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Freshwater cnidarian *Hydra*: A long-lived model for aging studies

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ABSTRACT

The recent increase in human life expectancy is accompanied with a more frequent occurrence of age-related diseases such as neurodegenerative diseases and cancers. An unprecedented effort has been dedicated to develop animal model systems to decipher the aging mechanisms, and the genetic study of short-lived animals, namely *Drosophila* and *Caenorhabditis*, brought important breakthroughs. Here we highlight the interest of additional model systems like the cnidarian *Hydra* polyp. *Hydra* provides a model where all tissues continuously renew from large stocks of proliferative adult stem cells, where amputation leads to regeneration with perfect replacement of any lost body structure and where signs of aging remain limited over the years. Aging is easily inducible in some *Hydra* species, providing a paradigm for deciphering the mechanisms that promote resistance to aging. A dynamic autophagic flux, a sustained proteostasis, the G2 pausing of stem cells and an efficient DNA-damage repair might play an essential role.

Keywords: adult stem cells, autophagy, cnidarians, colchicine and hydroxyurea antiproliferative agents, DNA-damage repair, epithelial plasticity, evolution, heatshock response, *Hydra* long-lived model system, proteostasis, resistance to aging, regeneration, telomere attrition

Short-lived versus long-lived model systems for aging studies

Lifespan largely varies among animal species: Butterflies or mayflies live for few days while elephants or turtles live for several decades, suggesting small animals are short-lived while large ones are long-lived. This correlation between animal body size and expected lifespan can also include the age of first reproduction and fertility (**Figure 1A**). Such analysis led to the "r and K" reproductive strategy theory proposed in the 60's (MacArthur, 1962; Cody, 1966; Pianka, 1970). Briefly, K-selected species are generally large size animals that exhibit low and/or delayed fertility. They usually produce few descendants to which they dedicate a specific parental investment, leading to a high survival rate of next generations. In other words, K-selected species invest in longevity. Human and elephant perfectly fit into this K-selected group. In contrast, r-selected species such as mice, flies and nematodes invest in reproduction. They become sexually mature at a young age, produce a high number of offspring, do not show parental investment and exhibit a short lifespan. However, an extended comparison of these life cycle characteristics actually shows a continuum among animal species (**Figure 1B**) (Martıńez, 1998) and this paradigm has been somehow challenged regarding the evolutionary mechanisms at work (Reznick et al., 2002). Still, the correlation between age of first reproduction and life expectancy remains valid.

In aging studies as in developmental biology, animals with short lifespans and large offspring were favored since they provide the conditions to perform genetic studies over several generations in a relatively short period of time. Indeed, despite differences in their life cycles with that of humans, the use of mice, nematode and fruit fly as model organisms efficiently contributed to dissect aging and developmental processes and led to the characterization of key hallmarks of aging that improved our understanding of human aging (López-Otín et al., 2013). However, in adult nematode as in fruit fly, adult stem cells are limited or absent, genetic losses are common when compared to mammals, and possible pausing during

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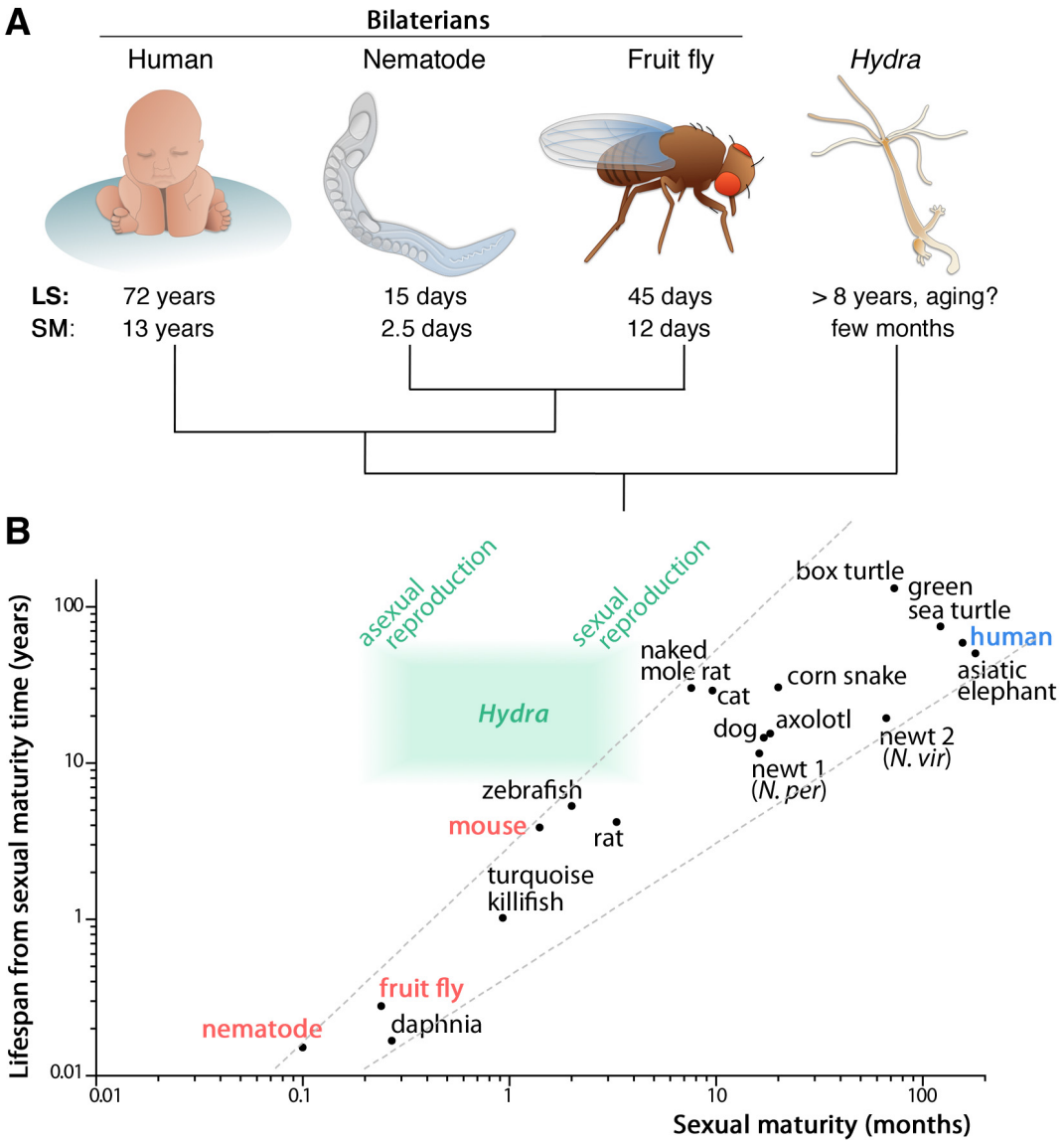

Figure 1: *Hydra* as long-lived model organism

(**A**) Scheme showing the phylogenetic positions of *Hydra* and model organisms classically used for aging studies. Lifespan (LS) and sexual maturity (SM) are provided for each. Note that the maximal age observed so far for individual *Hydra* polyps is eight years, a period corresponding to the duration of the study (Schaible et al., 2015). (**B**) Graph showing the correlation between sexual maturity and lifespan. *Hydra* appear above other animals, supporting them as long-lived animals as previously shown by Martinez (1998) with a distinct dataset. Data used here were retrieved from Schaible et al. (2015) for *Hydra* and from the AnAge website (genomics.senescence.info/species) for the other species. Abbreviations: *N. per: Notophthalmus perstriatus*, *N. vir: Notophthalmus viridescens*.

development might lead to similar phenomenon in adulthood, distinct from aging (Austad, 2009).

