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Secrets from immortal worms: What can we learn about biological ageing from the planarian model system?

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Abstract

Understanding how some animals are immortal and avoid the ageing process is important. We currently know very little about how they achieve this. While research in genetic model systems has revealed the existence of conserved genetic pathways and molecular processes that affect longevity. Most of these studies have been performed in established model organisms, amenable to both classical and molecular genetic approaches, with relatively short lifespans. Here we consider the use of planarians, with an immortal life-history that is able to entirely avoid the ageing process. These animals are capable of profound feats of regeneration fueled by a population of adult stem cells called neoblasts. These cells are capable of indefinite self-renewal that has underpinned the evolution of animals that reproduce only by fission, having disposed of the germline, and must therefore be somatically immortal and avoid the ageing process. How they do this is only starting to be understood. Here we suggest that the evidence so far supports the hypothesis that the lack of aging is an emergent property of being highly regenerative and the evolution of highly effective mechanisms for ensuring genome stability in the neoblast stem cell population. The details of these mechanisms could prove to be very informative in understanding how the causes of ageing can be avoided, slowed or even reversed.

Keywords: stem cells; neoblasts; self-renewal; genome stability; regeneration; tumour suppressor

Abbreviations

NB– Neoblast
 ROS- Reactive oxygen species
 TSG- Tumour suppressor gene
 PIWI- P-element-induced wimpy testis
 piRNA- Piwi interacting RNAs
 TEs- Transposable Elements
 GSC- Germline Stem Cell
 IR- Ionising Radiation
 Gy- Gray
 DDR- DNA damage response

1. Introduction

Obtaining as complete an understanding of the biological ageing process as possible is important. A census of the Animal Kingdom will reveal that while the ageing process is assumed to be a characteristic of most taxa, it is not a pre-requisite of being a metazoan [1]. Some seem to avoid the physiological decline that characterises ageing altogether and like Hydra [2,3] and planarians [4–8] are also capable of profound regenerative feats. Some of these regenerating animals can even be referred to as somatically immortal, based on the observation of a species life cycle that does not involve either sexual reproduction (and meiosis) or development from a zygote, but instead uses only regeneration to reproduce. Animals traditionally used to study the mechanisms of ageing have relatively short life cycles and age rapidly, making the study of the ageing process convenient. These studies have led to an understanding of some of the genetic regulation underpinning ageing and the realisation that some aspects of the ageing process are conserved [9,10]. In contrast to this, the study of long-lived or immortal animals has lagged far behind, as these organisms were not amenable to classical genetic approaches, and therefore studying the underlying mechanisms as to why ageing is apparently absent was clearly problematic. However, advances in molecular genetics have now made these animals experimentally tractable [11].

Understanding how some animals have evolved to avoid the ageing process may provide novel ideas about how we can eventually alleviate the problems associated with ageing human populations, and the many age associated diseases, like cancer and dementia, that accompany this [12]. Here we focus on discussing planarian flatworms [7,8]. They have been formally studied as a research system for well over a century and early on were recognised as potentially immortal [13,14]. These animals are famous for their powers of regeneration,

capable of regenerating all organs and tissues of the body from small starting fragments. This ability is fuelled by a population of pluripotent adult stem cells called neoblasts (NBs) capable of making all body tissues and undergoing indefinite self-renewal [15,16]. While current evidence supports the hypothesis that highly proliferative adult stem cells are a synapomorphy of the phylum Platyhelminthes [17,18], the extent of regenerative ability varies greatly across flatworms [19] and even within the Triclad planarians where head regeneration can be reactivated by genetic manipulation [20,21]. Planarians can reproduce sexually and in many cases, such as the major laboratory model species *Schmidtea mediterranea*, an asexual species that reproduces by exploiting regeneration has also evolved from the sexual state (Figure 1A and B) [22]. In these scenarios reproduction has become entirely a collective function of the of the NB population, which as well as powering regeneration effectively take on the role of the germline [15,23]. For these asexual species to persist as a continuous adult population these somatic NBs must be collectively immortal and underpin ever-ongoing homeostatic maintenance of healthy adult tissues. Thus, both the endogenous and exogenous causes of ageing in most animals must be dealt with by this cell population. Given we now know many potential causes of ageing in animals we can ask: what are the cellular and molecular mechanisms behind the planarian life history traits that avoid ageing?

In this discussion we begin by briefly introducing planarian stem cell biology and homeostatic tissue maintenance processes that underpin the planarian immortal life history. We then consider some of the fundamental causes and consequences of ageing in mammals and humans that are relevant to the planarian life history and what is known about how planarians might avoid them. One major conclusion from this is that a combination of emergent features from being able to regenerate, NBs having properties normally associated with the immortal germline of animals and the increased activity of processes that promote genome stability, together underpin the immortal planarian life history.

2. An overview of planarian stem cells, tissue homeostasis and regeneration

2.1 The experimental accessibility of planarians and their NBs

While there is rich body of planarian research throughout the late nineteenth and twentieth century [14,16,24–26] it was the discovery of RNAi that catalysed a major rejuvenation in planarian research [27–30]. Over the last twenty years the ability to study gene function in planarians has allowed a growing research community to make in-roads into understanding

the biology of these exciting animals [7,8,16]. Improvements in sequencing technology have also benefited planarian research leading to the sequencing of the *S. mediterranea* genome [31], many transcriptome studies [32–36], single cell expression profiling [37] and RNA sequencing [38–40] and genome-wide epigenetic studies [41–43]. The ability to accurately FACS sort NBs at different stages of the cell cycle and undifferentiated NB progeny from differentiated cells, based on nuclear to cytoplasmic ratios, has provided essential access to these cells to apply genome wide approaches [44,45]. These ‘omic approaches, combined with an ever-growing list of cell and tissue markers and improved protocols of visualising gene expression in whole animals, provides a powerful set of tools for planarian researchers [46–49]. One notable absence is an approach for mis-expressing genes or exploiting genome editing tools, and this deficit needs urgent attention to improve the depth of mechanistic insight that can be achieved in this model system.

