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

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Supplementary information

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Contents:

Animal cell survival and proliferation at hypoxia

We tested the effect of hypoxic conditions on cellular growth and survival of six animal cell lines (non-malignant from humans – fibroblast cells, perivascular brain cells, lung alveolar epithelial cells and breast epithelial precursor cells – from human tumour cells, and cells from the late embryo of the invertebrate *Drosophila melanogaster*) at normoxic (21% O_2) and hypoxic (1% and 0.1% O_2) conditions during 72 hours (Figure 2 and SI Table S2). The increase in cell numbers (proliferation) at the three oxygen conditions were, on average, 11 ±7 fold (21%), 5 (±3) fold (1%) and 4 (±2) fold (0.1%). The fraction of dead cells after 72 h incubation was on average 17% ±12 (21%), 20 ±13% (1%), and 21 ±8% (0.1%). In all tested cell types, proliferation continued under hypoxic conditions, and hypoxia did not substantially affect cell survival when compared to cells grown at 21% oxygen. Even at severe hypoxia (0.1%), survival rates resembled those of cells grown at 21% O_2. ANOVA tests demonstrate that while proliferation significantly differs between the settings (p-value 0.03) cell death rates does not (p-value 0.82), see SI Table S2. These results demonstrate that non-malignant and malignant human cells [1] as well as invertebrate cells survive and retain proliferative capacity at severe hypoxia and that oxygen shortage appears unproblematic at the cellular level.

Phylogenetic proteome analyses of HIF-α subunits and EPO

Results and comparison

In order to examine the , we compiled previously published proteome analyses[2-5] as well as performed our own (TBLASTN) analyses in a total of 39 proteomes (Main MS Figure 2; SI Tables 3A-B). We find that HIFs are common to all bilaterian animals, while HIF-2α appears exclusively in vertebrates (a chordate subphylum), including lampreys (Main MS Figure 2). Porifera (sponge), Cnidaria (jelly fish), and Ctenophora (comb jellies) express a HIF-like protein that lacks the oxygen sensing c-domain. Our HIF-results generally agree with previous findings, such as the lack of HIF-α expression in sponges[6] and ctenophora[5]. Our analysis of the cnidarian *Hydra vulgaris*, lacking the c-terminal oxygen sensing domain of its HIF-like protein, however differs from how the other cnidarian *Nematostella vectensis* has been

shown in three studies to express a HIF[3,5,6]. A loss of data, such as poor recovery or alignment of the *Hydra vulgaris* proteome, is more likely than a faulty gain of data. Therefore, we rely on the studies that the *Nematostella vectensis*, and thus Cnidarians, express a HIF-like protein[3,5,6]. However, we specifically investigated HIFs in animals around the bifurcation between protostomes and deuterostomes – and here our study differs from two previous studies. We observe that of out of the early branching deuterostomes – the Purple sea urchin (echinoderm) and the Acorn worm (hemichordate) – as well as deuterostomes in the chordate subphyla – Lancelet fish (cephalochordate), Vase tunicate (tunicate), and Northern lamprey (hyperoartia) – only Northern lamprey display a distinct HIF-2α orthologue. Here, our result differs from that of Rytkönen et al 2011 and that of Graham and Presnell 2017, since those studies include the same species but without detecting HIF-2α. All studies conclude that EPO appears first in the genomes of fish (e.g. *Latimeria chalumnae*). The results are visualized in a diagram simplified from Zrzavý et al 1998[7] with branch lengths not to scale.