Therefore complementary organisms as model systems for aging studies would be highly beneficial (Murthy and Ram, 2015; Valenzano et al., 2017; Cohen, 2018). Together with new promising short-lived models such as the African turquoise killifish (Kim et al., 2016), long-lived animals such as sponges, cnidarians (e.g. *Hydra,* corals) or planarians emerged. All these organisms show very low signs of aging over time, implying that they have naturally developed efficient mechanisms to postpone aging and death. Such models should be informative on the mechanisms that help escape the main hallmarks of aging, and should help identify new approaches to tackle aging-related issues in humans.

Hydra, a long-lived model system

Since the 18^{e} century, *Hydra* progressively became a classical model for developmental biology, cell biology, ecotoxicology and more recently aging (Steele, 2002; Galliot, 2012; Tomczyk et al. 2015;

Murugadas et al., 2016). *Hydra* polyps are one to two centimeters long freshwater animals that belong to Cnidaria, a phylum identified as the sister group of bilaterians (**Figure 1A**). These animals consist in a tubular body terminated by the head at the apical extremity and by the basal disc also named foot at the other extremity. The head is composed of a dome called hypostome, terminated by the mouth opening at its tip, and a ring of tentacles at its base. In favorable conditions (laboratory or temperate climate), *Hydra* polyps feed regularly and reproduce asexually by budding, while gametogenesis and sexual reproduction occur as a response to environmental fluctuations such as starvation for *H. vulgaris* or cold temperature for *H. oligactis* (Brien, 1953; Loomis, 1954; Yoshida et al., 2006; Nishimiya-Fujisawa and Kobayashi, 2012; Tomczyk et al. 2015).

Given the importance of asexual reproduction in *Hydra*, there are at least three different ways to consider the age of first reproduction, (i) the age of the primary polyp from hatching time (age *stricto sensu*), (ii) the age of a bud after detachment from the parent (bud age, ramet age), (iii) the age of the clonal culture from hatching of the founder polyp (culture age, genet age) (Cohen, 2018). For comparative analysis with lifespans of most animal species, which are based on sexual reproduction, we should consider the age *stricto sensu* measured after embryonic development. Unfortunately, such information is poorly documented in *Hydra* as the *Hydra* model system was developed for studying the mechanisms of adult regeneration or the biology of adult stem cells in large clonal cultures of wild-type or mutant strains submitted to cellular and molecular analyses, to loss-of-function assays, or since 2006 to transgenesis (see references in Galliot, 2012). Therefore, no effort has been made to monitor the age of individual animals from hatching time over several decades. Nevertheless, if we consider that primary polyps usually start budding one week after hatching and that the first occurrence of sexual reproduction occurs few months after hatching in clonal culture, *Hydra* definitely appear as long-lived animals (**Figure 1B**).

In his seminal article entitled "*La Pérennité Somatique",* Paul Brien reviews a series of examples taken from plants and animals that document the concept of "somatic perennity", i.e. tissues that persist or regenerate independently of sexual reproduction (Brien, 1953). He himself used *Hydra* to test experimentally the perennity of somatic tissues versus that of germ cells by measuring the impact of sexual reproduction on asexual budding in three distinct *Hydra* species. To do so, he maintained several original polyps individually and monitored their mortality and budding rates over the years, in the absence or the presence of gametogenesis. He found that original *H. vulgaris* or *H. viridissima* polyps remain as young and bud-productive as their own buds over a period of 4.7 years, with no impact of parallel gametogenesis. In the case of *H. oligactis*, he noted that asexual budding rapidly disappears in the presence of gametogenesis, and that animals become "exhausted" after having produced a series of oocytes (17). He concluded that *Hydra* maintained in culture is immortal as it can continuously reproduce and grow "*without ever passing through a sexual phase*". More recent studies confirmed the lack of aging signs in individual polyps from various *H. vulgaris* strains maintained in culture over the years, seven years in the longest follow-up, as well as in clonal cultures maintained over 37 years (Martıńez, 1998; Schaible et al., 2015). By contrast, some *H. oligactis* strains appear less resistant to stress and are inducible for aging (Brien, 1953; Brennecke et al., 1998; Yoshida et al., 2006; Tomczyk et al. 2015; Tomczyk et al. 2017).

Three continuously cycling stem cell populations

Hydra consists in two myoepithelial cell layers, named epidermis and gastrodermis, separated by an extracellular matrix named mesoglea (**Figure 2A**). The dozen of different cell types that populate the *Hydra* body derive from three distinct stem cell populations that cannot replace each other, the epithelial stem cells of the epidermal and gastrodermal layers (eESCs, gESCs), and the multipotent interstitial stem cells (ISCs). All epithelial cells along the body column are self-renewing stem cells, while ISCs are predominantly located in the central region of the body column. Both ESCs and ISCs show quite unconventional cell cycles with a short G1 and a pausing in G2 (Dübel and Schaller, 1990; Buzgariu et al., 2014). ESCs that divide every three to four days along the body column (Bosch and David, 1984), are multifunctional cells, constantly self-renewing but also performing functions usually restricted to differentiated cells such as autophagy, immune response, protection against environmental threats, mesoglea production, food digestion, patterning processes (Buzgariu et al., 2015).

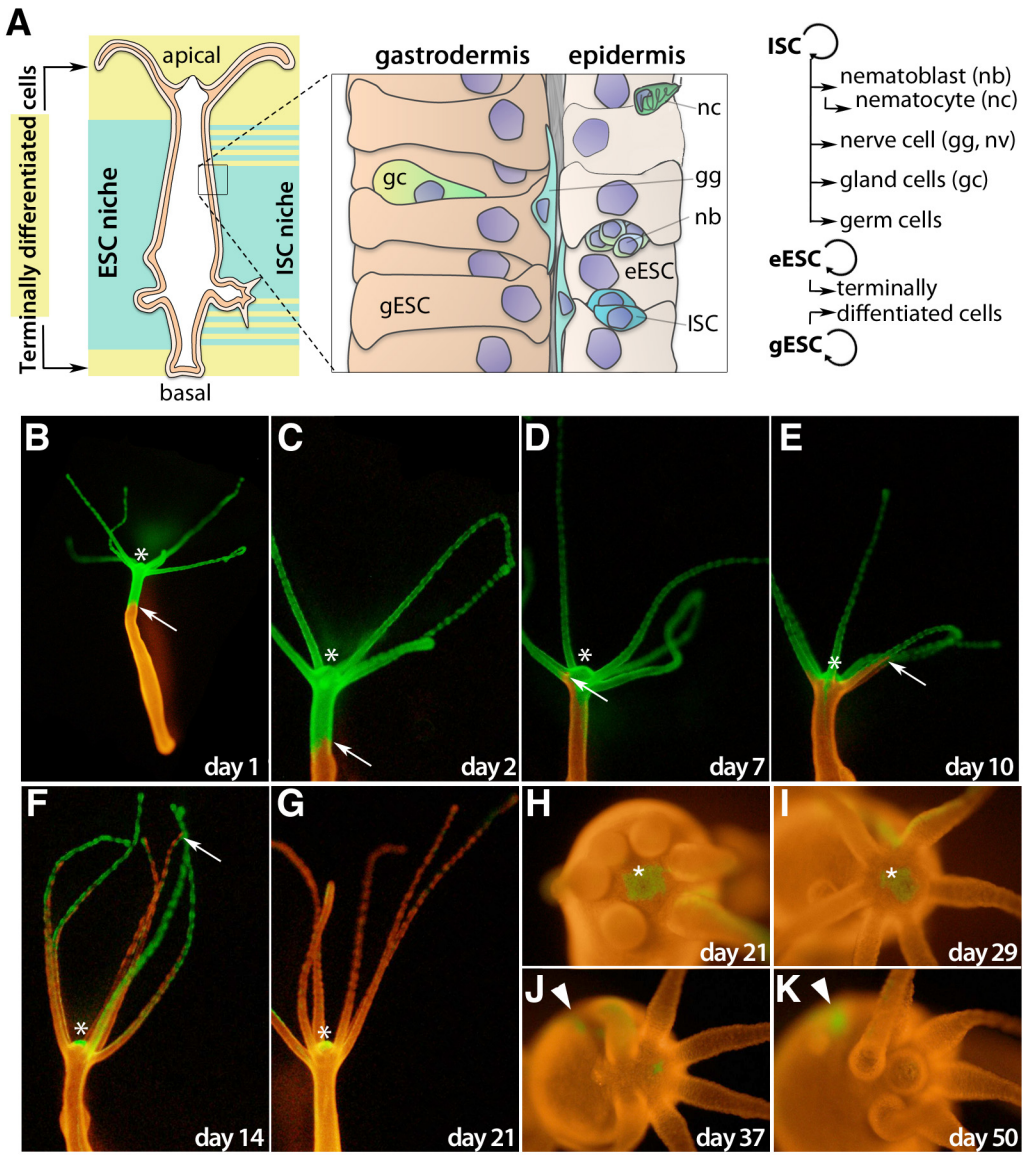

Figure 2: Adult stem cell populations support continuous tissue renewal in *Hydra*

