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# Mechanisms of cancer resistance in long-lived mammals

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## Preface

Cancer researchers have traditionally used the mouse and the rat as staple model organisms. These animals are very short-lived, reproduce rapidly, and are highly prone to cancer. They have been very useful for modeling some human cancer types and testing experimental treatments; however, these cancer-prone species offer little for understanding the mechanisms of cancer resistance. Recent technological advances have expanded research bestiary to non-standard model organisms that possess unique traits of very high value to humans such as cancer resistance and longevity. In recent years, several discoveries have been made in non-standard mammalian species providing new insights on the natural mechanisms of cancer resistance. These include mechanisms of cancer resistance in the naked mole rat, blind mole rat and elephant. In each of these species, evolution took a different path leading to novel mechanisms. Many other long-lived mammalian species display cancer resistance, including whales, grey squirrels, microbats, cows and horses. Understanding the molecular mechanisms of cancer resistance in all of these species is important and timely as ultimately, these mechanisms could be harnessed for the development of human cancer therapies.

## Introduction

"In search of cancer cures, it is time to move away from the 'streetlight'"

Cancer remains the second leading cause of death in developed countries[1]. While many treatments are now available for different types of cancer, most have serious side effects and

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are damaging to the normal tissues of the organism[2]. A major goal of cancer research is to develop a therapeutic or preventative strategy that is both efficient and nontoxic. Indeed, such naturally-occurring strategies have evolved multiple times during evolution, as animal species differ dramatically in their cancer rates and ages of disease onset. For example, 50 to 90% of aged mice die of cancer[3–5], while in humans this number is approximately 23%[6]. Less is known about cancer in wild animals. However, several species are known to be extremely cancer resistant. These include the naked mole rat, blind mole rat, elephant and bowhead whale. The age of onset of cancer also varies greatly depending on the lifespan of the species. While it takes a mouse, on average, two years to develop cancer, it takes decades for long-lived species.

The first demonstration that different species require different numbers of mutational 'hits' for malignant transformation was made by Rangarajan and colleagues[7] who showed that two hits are needed for transformation of mouse fibroblasts, namely inactivation of either *Trp53* or *Rb1* and an activating mutation in *Hras*, while five hits are needed to transform human fibroblasts (inactivation of *TP53*, *RB1*, protein phosphatase 2A (*PP2A*), and constitutive activation of telomerase and *HRAS*). Although tumors more frequently arise in epithelial cells rather than fibroblasts, this analysis suggests that humans have evolved much more robust anticancer defenses than mice.

Evolutionary pressure to evolve efficient anticancer mechanisms is very strong. An animal developing cancer prior to its reproductive age would leave no progeny. Thus, animals evolved efficient mechanisms to delay the onset of tumors until post-reproductive age. Hence, cancer becomes frequent in aged animals where it is no longer subjected to natural selection. As a consequence long-lived animals are expected to have more efficient anticancer defenses to keep them cancer-free for longer.

Another factor influencing the risk of cancer is body size. Larger animals have more somatic cells that have the potential to accumulate mutations, thus statistically their risk of developing cancer is higher. To counteract this risk large-bodied species must evolve more efficient tumor suppressor mechanisms. Therefore, novel and more sophisticated anti-cancer strategies are found in long-lived and large-bodied mammals.

It has often been proposed that diet may play a role in interspecies differences in cancer rates or lifespan[8,9]. However, whilst the effects of diet are very important for epidemiology of cancer within a given species, when comparing different species these environmental effects could be considered negligible. Moreover, a healthy vegetarian diet will not enable the mouse to live for 30 or 200 years, as long as the naked mole rat or whale, respectively.

The molecular mechanisms of natural cancer resistance are of intense interest to cancer research (Box 1). These mechanisms have been selected over millions of years of evolution and are safe and efficient. Understanding these mechanisms and then using this knowledge to engineer cancer resistance in humans will be necessary to improve upon current cancer preventative and therapeutic strategies.

Mice and rats are staple models for cancer research. These animals are easy to maintain and are highly susceptible to cancer. Mouse genetic models have provided spectacular advances

in our understanding of the process of tumorigenesis. However, mice and rats have less to offer for understanding the mechanisms of cancer resistance. In this Opinion article, we will discuss the progress achieved in identifying mechanisms of cancer resistance in 'unconventional' model organisms for cancer research. We argue that the studies of long-lived and cancer-resistant species of animals have the potential to bring about breakthroughs in cancer therapy and prevention. Studying these unconventional animal models may be less convenient, but ultimately very rewarding.

## Body mass, lifespan and cancer

As discussed above, more mutational hits are required for malignant transformation of human cells than of mouse cells. For example, telomerase, a ribonucleoprotein that functions to replicate the repetitive sequences at the ends of chromosomes, known as telomeres must be de-repressed to transform human cells. By contrast it is constitutively active in the mouse[10]. Replicative DNA polymerases cannot fully replicate chromosome ends, as they require an RNA primer to start. This is referred to as the 'end replication problem'[11]. Rebuilding chromosome ends is accomplished by telomerase, which carries its own RNA template[12]. In most human somatic cells, expression of the protein component of telomerase TERT is silenced during embryonic differentiation. Hence, when cells replicate, their telomeres shorten, which eventually leads to replicative senescence when cells with critically short telomeres enter permanent cell cycle arrest. Replicative senescence is an important tumor suppressor mechanism limiting cell proliferation[13]. TERT expression is de-repressed in most malignant human tumors[14]. Thus, mouse cells, where telomerase is active, are already one step closer to malignant transformation than human cells.

What then is the TERT status in other species? Analysis of the tissues of 15 rodent species with lifespans ranging from 3 to 32 years and body masses ranging from 30 g to 50,000 g revealed considerable diversity in the levels of telomerase expression[15]. Analysis of telomerase activity showed no correlation with maximum lifespan, but a very strong negative correlation to body mass[15]. There was a defined body mass threshold of 5,000-10,000 g after which telomerase was repressed in the majority of somatic tissues. Thus, increased cancer risk conferred by large body mass leads to evolution of repression of telomerase activity. Analysis of fibroblasts from rodents[16], as well as from a wider range of mammals[17], confirmed that species with large body masses evolved replicative senescence.

Longer lifespan would also be expected to increase cancer risk. However, small bodied species, even the longest-lived ones, did not display replicative senescence[16]. Instead, these species evolved diverse tumor suppressor mechanisms that in cell culture often manifest in very slow cell proliferation. For example, under standard culture conditions that support the growth of fibroblasts from species as diverse as mouse and human, fibroblasts from the longest-lived small rodents, naked mole rat, chinchilla and squirrels, proliferate very slowly with a doubling time of approximately 7 days, whereas human fibroblasts divide on average every 2 days[16]. The mechanisms that slow down cell proliferation were hypothesized to also act to restrict malignant growth. Indeed, the ability of fibroblasts from small rodents to form colonies in soft agar, upon inactivation of *Trp53* or *Rb1* using the SV40 large T antigen, and

exogenous expression of HRAS-G12V was found to be significantly negatively correlated with lifespan[18].

