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Refined control of cell stemness allowed animal evolution in the oxic realm

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Animal diversification on Earth has long been presumed to be associated with the increasing extent of oxic niches. Here, we challenge that view. We start with the fact that hypoxia (<1–3% O_2) maintains cellular immaturity (stemness), whereas adult stem cells continuously—and paradoxically—regenerate animal tissue in oxygenated settings. Novel insights from tumour biology illuminate how cell stemness nevertheless can be achieved through the action of oxygen-sensing transcription factors in oxygenated, regenerating tissue. We suggest that these hypoxia-inducible transcription factors provided animals with unprecedented control over cell stemness that allowed them to cope with fluctuating oxygen concentrations. Thus, a refinement of the cellular hypoxia-response machinery enabled cell stemness at oxic conditions and, then, animals to evolve into the oxic realm. This view on the onset of animal diversification is consistent with geological evidence and provides a new perspective on the challenges and evolution of multicellular life.

The long-lived idea that increasingly oxic conditions, in both the atmosphere and ocean, facilitated the diversification of animals in the so-called Cambrian explosion (555–500 million years ago (Ma))[1,2] has guided great efforts to explore when and how low oxygen limits animal life. Although these studies disagree on the synchronicity between changes in oxygenation and the initiation of the Cambrian explosion, they commonly share the assumption that the oxic niche is permissive and unproblematic for the evolution of large life forms. We challenge that assumption, based on the fundamental requirement for hypoxia (<1–3% O_2) to sustain cell stemness[3,4] and how oxic conditions (>1–3%) promote cell differentiation[5]. Here, we evaluate an evolution of cell stemness control in animals, as outlined in Table 1, and discuss how the model maps onto geological and biological evidence of stemness and the varying capacities of animals to live in the oxic realm.

A paradox and a solution

The apparent paradox between how oxic conditions demote cell stemness and the necessity of stem cells for viable animal life challenges our view on the oxic environment as permissive for animals with a lifelong need for tissue regeneration. For the generation and renewal of complex animal tissue and organs to occur, pools of stem cells—including tissue (adult) stem cells—must be maintained. Adult stem cells are committed to a certain range of cell fates, halted in their differentiation and found in all regenerating tissues. In contrast to those stem cells that retain their pluripotency by residing in hypoxic niches, such as the human bone marrow[6], adult stem cells can also maintain an immature phenotype in oxic settings[7,8] (see a note on the nomenclature in Box 1). Thus, given that oxygen promotes stem cell differentiation, the immature adult stem cell phenotype in vascularized and oxygenated tissues is counterintuitive, and needs an explanatory model that is lacking at present.

Central to how cells respond to oxygen concentrations are the hypoxia-inducible factors (HIFs) with their oxygen-sensitive α-subunits. The HIFs constitute the primary known animal cell transcription factors with an activity that is directly regulated by oxygen; high levels of molecular oxygen promote their degradation and thus mute hypoxia-driven cell responses[9] (Fig. 1a,b, Box 1 and Supplementary Information). One such HIF-driven gene is *EPO*, which encodes a hormone necessary for the production of red blood cells and thus oxygen transport[10] (Fig. 1c). Tumour biology demonstrates that HIF-2 in particular, although oxygen sensitive to degradation in many contexts, can be stabilized and active at physiological (that is, oxic) conditions[7,11], thus creating a cell phenotype referred to as pseudohypoxic (Fig. 1b and Box 2). This pseudohypoxic phenotype seems to facilitate the activation of pathways promoting stem cell-like features[7] and might therefore provide an explanation for the halted differentiation of adult stem cells under oxic conditions.

Animal life requires discrete periods of cellular hypoxia

Animals first evolved in a low-oxygen world[12] (estimated at approximately 2–4% O_2, Fig. 1d and Supplementary Table 1), which would result in hypoxic tissue by modern definitions[13]. The hypoxic state is conventionally described as a problem and challenge to animal functionality. For example, HIF activation is considered an adaptive response to counteract cellular and tissue damage caused by hypoxia. With a similar outlook, many animals are described to tolerate hypoxia, even if it is for months in the case of fish, crayfish or turtles[14,15]. Hypoxia, however, is also described as a necessity for animal tissue homeostasis that continuously balances oxygen concentrations around the threshold of hypoxia, whether in marine crustaceans[16] or humans[13]. The human embryo, furthermore, resides non-vascularized in a hypoxic uterus for at least two weeks, until the placenta begins to supply the fetus with oxygenated blood[17] and human blood cells originate from haematopoietic stem cells that require its hypoxic bone-marrow niche[4].

An association between hypoxia and specialized cells and tissue is also exploited and explored. Artificial growth of skin in so-called three-dimensional (3D) bioreactors is improved by a delicate balance of both hypoxic and oxic conditions[3]. Similarly, animal tumour cells are observed to survive and proliferate even at extreme hypoxia[18]. Here, we have cultured differentiated invertebrate and vertebrate animal cells at 0.1% and 1% O_2, and show that all cell lines retained proliferative and survival capacities at severe

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Box 1 | Hypoxia and the HIF system

Hypoxia—that is, oxygen shortage—is a context-dependent term. In marine biology, hypoxia refers to physiologically stressful oxygen concentrations[93] although its term and definition vary[90]. In cellular, developmental and tumour biology, oxygen levels that evoke an adaptive response through HIF activation are termed hypoxic. In experimental cell biology, an environment with 1% oxygen is generally used to create a hypoxic response and termed 'hypoxia'. In contrast, as physiological oxygen levels differ from one organ and cell type to another, normal end-capillary oxygen concentrations are sometimes termed 'physoxia'. In experimental cellular and molecular biology, the term 'normoxia' is frequently used and simply means that studied cells have been cultured at normal atmospheric conditions, that is, at an ambient oxygen level of 21%. Yet animal tissue is rarely exposed to the concentrations common in the atmosphere[4]. For context-dependent terms, thresholds, units and conversions, see Supplementary Table 1.