(A) Schematic view of the bilayered *Hydra* anatomy. The central body column is composed of adult stem cells (green) while extremities, basal disc and tentacles, only contain terminally differentiated cells (yellow). A closer view shows the cellular composition of the two myoepithelial layers, gastrodermis inside and epidermis outside. Progenies of the three adult stem cell populations: the multipotent interstitial stem cell (ISC) give rise to somatic and germ cells, while the epithelial stem cells, either epidermal (eESC) or gastrodermal (gESC), only give rise to epithelial cells that show specific features at the apical and basal extremities. **(B -K)** Homotopic transplantation experiment between two animals of distinct *Hv_AEP* transgenic strains showing over a 50-day period the progressive renewal of epidermal epithelial cells. The upper part (green) was dissected from an eESC-GFP animal (Ecto-1, Anton-Erxleben et al., 2009) and transplanted onto an eESC-RFP animal (Vogg et al., 2019). White arrows indicate the progressive passive displacement of the boundary between GFP and RFP positive cells; asterisks show the mouth region at the tip of the hypostome, arrowheads point to remnant patches of GFP positive cells.

ESCs get progressively displaced towards the extremities and stop proliferating at the boundaries of the body column, in G2 for the cells that reach the tentacles and the basal disc, in G1 for the cells located in the hypostome (Dübel and Schaller, 1990; Hobmayer et al., 2012; Buzgariu et al., 2014). In the tentacles, eESCs terminally differentiate as battery cells, each cell embedding several nematocytes. By contrast, ISCs that divide every 24-30 hours, are multipotent stem cells that give rise in G1 to somatic cells, i.e. neurons, mechano-sensory cells named nematocytes, gland cells, as well as germ cells (**Figure 2A**, right panel) (Watanabe et al., 2009; David, 2012).

The large pool of self-renewing stem cells and their constant differentiation allow the complete renewal of all tissues, including the nervous system, within few months (Steele, 2002). Such dynamic homeostasis also provides a well-documented experimental paradigm to evidence the plasticity of the epithelial cells. Briefly, ISCs that cycle faster than ESCs, can be easily eliminated by brief exposure to antiproliferative drugs such as hydroxyurea (HU) or colchicine (Col), or to heatshock (HS) in thermosensitive strains. In few days, all cycling interstitial cells have disappeared and after several weeks, most interstitial derivatives including the nerve cells are missing as no longer produced. Such "epithelial" *Hydra* survive well this loss when manually fed, evidenced for at least one year, and even more surprisingly keep intact their asexual developmental properties, i.e. budding and regeneration (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). Molecular analyses have shown that epithelial cells actually adapt to this loss, up-regulating a large number genes within one week, including genes devoted to neurogenesis and neurotransmission (Wenger et al. 2016).

The feasibility of transgenesis in *Hydra* was demonstrated in 2006 (Wittlieb et al., 2006) and since then, cell tracing strategies combining transgenesis and transplantation experiments could evidence the turnover of both stem cells and the differentiated cell populations (Wittlieb et al., 2006; Hobmayer et al., 2012). Here we performed a similar homotopic transplantation experiment where the upper part of a GFP positive polyp, i.e. head plus upper body column, was grafted onto a decapitated RFP positive animal (**Figure 2B-2C**). The daily imaging of this chimeric animal shows the evolving spatial distribution of the GFP+ and RFP+ cells. After one week, some red cells already reach the region below the head (**Figure 2D**), then spread into the tentacles and reach their extremities by day-14 (**Figure 2E, 2F**). At day-21, all tentacles are populated with RFP+ cells while the hypostome remains green (**Figure 2G-2I**). Finally, cells of the hypostome are renewed during the next month (**Figure 2J-2K**) in agreement with data produced by (Hobmayer et al., 2012). This complete and constant "rejuvenation" of the polyp undoubtedly contributes to the long lifespan of *Hydra,* helping cells to bypass some aging mechanism such as the progressive loss of proteostasis.

Cell cycle, proteostasis and autophagy

Protein folding is a key part of protein function and the global control of protein quality, named proteostasis, relies on three distinct mechanisms (Lamark and Johansen, 2012): (i) the appropriate protein folding achieved and maintained by chaperones such as Hsp70, (ii) the poly-ubiquitination of misfolded proteins that tag them for proteasomal degradation, (iii) the autophagy process responsible for the degradation of aggregates of damaged proteins, also named aggrephagy (**Figure 3A**). With time, the proportion of damaged proteins increases and cells enter into a vicious cycle where proteasomal degradation declines while the system is overloaded by damaged proteins (Bence et al., 2001; Grune et al., 2004; López-Otín et al., 2013). As a consequence, the global protein quality progressively declines within cells, affecting their function and behavior, thus inevitably leading to aging and eventually to death.

In this context, the constant cell division observed in *Hydra* appears as a great opportunity to get rid of damaged proteins. By dividing and continuously replacing the different cell populations, stem cells efficiently dilute the proportion of cells containing damaged proteins and putative aggregates, and thus limit the negative impact of misfolded proteins. The analysis of the mechanisms at work in polyglutamine (polyQ) neurodegenerative diseases such as Huntington's disease or spino-cerebral ataxias have demonstrated the importance of cell division in the maintenance of proteostasis. Indeed, in such diseases, patients suffer from protein mutations that affect protein folding, resulting in the formation of numerous toxic protein aggregates (Bates, 2003; Metaxakis et al., 2018). However, as their name indicates, such diseases mainly affect the nervous system. A recurring hypothesis is that mutant proteins more readily accumulate in neurons because these cells never divide and do not get readily replaced upon death. Interestingly, upon a variety of toxic insults an up-regulation of cyclins and cyclin-dependent kinases is observed in neurons, interpreted as an attempt to enter the cell cycle when cells enter neuronal programmed cell death (Lin et al., 2001; Currais et al., 2009). Whether this reactivation of a latent cell cycle triggers neuronal programmed cell death or corresponds to an unsuccessful ultimate attempt to protect the cell by diluting the damaging agent is not clear but it highlights the importance of dividing capacities.