When the requirements for malignant transformation were compared across 18 rodent species determined using mouse subcutaneous xenografts, a continuum of phenotypes was revealed with larger and longer-lived species requiring more hits[18]. For small and short-lived rodents, inactivation of *Trp53* or *Rb1* plus an activating mutation in *Hras* was sufficient to form tumors. However, longer-lived small rodents required both *Trp53* and *Rb1* to be inactivated. In addition to *Trp53*, *Rb1* and $Hras^{G12V}$ genetic alterations, larger rodents also required activation of telomerase. Interestingly, in rodents that were either shorter-lived (capybara) or not very large (paca, porcupine) telomerase was only required for malignant tumors, while benign tumors could form without telomerase. In the large and long-lived rodent the beaver, the requirements were identical to that of humans. Beaver cells must inactivate *Tp53*, *Rb1*, *Ppp2a* and constitutively activate telomerase and *Hras* for any tumors to be formed[18].

Thus, both body mass and lifespan shape the evolution of tumor suppressor mechanisms. The body size determines whether cells have limiting telomeres while lifespan determines other tumor suppressor mechanisms (Figure 1). Why did small species not evolve replicative senescence? We hypothesize that replicative senescence is not beneficial for small-bodied animals, as a benign tumor arising prior to short telomere-mediated growth arrest would be deleterious for a small sized body. For example, a 3 g tumor would substantially handicap a 30 g mouse but would be inconsequential for a 55 kg capybara (Figure 1). Hence, small-bodied long-lived species evolved mechanisms that restrict cell proliferation early, at the hyperplasia stage[18]. These mechanisms are diverse and each clade seems to have evolved them independently. In the subsequent sections, we will discuss the two best understood examples of such mechanisms in the two long-lived rodents, naked mole rat and blind mole rat.

## Cancer resistance mechanisms

### The longest-lived rodent, the naked mole rat

The naked mole rat (*Heterocephalus glaber*) is a mouse-sized rodent that inhabits subterranean tunnels in East Africa. Due to a constant temperature underground, and no need for insulation, naked mole rats have lost their fur resulting in their peculiar name and appearance. Being naked is not the most remarkable feature of this unique rodent. Naked mole rats are extremely long-lived, with a maximum lifespan in captivity of 32 years[19] (their lifespan in the wild being shorter), and are highly resistant to cancer[20,21]. Thousands of individual animals have been monitored over decades in biomedical research laboratories[20], as well as in zoo colonies[22], and only six cases of tumors, two of them possibly benign, have been found[23, 23]. All of the six reported neoplasms occurred in zoo colonies where animals are exposed to light and greater temperature ranges than in biomedical facilities.

Multiple mechanisms contribute to the remarkable cancer resistance of the naked mole rat (Figure 2). Indeed, to resist cancer over the 10-fold longer lifespan than that of mice, a single mechanism would be insufficient. The naked mole rat belongs to the group of small,

long-lived mammals that do not show replicative senescence and rely on early-acting, anti-hyperplastic tumor suppressor mechanisms.

Fibroblasts of the naked mole rat proliferate very slowly in culture due to the phenomenon of early contact inhibition (ECI)[24]. Contact inhibition is a property of most normal adherent cells. When normal cells come into close contact with each other they stop proliferating and form a dense monolayer. In contrast, cancerous cells lose contact inhibition and continue to proliferate on top of each other. The naked mole rat cells are more sensitive to contact inhibition than normal cells of other species; naked mole rat cells arrest cell proliferation at earlier stages prior to forming a dense monolayer, typical of fibroblastic cells. ECI is triggered by activation of $p16^{INK4A}$, rather than p27, typical of contact inhibition common to other species such as human or mouse. If *Cdkn2a*$^{INK4A}$ (encoding $p16^{INK4A}$) is silenced or mutated in naked mole rat fibroblasts, then normal contact inhibition is activated via induction of p27[24]. To completely abrogate contact inhibition in the naked mole rat requires the loss of both genes *Cdkn2a*$^{INK4A}$ and *Cdkn1b* (encoding p27). Since contact inhibition is lost in most solid tumours[25], having two levels of protection against the loss of contact inhibition increases cancer resistance.

Interestingly, the *Cdkn2a–Cdkn2b* locus in the naked mole rat has a unique structure[26] and is subject to positive selection[27]. *Cdkn2a–Cdkn2b* is a rapidly evolving locus that contains key tumor suppressor genes[28]. In humans and mice it encodes the cyclin-dependent kinase (CDK) inhibitors, $p15^{INK4B}$, $p16^{INK4A}$, and a p53-activator protein ARF that shares the coding sequence with $p16^{INK4A}$. However, in the naked mole rat alternative splicing results in a novel transcript fusing the first exon of p15 with the second and third exons of $p16^{INK4A}$ [26]. The resulting novel product, named pALT, acts as a potent CDK inhibitor, adding yet another level of cell cycle control to the naked mole rat cells[26].

The extracellular signal leading to activation of ECI has been shown to be a unique high molecular mass hyaluronan (HMM-HA) secreted by naked mole rat cells[29]. The signalling pathway requires the CD44 receptor leading to activation of expression of the *Cdkn2a–Cdkn2b* locus[29]; however, the intermediate signalling steps are unknown. Hyaluronan is a linear glucosaminoglycan that constitutes the major non-protein component of extracellular matrix (ECM). The longer molecules of hyaluronan have anti-proliferative, anti-inflammatory and anti-metastatic properties[30]. In contrast, shorter molecules are associated with inflammation, more rapid proliferation and metastasis[30]. In other mammals, including mouse and human, hyaluronan molecules are 6-10 times shorter than in the naked mole rat[29]. Two factors contribute to the high abundance of HMM-HA in the naked mole rat. First, the hyaluronan synthase 2 (*Has2*) gene in the naked mole rat has a unique sequence possibly contributing to higher production of hyaluronan; and second, hyaluronidases, the enzymes that degrade hyaluronan have very low activity in the tissues of naked mole rats[29]. Abrogation of HMM-HA in naked mole rat cells, through either gene silencing or overexpression of a hyaluronan degrading enzyme, makes them prone to forming tumors upon inactivation of *Tp53* and *Rb1* and activation of *Hras*$^{G12V}$ [29]. Thus, naked mole rat fibroblasts require four hits for malignant transformation. Three are shared with other rodents and the fourth, related to HMM-HA, is unique to the naked mole rat.

With regard to inactivation of *Tp53* and *Rb1*, the naked mole rat has another unique property. Inactivation of only one of these tumor suppressors causes apoptosis in naked mole rat cells[24]. This is in stark contrast to mouse or human cells, where inactivation of either *RB1* or *TP53* results in more rapid proliferation[7]. Similarly, inactivation of $Cdkn2a^{ARF}$, which leads to reduced activity of p53, was reported to trigger senescence in naked mole rat cells[31]. This suggests that the naked mole rat evolved mechanisms to 'sense' the loss of either *Tp53* or *Rb1* tumor suppressors and trigger apoptosis or senescence. Both of these tumor suppressors must be inactivated simultaneously for the cells to continue to malignancy[24].