Due to these developments and some elegant experimental designs, we now understand some fundamental aspects of regeneration. One emerging theme so far being that well-known signalling pathways conserved in embryogenesis have conserved roles and interactions during regeneration [50–57]. Additionally, many similarities between NBs and mammalian embryonic and germline stem cells have been discovered [22,35,58]. This, along with other studies across the Animal Kingdom [59–61], has contributed to an appreciation of surprising levels of molecular conservation across animal stem cells and the mechanisms that underpin ‘stemness’. One prominent view of ageing in mammals is that it can be viewed as a gradual accumulation of adult stem cell dysfunction [62–64], so this conservation of stem cell biology suggests that many discoveries made in planarians and other highly regenerative animals with stem cells will be directly relevant to our own ageing biology.

2.2 Planarian NBs are the driving force behind regeneration

Planarians are somewhat unusual in having adult stem cells that are collectively, and in at least some cases individually, pluripotent. Currently the consensus that NBs are those cells still within the cell cycle and that express transcripts considered to be pan-NB markers, with the most widely used being the *smedwi-1* (coding for a PIWI family ortholog) [65] and *Smed-histone2B* (encoding a Histone 2B protein) [34,66]. The injection of single NBs into lethally irradiated hosts, where all cycling cells have been killed, has been shown at low frequency to be able to rescue individual planarians. From these rescued individual animals it was also possible to establish new populations [67]. While the main conclusion from this work is that

individual NBs can be pluripotent, it also provides compelling evidence that an ‘individual’ somatic stem cell and its descendants generated by self-renewal are immortal.

Sampling expression in individual NB cells has revealed some heterogeneity in the population at both the level of gene expression and at the level of potency. These experiments reveal a NB population (called *sigma*) that gives rise to *zeta* and *gamma* NB populations (Figure 1C). Each of these three classes are enriched for expression for specific transcription factors. Functional experiments have revealed that *zeta*-NB are committed to the epidermal lineage, which is currently the best described lineage in planarians (Figure 1C) [37]. The expression profile of markers of the *gamma*-NB sub-population suggest that these NBs are very likely committed to making the gut and endodermal lineages. Both *zeta* and *gamma* NB cells do not appear to have any self-renewing capacity. Once transiting from the *sigma*-NB class to *zeta* or *gamma* during S-phase they appear go through mitosis and leave the cell cycle. This observation is based on gene expression so it is possible they switch off *zeta* and *gamma* marker expression and reactivate *sigma* marker expression to stay in the cell cycle. However, recent analyses of condensin knockdown phenotypes, which block mitosis and can result in endocycling, revealed that only the *sigma*-NBs have increased DNA content indicative of having attempted multiple rounds of division. These data suggest then only *sigma*-NBs are capable of self-renewal [68]. Another single cell RNA seq study has suggested the existence of a *smewi-1*-ve/low population of *nu*-NBs, committed to neural fate [40].

We now know that many aspects of stem cell biology in planarians are conserved with vertebrates and mammals. These include for example aspects of the overall molecular expression profile of NBs [35,69], control of splicing and intron retention [70], epigenetic regulation[41–43] and the mechanisms controlling stem cell migration [71,72]. Together this body of work has established planarians as a powerful model system for studying stem cells and fundamental aspects of regeneration. One way to explain the lack of ageing in planarians in fact would be to consider it entirely as an emergent property of a highly regenerative lifestyle. In this case any physiological decline, associated with molecular mechanisms responsible for ageing, in differentiated tissues or the stem cell compartment is immediately recognised as tissue damage requiring a regenerative response, and is quickly repaired by the activity of NBs.

2.3 Growing and shrinking: homeostatic plasticity in response to nutritional status

One underappreciated aspect of planarian biology often overlooked when new researchers first encounter planarians is their incredible homeostatic plasticity [73]. As well as being able to regenerate their whole bodies, animals are constantly undergoing a homeostatic program of differentiated cell replacement fuelled by differentiating NB progeny. Thus, all differentiated tissues and organs undergo a constant renewal. This in itself is not particularly unusual, for example many highly proliferative mammalian tissues and organs like the blood system, gut and skin do the same. What is unusual is that in planarians this can continue unchecked in the absence of nutrients. During starvation NB proliferation and tissue turnover continues as animals shrink by a controlled process, which can vary over a scale 1×10^3 in volume/size [74–76]. Consequently, during starvation dying differentiated cells continue to fuel NB differentiation. Both empirical data and mathematical modelling suggest that during the starvation process NBs shift the balance of symmetric and asymmetric division, such that more divisions are symmetrical [74,77].

This homeostatic plasticity is clearly a major tissue/cell level mechanism for avoiding ageing. Firstly, the constant turnover and renewal of differentiated cells, tissues and organs completely avoids the accumulation of natural physical wear and tear on differentiated tissues and probably ensures physiological function is not affected. Secondly, the evolution of the ability to withstand relatively long periods of starvation and be reactive to nutrient instability would go in hand in hand with the evolution of the ability to avoid ageing. An immortal life history would not be a successful strategy if short periods of time without food were fatal.