HIF transcription activity is rooted in ancestral oxygen-sensing molecular mechanisms[87,88] and, as of today, is the primary known family of animal cell transcription factors with an activity directly regulated by oxygen. These dimeric factors are activated at hypoxia through the inactivation of oxygen-dependent hydroxylases, which leads to the stabilization of HIF-α subunits and activation of the formation of functional HIF transcription complexes[9] (Fig. 1a,b). At hypoxia, stabilization and activation of HIFs result in the expression of several hundred genes that affect a plethora of fundamental biological responses[94,95], such as vascularization and angiogenesis, genome stability, switching between aerobic and anaerobic metabolism and differentiation processes[32,85,86] (Fig. 1c and see Supplementary Information for details). Gene duplications have resulted in HIF-α homologues that encode for three oxygen-sensitive subunits, of which we focus on the two most studied: HIF-1α and HIF-2α. HIF-1α, for example, is stabilized as an immediate response to hypoxia allowing the HIF-1 transcription complex to drive vascularization and a metabolic switch[9]. The regulation of HIF-2α stability is more complex, and through yet-to-be-described mechanisms, HIF-2α is not degraded under oxygenated conditions in specific cell types, such as tumour macrophages[96] or mesenchymal-like and stem cell-like neuroblastoma cells[7]. That transcription complexes involving HIF-2α can remain active at physiological oxygen tensions (~5% O_2) (Fig. 1b), is illustrated by how tumour cells that are positioned adjacent to blood vessels can express high levels of HIF-2α but no HIF-1α proteins[7,33] (Supplementary Fig. 3c,d). Therefore, although oxygen is present in the tissue setting, a HIF-2 dependent hypoxia-response machinery may induce several bona fide hypoxia-driven genes (such as *VEGF*, *HK2*, *BNIP3*)[11] and create a pseudohypoxic phenotype. HIF transcription activities are also regulated through the factor inhibiting HIF-1 (FIH1), which hydroxylates asparagine residues on the HIF subunits and blocks the formation of functional transcription complexes. One molecular explanation for the apparent paradox that HIF-2α can be active at physiological oxygen levels is that FIH1 hydroxylates HIF-2α less efficiently than HIF-1α[97].

hypoxia (Supplementary Fig. 2 and Supplementary Information). These results reinforce the idea that hypoxia does not pose a problem for animal cells when provided with otherwise proper growth conditions.

Although the general biological focus is on surviving hypoxia, an alternative view is that organisms with regenerating tissues instead survive the oxic setting. This alternative view aligns with previous work that emphasized the hypoxic ancestry of animal evolution[19,20] and has allowed us to even further explore whether a coupling remains essential. For example, could the hypoxia tolerance observed across metazoan phylogeny[15] actually be reflecting an ancestral capacity that is still necessary today? Even humans can survive hours in near-drowning cases, when cold conditions have temporarily shut off energy-expensive organs such as the brain[22]. Instead of describing this phenomenon as hypoxia tolerance, we argue that oxygen-requiring organs are late evolutionary innovations that bias our interpretation of how animals experience hypoxia.

Novel stemness control in the non-permissive oxic realm

The differentiation-promoting effects exerted by oxygen (>1–3%) are associated with tight chromatin and restricted gene expression[23,24]. For example, at oxic culture conditions (21% O_2) human breast epithelial precursor cells can spontaneously form polarized ducts, while hypoxia (1% O_2) impairs this differentiation[5] (Supplementary Fig. 1). Considering the strong differentiation-promoting effects exerted by oxygen, an efficient oxygen supply in regenerating tissues was probably disadvantageous before the establishment of mechanisms that protect tissue (adult) stem cell features. It has been suggested that somatic animal cells rely on mechanisms to block and limit oxygen-induced differentiation[20] but the central role of adult stem cells in tissue renewal emphasizes the importance of mechanisms that promote stemness (see further in Supplementary Information). Thus, animal stemness control in oxic milieus should have preceded the appearance of efficient oxygen-transport systems, involving erythropoietin (EPO)[10] and red blood cells, for example. EPO expression and the pseudohypoxic phenotype are HIF-2 driven, which led us to compare the phylogenetic occurrence of the HIF-α subunits and EPO.

A compilation of proteome analyses, by us and others[25–27], demonstrate that while HIFs are common to all animals but sponges (Porifera) and comb jellyfish (Ctenophora), HIF-2α appears exclusively in vertebrates (a chordate subphylum) and EPO first in the genomes of fish (for example, *Latimeria chalumnae*) (Fig. 2, Supplementary Information and Supplementary Table 3a,b for results, comparison and data). With a particular focus on HIFs in animals around the bifurcation between protostomes and deuterostomes, it seems that neither of the early branching deuterostomes—purple sea urchin (echinoderm)[25,27] and acorn worm (hemichordate) (this study, see Supplementary Information)—nor the early deuterostomes in the chordate subphyla—lancelet fish (cephalochordate)[25] and vase tunicate (tunicate)[27] (this study, see Supplementary Information and Fig. 2)—display a distinct HIF-2α orthologue. According to our results, as well as ENSEMBL proteome annotations, the first HIF-2α orthologue is detected in northern lamprey (Hyperoartia), suggesting that the first appearance of EPO in vertebrate evolution occurs after the appearance of HIF-2α. However, this has not been demonstrated in previous studies[26,27]. EPO is the key growth factor regulating red blood cell synthesis in response to systemic hypoxia, and hypoxia-induced EPO is driven by HIF-2 and not HIF-1 (ref. [10]). Taken together, these observations suggest: (1) that putative HIF-2-driven stemness control preceded the development of the distribution of tissue oxygen through red blood cells, a distinctly different mode of oxygen distribution compared with that of insects, for example, and (2) that the animal phyla that diversified in the Cambrian (with the exception of sponges and comb jellyfish) had the ability to access the cellular hypoxia machinery through the presence of HIFs. Below, we present and discuss a model of HIF-driven stemness control. In short, our model claims that HIFs (probably together with additional, not yet discovered mechanisms) allowed animals to enter and diversify in the oxic realm by promoting cell stemness despite oxic conditions and, therefore, that the Cambrian explosion was not directly driven by expanding oxic niches.