The process of induced pluripotent stem cell (iPSC) reprogramming has a lot in common with malignant transformation[32–34]. Interestingly, naked mole rat cells are resistant to iPSC reprogramming[31,35,36] and even when reprogrammed, naked mole rat iPSCs are very inefficient at forming teratomas (germ cell tumours composed of cells derived from the three germ layers)[31,35,36]. The low reprogramming efficiency of naked mole rat cells could be explained by a more stable epigenome, where the promoters of reprogramming genes (*Oct4*, *Sox2*, *Klf4* and *Myc*) are more deeply repressed than in mouse cells[30]. A more stable epigenome is likely to further contribute to tumor resistance of the naked mole rat.

Interestingly, naked mole rat cells are capable of fructose-driven glycolysis, which evolved as an adaptation to living in hypoxic environments[37]. Fructose-driven glycolysis is also found in tumors[38]. Hence, the evolution of this trait would make naked mole rat cells more prone to cancer, which must have been counteracted by the multiple tumor suppressive adaptations mentioned above. Additional mechanisms that may contribute to cancer resistance of the naked mole rat are high-fidelity protein synthesis[39], more active antioxidant response pathways[40], and more active proteolysis through autophagy[41] and the proteosome[42].

## The blind mole rat

The blind mole rat, *Spalax ehrenbergi* superspecies, is a group of related subterranean rodent species that inhabit forests and mountain valleys in the Middle East. Blind mole rats are more closely related to *Muridae* rodents (mice and rats) than to African naked mole rats[43]. However, unlike mice and rats, the blind mole rat is extremely long-lived, with a maximum lifespan of 21 years[44], and resistant to cancer[45]. As expected due to their small size, blind mole rats express telomerase in their somatic tissues and do not use replicative senescence as an anticancer mechanism[45]. Blind mole rats completely lack external eye structures, hence their name. The strictly subterranean lifestyle of blind mole rats resulted in a unique tolerance to hypoxia[46]. Possibly, to avoid hypoxia-induced apoptosis, blind mole rats evolved alterations in the *Tp53* sequence, such as an arginine to lysine substitution in the DNA binding domain (Arg-174 in human), which is also found in hypoxia tolerant human tumors[47]. However, despite the weakened p53, no cases of spontaneous tumors have been observed in blind mole rats even with decades of observation of several hundred animals. Furthermore, attempts to induce tumors using carcinogens in vivo revealed a strong initial necrotic response that was not followed by tumor formation[48]. The initial necrotic response

in the skin is associated with increased expression of interferon β (IFNβ) (V.G., unpublished observation).

At the cellular level, blind mole rat fibroblasts display a unique phenotype upon passaging in culture termed 'concerted cell death' (CCD)[45]. After 12-15 population doublings, the entire culture of blind mole rat cells dies within 3-4 days via a combination of necrotic and apoptotic processes. Cell death is mediated by a massive release of IFNβ into the medium[45]. The CCD phenomenon in cell culture is reminiscent of the *in vivo* necrotic reaction to carcinogens. The current model is that blind mole rat cells are acutely sensitive to hyperplasia. Rapid cell proliferation triggers the IFN response that results in elimination of cells at the site of hyperplasia via a combination of necrotic and apoptotic pathways (Figure 3). This is akin to a 'scorched earth' strategy rather than the pin-point elimination of rogue cells by apoptosis. Interestingly, genome analysis in the blind mole rat revealed duplications of genes in the IFN pathway[49]. Thus, the data so far indicates that the IFN-mediated CCD strategy might have evolved in the blind mole rat to counteract the weakened pro-apoptotic function of the p53 protein.

In addition to the IFN-mediated mechanism, the blind mole rat cells also produce HMM-HA[29]. However, there are important differences with the naked mole rat. The blind mole rat cells do not display ECI. Hence, the mechanism of hyaluronan-mediated tumor suppression differs between the two species. It has been reported that blind mole rat cells secrete a compound, that mildly slows proliferation of tumor cells[48]. The identity of the compound is not yet known, but is likely to be HMM-HA. Another ECM component that is modified in the blind mole rat is the heparanase enzyme[50]. Heparanase is an endoglycosylase that degrades heparin sulphate on the cell surface and in the ECM. The blind mole rat expresses a splice variant of heparanase that acts as a dominant negative, inhibiting matrix degradation[50]. This together with abundant expression of HMM-HA[29] may result in a more structured ECM that restricts tumor growth and metastasis.

## Long-lived bats

Bats account for a large fraction of species of mammals and have been studied extensively. It is then surprising that only a few cases of tumors have ever been described in bats[51–53]. Indeed, extensive pathological studies could not identify any tumors in a large international collaborative project that studied bats in Asia, Africa and Australia[54,55]. The low cancer incidence in bats is consistent with the observations of suppressed tumorigenesis in long-lived mammals, such as naked mole rats and blind mole rats. All bats are long-lived relative to their body mass (lifespan ranges from 7 to 42 years), but interestingly the longest-lived bats (Brandt's bat with the lifespan of 42 years) are also among the smallest.

Recent studies suggested a critical role for mitochondrial function in bat physiology that evolved to counteract oxidative stress resulting from metabolically costly activities, in particular flight[56]. Such mechanisms might have evolved pleiotropic effects responsible for tumor resistance as well as pathogen control in the bats. A recent comprehensive integrative gene expression study revealed bat-specific as well as differentially expressed (compared to other mammals) microRNAs (miRNAs) and mRNAs that function in previously described longevity pathways, revealing distinct bat gene expression patterns[57]. It has been shown that

the long-lived bats may possess unique regulatory mechanisms to resist tumorigenesis, repair cellular damage and prevent oxidative stress, which likely contribute to their extraordinary long lifespan[58,59]. In particular, 3 out of 4 up-regulated miRNA (miR-101-3p, miR-16-5p, miR-143-3p) in the greater mouse-eared bat, *Myotis myotis* (a microbat closely related to the Brandt's bat) appear to function as tumor suppressors against various types of human cancers, and one down-regulated miRNA (miR-221-5p) may act as a tumorigenesis promoter in human breast and pancreatic cancers[57].

Growth hormone (GH) insensitivity includes genetic abnormalities of the GH–insulin-like growth factor 1 (IGF1) axis[60]. Mutations in the single-transmembrane GH receptor (GHR), including in exon 8 coding the transmembrane domain, have been shown to result in human Laron-type dwarfism (short-stature)[60]. GHR mutations or GH signaling deficiencies including those associated with Laron-type dwarfism, have been associated with increased resistance to cancer in humans and mice[5,61]. It is then of interest that Leu284 in the transmembrane domain of GHR, which is highly conserved in tetrapods, is absent in long-lived *Myotis* microbats and several other bat species[58]. Thus, reduced GH–IGF1 signaling may be a contributing factor to cancer-resistance in long-lived bats. Other candidates to this phenotype are proteins involved in the DNA damage checkpoint such as ataxia telangiectasia mutated (ATM), RAD50, KU80 (also known as XRCC5) and DNA-dependent protein kinase (DNA-PK), and nuclear factor-κB (NF-κB) pathways, which were identified based on an unexpectedly high proportion of positively selected genes in bat genomes[59].