Materials and Methods

Phylogenetic analysis: Protein amino acid sequences (FASTA) for human *HIF1A*, *HIF2A* (*EPAS1*) and *EPO* were obtained from the NCBI gene database. FASTA protein functional domain conservation across organisms was examined with use of the BLAST function available for OrthoDB [8]with focused hierarchical searches on Metazoa, as well as domain descriptions obtained through The European Bioinformatics Institute (EMBL-EBI) tool InterPro[9]. For organisms not included in OrthoDB but with available genomes, the NCBI tBlastn function (allowing for alignment protein amino acid queries to translated nucleotide databases from available genomes) or the Ensembl Genome Browser[10] BLAST protein function was used to identify conserved protein domains that were then further characterized using InterPro. The analyses of *Pleurobrachi bachei* were performed using the neurobase database (http://neurobase.rc.ufl.edu/Pleurobrachia/blast), while *Octopus bimaculoides* were analyzed using the metazome database (http://www.metazome.net/Obimaculoides.php).

On atmospheric oxygen in the past, and its importance, in short

Deciphering ambient free oxygen during Earth's 4.5 Ga old history is non-trivial since geological tools and understanding can target either local or global conditions, and sometimes both. The dramatic appearance of animal fossils during their diversification during the so-called Cambrian explosion (0.5 Ga) is also biased in the sense that it was a diversification synchronized with the wide application of biomineralization. The diversification, hence, does not equal the time when the last common animal ancestor appeared and the extent and reason for a lag is debated. One can confidently propose, however, that 'oxic' environments would have been present, at least locally, in the vicinity of cyanobacteria since ~3 Ga[11] which means for most of Earth history[12]. Microenvironments with oxygen concentrations high enough to challenge eukaryotic cell stemness (>2%) could, therefore, have posed an evolutionary impediment for multicellularity since long before animals either evolved or entered the rock record. Such an evolutionary impediment would have expanded during global oxygenation of the atmosphere, noted to occur at e.g. 2.5 Ga[13], 1.4 Ga[14] and also towards the end-Neotproterozoic[15]. Meanwhile, the end-Neoproterozoic oxygenation has been predicted to link to animal evolution. This association is based on the observations that animals (vertebrates) die without oxygen (as explored by Joseph Priestly and the Lavoisiers in the late 18th century) and that animal fossils appear suddenly in the rock record[20]. A presumed oxygenation is suggested to have created the permissive environment for animals' energetic metabolism and life styles. Thus, the permissive oxic environment would have facilitated animal diversification, and as such control the onset of the Cambrian explosion[16]. Efforts to in detail test and quantify[17,18] the so-called *oxygen control hypothesis* have, however, resulted in a complex picture of changes in both biosphere and surface chemistry[15] (used to argue for global[29,30] or local oxygenation[19]) that stretch over considerable time in the end-Neoproterozoic. To this picture now adds that primitive animals appear to have low oxygen requirements[20,21], and that ecological knock-on effects also drive evolution[22-24]. Currently, the discussion encompasses a geochemically and ecologically turbulent end-Neoproterozoic era. No compelling evidence demonstrates a causality between a rise in atmospheric oxygen and the rise of animals. Based on these observations, and those of how cell stemness links to hypoxia we here join the efforts exploring the alternative option[17] as to why it took ~85% of Earth history for animals to evolve – that a biological innovation was needed for animal evolution to overcome some threshold. The innovation of delicate

control of stemness capacity in the oxic realm would, reasonably, give its hosts a significant evolutionary advantage.