Hydra. Regarding the autophagy degradation

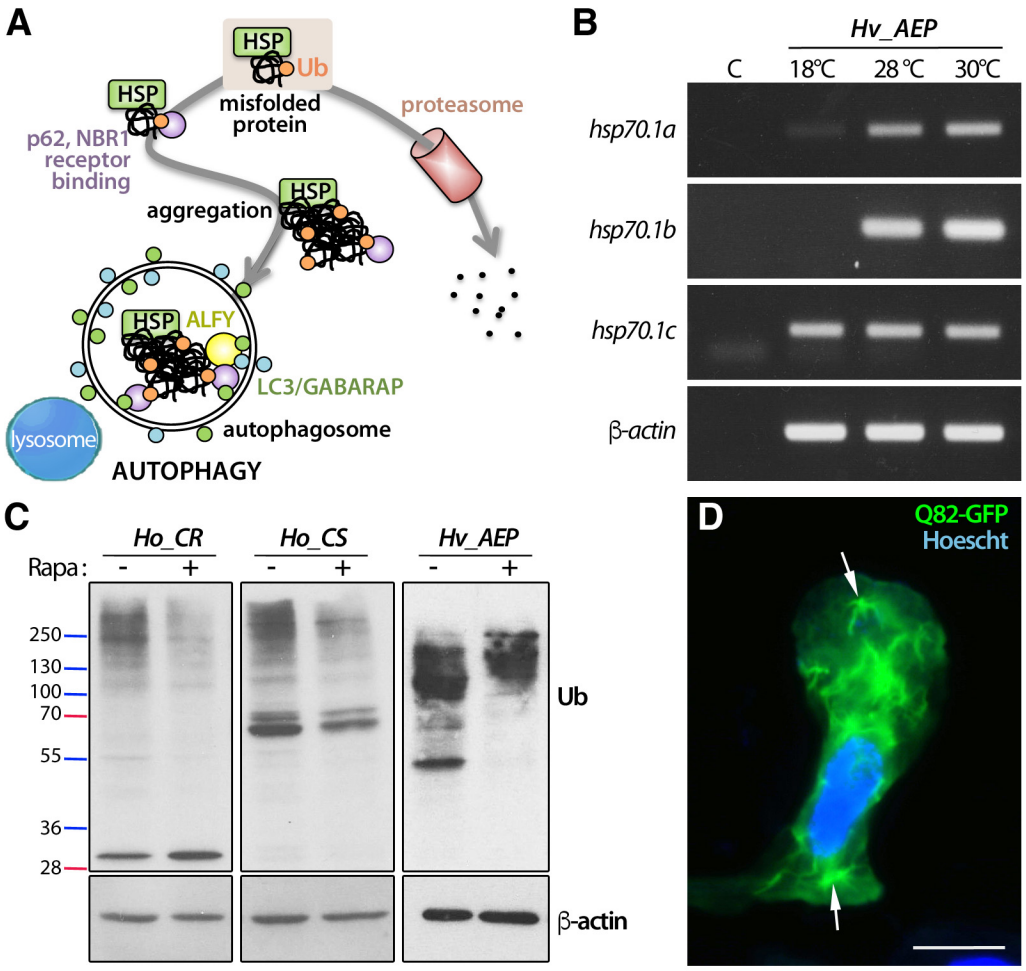

Figure 3: Proteostasis in *Hydra*

(A) Scheme showing the two pathways that degrade misfolded proteins that are poly-ubiquitinated (Ub) and bound to chaperones (HSP): either proteasomal degradation, or autophagic degradation after binding to cargo-receptors such as p62 and NBR1. **(B)** Semi-quantitative RT-PCR experiment (30 cycles) showing the expression level of *hsp70* genes in *H. vulgaris* (AEP strain) animals exposed to a two-hour heat shock. C: amplification in the absence of cDNA. **(C)** Western blot analyses showing the decrease in poly-ubiquitinated proteins (Ub) in rapamycin-treated animals (0.8 µM). *H. oligactis* (*Ho_CR* or *Ho_CS*) animals were maintained at 10°C and continuously treated for 13 days, *H. vulgaris* (*Hv*_AEP) were maintained at 18°C and treated for 10 days. **(D)** Detection of GFP-positive aggregates in epithelial cells of transgenic *Hv_AEP* animals over-expressing the aggregate prone protein Q82-GFP. Scale bar: 10 µm.

Beyond its sustained ability for cell division and cell differentiation, preliminary studies suggest that *Hydra* possess efficient mechanisms to maintain proteostasis. A lower RNA stability of *hsp70.1* in *H. oligactis* than in *H. vulgaris* might explain the higher sensitivity of *H. oligactis* to thermal stress (Brennecke et al., 1998). *H. vulgaris* actually expresses three different *hsp70.1* genes, two of them (*hsp70.1a* and *hsp70.1b)* are heat-inducible while *hsp70.1c* is constitutively expressed in standard conditions (**Figure 3B**). This result suggests that a pool of HSP70 proteins is permanently available, whatever the environmental conditions, likely contributing to the maintenance of an efficient proteostasis in machinery, all components of the autophagy pathway are present in *Hydra* and the autophagy flux is rapidly induced upon starvation in *H. vulgaris* (Chera et al., 2009). However, the autophagy flux is poorly inducible in *H. oligactis* undergoing aging, and blocking the induction of autophagy in *H. vulgaris* leads to a rapid loss of fitness of the animals (Tomczyk et al., 2017). These results highlight the importance of autophagy in the resistance to aging in *Hydra*.

H. oligactis polyps exposed to the proteasome inhibitor MG132 show an accumulation of their ubiquitinated proteins (Tomczyk et al., 2017), which are cleared when the animals are treated with rapamycin, an activator of autophagy (**Figure**

3C). Also, the aging strain of *H. oligactis (*named *Ho_CS)* more rapidly loses its fitness than the non-aging *Ho_CR* or *H. vulgaris* animals when exposed to MG132. These observations indicate that both the proteasome and the autophagy machineries are involved in the degradation of ubiquitinated materials in *Hydra*, either misfolded proteins, or aggregates, or dysfunctional mitochondria. However, no evidence of aggregates has been so far reported in *Hydra* cells. Although this lack of detection can be due to some technical limitations, it also suggests that proteasomal and autophagic degradations combined to an active cell cycle are sufficient to get rid of misfolded proteins under homeostatic conditions. To test the mechanisms of aggregate formation and aggregate turn-over in *Hydra* cells, we recently produced different transgenic lines expressing aggregate-prone polyQ proteins (Schenkelaars, unpublished), similarly to the approach performed in *C. elegans* (Satyal et al., 2000; Silva et al., 2011). By doing so, we revealed that *Hydra* can form aggregates (**Figure 3D**), validating an approach that should help test the limits of the strength of proteostasis in *Hydra*.

FoxO, a promoter of stem cell self-renewal in *Hydra*

Several decades ago, genetic screens performed in *C. elegans* identified the Insulin-like Growth Factor-1 receptor DAF-2 or the advanced glycation end product 1 (AGE-1) as pro-aging factors whose mutation significantly increases the lifespan of nematodes, an effect that relies on the activation of the transcription factor DAF-16 (Kenyon et al., 1993), the vertebrate orthologue being named FoxO (Forkhead-box protein O). The conserved role of the Insulin/IGF-FoxO signaling pathway in the control of aging was subsequently demonstrated in *Drosophila* and mammals, either by inhibiting the nuclear translocation of FoxO that activates the pro-aging effect of the pathway or by inhibiting the pathway, for instance by promoting the activity of the phosphoinositide 3-phosphatase PTEN that allows the nuclear localization of FoxO and the activation of cascades that promote longevity (Martins et al., 2016).

In *Hydra* all components of the pathway were identified (see references in Martins et al., 2016; Schenkelaars et al. 2018), three insulin-like peptides (ILPs), the HTK7 receptor similar to the vertebrate insulin/IGF receptors, the PKB/AKT, PDK2 and Pi3K kinases, the antagonizer PTEN and the transcription factor FoxO, which is expressed in all stem cell populations (Boehm et al. 2012). Quantitative RNA-seq data designed to provide spatial, cell-type and ISC-loss response expression patterns proved to be informative (Wenger et al., 2016; Wenger et al., 2019). Briefly, RNA-seq data were produced in independent replicates (i) from slices dissected along the body column (**Figure 4A**), (ii) from FACS-sorted cells of transgenic lines constitutively expressing GFP in one or the other stem cell populations (**Figure 4B**), (iii) from the body column of animals having lost their ISCs after drug or heatshock treatment (**Figure 4C**). These analyses confirmed that these genes are heavily expressed along the body axis as well as in ESCs, an expression that persists when ISCs are eliminated (**Figure 4D** and not shown).