## The largest mammals: elephants and whales

In 1977, Peto[62] noted that it is surprising that while humans have 1000 times more cells than a mouse and are much longer-lived, human cancer risk is not higher than that in the mouse. This observation was seemingly inconsistent with the multistage carcinogenesis model[63] according to which individual cells become cancerous after accumulating a specific number of mutational hits. This contradiction became known as Peto's paradox[64,65]. It has been proposed that an answer to Peto's paradox is that different species do not need the same number of mutational hits. In other words, large-bodied and/or long-lived animal species have evolved additional tumor suppressor mechanisms to compensate for increased numbers of cells. Furthermore, many large animals are also long-lived, hence they need additional protection from cancer over their lifespan. As discussed above, animals larger that 5-10 kg of body weight evolved replicative senescence as an anticancer defense. But what additional tumor suppressors have evolved in animals with body masses a thousand times bigger, such as elephants and whales?

Recently, two groups simultaneously identified 19 extra copies of the *TP53* gene in the elephant genome[66, 67] (Figure 4). All additional copies of *TP53* appear to be pseudogenes and contain various deletions. Some of these novel forms of the *TP53* gene are transcribed from neighboring transposable element (mobile DNA) derived promoters. Transcripts from two of the 19 *TP53* pseudogenes are translated in elephant fibroblasts[66]. However, all the additional copies of *TP53* are missing DNA binding domains and the nuclear localization signal and, therefore, cannot function as transcription factors.

Remarkably, elephant cells have an enhanced p53-dependent DNA damage response leading to an increased induction of apoptosis, compared to smaller members of the same family, such as armadillo, hyrax and aardvark[66]. Although the precise mechanism of action of the novel forms of *TP53* is not known, it was proposed that their protein products may act to stabilize the wild type p53 protein by binding to either the wild type p53 molecule itself or to its endogenous inhibitors, the MDM2 proteins[66, 67]. It is also possible that the extra copies of *TP53* have some novel functions. Notably, the elephant *TP53* copies appear to be under positive selection further suggesting they play a functional role (V. Lynch, personal communication).

The increased sensitivity of elephant cells to genotoxic stress may act as an anticancer mechanism through a more aggressive elimination of damaged cells prior to them becoming precancerous. However, cell death by apoptosis occurring more frequently would deplete the stem and progenitor cell pools in the tissues by increasing the need for cell replacement. Therefore, an enhanced apoptotic response needs to be balanced by other adaptations to ensure that it does not lead to premature stem cell exhaustion. Mouse models with constitutively active p53 display premature aging and loss of stem cells and tissue cellularity[68,69]. Therefore, elephants may hold additional adaptations related to stem cells and tissue maintenance. Interestingly, mice engineered to carry extra copies of wild type *Trp53* transgenic alleles consisting of large genomic segments containing the intact *Trp53* gene were protected from cancer and did not display premature aging[70]. When these transgenic *Trp53* alleles were combined with a transgenic *Cdkn2* allele the mice showed cancer resistance and even an increased median lifespan[71].

Interestingly, sequencing and characterization of several whale genomes[72, 73,74], including the longest-lived bowhead whale[75], which has a maximum lifespan of 211 years[76], did not reveal similar duplications of *TP53* as in elephants. Whales are very large creatures with an adult bowhead whale weighing 100 tons, compared to only 3 tons for an adult elephant, and just under 0.1 ton for an adult human[77]. Thus, whales have likely evolved novel anticancer adaptations that are not even found in elephants or humans.

Comparative genomic and transcriptomic studies[75,78] in the bowhead whale identified genes under positive selection linked to cancer and aging, as well as bowhead whale-specific changes in gene expression, including genes involved in insulin signaling[78]. Notable examples of positively selected genes are excision repair cross-complementation group 1 (*ERCC1*), which encodes a DNA repair protein and uncoupling protein 1 (*UCP1*), which encodes a mitochondrial protein of brown adipose tissue[75]. In addition, these studies identified copy number gains and losses involving genes associated with cancer and aging, notably a duplication of proliferating cell nuclear antigen (*PCNA*)[75]. Since both ERCC1 and PCNA are involved in DNA repair, these proteins may protect from cancer by lowering mutation rates; thus whales may not need extra copies of *TP53* because their cells do not accumulate cancer causing mutations and do not reach a pre-neoplastic stage.

Slower metabolism of the largest mammals may lead to lower levels of cellular damage and mutations, and thus contribute to lower cancer incidence. However, no data is yet available on how or whether metabolism indeed contributes to the cancer incidence in these species. It

would be of great interest to understand the molecular mechanisms of cancer resistance in elephants and whales as these could potentially be translated to improve cancer resistance in humans.

## Mutation rates in animal species

Already in the 1950s DNA mutations were suggested to be the main cause of cancer along with aging[79]. Hence, it is reasonable to assume that species-specific differences in the DNA mutation rate are critical determinants of cancer risk, which is intricately linked to longevity. The germline mutation rate has been found to vary greatly between species[80,81]. When comparing DNA sequence changes in phylogenetic lineages it was noted that the rate of such mutational changes was much slower in long-lived species such as primates as compared to short-lived rodents[82]. In these early experiments, evolutionary change was measured only in a fraction of the genome and these results are somewhat controversial as they did not account for differences in generation time or metabolic rate. However, the most likely explanation for species-specific differences in cancer risk remains differences in genome maintenance[83]. Indeed, a comparison between mouse, human and naked mole rat revealed significantly slower nucleotide substitution rates in the longer lived species as compared to the mouse[84]. Direct analysis of the germline genome of mouse and human for *de novo* base substitutions in offspring showed a significantly lower germline mutation rate in humans as compared to mouse[85]. Interestingly, using a novel single cell approach the somatic mutation rate was found to be much higher than the germline mutation rate in both species; yet somatic mutation rates were also lower in cells from humans as compared to those of mice[86]. Importantly, these observed differences between species were not due to differences in generation time[86]. Since mutations are a major contributor to cancer development, species-specific differences in spontaneous mutation rates may well contribute to the differences in cancer risk between short-lived and long-lived species.

A major determinant of mutation rate is DNA repair fidelity. Evidence begins to accumulate that cancer-resistant and long-lived species may have more efficient DNA repair[87]. Long-lived species were reported to more efficiently form p53-binding protein 1 (53BP1) foci for a given amount of DNA damage suggestive of a greater capacity to detect DNA damage[88]. Furthermore, genome and transcriptome sequencing of long-lived animals show that multiple genes involved in DNA repair are expressed at higher levels[89,90], or display the signature of positive selection[59,91].

## Concluding remarks

Mammalian species evolved a diverse set of anticancer mechanisms. Not all species have equal protection. Large and long-lived animals are more resistant to cancer. Some of the mechanisms that evolved are common among multiple cancer-resistant species while others only evolved in individual clades. For example, mammals with body mass greater that 5-10 kg have all evolved repression of telomerase activity and replicative senescence. The mechanisms which have evolved in even larger species are only beginning to be understood. Elephants have evolved pseudogene duplications of the *TP53* gene that may lead to an increased apoptotic response, while much larger whales do not seem to use that strategy.