Cellular responses to hypoxia

Some of the cellular mechanisms activated in response to hypoxia are central to the discussions of this study, in particular regulation of the HIFs. The HIF transcription factors comprise one of three different oxygen sensitive α-subunits (HIF-1α to HIF-3α), encoded by separate genes, *HIF1A*, *HIF2A*/*EPAS1*, *HIF3A*/*IPAS*. The α-subunits work in complex with an oxygen insensitive HIFβ (ARNT) subunit[25]. Their regulation occurs primarily post-translationally where the α-subunits become hydroxylated by oxygen-dependent prolylhydroxylases (PHDs) at two conserved proline residues (Main text Figure 1 a-b) resulting in targeted (ubiquitinated) proteasomal degradation involving the von Hippel Lindau (VHL)-ubiquitin ligase complex[26-28]. At hypoxia the PHDs are inactive and the HIF-α subunits are stabilized and form complexes with ARNT and other co-activators, activating the transcription of hypoxia-driven genes containing one or several hypoxia responsive elements. The degradation rate of HIF-2α at physiological conditions has not been investigated in detail, but there are vast experimental evidences that HIF-2α can be stable and active at physiological oxygen conditions[29,30]. A second level of oxygen dependent regulation of the HIFs is mediated via a protein that inhibits HIF (FIH-1). Conserved HIF asparagine residues become hydroxylated by FIH-1 in the presence of oxygen, leading to impaired HIF - coactivator interaction and impaired formation of active transcription factor complexes[31]. FIH1 is less active towards HIF-2α, which might explain why HIF-2 can be active at physiological oxygen conditions (as indicated by large arrows leading to transcription (DNA symbol) for HIF-2α only, in Main Text Figure 1b).

Other pathways relevant for stemness

Cell stemness control, most likely, could be achieved through other means than HIF-2-driven pseudohypoxia. Currently, however, HIFs are known to have a most significant role in oxygen sensing and regulation during tissue homeostasis, and so is the particular role of HIF-2 for stemness. A key observation,

we claim, is that mechanisms promoting pseudohypoxia and HIF-2 activation in solid tumors are inheritable, i.e. gain-of-function mutations in HIF2A and loss of genes like, *VHL*, *SDHx* etc are found in familial cancers. This means, according to our view (and hypothesis), that these genetic aberrations are fundamental drivers and that they hit cellular stem cell pools. We further emphasize that HIFs are as of today the primary cellular system that responds to external oxygen concentrations. In addition, multicellular organisms encounter environments with varying oxygen concentrations and, according to the association between oxygen driven cell differentiation, could not easily enter the oxic niche. Therefore, it is reasonable to start the discussion of access to the cellular hypoxia machinery by exploring HIFs. Which are, in addition, unique to animals and possible to trace specifically within animals; our focus here.

A key difference between our view and that of a previous contribution on stemness during evolution ('Anaerobiosis and Stemness' by Ivanovic and Vlaski-Lafarge, 2015) is whether evolution has provided animals with the ability to access the hypoxia-driven cell stemness (us) or an ability to put brakes on cell differentiation (Ivanovic and Vlaski-Lafarge, 2015). In Ivanovic and Vlaski-Lafarge (2015), the authors reason that evolution has resulted in a range of 'lock' mechanisms that inhibit cell differentiation. A complex network of 'lock' mechanisms and factors restrict cell differentiation options[32]. Among these mechanisms and factors are both transcription factors (Fox, Hox, Sox, Oct4, and thus also HIF) and signaling pathways (Wnt, Notch, and TGF β) brought forward[32]. It is beyond our scope here to evaluate the evolutionary role of e.g. Notch for stemness other than observing that most of the mentioned pathways associate with that of HIFs. We do, however, claim that our view of that HIFs (or similar mechanism) provide keys to the hypoxia-machinery is a simple model to explain the observations. Especially since the HIF-a subunits are degraded by oxygen and since other markers, e.g. Notch, can mark both stemness and differentiation (in e.g. skin). While both views are reasonable, it appears that many mechanisms are known to assist in cell specialization while only – as of today – hypoxia and pseudohypoxia are known to provide access to the immature phenotype of animal cells.

Table 1. Oxygen thresholds and estimates in atmospheric saturation (%). Original reported values (marked in bold) are converted to units convenient in paleontology (% PAL)*, geochemistry (µM), ecology (ml/l) and physiology (mm Hg).