3. Planarians as a model for ageing research

3.1 Which causes of ageing in mammals are relevant to the planarian life history?

If studying planarians is to be a useful model ageing research we first need to establish which specific causes of ageing are relevant to an animal's life history and have been solved by the evolution of appropriate cellular and molecular mechanisms. If we consider what are currently thought to be the likely causes of human ageing we can see that many of the primary molecular causes must also be relevant to planarians. In order to achieve their immortal life histories planarian NBs must protect their DNA against both endogenous and exogenous mutagenic agents as well as circumvent the issue of telomere attrition [78,79] common to all eukaryotic cells with linear chromosomes. In addition to avoiding mutation to the genome sequence itself, NBs must avoid adverse effects on other necessary cellular activities such as the maintenance of a tightly regulated genome-wide epigenetic state [80,81]

and correct cellular proteostasis [82,83], both of which can have long term effects on cellular function. Another relevant fundamental cause of ageing is mitochondrial dysfunction leading to increased production of mutagenic and damaging reactive oxygen species (ROS) [9,84]. All these causes of ageing are not independent and can be responsible for causing each other, for example mitochondrial dysfunction and ROS production can damage proteins and genetic mutations [85,86]. It maybe that planarian NBs have evolved efficient and/or novel pathways for dealing with these harmful processes.

Loss of cellular function or suboptimal cellular function caused by these causes of ageing in turn leads to broader physiological signs of ageing at the level of tissues and organs. For example, stem cell pools can eventually becoming exhausted as cells become senescent as a result of elevated levels of DNA damage or simply due to reaching the end of their normal replicative lifespan, and as a consequence are unable to renew organs and tissues [64,87]. Alternatively transformation of stem cells leads to over-proliferation and cancer. Poorly functioning stem cells will produce poorly functioning differentiated cells in tissues and organs that affect broader physiological function. This can also feedback to the stem cell pool through poor stem cell niche signalling affecting both stem cell self-renewal and differentiation [88,89]. Clearly all these tissue, cell and molecular levels of ageing are entirely avoided by planarians, and investigating the underlying processes bestowing this ability will be informative for developing anti-ageing strategies in the future.

3.2 The immortality of the NB population and regenerative mechanisms act together to avoid ageing in planarians

A useful way to consider the mechanisms that avoid ageing in planarians is to split them into two levels. The first are the molecular mechanisms within NBs that maintain their integrity by dealing with the molecular causes of ageing. We might predict mechanisms that maintain genome integrity would be very efficient and a core part of NB function and that high risk processes (like ROS forming energy production) would be limited or removed from NBs wherever possible. As discussed below we now know that NBs appear to be able to avoid telomere attrition in some contexts, have powerful protection against endogenous sources of genome instability, and are remarkably resistant to exogenous sources of DNA damage. This suggests that, as has been found in other long-lived animals like the naked mole rat [6,90], that mechanisms to maintain genome integrity and reduce errors in other cellular processes have been optimised during evolution compared to animals that do age [91–93]. Together these data and insights (discussed below) begin to provide some explanation as to how

planarians avoid ageing. Currently there is little known about the regulation of mitochondrial function in NBs, except that NBs have a very few immature mitochondria [94,95]. This suggests that one way NBs protect against this source of ageing is they avoid ROS production, instead generating energy by other means or perhaps importing it from other shorter-lived differentiated cells where the effects of ROS are less critical. Similarly, as yet little insight into proteostasis is available for planarians.

The second level at which planarians avoid ageing is through regeneration itself. The ability to repair any kind of cell and tissue damage caused by physical wear and tear, pathogens or indeed the cumulative effects of molecular events that lead to ageing protects against the physiological aspects of ageing. As previously mentioned a constant process of homeostasis replaces all differentiated tissues at a regular rate, meaning that no individual differentiated cell is old. All of these processes are controlled by an interplay of nutritional and positional signals in the animals that regulate the pluripotent NB population [74]. This means that if any single NBs do acquire mutations or damage to cellular processes that might lead to ageing, for example through the production of poorer quality differentiated cells, this will eventually trigger a response from those NBs that are still fully functional. While the damaged NB and its progeny may not survive the remaining NBs are more than sufficient to maintain organismal function without missing a step.

Based on this then there are two avenues for investigation for using planarians as a model system of ageing. Firstly, we can study the regeneration process and how this is orchestrated. Learning about regenerative mechanisms, including how NBs are controlled to respond appropriately to damaged tissue, how new tissue is formed and patterned, and how homeostatic turnover is maintained are all broadly relevant to developing regenerative medicine based approaches to counteract the ageing process. Secondly, we can investigate those processes in NBs that protect against mutation and ensure genome stability and from these gain insight into exactly how the canonical causes of ageing are avoided. For the rest of this discussion we focus on what is currently known about genome stability mechanisms in planarian NBs.

4. Telomere dynamics in planarians are adapted to an immortal life history

4.1 Telomeres, telomerase and the end replication problem

Telomere shortening is one of the most highly discussed phenomena regarding organismal ageing, with many different aspects of telomere biology now implicated as contributing to the aging process [79,96,97]. Telomeres are a repetitive DNA sequence that protects the

chromosomal ends and are eroded after every cell division due to “end replication problem” [98]. This telomere erosion is repaired by the action of the enzyme telomerase in actively dividing proliferative cells [99,100]. Most adult cells do not have telomerase at high levels, and thus telomeres shorten with age as cells divide [101,102]. Inappropriate telomere attrition leads to several age-related disorders and is considered to be one of the hallmarks of ageing, while inappropriate telomerase activity is a hallmark of many cancers [9,103–105]. This suggests that telomeres themselves and cellular senescence induced by telomere shortening may have evolved in humans as a protection mechanism against cancer. Given the weight of available evidence it is not surprising that reactivation of telomerase to counteract telomere attrition has been suggested as an intervention to counteract ageing [106,107]. Given the need to maintain telomere ends in dividing cells this raises the question as to how the end replication problem is solved in planarians, that do not age, and have immortal somatic stem cell populations.