Mechanisms of cancer resistance found in small-bodied, long-lived animals are very diverse but all act at the early stages of cancer progression. Naked mole rats have evolved HMM-HA that restricts cell proliferation and arrests growth of premalignant cells. Blind mole rats also express HMM-HA, but do not display ECI and instead have evolved the CCD mechanisms that trigger cell death mediated by IFN secretion in response to hyperplasia.

The reason for such diversity in tumor suppressive mechanisms is that the need for more efficient anticancer defenses has arisen independently in different phylogenetic groups. As species evolved larger body mass and/or longer lifespan, depending on their ecology, the tumor suppressor mechanisms had to adjust to become more efficient. In each case, the ecology and unique requirements of individual species would determine the outcome. The evolutionary process works with what is available; for example, a bird's wing has evolved from an upper limb of a terrestrial animal rather than by creating a new appendage[92]. Similarly, in the case of two subterranean rodents, the naked mole rat and the blind mole rat, these species independently evolved HMM-HA, likely as an adaptation to subterranean lifestyle to confer stronger and more flexible skin, which constantly rubs against the walls of their burrows. Later this adaptation may have been co-opted to confer tumor resistance and longevity.

While the ultimate goal of cancer research is to develop safe and efficient anticancer therapies as well as preventative strategies, what can be learnt from tumor-prone models has its limitations. Mice simply do not possess anticancer mechanisms that humans do not already have. With regard to inherently cancer resistant species, the potential for improving the development of anticancer therapies is much greater. Anticancer adaptations that evolved in these species may be missing in humans and if introduced into human cells could result in increased cancer resistance. For example, humans did not evolve HMM-HA, as they do not lead a subterranean lifestyle; hence, activating similar mechanisms in humans may be beneficial. HA is a natural component of human bodies and is well tolerated. Therefore, identifying strategies to systemically upregulate HMM-HA in human bodies may serve in cancer prevention for predisposed individuals or as a cancer treatment.

Nature has a lot to offer in the search for novel tumor suppressor strategies, as there are many naturally cancer-resistant species outside of the common laboratory bestiary. In addition to the naked mole rat, the blind mole rat, microbat and elephants described here, horses and cows were reported to be highly resistant to mammary cancer[93]. The secrets of cancer resistance in other large mammals such as hippopotamuses, walruses and whales are waiting to be uncovered. Other species of interest are squirrels. These animals have extremely high telomerase activity[15], which in humans is associated with tumorigenesis, yet squirrels are long-lived and cancer resistant (Figure 5). Understanding the molecular mechanisms of multiple anticancer adaptations that evolved in different species and then developing medicines reconstituting these mechanisms in humans could lead to new breakthroughs in cancer treatment and prevention.

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**Box 1**

### Finding new tumor suppressor mechanisms

Animal lineages have evolved a diverse array of tumor suppressor mechanisms. Some of these mechanisms are conserved, while others are unique and are shaped by the species' lifestyle and ecology. What then might be the strategies to identify such unique mechanisms? First, one can start with a long-lived and/or cancer resistant species. Next, establish cultures of primary cells from this species and observe the behavior of the cells and propensity for malignant transformation. One can try to introduce the known sets of mutational 'hits' into these cells such as inactivation of tumor suppressors and activation of oncogenes to see if additional hits, beyond what is known from human and mouse studies, are required for tumor formation. However, this strategy may override the unique mechanisms or result in tumor suppressive strategies that act upstream of the known tumor suppressors being overlooked. For example, in a species with extremely accurate DNA repair, mutational hits would not occur naturally and the forced inactivation of tumor suppressors would still lead to malignancy.

Another way to find novel tumor suppressor mechanisms is harder to define; one has to observe cell behavior and look for anything unusual. For example, this is how we found early contact inhibition (ECI) in the naked mole rat and concerted cell death (CCD) in the blind mole rat. Once the unique cellular phenotype is found, one can proceed to identify its molecular underpinnings. In addition to intrinsic cellular mechanisms, cancer resistant species may possess systemic mechanisms of tumor suppression, such as more efficient elimination of malignant cells by the immune system. Identification of such mechanisms is an exciting new avenue that requires working with the whole animal and maintaining animal colonies.