O_2	atm %	% PAL*	µM	ml/l	mmHg**
Oceans					
Oxic	5-21	26-100	64-250	**1.43-6.00**[33]	41-160
Hypoxia	2-5	9-26	22-64	**0.50-1.43**[33]	14-41
Severe hypoxia	<2	<9	<22	<**0.50**[33]	<14
The late Neoproterozoic	3-4	**15-20**[15]	38-50	0.84-1.12	24-32
The early Neoproterozoic	0.2-2	**1-10**[15]	3-25	0.06-0.56	1.6-16
Operational oxic-hypoxic boundary	5	26	64	**1.43**[34]	41
Biology					
Bilateral animal (e.g. polychaete)	<0.4	<2	<5	**0.02**[35]**-0.10**[21]	<3
Chromatin O_2 response	**1-3**[3,36]	5-14	12-36	0.27-0.80	8-23
HIF-1[#1] activation	**1-2**[29]	5-10	12-24	0.27-0.53	8-15
HIF-2[#2] activation	**5 (7)**[29]	24 (33)	60 (83)	1.33 (1.87)	38 (53)
Physiological (organ dependent)	**3-7**[30]	14-35	36-88	0.80-1.87	23-56
avg	5[30]	24	60	1.33	38
-Hypoxia	**<2**[30]	<10	<24	<0.53	<15
-Severe hypoxia	**<0.2**[30]	<1	<2	<0.05	<2
-Solid tumors	**0-7**[30]	0-25	0-88	0-1.87	0-56

* Percent of present atmospheric level 21% (a).

** At sea level.

#1Hypoxia inducible factor 1 (the active dimer of oxygen sensitive subunit HIF-1α and ARNT/HIF-1α)

#2Hypoxia inducible factor 2 (the active dimer of oxygen sensitive subunit HIF-2α and ARNT/HIF-1α)

Supplemental Table 2 (see excel sheet). Cell growth data after 72 h incubation at 21%, 1% and 0.1% O_2. Proliferation is estimated as increase of living cells, compared to the seeded number. Death rate is estimated as the percentage dead cells of all cells after incubation.

Supplemental Table 3a (see excel sheet). Summary of presence of HIFs and erythropoietin (EPO) across multiple organisms. Presence of the protein is marked by X. The study in which the presence of the HIF proteins is analyzed, in this or other efforts[2-4,6], is provided under the column *Study*. All EPO analyses were performed in the present study. For proteins described in our study (bold) see extended information SI Table 1b.

Supplemental Table 3b (see excel sheet). Extended gene and protein information in Organisms investigated in our study. Gene and Protein DatabaseIDs are provided for NCBI or ENSEMBL databases. Corresponding FASTA amino acid sequences are provided. Genes and proteins were identified using tBlastn with human HIFs or EPO amino acid sequences as reference.

Figure S1. Hypoxia affects many features at the cellular as well as organ level (modified from[37]) and prevents cellular differentiation. (A-B) Primary human breast epithelial precursor cells form (A) differentiated, polarized acini-like structures when cultured at 21% for 21 days as visualized by nuclear (blue - DAPI) and actin (red - phalloidin) staining. (B) At hypoxic conditions (1% O_2), these cells do not differentiate or form acini-like structures (size bar 20 µm, modified from[38]). (C-D) Breast ductal carcinoma *in situ* (DCIS) grows non-invasively and non-vascularized within a breast duct, sometimes with a central necrosis (as indicated by *). (C) Oxygenated cells at the edge of lesions form intra-ductal, differentiated duct-like structures (arrow) as demonstrated by hematoxylin - eosin staining, while (D) HIF-1α immunoreactivity (red-brown) reflects hypoxia at the tumor center and show immature cells that do not form intra-ductal structures and have a low cytoplasm to nucleus ratio, two hallmarks of undifferentiated breast epithelial cells (modified from[39]). E) A selection of gene expressions and biological responses after the activation of HIFs[40,41].