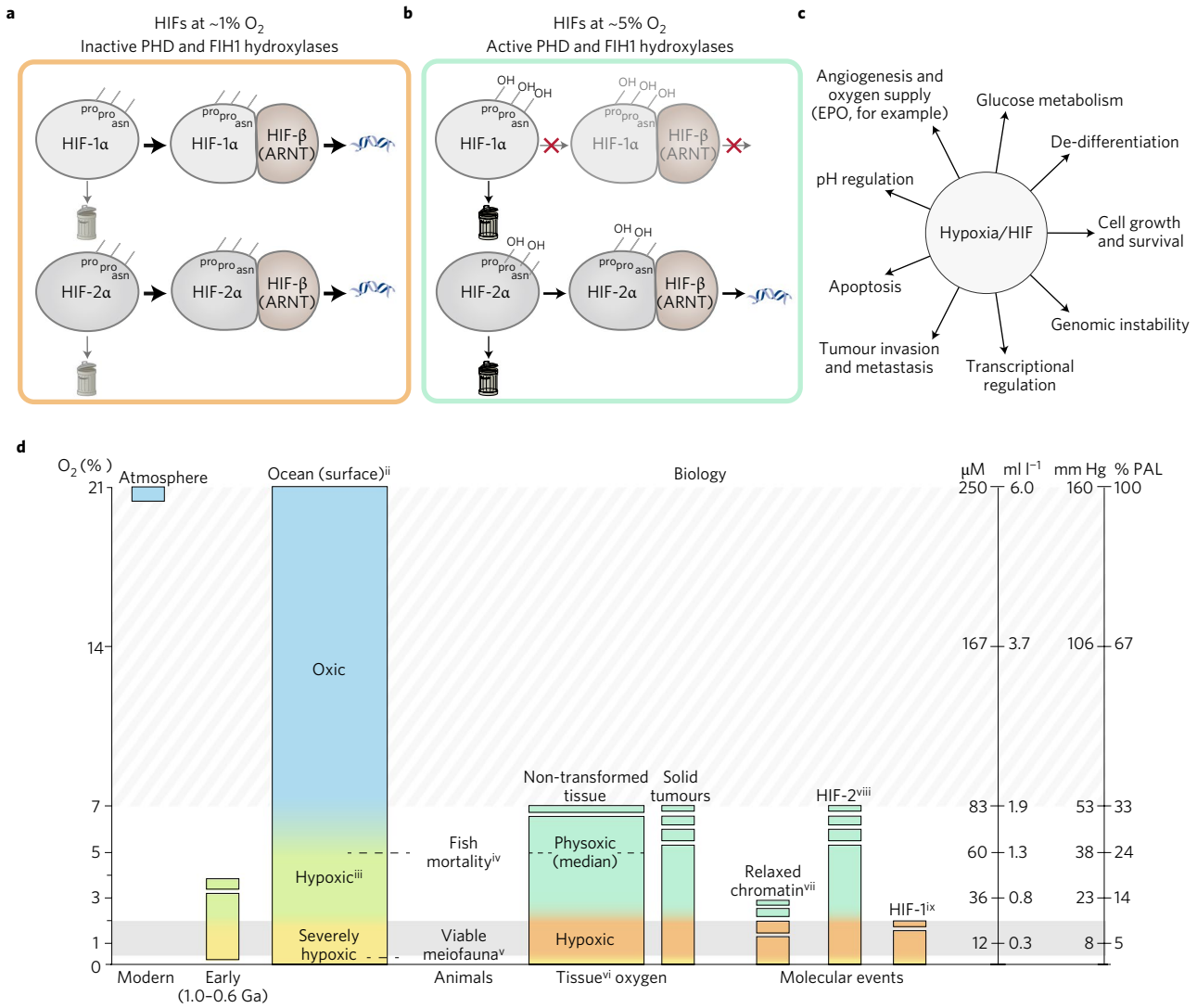

Fig. 1 | Environmental and biological oxygen thresholds and a simplified depiction of activation and degradation of HIFs. a,**b**, Activation and degradation of HIFs at hypoxia (~1% O_2; **a**) where both HIF-1α and HIF-2α are stabilized and in response to human end-capillary oxygen pressure (~5–7% O_2; **b**) when primarily HIF-2α is stabilized. Hydroxylation of the prolyl (pro) and aspargyl (asn) residues by prolyl hydroxylase (PHD) and asparagyl hydroxylase (FIH1) in the presence of oxygen allows recognition by the VHL-ubiquitin ligase complex leading to proteasomal degradation (waste bin), see also Supplementary Information. **c**, Generalized overview of the biological responses governed by hypoxia and HIFs in vertebrate tissue[89]. **d**, Oxygen concentrations in the atmosphere today and in the Neoproterozoic[54] (i), and corresponding shallow ocean water oxygenation (12 °C, 1 atm, 35‰ salinity; (ii)). Thresholds here depict operational definitions adapted from ref. [90] (iii), below which most fish die[91] (iv), above which benthic bilaterian animal diversity increase[90] (v), that of mammalian tissue, non-transformed and solid tumours[13] (vi), where chromatin is relaxed[25,53] (vii), and the operational spans of HIF-2α (viii) and HIF-1α[11] (ix). Corresponding styles and units used in geochemistry (μM), ecology (ml l^{-1}) and physiology (mm Hg) and palaeontology (%PAL) or to describe partial pressure (per cent saturation) (see Supplementary Table 1 for conversions). The hypoxic field, defined by biological thresholds (grey), is distinct from that of physoxic threshold (white), while oxygen concentrations above ~7% (striped) are largely irrelevant for tissue functions[4].