Transgenesis helped understand FoxO functions in *Hydra.* FoxO responds to stress but not to starvation when overexpressed in interstitial cells and as expected, its sub-cellular localization is phospho-dependent (Brigde et al. 2010). When overexpressed in ESCs, FoxO is pro-apoptotic, an effect antagonized when animals also overexpress ILP-1, suggesting that the insulin/IGF pathway controls FoxO activity at least in epithelial cells (Lasi et al. 2010). Loss of function assays showed the key role played by FoxO in the maintenance of stem cell self-renewal and in the inhibition of cell differentiation (Boehm et al., 2012). This function might represent the ancestral function of FoxO, from which additional ones evolved as observed in bilaterians (Schaible, 2013). Finally, FoxO is also involved in *Hydra* immunity (Mortzfeld et al., 2018). Both functions, stem-cell renewal and immune response to environmental changes likely play a role in the resistance to aging, but direct evidences for a master role of FoxO on the resistance to aging in *Hydra* are currently missing.

Telomere attrition

In *Hydra*, the maintenance of a continuous cycling activity appears beneficial to remain young. However, the divide-or-age dictum implies heavy constraints regarding the maintenance of genome integrity as the replicative DNA polymerases are not able to replicate linear DNA at chromosome ends. These extremities are protected by non-coding sequences named telomeres, TTAGGG in mammals as in *Hydra* where some TCAGGG variants were also found, TTAGGC in nematodes, TTAGG in arthropods (Traut et al., 2007; Chapman et al., 2010). Telomeres tend to shorten division after division until the chromosome ends get dangerously closer to genomic regions containing

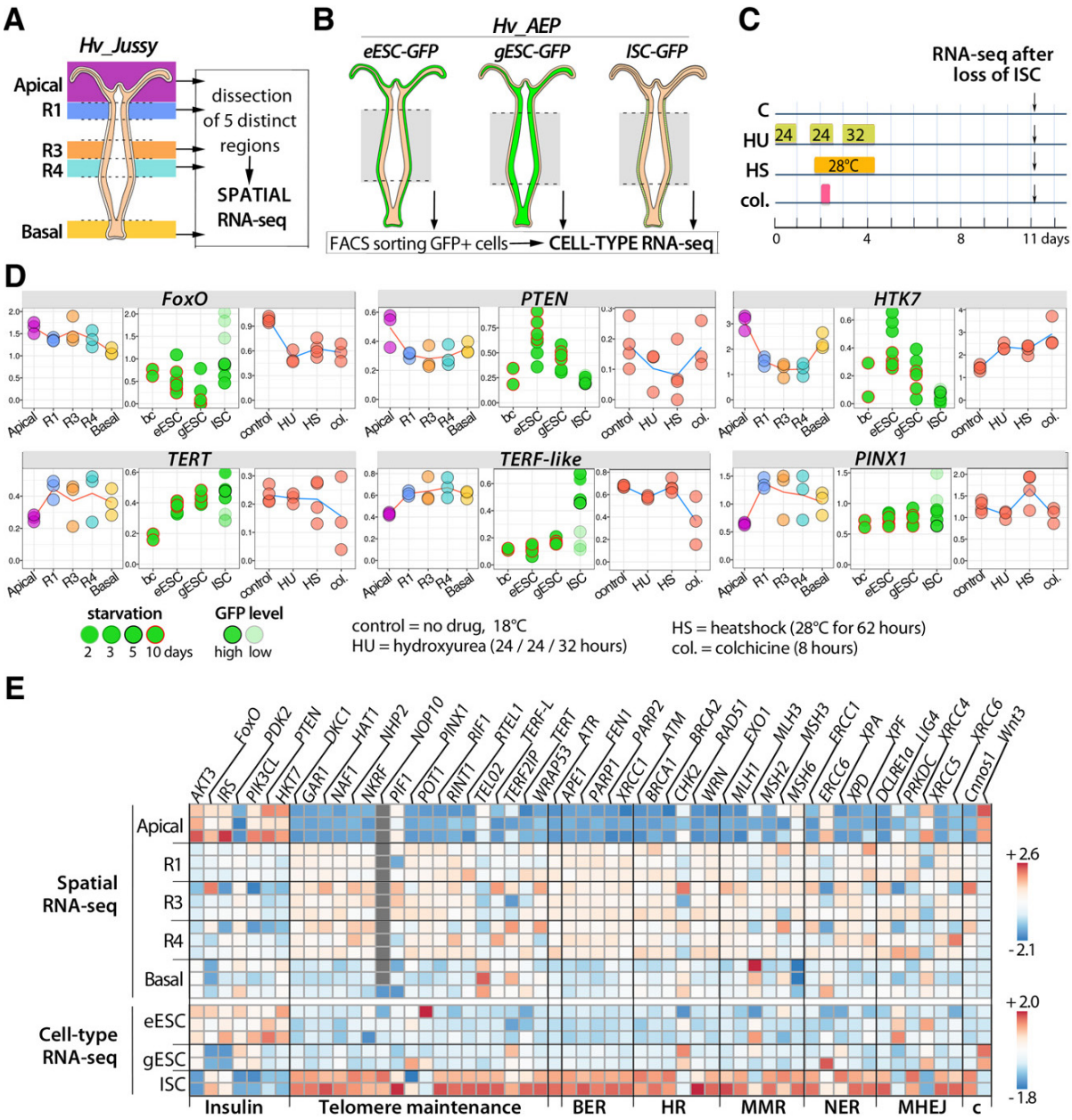

Figure 4: Spatial and cell-type regulation of the DNA repair and telomere attrition genes in *Hydra*

(A-C) Procedures for RNA-seq analysis in three independent experiments as in (Wenger et al., 2016; Wenger et al., 2019). **(A)** Spatial expression measured in five distinct regions: apical, body column (bc) at R1, R3, R4 positions, and basal. **(B)** Cell-type expression measured in GFP-positive cells sorted by flow cytometry from dissected body columns of transgenic *Hv_AEP* animals that constitutively express GFP in eESCs (eESC-GFP) (Anton-Erxleben et al. 2009), gESCs (gESC-GFP) (Wittlieb et al., 2006), or ISCs (ISC_GFP) (Hemmrich et al. 2012). **(C)** Epithelial bc expression after ISC loss upon heatshock (HS), hydroyurea (HU) or colchicine (col.) treatment. **(D)** Spatial, cell-type and ISC-loss RNA-seq profiles of *FoxO, PTEN* and *HTK7* presumably involved in insulin-like/IGF/FoxO signaling (upper); *TERT, TERF-like* and *PINX1* genes presumably involved in telomere maintenance (lower). Values on the y axis correspond to Estimated reads $x10^{-3}$. **(E)** Heatmaps showing the relative expression level of genes involved in insulin signaling, telomere maintenance, based-excision repair (BER), homologous recombination (HR), mismatch Repair (MMR), nucleotide excision repair (NER), non-homologous end joining (NHEJ) along the body axis (spatial RNA-seq) and in stem cell populations (cell-type RNA-seq). Note the lower expression of most genes at the Apical and Basal extremities except *LIG3* – not shown-, *MSH3, XRCC4* that show a rather "flat" spatial expression. All genes are expressed in all stem cell populations, usually at higher level in ISCs, except *HTK7* and *XRCC4*. Two developmental genes, *Wnt3* and *Cnnos1*, are shown as control (c). Heatmaps and clustering were produced using Clustvis (biit.cs.ut.ee/clustvis/). RNA-seq profiles available on HydrAtlas.unige.ch, for Accession Numbers see Table-1.

information. Telomere shortening also leads to chromosome fusion and genomic instability. Below a certain threshold of telomere size, cellular senescence and apoptosis occur, leading to organismal aging (Levy et al., 1992). The telomere-specific reverse transcriptase named telomerase (TERT) together with the telomerase RNA component TERC and the proteins Dyskerin (DKC1), TCAB1 (renamed WRAP53), NOP10, NHP2, NAF1, GAR1 form the telomerase holoenzyme, a ribonucleoprotein complex that allows the reversal of this process by adding the repetitive nucleotide sequences (Wu et al., 2017). The genes encoding these proteins are all present in *Hydra,* all expressed in stem cells, *DKC1, NAF1, NHP2, NOP10* at high levels (**Table-1** and not shown).