4.2 Asexual planarians maintain telomere length during regeneration

Planarians have the same conserved TTAGGG telomere repeats as humans and the NBs in *S. mediterranea* have telomerase activity, which gets upregulated during regeneration [5]. Comparing the asexual and sexual strains of *S. mediterranea* (Figure 2A and B) revealed that the asexual strain can maintain their telomere ends over successive rounds of regenerative reproduction by fission, but sexual worms show an age-correlated decline in telomere length compared too young hatchlings [5]. In addition, while both strains are equally competent at regeneration, repeated regeneration of the asexual strain did not erode telomere length while it does in the sexual strain. This suggested that telomerase activity in NBs is different according to reproductive strategy and that NBs in the asexual strain may have evolved a robust telomere maintenance system.

One caveat of these findings is that they were conducted over a necessarily limited time frame and on populations of cells from the whole body. This means that, although different from asexual animals, telomerase activity may reactivate in sexual animals when a yet unknown critical telomere length is reached and/or that telomere length is maintained in a small subset of NBs and this has not been detected by the current experimental approaches.

4.3 Asexual planarians have increased telomerase activity controlled by alternate splicing

In *S. mediterranea*, *Smed-tert*, the gene encoding the catalytic protein subunit of telomerase, was found to be alternatively spliced into 4 different isoforms, with only the full-length

isoform predicted to be capable of extending telomeres by adding repeat units (Figure 2C) [5]. Both increased expression and control of alternative splicing to upregulate the active isoform of *Smed-tert* in asexual NBs during regeneration appears to be the adaptation that allows asexual animals to maintain somatic telomere length. This does not happen to the same extent in sexual NBs, and so telomere length appears to decrease when proliferation is increased by inducing regeneration in these animals. Recent work looking across all animals has revealed that alternate splicing of the *tert* gene is also prevalent in many closely-related planarian species and also in other metazoans suggesting evolution of splicing of telomerase may be a frequent event for adapting telomerase activity to different life histories [108].

4.4 Other telomere maintenance mechanisms await discovery in planarians

It remains unclear as to whether telomere/telomerase independent mechanisms exist to explain why shortening telomeres induced by regeneration in sexual planarians haven't (yet) been demonstrated to lead to eventual ageing or senescence. Even long-term knockdown of *Smed-tert* by RNAi has not yet lead to any observable phenotypes [5]. Intact telomeres themselves though are likely essential for normal NB function. Telomeres are bound by a 6-subunit protein complex called shelterin [109] that protects the telomeres from DNA repair machinery. The knockdown of POT1 (protection of telomere 1) in planarians, a shelterin complex component, leads to regenerative defects, activation of DNA damage in planarian cells and is lethal [110] suggesting intact telomere structures are essential. Further functional studies of telomeres and telomerase in planarians should give more mechanistic insights into how telomere maintenance in these potentially immortal worms is regulated to avoid NB senescence.

5. Protection against stem cell transformation and cancer.

5.1 Evolution of high regenerative capacity protects against the pathology caused by cancer

One major risk associated with longevity (and size) is the balance between having the proliferative capacity for extensive tissue homeostasis (and growth) and the risk of proliferative cells becoming transformed leading to the formation of tumours [93,111–114]. The highly proliferative nature of NBs and the constant homeostatic process offer an opportunity to study this balance in a highly accessible context. It is tempting to hypothesise that planarians will have evolved very effective tumour suppressive mechanisms, and that although NB are amazingly proliferative they are also kept under strict control. We know that NB proliferative rate is tightly regulated. For example, it increases upon wounding with two

characteristic peaks, before returning to basal levels [115], demonstrating fine temporal control that is able to both accelerate and brake NB proliferation as necessary. This control makes planarians an excellent model system to study the mechanism controlling stem cell activity relevant to cancer (see below).

However, while molecular mechanisms to control NB activity are crucial to prevent over activity, planarians are protected against the occurrence of cancers by the very fact they are highly regenerative. This can be demonstrated by a simple thought experiment that walks through what would happen when a single NB first becomes transformed and cycles out of control (Figure 3). Imagine a single NB becomes transformed through the acquisition of mutations that allow it to avoid both proliferative control and any cell death mechanisms normally triggered by genome instability. Subsequently, a clone of cells potentially consisting of both self-renewing and post-mitotic cells will form. As this clone of cells begin to cause any physiological damage, for example by disturbing the integrity of an epithelial layer or an organ, this damage would be sensed by regenerative mechanisms. As a result of this normally functioning NBs would be mobilised to repair the damaged tissue and eventually a fragment containing the tumour like tissue would separate from healthy tissue (Figure 3). Given it is highly unlikely that a large proportion of NBs would be transformed simultaneously we can assume that naturally occurring cancers are not a major concern in a highly regenerative system. Other highly regenerative animals may benefit from the same protection against mutation and transformation of their stem cell populations.

5.2 Studies investigating NB proliferation can provide novel information about cancer related mechanisms

The chances that cancer can arise through the transformation of stem cells obviously increases with age and the primary function of some of the mechanisms that lead to ageing through senescence is to prevent cancers earlier in life. Intuitively planarians and their NBs could be an excellent model system to study fundamental aspects of stem cell activity that are common to both regeneration and cancer. While the control of proliferation is central to this, mechanisms that regulate migration and differentiation (or failure to differentiate) are also important. As planarian NBs are always proliferating both potentially oncogenic drivers of proliferation and tumor suppressive mechanisms are likely key to allow proper NB regulation and provide protection against neoplasia [116,117]. Use of RNAi allows a direct method to screen for both oncogenic and tumour suppressor gene (TSG) function *S. mediterranea*.

These studies have revealed both conservation and divergence of function between planarians and mammals and have clearly demonstrated planarians can be used to make novel insights relevant to cancer [43,116–119].