Stemness in solid tumours analogous to stemness in animals

Tumours manage the leap from single cells to multicellular entities and resemble developing organisms in their abilities to maintain stem cell pools and to manage cell differentiation, phenotypic plasticity, cell migration and inter-cellular communication. However, tumours do so with amplified, silenced or deficient control mechanisms[28]. As tumours exploit normal cell mechanisms, their progression has been suggested to reflect evolution in reverse[29]. Importantly, tumours also 'hijack' the maintenance of mechanisms of (cancer) stem cells at oxic conditions[30,31].

Cancer stem cells are non-equivocal to adult stem cells, but share the self-renewal capacity and the ability to form tissue. Thus, cancer stem cells seem to be central in the evolutionary 'success' of tumour tissue. Cancer cell stemness, and cell responses to low-oxygen concentrations in general, couples to the HIFs[9] and their oxygen-sensitive α-subunits, which are also essential for normal embryogenesis and tissue development[32]. Whereas the HIF-2 complex, with the subunit HIF-2α (encoded by *EPAS1*)[11,33], often evokes similar responses to HIF-1, HIF-2 seems to also promote stemness in some oxygenated tumour tissue[34]; a condition defined as pseudohypoxia[35]. Notably, high levels of HIF-2α are found in stem-cell-like tumour cells in proximity to blood vessels (perivascular) in neuroblastoma and glioma, and the presence of these cells correlates to aggressive disease[7,11,33]. The fact that gain-of-function mutations in *EPAS1*/*HIF2A* is found in hereditary and spontaneous sympathetic nervous system (SNS) tumours further underscores a pivotal role of HIF-2

Box 2 | The power of pseudohypoxia

The power of pseudohypoxia is exemplified by tumours with mutations and/or deletions that result in altered gene programs that mimic a hypoxic state. In one kind of kidney cancer (clear cell renal cell carcinoma) and several other tumour forms, deletion of the von Hippel Lindau gene (*VHL*) is frequent and leads to constitutive expression of HIF proteins as they do not become hydroxylated on prolines in the presence of oxygen, which requires the VHL protein, and the HIFs will not be degraded in the proteasome[98] (see Supplementary Information for details). These tumours seem to hijack the pseudohypoxic phenotype through activation of HIF-2 and its associated stemness control, which proves 'successful' for the multicellular entities of tumours. In this context, two tumours of the peripheral nervous system (paraganglioma and pheochromocytoma) are particularly interesting, as a major subset of these tumours have deletions (in *VHL*, *SDHx* and *FH*, for example) or gain-of-function mutations (in *EPAS1/HIF2A*), resulting in HIF-activation and pseudohypoxic phenotypes[34,99]. The losses and gains in these tumours frequently associate with hereditary forms[100], and we therefore conclude that there is a fundamental link between pseudohypoxia and tumorigenesis. This further implies that pseudohypoxia, cancer stem cells and tumour-initiating capacity are connected. As detailed in the text, pseudohypoxia seems to be required also for normal development of the SNS, for example, and physiological tissue regeneration in skin.

in promoting a pseudohypoxic stem cell phenotype. The power of pseudohypoxia is further supported by how it relates to tumorigenesis (Box 2). Taken together, HIF-1 and HIF-2 both activate large sets of partially overlapping genes[36] but under different time intervals and at different oxygen thresholds[11], where HIF-2α in particular associates with stemness and the pseudohypoxic phenotype.

In normal animal development, the link between HIF-2/HIF-2α and pseudohypoxic promotion of cell stemness is less studied. HIF-2α is present in the human basal skin stem cell layer[37] adjacent to blood vessels supplying oxygen, for example, and therefore indirectly suggests a pseudohypoxic phenotype. In addition, HIF-2α is transiently expressed during early human and rodent SNS development, under conditions where HIF-1α is not present[8,32,38]. The HIF-2α-positive SNS cells are immature and stem-cell-like and HIF-2A knock-out animals have an underdeveloped SNS[32], suggesting that HIF-2α marks SNS stem cells located in a pseudohypoxic niche. Furthermore, haematopoietic stem cells, although residing in hypoxia, depend on HIF-2 for their long-term re-populating capacity[39]. A key feature of stem cells is their strict control of the genome through genome stabilization[40], which becomes less tight as cells differentiate. When HIF-2α is eliminated in vivo, the production of reactive oxygen species (ROS) increases[40], which is known to induce DNA damage. These data indirectly indicate that HIF-2 promotes genome-stabilization mechanisms. A direct role of HIF-2 in maintaining embryonic multipotency is also implicated by how HIF-2 (not HIF-1) promotes the expression of stem cell marker genes (such as *Oct4*, *Nanog* and *Sox2*) in hypoxic rodent embryo cultures[41,42]. In light of how tumours exploit normal cell functions[28] and the indications of an association between HIF-2 and stemness in normal tissue, it is reasonable to presume that the maintenance of adult stem cell pools in oxygenated animal tissue involves similar mechanisms to those described in tumours.