TERT is usually not expressed in mammalian somatic cells, explaining why telomere length can help predict life expectancy (Heidinger et al., 2012). Strikingly, ectopic expression of TERT in somatic cells appears sufficient to confer them immortality *in vitro* (Bodnar et al., 1998), while its constitutive overexpression in cancer-resistant mice suffices to delay aging, an effect also obtained when TERT is delivered through gene therapy in adult mice (Tomas-Loba et al., 2008; de Jesus et al., 2012). If TERT activity is an informative proxy of the aging rate, what about TERT activity in long-lived animals? In asexual planarians, TERT appears to be active in somatic cells (Tan et al., 2012), confirming that telomere protection is a corner stone for low aging. In *Hydra*, TERT is expressed along the body axis, enriched two-fold in each stem cell population when compared to the complete tissue from the body column, at a sustained level after the elimination of ISCs (**Figure 4D**). These results suggest a constitutive TERT activity in *Hydra* stem cells, likely up-regulated in ESCs upon ISC elimination.

Telomeres are protected from excessive DNA-repair or telomerase activity by a complex named *shelterin* or *telosome*, made of six telomere-binding proteins in humans: TRF1, TRF2, Rap1, TIN2, TPP1, POT1 (De Lange, 2005). TRFs, Rap1 and TPP1 are now named TERFs (TElomeric Repeat binding factor), TERF2IP (TERF2-interacting protein) and ACD (Adrenocortical dysplasia protein homolog) respectively. *Hydra* does express orthologs to *TERF1/TERF2* (a single gene named *TERF*), *POT1* and *TERF2IP* (distantly-related) but no *TIN2* or *TPP1/ACD,* actually known as vertebrate-specific. This suggests that the *Hydra* telosome is made of three evolutionarily-conserved subunits, TERF, TERF2IP, POT1, potentially able to recruit the telomerase inhibitor PINX1 as well as the DNA-repair factors that associate with the telosome in mammals, namely the ATM and ATR kinases, the DNA-dependent protein kinase PRKDC, the Ku70/80 factors now named XRCC6 and XRCC5, the nucleases EXO1 and FEN1, the DNA excision repair protein ERCC1, the Werner syndrome DNA helicase WRN, the Poly [ADP-ribose] polymerases PARP1 and PARP2, the Breast cancer type 1 susceptibility protein BRCA1, the DNA repair protein RAD51 (de Lange, 2005; Jackson and Bartek, 2009). These factors are all well conserved in *Hydra* and expressed at higher levels in the body column and in stem cells (**Table-1** and data not shown).

In addition to DNA-repair factors, a series of proteins positively or negatively regulate the activity of TERT like *HAT1, NKRF, PIF1, PINX1, RIF1, RINT1, RTEL1, TELO2,* which are all present and expressed in *Hydra* (**Table-1**). Except few cases, most of them including *TERT, TERF, POT1, PINX1* are expressed at higher levels in the body column than in the apical region (**Figure 4D, 4E, 4F** and data not shown), suggesting a higher activity in proliferative regions. In fact, all these genes are expressed in the three stem cell populations, most often at a two- to three-fold higher rate in ISCs than in ESCs, possibly reflecting the faster self-renewal of ISCs (**Figure 4G**). As exceptions, *PINX1*, *TERF2IP* and *TERT* show similar levels of transcripts in ESCs and ISCs. The expression of all these genes one week after ISC elimination is either globally maintained in the body column, or less down-regulated than expected from the expression level observed in ISCs, indicating that ESCs adapt to the ISC loss by up-regulating the components of both the telomerase complex and the telosome as well as the telomerase regulators. Studies monitoring in aging versus non-aging *Hydra* tissues the activity of the TERT complex or the regulation of the telosome are needed to characterize the status of the telomeres in these two contexts, to dissect the mechanisms that potentially maintain telomeres intact in non-aging *Hydra* as well as the impact of TERT activity on the resistance to aging.

DNA-damage response et DNA-repair pathways

In addition to telomere attrition, genomic stability needs to be protected from accidental environmental damages as well as from alterations that occur during the replication process (Branzei and Foiani, 2008; Jackson and Bartek, 2009; Husted and Durocher 2017).