The first conservation of tumor suppressor function was demonstrated for PTEN (Phosphatase and tensin homolog), a frequently mutated TSG in most human cancers, that governs a plethora of biological processes including cellular proliferation, migration and differentiation[120]. Combined RNAi of two homologs of PTEN in planarians (*Smed-PTEN1 and 2*) resulted in a hyper-proliferative phenotype with ectopic cellular outgrowths [121]. This RNAi approach can also be used to implicate novel tumor suppressors. Knockdown of the planarian ortholog of SMG-1, a member of Pi3k family with similarity to TOR, was found to increase NB proliferation and lead to ectopic outgrowths providing evidence that SMG-1 was a novel tumor suppressor [118]. Rapamycin treatment reduced cellular proliferation in both PTEN or SMG-1 RNAi worms suggesting that for these phenotypes planarian tumorigenesis is driven by over-activity of the canonical Pi3k-AKT-mTOR signaling pathway [118,121]. Another recent study has also shown potential conservation of genome wide epigenetic regulation of NB proliferation. Knockdown of the planarian orthologs of the histone methyltransferases human MLL3 (*Mixed Lineage Leukaemia*) and MLL4 leads to ectopic outgrowths caused NB over-proliferation (Mihaylova et al., 2017). Interestingly, the analysis of this phenotype reveals that only some NBs become transformed indicating that the epigenetic effects caused by RNAi mediated loss MLL3/4 of function vary between cells. Combined RNA-seq and ChIP-seq of NBs after knockdown reveal a selection of conserved oncogenes and TSGs that are epigenetically mis-regulated after MLL3/4 knockdown, and this likely drives the tumor-like phenotype [43].

The function of other tumor suppressor pathways in planarians is not clearly conserved, and this may be a necessary adaption to allowing the high rates of NB proliferation that underpin their regenerative life history. For example, planarians have a single homolog of the p53/63/73 gene family and of the Retinoblastoma (Rb) gene, which are amongst the most commonly mutated TSGs in human cancer [119,122]. Studies of these two genes suggests that in planarians they have a minor, if any role, in limiting stem cell proliferation. The planarian p53/63/73 orthologue is mainly expressed in the early post-mitotic progeny of NBs, rather than in cycling NBs. Knockdown of *Smed-p53* results in loss of both NBs and post-mitotic progeny after a brief initial increase in proliferation, which is perhaps compensatory response for the disappearance of progeny. The loss of NB progeny and potential defects in differentiation are lethal [119]. The *Smed- Rb* gene is mostly expressed in NBs and

knockdown of Rb showed hypo-proliferation of mitotic cells leading to stem cell depletion, suggesting conservation of a role in cell division but not a role analogous to tumour suppressor function [122].

Overall, our present understanding of the role of different oncogenes and TSGs is still rather limited. What we know so far suggests that while NBs can efficiently regulate proliferation to ensure regeneration is controlled there is preliminary evidence that some control mechanisms are comparatively relaxed when compared to mammals. This may be necessary to achieve the balance between the anti-aging effects of a constant homeostatic NB activity and anti-cancer pathways to maintain genome stability. While all metazoans must achieve this balance as appropriate to their normal lifespan this will be most extreme in animals that have high regenerative potential.

6. Guarding the genome from endogenous DNA damage and mutational processes

6.1 Planarian NBs use the piwi/piRNA system to silence transposable elements

Genome sequencing has revealed that transposable elements (TEs) constitute a large portion of eukaryotic genomes [123,124]. As active TEs are highly mutagenic, and may target protein-coding genes for insertion, their repression is necessary for the maintenance of genomic stability. This is particularly true of germline stem cells (GSCs), which must repress TE activity in order to maintain a long-term ability to proliferate and produce both new stem cells (self-renew) and differentiating progeny. To combat the invasion and expansion of these selfish elements in the germline, metazoans have evolved a novel class of small RNA molecules called piwi interacting RNAs (piRNAs), that are typically 24-31nt long and that bind to members of the PIWI (*P-element-induced wimpy testis*) subclass of the Argonaute superfamily of proteins. These PIWI-piRNA effector complexes bind to complementary TEs and silence them both epigenetically, by the recruitment of H3K9me3 chromatin marks and/or DNA methylation [125–129] and through post-transcriptional mechanisms, via direct cleavage of TE transcripts in a self-amplifying ‘ping-pong’ cycle [125,130–132].

Although the PIWI-piRNA pathway has been best characterized in the germlines of classical model systems, observations in other organisms suggest that PIWI proteins are present in somatic stem cells and in many cases are necessary for stem cell function. Moreover, phylogenetic analysis suggests an intriguing correlation between the presence of multipotent and pluripotent stem cells that confer the ability for extensive regeneration, and the level of PIWI expression [133]. Sponges [134], acoels [135], cnidarians [136,137], and tunicates

[138,139] are all capable of whole-body regeneration, and contain pluripotent adult stem cells that express one or more PIWI proteins. The correlation between PIWI and potency can be explained by the fact that these stem cells must protect their genome from the malevolent effects of endogenous TEs in order to maintain a long-lived stem cell program consisting of differentiation and self-renewal.