A model of improved stemness control

Based on published data and our own observations, we propose three modes of stemness control within animals and their multicellular predecessors. In the most rudimentary mode, diffusion or cell metabolic activity create oxygen gradients in the tissue, which results in hypoxia and cell stemness in its core while cell differentiation is promoted by higher oxygen concentrations at its outer boundaries (Fig. 3a); a mode of stemness control that permitted simple cell organization before body-plan instructions or regulated apoptosis were in place. The organisms could either regulate the gradient, through the rate of cell proliferation, for example, or they required a predictable external environment. In the next developmental mode, introducing the capacity of a HIF switch between aerobic and anaerobic metabolism, organisms are freed from the necessity to live in environments with predictable oxygen gradients. To maintain stemness, these organisms induce hypoxic conditions either internally by decreased ventilation, increased respiration or cell proliferation, or externally by moving into hypoxic conditions. Invertebrate animals would possess the rudimentary control of stemness complemented—for most of them—by a HIF-1 mechanism. Finally, pseudohypoxia driven by HIF-2, and probably additional pathways, allows organisms—here vertebrates—to maintain hypoxia-associated stem cell features in proximity to well-vascularized and oxygenated (>1–3% O_2) tissue (Fig. 3b).

Stemness control offered through the HIF machinery (Fig. 3c) provided a crucial developmental key to sustain complex tissue in the oxic realm. The benefits of the high-energy yield of aerobic metabolism and the advanced potential for cell differentiation and tissue specialization make stemness control through HIF substantially advantageous. While HIF-1 provided a key to the hypoxia machinery so that animal evolution could occur in the oxic niche, HIF-2-driven pseudohypoxia gave vertebrates additional competitiveness by decoupling tissue generation from true hypoxia. We propose that this developmental event, occurring in two steps, together with subsequent ecological interactions[43], led to dramatic animal diversification.

Notably, a high capacity to change cell fate without any obvious association with hypoxia or HIFs is noted among multicellular life such as sponges[44] or fungi[45], where cells are totipotent and can transdifferentiate such that tissue renewal appears plastic. Modern sponges are observed to arrest canal ventilation for hours or days while their tissue turns anoxic[46]. This behaviour is consistent with the rudimentary mode of stemness control proposed in our model, where organisms manage their internal gradients by creating a predictable local environment. Totipotency is also observed in cnidarians, some worms, and echinoderms[47]. A common trait of animals with totipotency is that they generally have fewer cell types than most other animals[48]. A relationship between low cell specialization and high totipotency[49] suggests a developmental threshold to the reversibility of cell differentiation. Beyond that threshold, tissue and its specific stem cells would be increasingly specialized, so that cells could no longer transdifferentiate. The human red blood cell is an example of cell specialization beyond a point of no return, as its nucleus is expelled. Although alternative ways for stemness seem to be available, current data suggest that totipotency was traded for specialization during evolution[47].

Comparison with the geological record

The model of evolving stemness control can be compared to the Precambrian rock record, where macrofossils with presumed cell differentiation are rare but occur as early as 2.1 billion years ago (Ga)[50]. The simple morphology of the 2.1-Ga-old fossils[50] is consistent with cell fate management along oxygen gradients (Fig. 3a). However, the fact that Precambrian macrofossils are so rare is more notable when, principally, both hypoxic and oxic niches have existed since long before animals diversified (see Supplementary Information for details). Oxic niches were probably available for experiments in multicellularity in shallow settings (and in the vicinity of oxygen-producing cyanobacteria[51]) since perhaps 2–3

Table 1 | Overview of observations that construct the hypothesis of hypoxia as ancestral normality for tissue where HIFs provided a majority of animal phyla the control of hypoxia-machinery in the oxic realm

	Critical mechanisms and events associated with hypoxia		Adaptations to the oxic world
Cell biology	Stemness at hypoxia[3], where chromatin is relaxed[23,24]	Animal cells proliferate at extreme hypoxia[a]	Early vertebrate embryogenesis in true hypoxia[17]
Tumour biology	Basal tissue functions at hypoxia or with HIF, such as angiogenesis or stemness[32,85,86]	HIF unique to all bilaterian animals, plus Placozoa and Cnidaria, and HIF-2 unique to vertebrates[25,27,87,88,a]	HIF-2-driven pseudohypoxia promotes stemness[7]
Geobiology	Animals diversified when atmospheric oxygen levels were, presumably, <2–4% O_2 (or 10–20% PAL)[12]	Cambrian explosion of primarily bilaterian animals[55] not conclusively linked to increased extent of oxic niches[84]	Invertebrate adulthood relatively short[57] or associated with hypoxia[14,70,77], while tissue renewal remains fragile[64,65]

[a]This study.

