under these conditions.

Membrane bound HJV is regulated by furin cleavage which generates sHJV and by a membrane protease TMPRSS6 which degrades HJV both of which lead to reduced hepcidin levels. Loss of TMPRSS6 leads to continual stimulation of the BMP signalling pathway and increased plasma hepcidin and a condition known as refractory iron deficiency anaemia since the condition is not treatable by oral iron [37, 38]. However fragments of HJV produced by this cleavage do not appear to inhibit BMP signalling or hepcidin production themselves as shown for sHJV [39]. There is evidence that both furin and a membrane protease TMPRSS6 are regulated by HIFs and thus could be increased under hypoxia [40, 41].

In summary the idea of co-ordinated regulation of HJV or sHJV by changes in iron demand in vivo remains unclear.

4. Direct regulation of hepcidin in liver by HIFs

Some studies have suggested direct of regulation of hepcidin by HIF1α. Peyssonnaux et al [42] found that in liver HIF1α was increased in mice under iron deficient conditions and liver specific VHL-/- KO mice (the E3 ligase responsible for degradation of HIF1α and HIF2α) had increased HIF1α levels and reduced liver Hamp1 suggesting HIF1α might inhibit Hamp transcription. They further characterised several HREs within the Hamp1 promoter and showed that HIF1α bound to these HREs and that mutation of the HREs resulted in increased Hamp1 promoter activity. The conclusion was that HIF1α binding to Hamp1 could inhibit hepcidin production and thus explain in part how hypoxia or iron deficiency lead to reduced Hamp1 . However another study which used a mouse with liver specific HIF1α KO found that liver Hamp1 levels were not affected and responses to iron deficiency were similar in control and Hif$\alpha^{-/-}$ mice[1]. Indeed the same study used liver specific KO of ARNT the binding partner of both HIF1α and HIF2α. ARNT KO mice had virtually normal hepcidin responses to iron deficiency. This has more recently been confirmed in other studies [20]. Thus it appears that HIFs do not play a major direct role in regulating hepcidin.

5. The HIF2α IRP network.

Significant advances have recently been made towards unravelling the complexities of the iron/ oxygen sensing system. Interacting regulatory pathways for iron and oxygen metabolism form a complex network, however work with mice tissue selectively and conditionally knocked out in specific regulatory proteins have revealed ways to understand these complexities. The driver for these studies has been to develop an understanding of the interplay of iron metabolism (IRPs and HIF2a) with regulation of erythropoiesis (HIFs and erythropoietin (EPO)).

Galy et al [43] knocked out both IRP1 and IRP2 in intestinal enterocytes of adult mice using Cre-Lox intestinal specific conditional knockouts, triggering the knockout with injections of tamoxifen that activated tamoxifen dependent CRE recombinase targeted to enterocytes by a Villin promoter in adult (10-12week old) week old mice bred with floxed IRP1 and IRP2 gene insertions. This made it possible to study iron metabolism especially iron absorption regulation in mice lacking both IRPs in their duodenal enterocytes. The study results were interpreted as a) supporting the ability of ferritin

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to block iron absorption in a revival of the 'mucosal block' hypothesis; b) showing that the IRP system is not required for regulation of iron absorption by erythropoiesis or hepcidin and c) suggesting that IRPs set a basal rate of iron absorption. Collectively they suggest this supports a concept of 'layering' of regulatory mechanisms. Such a hierarchical structure, if correct, simplifies understanding of these regulatory networks [43].

In another paper the same group [44] showed that mice globally (ie in all tissues) expressing a constitutively activated IRP1 protein had impaired erythropoiesis (as well as widespread iron overload and overproduction of ferritin). They suggested but did not test whether suppression of HIF2α, known to have an IRE in its mRNA [44], was a factor in the erythropoiesis effect. Three other groups working in parallel showed that IRP1 (but not IRP2) regulates erythropoiesis by controlling translation of HIF2α mRNA [45-47]. These groups investigated a previously ignored transient polycythaemia in IRP1 global knockout mice at age 4-6weeks and showed that IRP1 but not IRP2 knockouts have increased HIF2α mRNA translation in kidney leading to increased EPO and consequent increased erythropoiesis. Anderson et al [45] further showed increased HIF2α dependent gene expression in intestine including increased transcription of genes for intestinal iron transporters DMT1 and ferroportin as well as the reductase Dcytb. Wilkinson and Pantopoulos [46] in contrast showed decreased hepcidin expression in liver. Ghosh et al [47] fed iron deficient diet to IRP1 knockout mice and observed increased mortality. They tracked this effect down to exacerbation of the polycythemia and associated haemorrhaging when iron deficient diet was fed to the knockout mice. These mouse studies confirmed in vivo an earlier suggestion by Zimmer et al 2008 [48] who worked with cultured cells to show that IRP1 but not IRP2 regulates HIF2α translation.