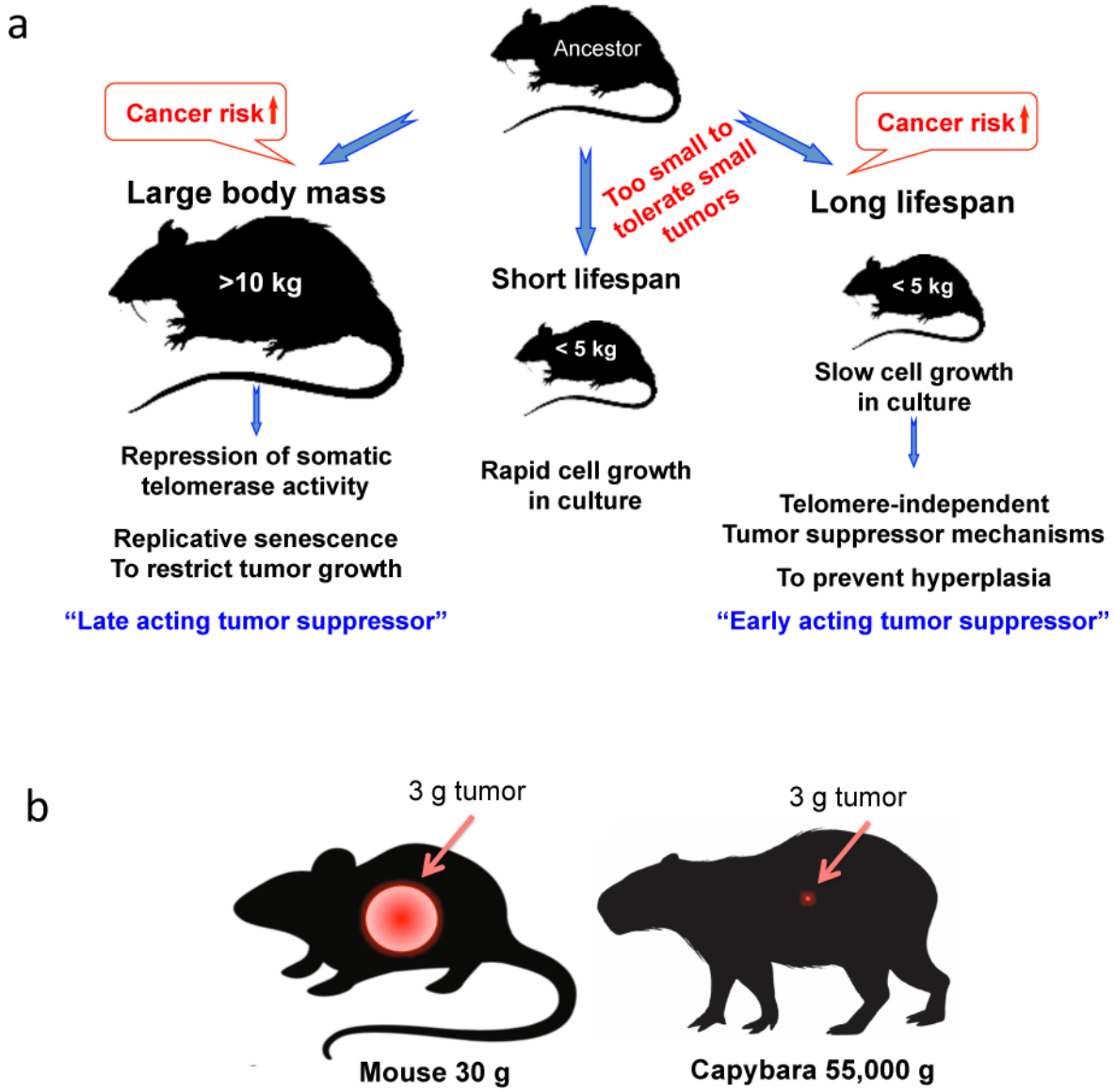


**Figure 1. Evolution of anticancer mechanisms shaped by lifespan and body mass**

(a) As species evolve a large body mass, their cancer risk increases due to the greater number of cells in the body that may acquire oncogenic mutations. To counteract this risk, large-bodied species, with body mass greater than 10 kg, evolved repression of somatic telomerase activity and replicative senescence as an additional tumor suppressor mechanism. Replicative senescence represents a late-acting barrier for tumor progression, since it allows the formation of small tumors prior to the activation of the telomere checkpoint. A long lifespan also increases the risk of cancer, and small (body mass less than 5 kg), long-lived species, which cannot tolerate the formation of small tumors, evolve telomere-independent tumor suppressor mechanisms. These mechanisms offset hyperplasia and manifest in slow cell proliferation *in vitro*. (b) Small- and large-bodied animals have a different tolerance to tumor size. Mouse and capybara are drawn to scale with a 3 g tumor. Such a tumor would likely affect fitness of a 30 g mouse but would be inconsequential for a 55 kg capybara. Part

**a** reproduced with permission from Ref. 15, 2008 Seluanov, A. et al. Aging Cell © Blackwell Publishing Ltd/Anatomical Society of Great Britain and Ireland 2008.

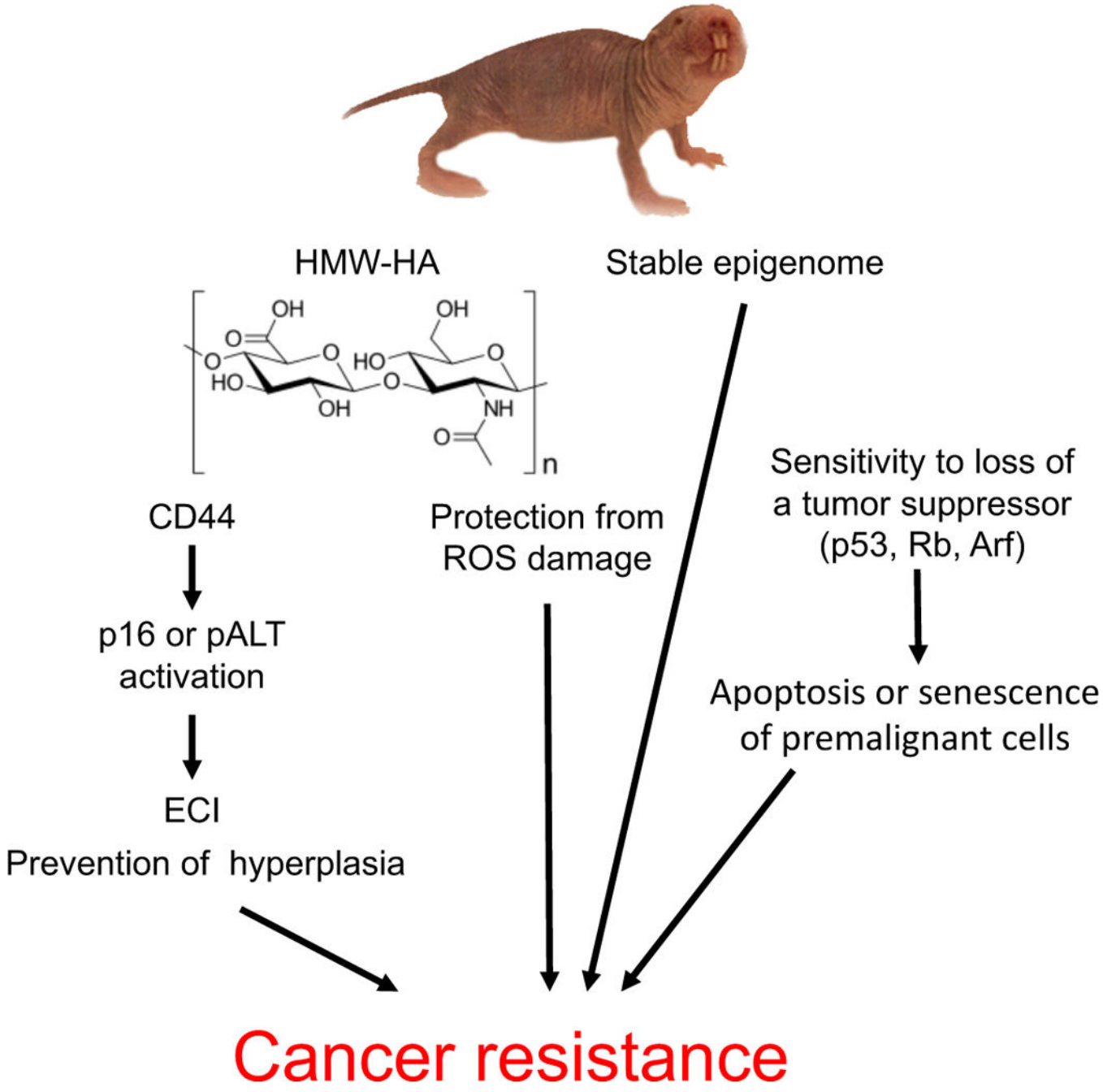


**Figure 2. Anticancer mechanisms in the naked mole rat**

Naked mole rat cells and tissues produce large quantities of high molecular mass hyaluronan (HMM-HA). HMM-HA interacts with CD44 receptors and triggers early contact inhibition (ECI) of naked mole rat fibroblasts via activation of $p16^{INK4A}$ or the naked mole rat specific product of the INK4 locus, pALT. ECI provides protection from cancer by arresting the cell cycle at a low cell density and preventing hyperplasia. HMM-HA may also provide protection from metastasis by maintaining a stronger extracellular matrix. HMM-HA also acts as an antioxidant thereby reducing reactive oxygen species (ROS)-induced damage to nucleic acids and proteins. In addition, naked mole rats have a more stable epigenome than

mouse cells, which can resist reprogramming by Yamanaka factors (*Oct4*, *Sox2*, *Klf4* and *Myc*) and may similarly resist reprogramming associated with malignant transformation. Furthermore, naked mole rat cells have a unique ability to 'sense' the loss of a single tumor suppressor such as p53, RB or $p19^{ARF}$ and undergo apoptosis or senescence.