TEs are predicted to constitute in excess of 31% of the planarian asexual genome [140], and studies have identified three major planarian PIWI proteins: SMEDWI-1, SMEDWI-2, and SMEDWI-3 in *Schmidtea mediterranea* [65,141] and their respective orthologs Dj-PIWIA, Dj-PIWIB, and Dj-PIWIC in *Dugesia japonica* [142]. The transcripts for all three of these genes are highly expressed in neoblasts, and inhibition of *smedwi-2* and *smedwi-3* (but not *smedwi-1*) causes significant reductions in organismal piRNA levels, and results in regenerative defects and lethality [141]. This phenotype was investigated in greater detail by Shibata et al (2016) who showed that following RNAi of DjpiwiB, neoblasts are able to proliferate normally and TEs continue to be silenced in DjpiwiB depleted neoblasts 7 days post-RNAi. By contrast, TEs were de-repressed at the onset of differentiation, as observed by the up regulation of a gypsy element in differentiated somatic cells. Unlike DjPIWIA and DjPIWIC that have cytoplasmic expression patterns restricted to neoblasts, DjPIWIB is expressed at the protein level in the neoblast nuclei and continues to be expressed in neoblast progeny and differentiated cells (Figure 4). This suggested that DjPIWIA and DjPIWIC compensate for the loss of DjPIWIB to maintain genome integrity in neoblasts, but since only DjPIWIB is inherited by the neoblast descendants, knockdown of DjPIWIB results in a loss of neoblast progeny during the differentiation process. Since DjPIWIB expression is nuclear, whereas DjPIWIA and DjPIWIC are cytoplasmic, a convincing parallel may be drawn between the planarian and *Drosophila*/mouse germline PIWI-piRNA pathways. DjPIWIB may be the counterpart of *Drosophila* Piwi, transcriptionally silencing TEs in the nucleus via epigenetic processes, while cytoplasmic DjPIWIA and DjPIWIC might be the counterparts of *Drosophila* cytoplasmic Piwi proteins Aub and Ago3 and implement transposon silencing at the post-transcriptional level by functioning as part of the ‘ping-pong’ pathway.

The hypothesis that PIWI is necessary for the maintenance of genomic integrity and cellular longevity is not restricted to stem cell systems. For example, the presence of a functional somatic PIWI-piRNA pathway in differentiated cells has been discovered in the adult *Drosophila* fat body, where loss of this pathway leads to TE mobilization and DNA damage in these cells and manifests in mutants being starvation sensitive, immunologically compromised, and short-lived [143]. The PIWI-piRNA pathway has been found to play a role

in the suppression of TE mobilization in certain neurons of the *Drosophila* brain, most likely as a method to maintain a long lifespan of specific neuronal populations. This study also proposed that neurons lacking PIWI proteins and which permit TE activity may confer a degree of neuroplasticity that may be useful for learning and memory [144]. Interestingly, in planarians, *smedwi-2* has been shown to be constitutively active in an irradiation insensitive population in the CNS, future investigations will look for role for TE silencing in regulating the longevity of this neuronal population [34].

6.2 High resistance to exogenous damaging agents suggests highly efficient DNA repair mechanisms

Defects in DNA repair mechanisms in adult stem cells leads to genome instability that causes ageing, stem cell exhaustion and carcinogenesis [145], suggesting that they protect against ageing by maintaining genome stability. They must act to repair damage from endogenous sources (e.g replicative error or damage by ROS) and exogenous damage from environmental sources [146,147]. Planarians NBs can withstand relatively high doses of ionizing radiation (IR), suggesting they have very efficient DNA repair mechanisms. Very little is known about the DNA damage response (DDR) in normal adult stem cells *in vivo* and planarians provide a potential system to study this.

While doses of IR around 60 Gray (Gy) kill all NBs by 24 hours after exposure and are therefore lethal to planarians, lower doses of up to 17.5 Gy have been reported to be non-lethal as the animal can rescue its NB population from the few remaining stem cells that survive [67,148,149]. These surviving NBs are presumably able to repair any damage and repopulating the animal. Planarians are not exposed to high levels of IR, UV or genotoxic agents in their natural environment at the bottoms of lakes and rivers. Animals that do experience highly stressful environment or conditions, like tardigrades, tend to have even higher IR resistance (>200 Gy) [150–152]. Based on this it seems highly likely that the relative resistance to IR of planarian NBs compared to mammalian cells in culture is an emergent property of having highly efficient DNA repair and other cellular machinery to avoid genome instability over long time periods. This would help explain how planarians avoid this cause of ageing.

A survey of the planarian genome shows that they possess many members of the different DNA repair pathways for different types of damage (Table 1). It should be possible to study these to see if they are indeed capable of very efficient repair, that also provides the observed

experimental IR resistance and if other novel mechanisms are involved that allow sufficient genome stability to avoid ageing and allow NBs to be an immortal stem cell population. So far relatively little is known about repair mechanisms or genes associated with DNA repair. For example, RNAi of *Smed-msh2*, the ortholog of the mismatch repair gene MSH2, was able to confer NB resistance to a cytotoxic DNA alkylating agent compared to control worms [153]. While, Knockdown of a Rad51, a core component in homologous recombination mediated DNA repair leads to stem cell defects and also induced genome instability in *S. mediterranea* [154], it is not known yet whether other components of DDR also play a major role in genome maintenance in planarians.

7. Future Perspectives and Conclusions

We are now gaining a better understanding of how planarian avoid the ageing process. Firstly, it appears that a high regenerative and homeostatic capacity acts to maintain tissue and organ integrity and avoid the physiological effects on any molecular events associated with ageing. Secondly, the adoption of PIWI-piRNA mediated genome surveillance mechanisms, normally associated with metazoan germlines, that silence endogenous mobile elements in NBs. Thirdly, increased activity and efficiency of the DNA damage response, which is assumed by observing the high resistance to experimentally applied exogenous damage, promotes genome stability. Taken together, these features underpin the immortal planarian life history. Other processes like the stability of the epigenome or proteostasis are understudied in planarians, but this should now be experimentally tractable to do so. Studying these different processes in planarians may well reveal novel insights into the underlying biology of ageing, and how the ageing process may be avoided or reversed.