The specific role of IRP1 and not IRP2 in regulation of HIF2α (but not other HIFs) expression may help to simplify the interactions of the iron and oxygen regulatory networks. The mRNA binding by IRP1 and IRP2 are both increased by low iron conditions consistent with at least partially overlapping roles in iron regulation, however the effect of oxygen differs between the two as hypoxia decreases IRP1 activity but increases IRP2 activity. It is known that IRP2 is important for physiological mRNA-level regulation of iron metabolism in vivo [49], however its apparent role was inconsistent with the 5' IRE in HIF2α which would block translation of HIF2α in low iron conditions and hypoxia where Hif2a is known to be induced. IRP1 has now emerged as being critical in specific tissues for specific functions in particular HIF2α mRNA translation control. IRP1 regulation will enhance HIF2α derepression in hypoxia, there remains, however a contradictory effect of blocking translation in iron deficiency [50]. This may act as block to limit activation of HIF2α dependent iron transport in specific cell types, thereby protecting those cells from excessive iron depletion. Selectivity of IRP1 for specific IREs has been suggested before [51-54], but early selective IRP knockout work suggested the two IRPs could largely substitute for each other [55]. Differences in protein expression attributable to variations in the two IRPs binding to IREs have been reported (reviewed in [7]) previously but the work reviewed above provides a first clear role for IRP1 in erythropoiesis and iron metabolism. Scientists are only beginning to elucidate the physiological significance of variations in IRP binding to IREs. These variations arise from differing affinities of the two IRPs and also variations in IRE structure that affect binding of both IRPs [7].

6. Other oxygen/iron sensing mechanisms in mammals

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Iron or oxygen levels can also affect iron metabolism in more indirect ways. A primary product of the reaction of iron and oxygen in physiological systems is the production of reactive oxygen species (ROS, [56]). Mechanisms have evolved to sense levels of reactive oxygen species (especially H_2O_2) and control various biological processes including iron metabolism [57-59]. In the case of iron excess leading to increased ROS, control of iron metabolism can be viewed as a form of feedback inhibition to lower local excess iron in the liver and therefore reactive oxygen species production. On the other hand a signalling role relevant to hepcidin's systemic functions is possible in response to hypoxia. ROS are a normal by-product of oxidative phosphorylation and ROS production by mitochondria increases in hypoxia (see above) and therefore provides an intermediate that links oxygen supply to ROS signalling pathways. ROS are also a by-product of detoxification reactions involving cytochromes p450, are produced during protein folding in endoplasmic reticulum and can also be produced by phagocytic cells during inflammation[59, 60] ER stress alters ROS production and there is integration between ER stress signalling and ROS signalling pathways [60].

NF-κB is a well-established activator of transcription of inflammation and apoptosis- linked genes and thereby a major mediator of inflammatory responses. NF-κB has long been known as to be affected by redox-related factors including ROS, iron and oxygen. More recently, NF-κB has been established to be hypoxia responsive through regulation by oxygen sensitive hydroxylases [61-63]. Regulation of hepcidin expression in macrophages is partly dependent on NF-κB [64, 65]. Iron has been shown to activate NF-κB in macrophages [66]. Recently a nuclear redox sensitive activator of NF-κB, pirin has been characterised as a non-heme iron protein that allows nuclear redox to regulate NF-κB mediated inflammatory responses [67]. Clearly multiple mechanisms by which iron and oxygen can affect NF-κB activity are known and further work is needed to clarify their significance for hepcidin regulation.

C/EBP alpha is an important developmentally regulated transcription factor controlling energy metabolism genes in liver and other tissues [68]. C/EBP alpha was shown in 2002 to be regulated by iron and to act on the CCAAT elements of the hepcidin promoter [69]. Liver specific C/EBP alpha knockout mice develop iron overload due to low hepcidin production [69]. A recent screen for hepcidin regulators confirmed CEBP alpha as an important regulator of hepcidin expression [70]. C/EBP alpha was linked to regulation of hepcidin by hypoxia in a study that suggested ROS were intermediates in the sensing mechanism that led to dissociation of C/EBP alpha (and also STAT3) from the hepcidin promoter and therefore downregulation of hepcidin [71]. Details of the signalling mechanism were not clarified by this study. Soon after, Pinto et al reported that the EPO receptor could signal to control levels of C/EBP alpha bound to hepcidin promoter [15], thus providing an alternative mechanism to link hypoxia to decreased hepcidin expression, however details of the mechanism were again not provided, except that the inhibitory C/EBP homologue, CHOP, was not involved. Several other triggers have been identified that seem to target C/EBP alpha levels to alter hepcidin expression, namely Alcohol [72, 73], hepatitis C infection [74] and ER stress [75]. The mechanism(s) linking these triggers to downregulation of C/EBP alpha activation of hepcidin promoter are not yet fully worked out but ROS and the effect of the C/EBP homologue CHOP which binds to and deactivates C/EBP alpha have been implicated [76].