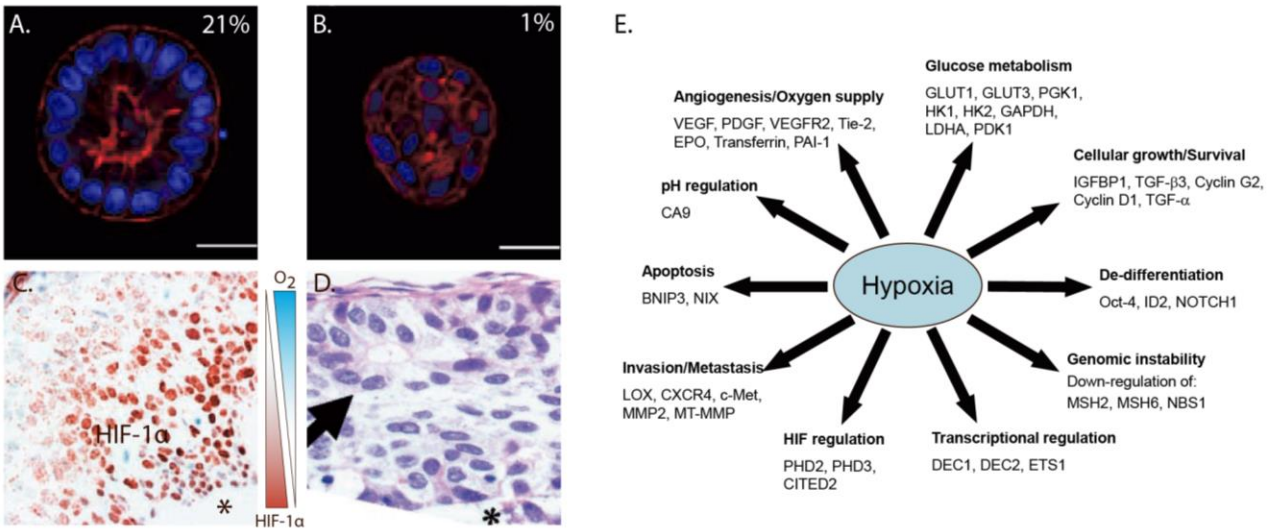


Figure S2. Cell viability in human non-malignant and tumour cells and Drosophila cells after growth during 72 hours at hypoxia and severe hypoxia. A) Human non-malignant fibroblast cells, perivascular cells (HBPV), lung alveolar epithelial (P2GH), and breast epithelial precursor (MCF10A) cells; B) human tumour neuroblastoma cells (SK-N-BE(2), and C) *Drosophila melanogaster* cells (S2) were grown at ambient oxygen (21% O_2), hypoxia (1% O_2) and severe hypoxia (0.1% O_2) for 72 h. after which the number of live and dead cells were counted. The number of cells seeded varies from 200'-2000' (*), in minimum 3 replicates. For experimental details, see SI and references[29,38].