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Figure legend

Fig.1. Mode of reproduction in *Schmidtea mediterranea*. (a) Asexually reproducing planarians stretch their body post-pharyngeally and split into two pieces. Each piece regenerates the missing parts resulting in two worms, thus exploiting regeneration as a mode of reproduction. (b) Sexually mature hermaphrodites copulate and lay cocoons. These cocoons give rise to hatchlings, which eventually develop the germline and become sexually matured. Sexually reproducing planarians are also capable of whole body regeneration, but do not do this naturally by fission, but use the germline as their mode of reproduction. (c) Neoblast heterogeneity in planarians. Stem cells in planarians express *smedwi-1* (coding for a PIWI family ortholog) and *Smed-histone2B* (encoding a Histone 2B protein). *Smedwi-1*⁺ cells are considered to be collectively pluripotent and contain a population of sigma class NBs (expressing relatively high levels of *SoxP1*, *SoxP2*) giving rise to Zeta (expressing *Zfp1*, *SoxP3*, *egr1*) NBs, Gamma NBs (expressing *hnf4*) and nu NBs (expressing *smedwi-2*, *elav2*, *ston2*, but with low/no *smedwi-1*). This subset of lineage committed NBs differentiate to give rise to epidermis, gut and the neuronal cell types.

Fig.2. Telomere dynamics in planarians are adapted to an immortal life history. (a) Asexually reproducing planarians upregulate telomerase activity during regeneration and can therefore maintain the telomere ends over successive rounds of regeneration. (b) Neoblasts of sexual worms show a progressive decline in the length of their telomere ends as they get older, they also shown decline if challenged to regenerate. (c) *Smed-tert*, the catalytic subunit of planarian telomerase, is alternatively spliced as 4 separate isoforms. During asexual regeneration, the full -length isoform is upregulated compared to the shorter form isoforms, and contains a functional reverse transcriptase (RT) and telomerase RNA-binding (TRBD) domain. Other isoforms have one or both of these domains disrupted.

Fig.3. Evolution of a high regenerative capacity can protect against the pathology caused by cancer. A single neoblast (brown cell) acquires mutations that cause it to escape proliferative control and avoid cell death mechanisms that are ordinarily triggered by genome instability (green cell). As these cancer-like cells begin to grow in number, this colony may disrupt normal tissue patterning, for example disrupting the epithelial layer (blue cells). This damaged is sensed by a currently unknown signaling mechanisms that trigger a regenerative response. As a consequence, NBs and their progeny are mobilised to the damaged site, where

they repair tissue and eliminate the over-proliferative NBs and any aberrant progeny. Conversely, in mammals for example, a regenerative response is not able to resolve the tissue pathology caused by a tumour.

Fig.4. The PIWI-piRNA pathway suppresses transposon activity in neoblasts and during differentiation. Depiction of a hypothetical PIWI-piRNA pathway in NBs. PIWIA and PIWIB proteins participate in the ‘ping-pong’ pathway to repress transcribed TEs, whereas PIWIB represses transposons via epigenetic regulation in the nucleus. PIWIB is inherited through differentiation, and acts to silence a subset of TEs in neoblast progeny. Following *piwib* RNAi, TEs escape epigenetic regulation and TE activity increases in both neoblasts and their somatic descendants, leading to an increase in genome instability. (Adapted from [142])

Table legend

Table 1: A survey of the *Schmidtea mediterranea* transcriptome (Jochen Rink lab, Dresden) identified putative orthologs to the mammalian DDR genes, and contig IDs are given in brackets as dd_Smed_v6. [36] used the NCBI BLAST+ tool to search for transcript homologs in the ref_seq protein database, and only transcripts were annotated that fulfilled the following criterion: (subject_coverage>0.2 & query_coverage>0.2) | e_value< 1E-30) & (PC_similarity > 40)). We interrogated the Planmine database (<http://planmine.mpi-cbg.de/planmine/begin.do>) for planarian transcripts of DDR genes that had been BLAST annotated by this process. We then performed a protein alignment of the human and *Schmidtea mediterranea* sequences, and verified orthology by looking for conservation of key domains. Published planarian DDR genes (Mre11, RAD51, BRCA2, Ku70 [154], MSH2, MSH6, MLH1 [153]) are given with their GenBank IDs in parentheses.