Several studies have shown that alcohol treatment decreases hepcidin expression [77-79]. More than one possible mechanism linking alcohol to hepcidin has been proposed. One recent study showed that ethanol suppressed hepcidin expression via induction of hypoxia with Hif1a and Hif2a

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both contributing to reduction of C/EBP alpha expression [72]. Others suggested ROS [78] or SMAD signalling[80] were involved.

The endoplasmic stress (ER) response (UPR- unfolded protein response) has been shown to affect hepcidin synthesis [75] and operates early in the stress response via alteration in C/EBPalpha thought to be mediated by the C/EBP homologue CHOPS [81]. Others, however proposed that CREBH acts on the hepcidin promoter to induce hepcidin presumably later in the stress response [82].

The mechanisms linking ROS to hepcidin expression also seem to be complex. An early study by Choi et al [71] suggested that both C/EBP alpha and STAT3 were involved in ROS signalling to hepcidin expression. Millonig et al [83] also suggested a role for STAT3. CHOPS upregulation in response hepatitis B virus infection has been implicated in ROS-mediated hepcidin downregulation via C/EBPalpha [74]. It is noteworthy that C/EBPbeta has been implicated in regulation of macrophage hepcidin expression [64] highlighting tissue or cell type specific factors in CCAAT element regulation of hepcidin promoter activity.

Furin is a proprotein convertase involved in processing of cell surface and extracellular proteins by cleavage after basic residues [84]. Furin has been implicated in hepcidin synthesis and regulation at two points, namely conversion of prohepcidin into active hepcidin [85] and cleavage of Hjv to release a soluble Hjv (s-Hjv) which is an inhibitor of BMP signalling [41]. Furin itself is regulated by hypoxia via Hifs [41] or via Tfr2 and Hfe [86]. Furin therefore is a link in a possible mechanism of hypoxic- modulation of hepcidin synthesis.

7. Do chronically anaemic mice show evidence of hypoxia in liver or duodenum?

In chronically anaemic mammals, tissue hypoxia does not necessarily occur as compensatory responses to blood flow, vasculature, oxygen diffusional path length and other oxygen delivery mechanisms can mitigate the effect of anaemia[87, 88]. In hypotransferrinaemic mice early studies found normal tissue oxygen levels in duodenal mucosa [87] with evidence for increased tissue haematocrit in the presence of normal blood flow rates [88]. In liver, tissue iron levels are increased so any alteration in gene expression in HIF- regulated genes can be attributed to tissue hypoxia rather than reduced iron levels. In duodenum studies of iron proteins suggested the enterocytes were not iron deficient [89] again suggesting that HIF responses might indicate local hypoxia rather than be attributable to reduced iron levels. Recently the availability of hypoxia reporter mice (OD-luc mice, see [90] has demonstrated that specific tissues important in iron metabolism namely duodenum, liver and kidney can be hypoxic in anaemic mice even though other tissues are spared from hypoxia in the same mice [91].

Such elegant studies have not yet been performed in hpx mice, however gene expression studies can illuminate the situation therefore we examined microarrays prepared from RNA extracts from liver and duodenum of hpx mice for altered gene expression. These microarrays were compared to published microarrays of gene expression in hypoxic mouse tissues. We found that 49% of 84 genes elevated in hpx duodenum were also elevated in hypoxic tissue arrays ([92], see GSE15891 at GEO profiles of Pubmed website; [93], GSE17796 at GEO profiles of Pubmed website, [1, 94, 95]). Similarly in liver, most of the highly altered mRNA probes (9 of the top 14 increased and 4 of the 13 most decreased) were found to be altered in similar ways in chronic or acutely hypoxic mouse liver.

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Iron loading, on the other hand was also evident from the finding that 7 of the 15 most decreased mRNA probes were also decreased in iron loaded mouse liver. Note that hypoxia predominantly increases mRNA expression while iron loading predominantly decreases gene expression. One can conclude therefore that irrespective of compensatory changes to oxygen delivery in chronically anaemic hpx mice, gene expression profiles in both liver and duodenum show evidence of tissue hypoxia and increased Hif activity.

Concluding remarks

The evolution of iron and oxygen regulatory mechanisms has proceeded hand in hand as the availability of iron is critically dependent on oxygen levels and enzymatic functions of iron involve oxygen [56]. When organisms developed circulatory systems with iron-containing proteins for oxygen delivery and storage, a further metabolic interaction between these elements was added. It is no surprise to find that regulatory interactions between iron and oxygen are fundamental to the metabolism of iron. The rapid advances of understanding of the molecular processing underlying these interactions can be expected to continue, however, constructing coherent models of this regulatory network may prove more challenging. An evolutionary perspective may prove helpful in understanding the complexities as it provides a context for the sequential acquisition of regulatory mechanisms through evolutionary history, leading to a layering of structures within the regulatory network.

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