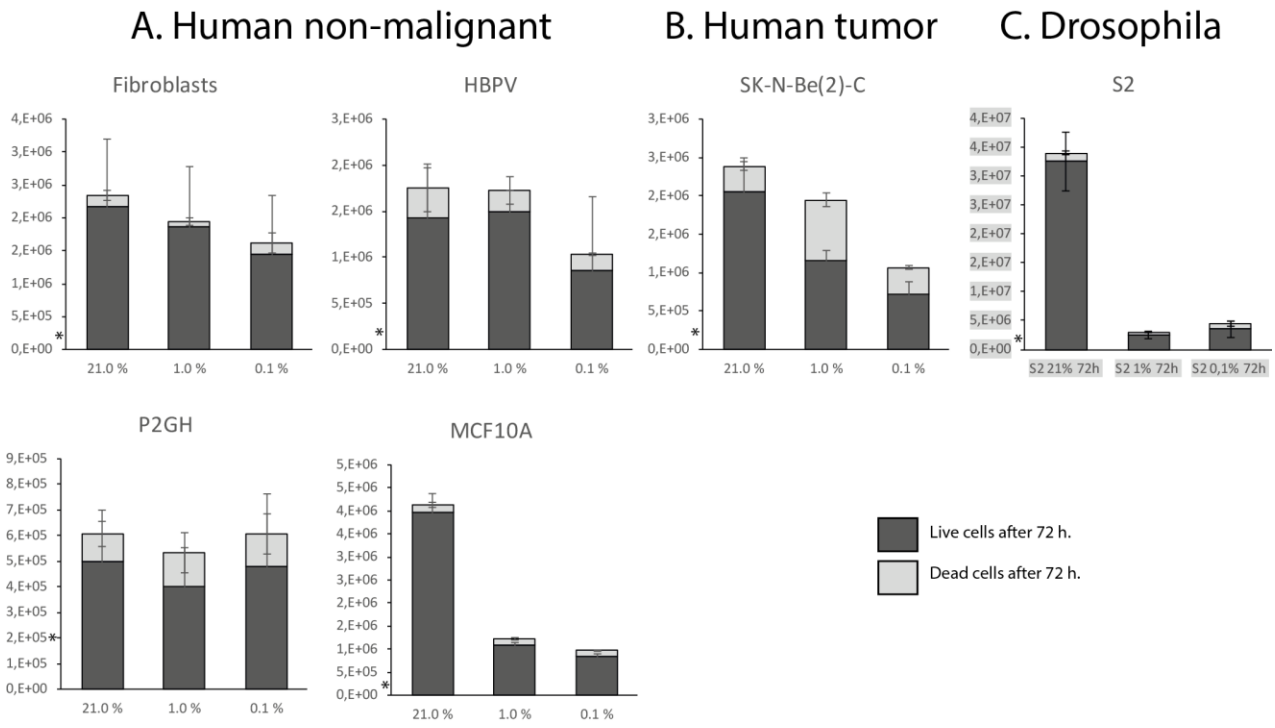


Figure S3. Hypothetical model of stemness and differentiation, in multicellular life forms, under *bona fide* hypoxic and pseudohypoxic conditions. A) In a model organism of multicellularity, lacking the ability to manage pseudohypoxia, stemness would reside in the least oxygenated tissue (due to many cell layers or cell proliferation) and differentiation occurs along a gradient towards a more oxygenated surrounding. B) The model organism could resemble the 2.1 Ga old macrofossils from Gabon, which demonstrate rudimentary cell differentiation (specimen G-FB2-f-mst1.1[42], 2010, scale bar 5 mm), and where intra-tissue oxygen gradients would facilitate stemness at the center of the organism. C) Vertebrate animals today manage stemness and differentiation in time and place in oxygenated tissues through pseudohypoxia, as demonstrated by e.g. immature cells (high stemness) near blood vessels. D) The pseudohypoxic niche defined by HIF-2α (brown-red) expression in vascularized neuroblastoma tissue (red) as interpreted from CD31-positive vascular endothelial cells detected in a consecutive section (modified from[29], scale bar 100 um).