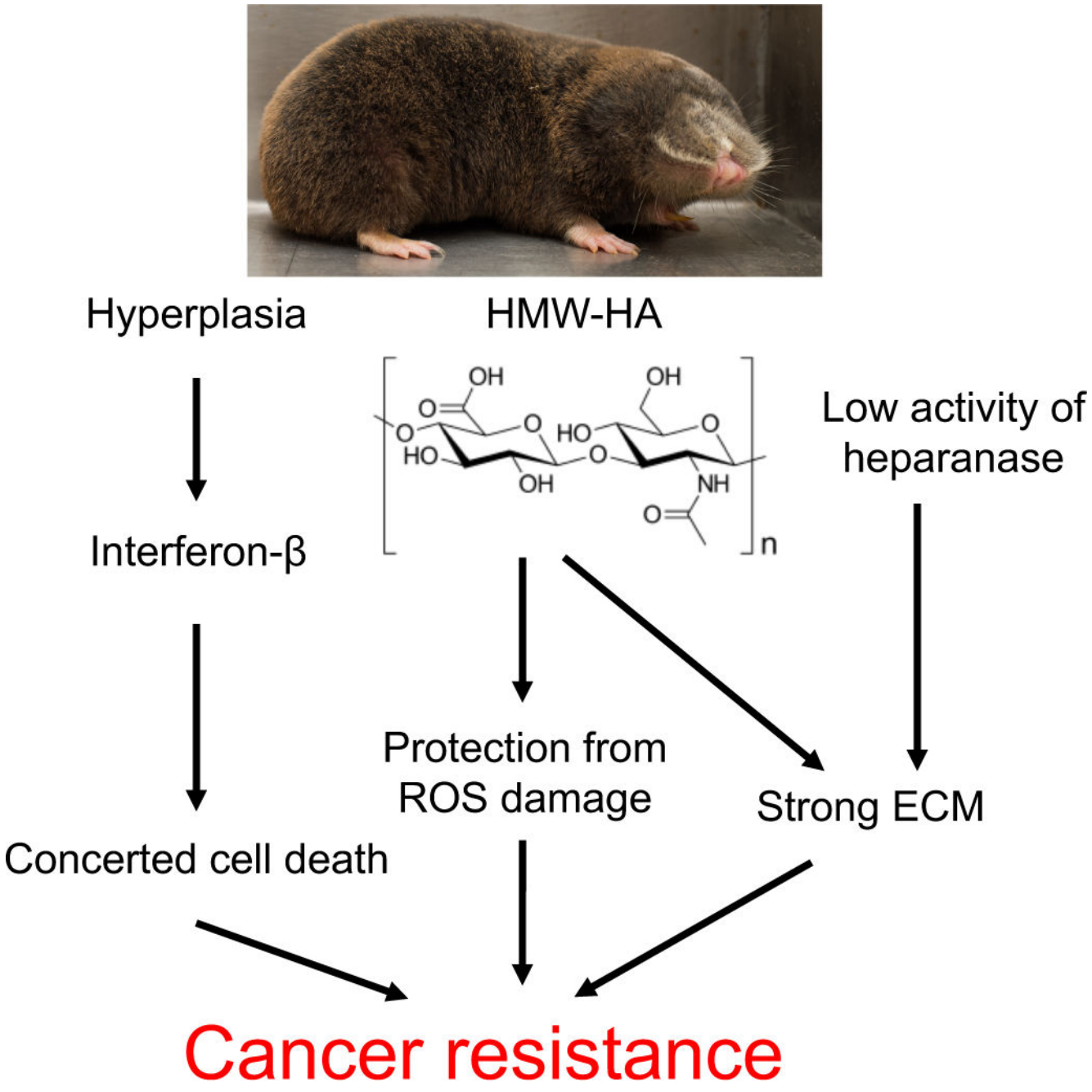


**Figure 3. Anticancer mechanisms in the blind mole rat**

In response to hyperplasia caused by hyper-proliferation of cells in vitro or carcinogens in vivo, blind mole rat cells secrete interferon β (IFN β) that triggers concerted cell death by necrotic and apoptotic mechanisms. Concerted cell death serves as an efficient way to eliminate pre-malignant hyperplastic cells. Additionally, similarly to the naked mole rat, blind mole rat cells secrete abundant high molecular mass hyaluronan (HMM-HA). However, unlike naked mole rat cells, the blind mole rat cells do not display early contact inhibition (ECI). HMM-HA in the blind mole rat may contribute to cancer resistance by protecting the cells from reactive oxygen species (ROS)-induced damage. Blind mole rats

express a dominant negative splice variant of heparanase that, together with HMM-HA, may contribute to stronger extracellular matrix (ECM) and prevent tumor growth and metastasis.