Figure 1

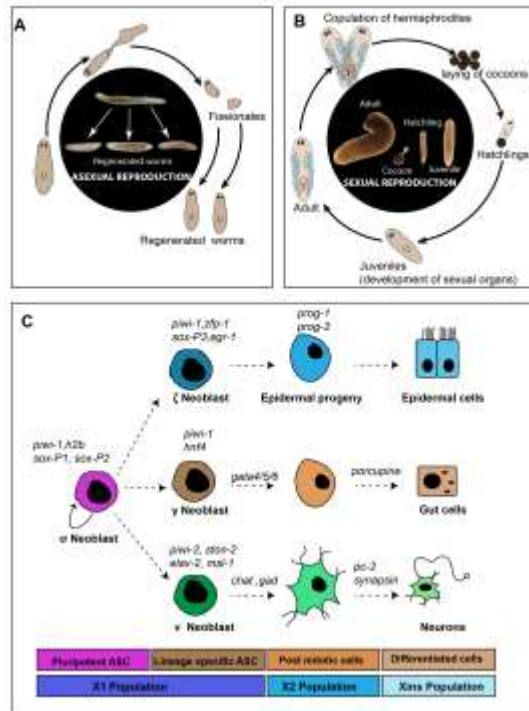


Figure 2

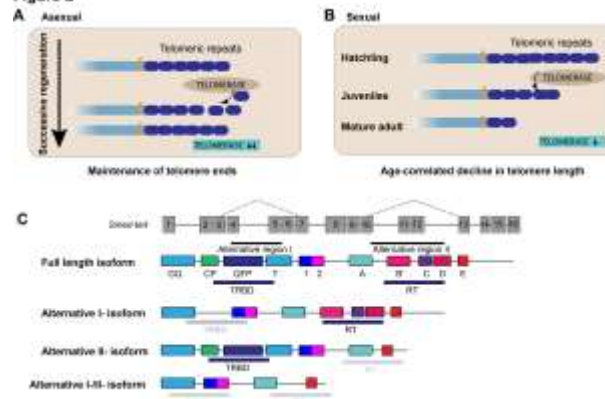


Figure 3

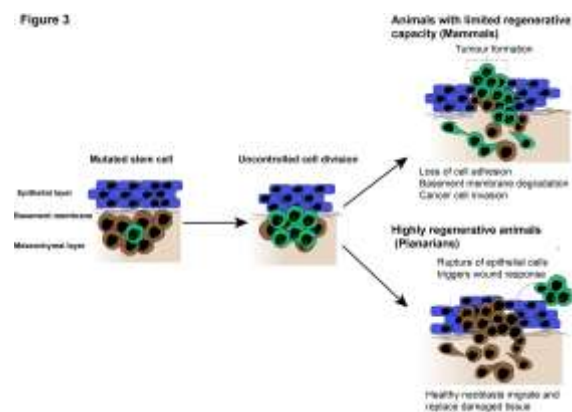


Figure 4

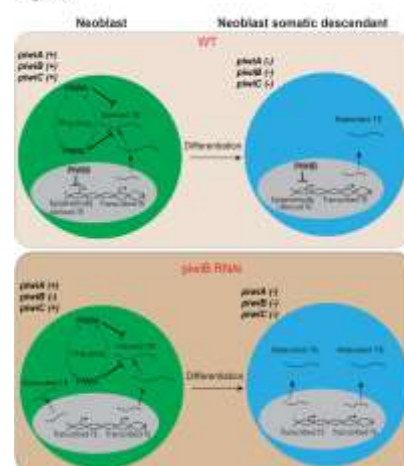


Table 1. Planarian putative orthologs of mammalian DDR genes

Source of damage	Activated DNA repair pathway:	DNA repair genes found in:	
		Mammals	Planarians
Ionizing radiation (X-ray/Gamma-ray) Anti-tumour agents	<i>Double stranded break repair sensors</i>	MRE-11, RAD50, NBS1, PRKDC, ATR, ATM	MRE-11 (AMA21730.1), RAD50 (ACT98282.1), NBS1 (dd_Smed_v6_13269_0_1), PRKDC (dd_Smed_v6_8462_0_1), ATR (dd_Smed_v6_8754_0_1), ATM (dd_Smed_v6_14586_0_1), PARP1 (dd_Smed_v6_10338_0_1)
	<i>Double stranded break repair mediators</i>	γ -H2Ax, MDC1, 53BP1, TopBP1	γ -H2Ax, MDC1 (dd_Smed_v6_4697_0_1), 53BP1 (dd_Smed_v6_12961_0_1), TopBP1 (dd_Smed_v6_14737_0_1)
	<i>Homologous recombination (HR) genes</i>	Rad51, BRCA1, BRCA2, RPA, PALB2, BARD1, CtIP, BLM	Rad51 (AIW60888.1), BRCA2 (AMA21729.1), RPA(dd_smed_v6_3342_0_1), BLM (dd_smed_v6_10119_0_1).
	<i>Non-homologous end joining (NHEJ) genes</i>	Ku70, Ku80, Artemis, Ligase IV, PRKDC, XRCC4	Ku70 (AMA21728.1), Artemis (dd_Smed_v6_12096_0_1), Ligase IV (dd_Smed_v6_11363_0_1) PRKDC (dd_Smed_v6_8462_0_1)
ROS, X-Ray, Alkylating agents	<i>Base-Excision repair/Alt-NHEJ</i>	OGG1,PARP1, PARP2, PARP3, PARG, Tankyrase, XRCC1, PNK	OGG1 (dd_smed_v6_14471_0_1), PARP1(dd_Smed_v6_10338_0_1), PARP2(dd_Smed_v6_6154_0_1), PARP3(dd_Smed_v6_2611_0_1), PARG (dd_Smed_v6_8617_0_1), Tankyrase, XRCC1(dd_Smed_v6_18657_0_1), PNK (dd_Smed_v6_1211_0_2)
Replication fork stalling	<i>Intrastrand crosslink repair (Fanconi Anaemia pathway)</i>	FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCI, FANCI, FANCI, FANCM,	FANCI (dd_Smed_v6_8024_0_1), FANCD2 (dd_Smed_v6_12881_0_1), FANCM (dd_Smed_v6_11718_0_1), FANCI (dd_Smed_v6_16638_0_2)
UV radiation	<i>Nucleotide excision repair</i>	XRCC1, APE1, FEN1,PARP, APTX	XRCC1 (dd_Smed_v6_18657_0_1), APE1 (dd_Smed_v6_11417_0_1), FEN1 (dd_Smed_v6_8206_0_1)
Replication errors	<i>Mismatch Repair pathway</i>	MSH2, MSH3, MSH6, MLH1	MSH2 (AEK64794.1), MSH6(AEB20401.1), MLH1(AEK64795.1)

Table legend

Table 1: A list of important mammalian genes involved in the canonical DDR pathways and their putative orthologs in planarians. A survey of the *Schmidtea mediterranea* transcriptome in the Planmine database (36) identified putative orthologs to the mammalian DDR genes, and only transcripts were annotated that fulfilled the following criterion: (subject_coverage>0.2 & query_coverage>0.2) | e_value< 1E-30) & (PC_similarity > 40)). We then performed a protein alignment of the human and *Schmidtea mediterranea* sequences, and verified orthology by looking for conservation of key domains. Published planarian DDR genes are given with their GenBank IDs in parentheses.