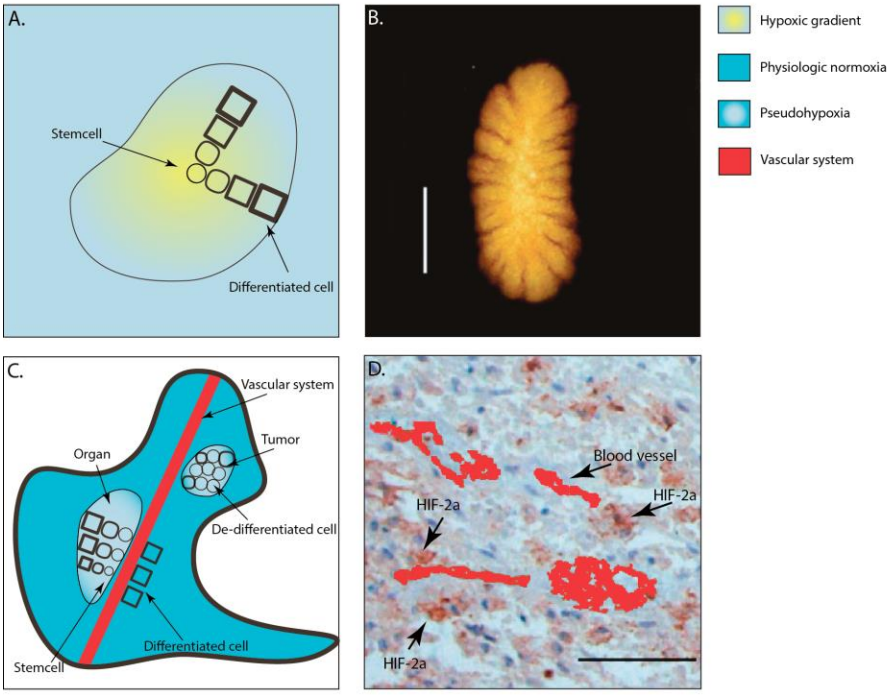
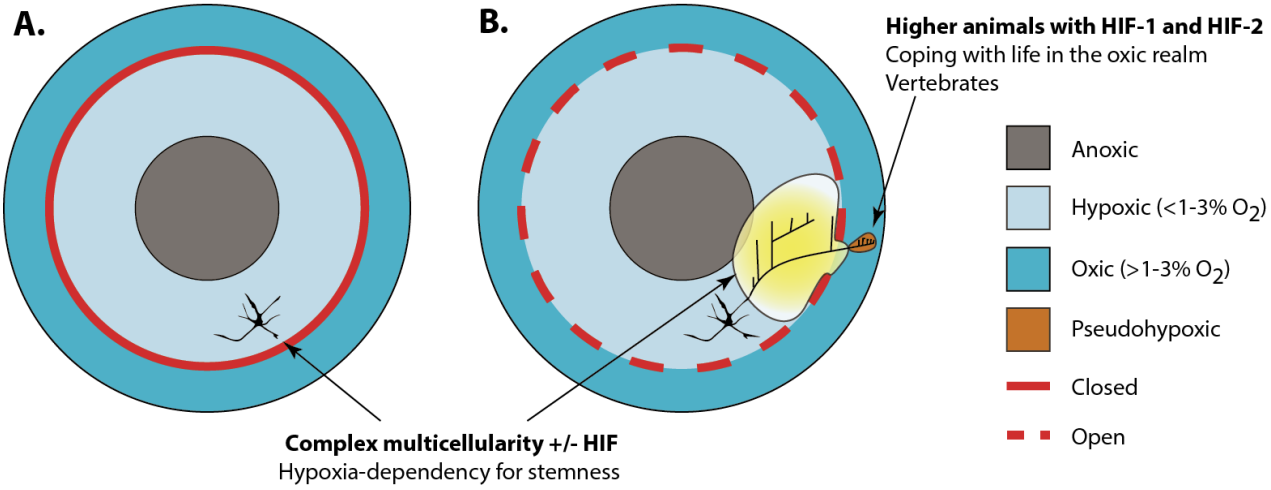


Figure S4. Multicellularity within (non-proportional) Earth habitats before (A) and after (B) the appearance of HIF-2. A) The oxic realm was locked to organisms with complex multicellularity as stemness could only be maintained by hypoxia. B) HIF-2 unlocked the oxic realm for vertebrate animals. Animals with only HIF-1 (bilateral invertebrates) ties to hypoxia for stemness in part of their life cycle, or generally live short in the oxic environment. Animals with HIF-2 cope for a long life in the oxic environment but constitute only ~3%[43] of all species (orange). The grading within invertebrate animals (graded yellow) denotes that other modes of stemness and terminal differentiation (see e.g. *Hydra* and *Planaria*) are available.



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