Gene name	Protein molecular function	Alternative names	Processes Pathways	Uniprot / NCBI (*H. vulgaris*)	HydrAtlas (*Hv Jussy*)	HydraAtlas (*Hv AEP*)
APE1	endonuclease	APEX	**BER**	A0A2H4CEP8	seq74053_loc24449	c22024_g1_i02
ATM	Ser/Thr kinase	A-T mutated	**DDR, SHELT-AP**	Q62388_HYDVU	seq17340_loc07515	c20790_g1_i02
ATR	Ser/Thr kinase	RAD3, MHK7.5	**DDR, SHELT-AP**	T2MDN7_HYDVU	seq75437_loc24784	c15300_g1_i01
BRCA1	E3 ubiquitin ligase	RING finger prot53	**DDR, SHELT-AP**	T2MGE5_HYDVU	seq50770_loc18060	c24899_g1_i01
BRCA2	RAD51-binding protein	FACD, FANCD1	**HR**	T2MFA1_HYDVU	seq44641_loc16293	c20224_g2_i01
CHK2	Ser/Thr kinase	CHEK2	**DDR, DSBR, HR**	T2MH34_HYDVU	seq52131_loc18467	c19643_g1_i01
DCLRE1a	hairpin endonuclease	DCR1a, Artemis	**NHEJ, Fanconi**	T2MB49_HYDVU	seq26285_loc10569	c21692_g1_i02
DDB1	damaged DNA binding	XAP1, XPCE	**NER**	T2MD81_HYDVU	seq19468_loc08276	c17486_g1_i01
DKC1	Pseudouridine synthase	NAP57, NOLA4	**TERT_C**	T2MIB3_HYDVU	seq38081_loc14435	c24655_g1_i01
ERCC1	ssDNA repair endonuclease	(-)	**NER, NHEJ, SHELT-AP**	T2MGR9_HYDVU	seq74558_loc24549	c24246_g1_i02
ERCC2	TFIIH basal transcription factor complex helicase	XPD	**NER, BER**	TG8H6N5_HYDVU T2MH99_HYDVU	seq74028_loc24445	c21610_g1_i03
ERCC3	idem ERCC2	XPB	**NER, BER**	G8H6N7_HYDVU	seq17777_loc07662	c18003_g1_i02
ERCC4	DNA repair endonuclease	XPF	**NER, NHEJ, HR, TERT_inh**	E5L9E3_HYDVU	seq64330_loc21834	c44705_g1_i01
ERCC5	ssDNA repair endonuclease	ERCM2, XPG, XPGC	**NER**	T2MGU5_HYDVU	seq39809_loc14954	c24104_g1_i10
ERCC6	ATP-dependent helicase	CSB	**NER**	T2MEM5_HYDVU	seq66952_loc22508	c23475_g1_i02
EXO1	5'->3' dsDNA exonuclease	HEX1	**MMR, SHELT-AP**	T2M3G4_HYDVU	seq31827_loc12381	c24495_g3_i01
FEN1	FLAP endonuclease	DNase IV, MF1	**BER, HR, SHELT-AP**	T2MGD3_HYDVU	seq18343_loc07881	c17668_g1_i01
GAR1	telomerase RNA binding	NOLA1	**TERT-C**	T2M4R4_HYDVU	seq32313_loc12542	c19630_g1_i02
HAT1	histone acetyltransferase B	KAT1	**Tel-MoR**	T2MIM7_HYDVU	seq07765_loc03840	c13305_g1_i01
LIG3	DNA ligase	DNA Ligase III	**BER**	XP_012555297.1	seq57739_loc20073	c25086_g1_i03
LIG4	DNA ligase	DNA Ligase IV	**NHEJ**	T2MFF1_HYDVU	seq60467_loc20814	c25247_g1_i01
MLH1	G/T mispair binding, ATPase	COCA2	**MMR**	T2MFX8_HYDVU	seq49402_loc17665	c20627_g1_i01
MLH3	mismatch DNA binding, ATPase	MutL protein 3	**MMR**	T2M389_HYDVU	seq37709_loc14314	c10492_g1_i01
MSH2	mismatch DNA binding, ATPase	MutS homolog2	**MMR**	T2MGB2_HYDVU	seq76842_loc25138	c21107_g1_i01
MSH3	mismatch DNA binding, ATPase	DUP, MRP1	**MMR**	T2MGN5_HYDVU	seq58483_loc20263	c11822_g1_i01
MSH6	mismatched DNA binding, chromatin binding, ATPase	GTBP, MutS homolog6, p160	**MMR**	T2MF78_HYDVU	seq14968_loc06647	c23333_g1_i01
NAF1	telomerase RNA binding	(-)	**TERT-C**	T2M7V4_HYDVU	seq32753_loc12684	c24225_g1_i02
NHP2	telomerase RNA binding	NOLA2, HSPC286	**TERT-C**	T2M3W1_HYDVU	seq74554_loc24547	c23203_g1_i01
NKRF	ATP-dependent DNA helicase	ITBA4, NRF	**Tel-MoR**	T2MIW6_HYDVU	seq78028_loc25439	c20279_g1_i01
NOP10	telomerase RNA binding	NOLA3	**TERT-C**	T2M4Y2_HYDVU	nd	c32598_g1_i01
PARP1	Poly ADP-ribose polymerase	ADPRT1, PPOL	**BER, HR, SHELT-AP**	T2MH54_HYDVU	seq25774_loc10388	c16864_g1_i01
PARP2	Poly ADP-ribose polymerase	ADPRT2, ARTD2	**BER, HR, SHELT-AP**	T2M499_HYDVU	seq26291_loc10572	c15483_g1_i01
PIF1	DNA-binding, helicase	RRM3, C15orf20	**SHELT-AP**	T2M5L3_HYDVU	seq21919_loc09102	c21238_g1_i01
PINX1	PIN2/TERF1-interacting telomerase inhibitor	TRF1-IP1, LPTL	**TERT_inh**	T2M7F7_HYDVU	seq17743_loc07648	c12081_g1_i02
PKD1						
POT1	telomeric DNA binding, telomerase inhibitor	(-)	**SHELT-C, Tel-MoR**	T2M447_HYDVU	seq56911_loc19865	c8446_g1_i01
PRKDC	DNA-dependent protein kinase catalytic subunit	DNPK1	**NHEJ, SHELT-AP**	T2MEX7_HYDVU	seq77944_loc25431	c25618_g1_i02
RAD51	DNA-dependent ATPase	RAD51A, RECA	**HR, SHELT-AP**	T2MEE7_HYDVU	seq65713_loc22187	c12360_g1_i01
RAD51C	DNA-dependent ATPase	R51H3, RAD51L2	**HR**	T2MJ61_HYDVU	seq77743_loc25370	c19027_g1_i01
RAD51D	DNA-dependent ATPase	RAD51L3, TRAD	**HR**	T2MIJ3_HYDVU	seq64014_loc21737	c18909_g1_i02
RIF1	DSB and telomere binding	Rap1-interacting F	**Tel-MoR**	T2MBS6_HYDVU	seq70541_loc23479	c25181_g1_i01
RINT1	Rad50 interaction	Rad50 interacting P	**Tel-MoR**	T2MBW2_HYDVU	seq42295_loc15605	c24278_g1_i01
RTEL1	regulator of telomere elongation helicase 1	C20orf41, KIAA1088, NHL	**Tel-MoR**	T2M3Q2_HYDVU	seq72663_loc24052	c21412_g1_i05
TELO2	HSP90 binding TTT complex	CLK2 homolog	**DDR, Tel-MoR**	T2M274_HYDVU	seq42743_loc15740	c8206_g1_i01
TERF	telomeric DNA binding	TRF2, TRBF2	**SHELT-C**	XM_012703518.1	seq37588_loc14273	c22977_g1_i02
TERF2IP	telomeric DNA binding	DRIP5, RAP1	**SHELT-C**	XP_012557755.1	seq44248_loc16179	c20301_g1_i01
TERT	telomerase RNA reverse transcriptase	EST2, TCS1, TRT	**Tel-MoR**	T2MJ53_HYDVU	seq75331_loc24757	c8070_g1_i01
WRAP53	telomerase RNA binding	TCAB1, WDR79	**TERT-C, HR, NHEJ**	T2M6A4_HYDVU	seq46080_loc16721	c24874_g1_i04
WRN	ATP-dependent helicase	RecQ3, RecQL2	**DDR, HR, SHELT-AP**	T2MDX0_HYDVU	seq39899_loc14971	c16203_g1_i02
XPA	damaged DNA binding	XPAC	**NER**	G8H6N8_HYDVU	seq76756_loc25114	c16302_g1_i01
XPC	DNA-binding, DNA repair	p125	**NER, MMR**	T2MEL3_HYDVU	seq19822_loc08410	c6698_g1_i01
XRCC1	ssDNA DNase, DNA ligase	(-)	**BER, NER, HR, NHEJ**	T2MGQ5_HYDVU	seq44333_loc16205	c16499_g1_i01
XRCC2	DNA-dependent ATPase	(-)	**HR**	T2MIN0_HYDVU	seq58892_loc20387	c12864_g1_i02
XRCC3	DNA-dependent ATPase	(-)	**HR**	T2MJ71_HYDVU	seq27528_loc10953	c22358_g1_i02
XRCC4	regulator of LIG4 activity	(-)	**NHEJ**	T2ME53_HYDVU	seq57287_loc19959	c21035_g1_i03
XRCC5	ssDNA-dependent helicase	CTBF, Ku80, Ku86	**SHELT-AP**	T2MHH9_HYDVU	seq58375_loc20242	c18931_g1_i01
XRCC6	telomeric DNA binding, helicase	CTC75, Ku70, TLAA	**SHELT-AP**	T2MGH8_HYDVU	seq42734_loc15738	c23742_g1_i01

Table 1: List of 55 DNA-repair and telomere regulation genes expressed in *Hydra*.
Protein function and protein names of human ortholog pages was retrieved from (de Lange, 2005; Jackson and Bartek, 2009) and Uniprot. DSBs: DNA double-strand breaks; DSBR: DSB Repair; ERCC: Excision Repair Cross-Complementing protein; SHELT-AP: shelterin-associated protein; SHELT-C: shelterin-complex; Tel-MoR: maintenance or repair of telomeres; TERT_C: telomerase ribonucleoprotein complex (holoenzyme); TERT_inh: negative regulator of telomerase; WRN: Werner syndrome protein; XP: *Xeroderma Pigmentosum* protein; XRCC: X-Ray Cross-Complementing protein. For other abbreviations see Figure 4 and main text. Websites: HydrAtlas: hydratlas.unige.ch; Uniprot database: www.uniprot.org, NCBI protein database: www.ncbi.nlm.nih.gov/protein.