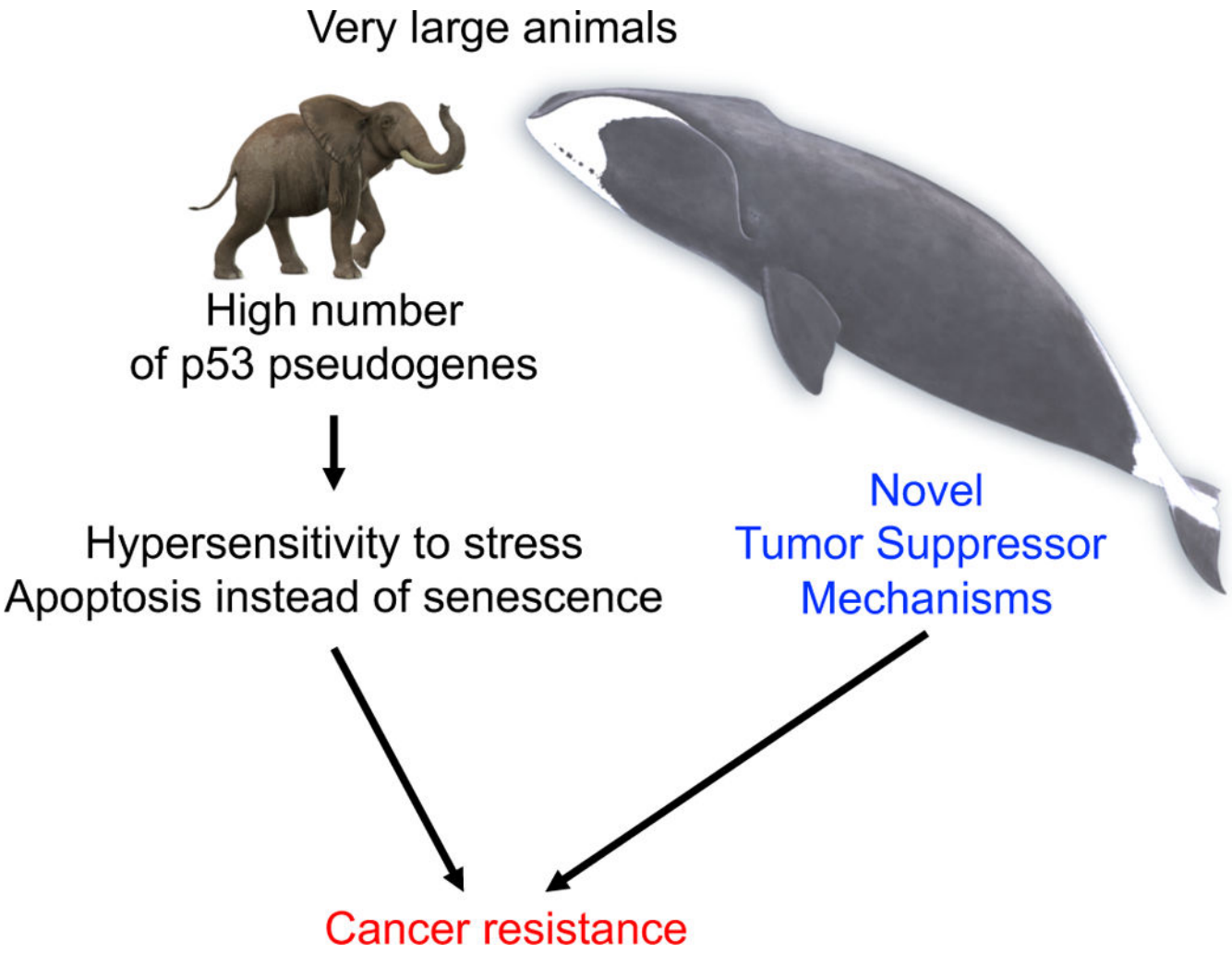


**Figure 4. Anticancer mechanisms in the largest mammals, elephants and whales**

Large animals have more cells in their bodies and statistically have a higher risk of developing malignancy. However, in reality, cancer incidence does not increase with the body mass of a species. This is because large animals have evolved additional tumor suppressor mechanisms. Elephants have evolved multiple copies of the *TP53* gene (pseudogenes) that are associated with an increased apoptotic response. Anticancer mechanisms in the largest mammals, whales, are not yet known, but they do not involve *TP53* duplications.

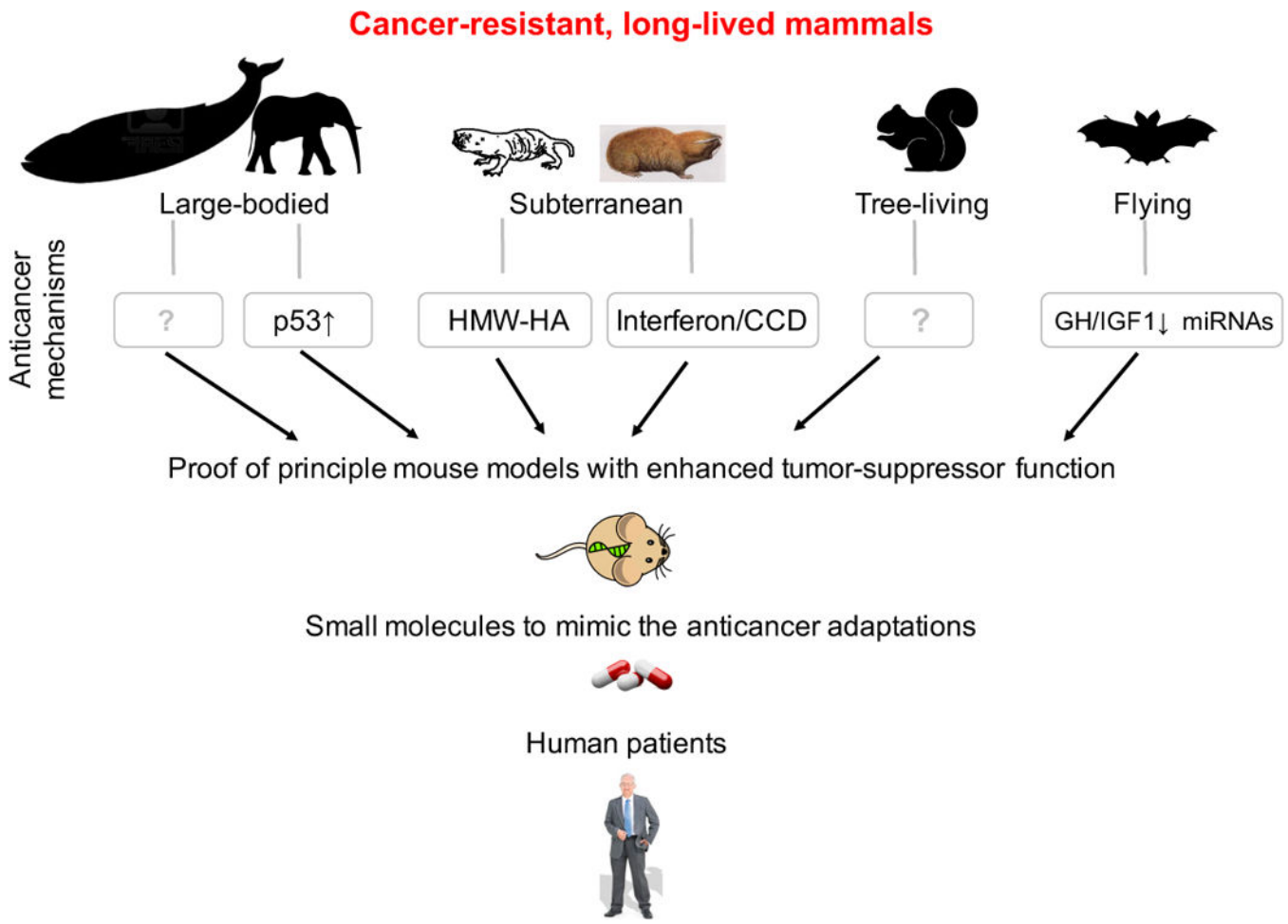


**Figure 5. Developing anticancer treatments based on naturally evolved cancer resistance**

Cancer resistance has evolved multiple times in mammals. Species that display cancer resistance include the largest mammals such as whales and elephants, subterranean long-lived mammals (the naked mole rat and the blind mole rat), long-lived squirrels and bats. The specific mechanisms differ and were shaped by species ecology, lifestyle, and body characteristics. These mechanisms are beginning to be understood. The known mechanisms include duplications of the *TP53* gene in elephants, overproduction of high molecular mass hyaluronan (HMM-HA) in the naked mole rat, interferon-mediated concerted cell death in the blind mole rat, and reduced growth hormone (GH)–insulin-like growth factor 1 (IGF1) signaling and microRNA (miRNA) changes in bats. Once the molecular underpinnings of these mechanisms have been identified, they can be engineered in mice. For example, mice overexpressing the naked mole rat hyaluronan synthase gene can be generated. If these mouse models then show improved tumor resistance, pharmacological interventions can be developed to mimic the anticancer adaptations from cancer-resistant species in human patients. Question marks indicate anticancer adaptations for which the exact molecular mechanisms are unknown.