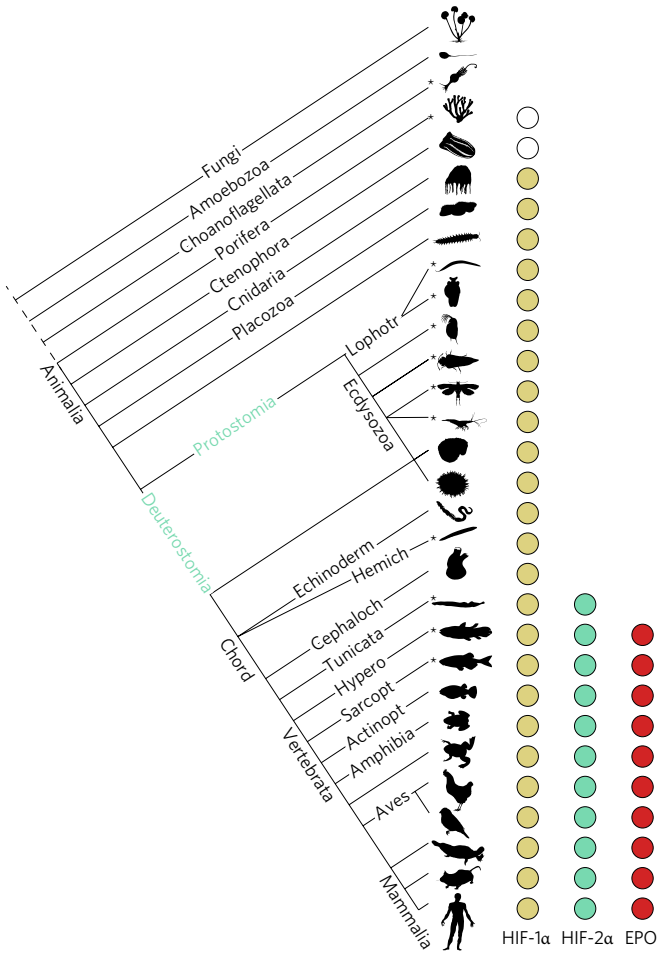

Fig. 2 | The appearance of HIF and EPO proteins in a phylogenetic tree of multicellularity. The occurrence of HIF-1α (filled orange circles), HIF-2α (filled green circles) and EPO (filled red circles), compiled from previous studies[25–27,87,88,92] and our analyses in a simplified diagram; the branch lengths are not to scale and for branch labels see Supplementary Table 3a. *Previously unpublished HIF proteomes, see Supplementary Information for methods and details. While HIF-1α is unique to Placozoa, Cnidaria (jellyfish) and all bilaterian animals (here represented through Protostomia and Deuterostomia), it seems that Porifera (sponges) and Ctenophora (comb jellyfish) host only the domain of Per-Arnt-Sim (PAS) but no oxygen-sensitive domain (ODD) such that the protein appears HIF-like but is not a true HIF (empty circle). HIF-2α occurs first in vertebrates (lampreys; belonging to the class Hyperoartia), while EPO occurs after lampreys. See Supplementary Information for details on the presence of HIFs and EPO (Supplementary Table 3a) and for extended gene and protein information (Supplementary Table 3b).

Ga[52]. Still, no sizeable diversification of multicellularity occurs until approximately 0.6 Ga. Although both the evidence and timing of Precambrian multicellularity are debated, the lag that separates the first eukaryotic ancestor and the pronounced diversification of multicellularity is consistent with the view that the oxic niche is non-permissive of tissue renewal. Without other biological tools for stemness, large life would have been unable to exploit the oxic realm and its energy gain. The threshold, above which macroscopic life would struggle with stemness and controlled differentiation, based on oxygen-promoted cell differentiation and tightening of chromatin[23,24], would be around 1–3% O_2 (5–14% of present atmospheric levels (PAL))[25,53]. Such non-permissive oxic niches (>5–14% PAL) were probably common during nascent animal diversification in the Neoproterozoic, where atmospheric oxygen is estimated to be 1–20% PAL[54] . Similarly, the equivocal evidence of any increasing atmospheric oxygen when animals diversified (for example, ref. [84]) is also consistent with the view that a biological innovation instigated the Cambrian events (see Supplementary Information for details).

In the blaze of biological innovation during the Cambrian, animals with organs and appendages appeared[55] and, details aside, the HIF-driven hypoxia-response machinery is versatile in that it affects cell specialization through many pathways (Fig. 1c). All modern bilaterian animals possess the abilities adjoined by the HIF-driven machinery and the Cambrian explosion was a diversification event of primarily bilaterian animals[1,2]. Furthermore, estimates of Cambrian atmospheric oxygen concentration (15–20% PAL or 3–4% O_2)[12] overlap with the functional spans of both HIF-1 and HIF-2 (5–24% PAL or 1–5% O_2)[11]. Although vertebrate fossil abundance is low during the Cambrian explosion, their mere presence[56] shows that the blueprint for pseudohypoxia was in place. Indeed today, vertebrate animal diversity is still far lower than that of invertebrate animals, creating an almost gated community with exclusive tools for life in the oxic realm (Supplementary Fig. 4).

Implications

Our model of evolving control of cell stemness through HIF implies that animals host fundamentally different abilities for tissue renewal, and therefore life, in the oxic realm. In general, the model implies that tissue renewal is unceasingly challenging for animals with only HIF-1α (invertebrates) while animals with HIF-2α (vertebrates) host unravelled abilities that have resulted in to both gain and risks.

The power of pseudohypoxia is uniquely given to vertebrates. Vertebrates generally live longer[57], grow larger, sustain more specialized tissue[48], and develop cancer more often than invertebrates[58]. We claim this is no coincidence, but related to their unprecedented stemness control through pseudohypoxia. Rigorous vertebrate management of stemness in both hypoxic and oxic tissue can be demonstrated with the example of human skin. The skin of adult mammals