)Briefly, Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) pathways are both involved in the repair of Double-Strand Breaks (DSBs). NHEJ activity is restricted to the G1 phase, processing the DSBs resulting from ionizing radiations, while HR is active during the S and G2 phases on DSBs that result from the collapse of replication forks. Three additional pathways take in charge single-strand damages: (i) the Nucleotide Excision Repair (NER) pathway that repairs during G1 the bulky lesions, such as those induced by UV; (ii) the Based-Excision Repair (BER) pathway that removes in G1 the oxoguanine (OxyG) residues induced by ROS that lead to transversion mutations as observed in cancer cells (G to T and C to A), and to correct during S phase uracil mis-incorporation; (iii) the Mismatch Repair (MMR) pathway that removes mismatches, small insertion and small deletions during the S phase. In summary, accidental damages are usually processed before the onset of replication by the NHEJ, NER and BER pathways while damages generated during replication are repaired by the HR, BER and MMR pathways that often extend their activity to the G2-phase. Even if their activity is milder during G2, the systematic G2 pausing of *Hydra* stem cells likely contributes to the quality control of their genome.

The components of these five pathways are well conserved in *Hydra* (Barve et al., 2013a, 2013b; Wenger and Galliot, 2013; Pekhale et al., 2017; Galande et al., 2018; Schenkelaars et al., 2018) (**Table-1**). As for their spatial expression, we noted on the qRNA-seq analysis that all samples from the body column group together regardless of their origin, while basal samples that also contain part of the peduncle region (where cells are still cycling) appear closer to the apical samples (**Figure 4E**). 17 genes from these five pathways (*APE1, ATR, ATM, BRCA1, BRCA2, DRCE1a, ERCC1, ERCC4/XPF, MLH1, MSH2, MSH6, PARP1, PARP2, PRKDC, XPA, XPD, XRCC1*) are expressed at lower levels at the extremities, lowest in the apical region except for *MSH6*, which shows the lowest levels basally. By contrast, *LIG3* (BER complex), *MSH3* (MMR pathway) and *XRCC4* (NHEJ complex) show rather stable levels along the body column (**Figure 4F**). Since the body column is enriched in stem cells, higher expression levels in the body column suggest an enhanced DNA-repair activity in the cycling cells, either stem cells or progenitors. In agreement with this hypothesis, the cell-type analysis confirms an enrichment of most transcripts in the stem cell populations when compared to the whole body column tissue, 2x to 6x higher in ISCs for most when compared to the ESCs, confirming previous data about *ERRC4/XPF* (Barve et al., 2013a) (**Figure 4G**). Only a limited number of genes are either equally expressed between ESCs and ISCs (*ATM, CHK2, DCLRE1a, MLH3, RAD51C*) or predominantly expressed in ESCs (*ERCC6, LIG3, LIG4, MSH3, PRKDC, XRCC4)*. As previously noted for the telomere machinery, the expression of most DNA-repair genes is maintained after the elimination of ISCs at a higher level than anticipated (not shown), indicating that these genes are up-regulated in epithelial cells in response to the dramatic changes undergone by the tissue, a response that is part of the epithelial plasticity. Functional analyses should tell how the DNA-damage response machinery is used to prevent DNA-damages in each of the three stem cell populations to protect the genome integrity and consequently limit the stem cell exhaustion over divisions (**Figure 5**).

CONCLUSIONS

In *Hydra*, adult homeostasis relies on adult stem cell populations that continuously self-renew in the central part of the animal, providing the conditions for a constant replacement of all differentiated tissues, predominantly located at the extremities. This rejuvenation process likely explains the lack of aging of the animal and the maintenance of its developmental capacities such as asexual reproduction through budding and regeneration. In addition, in *Hydra*, like in other animals with low aging (sponges, planarians), the germ cells arise from multipotent adult stem cells that continuously provide somatic lineages and transiently produce germ cells. From the data currently available, we propose an integrative model that assumes that the lack of barrier between the somatic and the germ cells gives significant advantages in terms of longevity.

The absence of segregation between the germline and the soma makes available evolutionarily-conserved mechanisms that protect the integrity of stem cells that give rise to germ cells. We anticipate that ISCs benefit from such mechanisms to maintain the integrity of their genome. That way, this stem cell population that most of the time gives rise to somatic cells, would be highly protected when compared to organisms where somatic stem cells and germ cells get separated early during embryogenesis. We identified here a series of evidences that support this hypothesis.

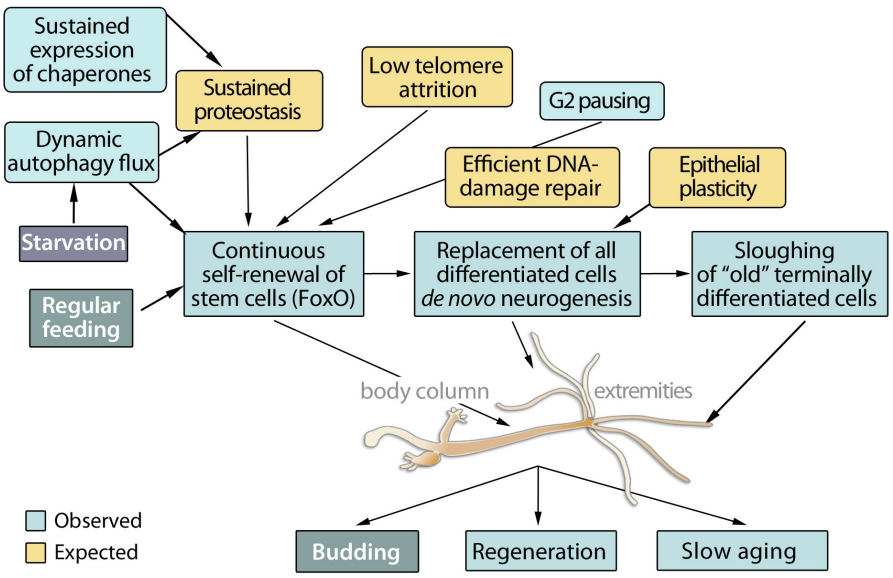

Figure 5: Schematic view of the cellular properties that contribute to slow aging, sustained budding and regeneration in *Hydra*

G2 pausing of stem cells and a dynamic autophagy flux in response to starvation are two properties well characterized in *Hydra* (see text). A low telomere attrition joined to a sustained proteostasis and an efficient DNA-damage repair are expected as deduced from the analysis of the expression of the genes involved in these pathways. In parallel or in synergy with a sustained FoxO activity, these five properties contribute to maintain large stocks of self-renewing stem cells in the body column to provide terminally differentiated cells at the extremities, which are regularly replaced. As a consequence of this dynamic homeostastis, the central part of the *Hydra* body column does not age while the extremities do.

The fact that 54/55 components of the telomerase machinery and the DNA-repair pathways are expressed in ISCs, 40 of them at higher levels in ISCs than in ESCs, suggest that conditions for a low attrition of telomeres and for the maintenance of genome integrity over divisions are optimal in these stem cells. The accumulation of DNA damage and telomere attrition might also be limited in the interstitial progenitors, at least until they enter their differentiation. As a consequence, cells produced through *de novo* neurogenesis, nerve cells and nematocytes, but also secretory cells, might benefit from this protective effect.

However, a large number of functions are carried by the epithelial cells in *Hydra*. These cells also show properties that favor longevity, they constitutively express TERT and its machinery as well as the DNA-repair genes, two tools they can up-regulate when necessary as observed after ISC elimination. This epithelial plasticity favors adaptation to environmental changes and thus likely contributes to longevity. In addition, ESCs exhibit a dynamic autophagy flux that together with a constitutive expression of some HSPs are thought to foster proteostasis and prevent cellular aging (**Figure 5**). The absence of exhaustion of these multifunctional epithelial cells through the maintenance of their self-renewal properties can be considered as a read out of this sustained proteostasis. In summary, *Hydra* shows an amazing combination of biological properties that likely promotes infinite renewal of stem cells, asexual reproduction, regeneration and longevity, some of these properties result from an absence of barrier between the soma and the germline, and others from the multifunctionality and the plasticity of the epithelial cells.

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