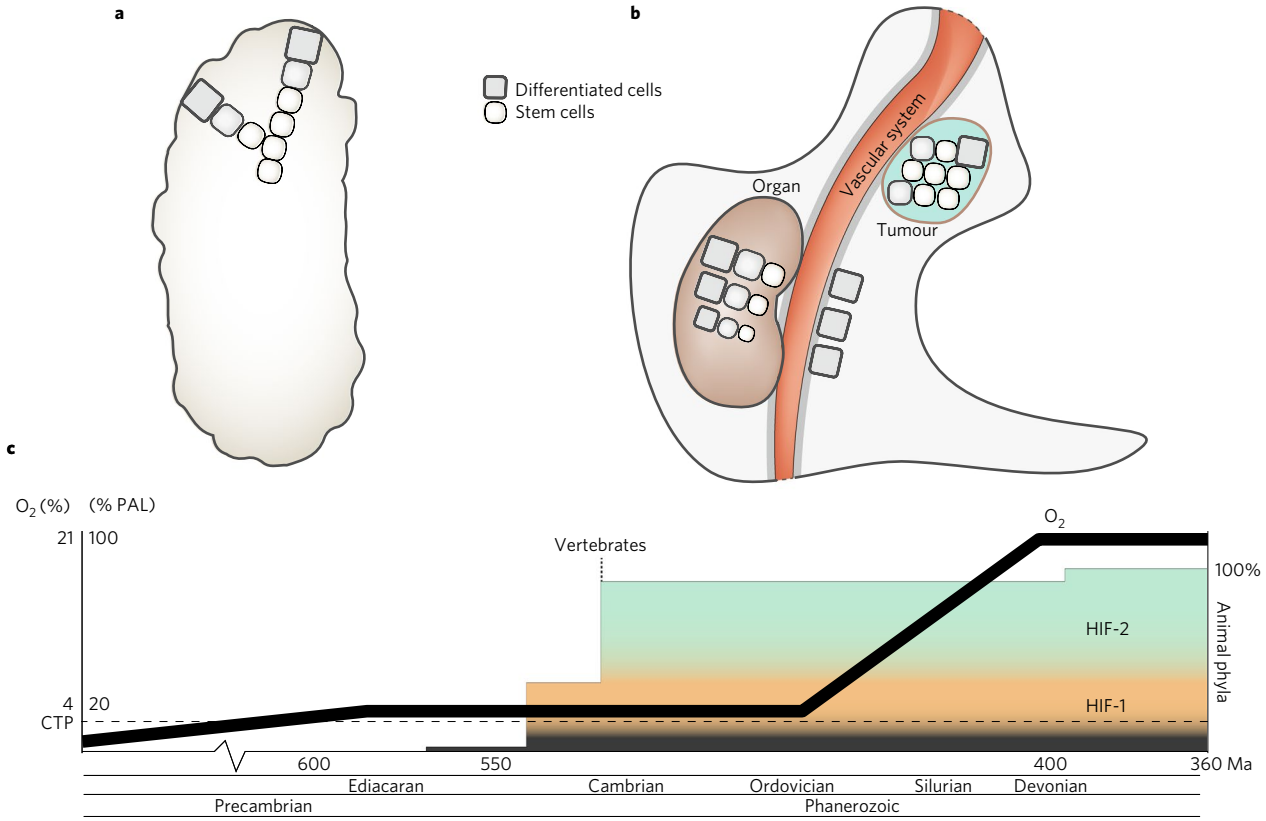

Fig. 3 | Hypothetical models of cell stemness control within multicellularity and refined stemness control in relation to atmospheric oxygen and animal diversification at the Precambrian–Cambrian boundary. a, In a primitive organism (a 2.1-Ga macrofossil, before the equivalent of HIFs), stemness would reside in the least oxygenated core and differentiation would occur along gradients, towards the relatively oxygenated surrounding tissue. Figure adapted with permission from ref. [50], Macmillan Publishers Ltd. **b**, Modern vertebrates manage stemness and differentiation in time and place, also in oxygenated tissues through pseudohypoxia, as demonstrated by immature cells (high stemness) near blood vessels, for example (see also Supplementary Fig. 3d). **c**, Schematic of increased stemness control through HIF-1 (orange) and HIF-2 (green), on top of the ability for cell totipotency (black) at the Precambrian–Cambrian boundary. Environments with high enough oxygen levels to challenge stemness, as indicated by the chromatin tightening point (CTP, dashed line), were present since the Precambrian and the evolution of improved stemness control is decoupled from immediate fluctuations in atmospheric oxygen (black line; the left *y*-axis gives percentage by volume and percentage of PAL). Improved stemness control through HIFs resulted in a dramatic increase in the number of animal phyla (right *y*-axis).

is considered a hypoxic organ, where neither underlying blood vessels nor diffusion from the atmosphere allows oxygen to reach the entire skin thickness[59]. Adult stem cells of the skin, however, reside near underlying vasculature and, presumably, in a pseudohypoxic setting suggested by their expression of HIF-2a[37]. These immature precursor cells sustain continuous skin renewal, as they differentiate and die when moving towards the oxic skin surface[60]. The SNS also requires HIF-2 for proper development[32] and high HIF-2α expression is shown to associate with aggressive tumours and postulated to mark cell stemness in the SNS-derived tumour neuroblastoma[7,11]. As this tumour setting is pseudohypoxic, we argue that the fundamental ability to develop the SNS is associated with HIF-2-driven pseudohypoxia.

The effects of exceptional control of stemness and homeostasis in the oxic environment, granted to vertebrates, goes hand-in-hand with energy-demanding capacities. Vertebrates regulate their salinity and osmotic balance of internal fluids, which invertebrates do not[61]. This regulation occurs through the kidneys, which are particularly energy-expensive organs. Kidneys would be one of several energy-expensive investments that vertebrates could acquire, or 'afford', after managing stemness in the oxic realm. Equally expensive innovations are skeletal muscles, versatile brain capacity, and a functional nervous system. These energy-expensive investments, however, leave the organism vulnerable to oxygen shortage. In addition, cellular specialization reduces DNA repair capacity[62], and a high number of cells and lifetime cell divisions all increase the risk of tumour formation, particularly through aberrant mechanisms exploiting pseudohypoxia.

Hypoxia-driven stemness is less versatile. If vertebrates host an elevated capacity to sustain tissue-specific adult stem cells and organ renewal through pseudohypoxia and HIF-2 compared with invertebrates, adult invertebrates would manage their tissue renewal differently. This particular comparison has, to our knowledge, not been done before and the capacity of invertebrate adult stem cells in particular remains to be explored. However, studies in the fruit fly (*Drosophila melanogaster*) provide some insight. Out of its four described stem cell pools, three belong to the germ line (post-mitosis) and only the intestinal stem cells (ISC) compare to adult vertebrate stem cells[63]. Influence of oxygen on ISC can be inferred indirectly, through the effects of aging in the oxic environment. When fruit flies age, their intestinal tissue renewal becomes less robust, as ISC over-proliferate due to loss of asymmetric division[64]. Stem cells, per definition, divide asymmetrically and give rise to one self-renewing stem cell and one daughter cell with a limited lifespan. Therefore, the loss of asymmetric division of ISC in *Drosophila*—living in oxic settings—will result in the loss of the regenerating capacity of their intestines. Tissue renewal

in terms of wound healing, furthermore, seems to be different in invertebrates than in vertebrates. For example, the wounds in the exoskeleton of insects are not completely healed[65] and the healing involves enlargement of the surrounding somatic cells, rather than migration and differentiation of the stem cells progenies[66]. Thus, we interpret these results to suggest that fruit flies, here a representative of invertebrates, have a weaker ability than vertebrates to maintain stem cell pools and tissue renewal through their life in the oxic settings.

It is notable that invertebrates constitute 97% of all animal species on Earth today[67]; some live for decades (lobsters, for example) or even centuries (freshwater mussels). Without HIF-2 and the ability to manage pseudohypoxic stemness, however, we ask if invertebrate tissue renewal requires true hypoxia? The majority of insect life, as larvae, is spent in hypoxic environments, such as decomposing trunks, mud or dung[68], where the nutritional precondition determines larvae size, which then decides the size of the adult insect[69]. Furthermore, the conundrum among resting insects is that they ventilate discontinuously with tracheal valves mostly closed (down to 1 ventilation per 24 h)[70], implying that they breathe rarely. The closed trachea conundrum has been explained as a protection from either water loss, hypoxic conditions or ROS[71]. We, however, argue that the closed trachea could also be attributed to insects managing internal oxygen gradients and stemness. Management of delicate oxygen gradients could also be inferred from how ants, in their subsoil nests, control ventilation. Intense microbial respiration of wood ant (*Formica polyctena*) nest material demonstrates significant oxygen consumption[72] and therefore probably decreasing oxygen concentrations with increasing distance from the entrance. Honeybees also seem to manage hypoxic conditions in their wintertime clusters[73]. In contrast, ample oxygen shortens the life span of adult worker bees[73] and correlates with higher death rates in both house flies and fruit flies[74,75].

Hypoxia can also be associated with the life cycles of molluscs and crustaceans. Bivalves generally shut their shells tightly when metabolically inactive[76], and subsequent hypoxia follows[21]. Near-anoxic blood (where oxygen normally is transported by a suspended copper protein) is observed during moulting of the lobster *Hommarus gammarus*[77] and hyperglycaemia in the crab *Carcinus maenas*[78]. Furthermore, reduced crustacean metabolic rates are associated with the expression of HIF-1α[14] during moulting as well as during so-called hypoxia-behavioural hypothermia; when crayfish seek out colder conditions[14], for example. The observation that phases of tissue hypoxia and HIF expression are controlled and predictable among these invertebrate animals suggests that they act to replenish their stem cell pools.

Candidates for a negative test of a hypoxia dependency among invertebrates are copepods; abundant and widespread in the world's oxygenated oceans. During their month-long life span, copepods migrate vertically in the water column on a daily basis, sometimes several hundred metres or through oxygen minimum zones. The rationale for the migration includes that they avoid predation or experience well-being by varying temperature[79]. However, other observations pertain to an association between copepods and hypoxia. Glycolytic metabolism is noted among copepods that hibernate at depth in the oxygenated ocean[80], copepods larvae seem to seek out hypoxic aggregates of sinking biomass (marine snow)[81], and the gut tract of copepods has been measured to be hypoxic to anoxic[82]. Localized or temporal hypoxia is what our model requires for the stemness and extended life span of invertebrates in the oxic realm, which even copepods demonstrate.

In summary. The ubiquitous need for hypoxia-driven stemness in animal tissue renewal, and hence animal evolution, makes us argue that the Cambrian diversification of animals was not driven by expanding oxic environments. Instead, the way cell stemness can be controlled by HIFs emphasizes that molecular tools have a key role in the control of tissue renewal for life in the oxic realm. The necessity for large life forms to maintain and control hypoxia-response machinery in the oxic realm reconciles otherwise puzzling observations, such as the presence of macrofossils in the Precambrian that did offer oxic niches[51], the low oxygen demand for simple animals that seems to be met before the Cambrian[83], and the equivocal evidence of increasing atmospheric oxygen at the onset of animal diversification[84]. Although animal diversity probably couples to the energy yield of aerobic respiration, the Cambrian explosion seems to be decoupled from the increased availability of free oxygen. Our view on how evolving stemness control led to the Cambrian explosion thus aligns with other events when biological innovations, such as photosynthesis, the eukaryotic cell and decay resistant tissue of plants, led to revolutionary changes in Earth's surface environment.

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Author contributions

E.U.H. initiated the study. E.U.H. and S.P. developed the concept and designed the experiments. E.U.H. and K.v.S. performed the experiments. E.U.H., K.v.S. and S.P. analysed the data and wrote the paper.

Competing interests

The authors declare no competing financial interests.

Additional information

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