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Peto's Paradox and the Evolution of Cancer Suppression: Lessons From Flies, Humans, and Elephants

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by

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Dedication

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ABSTRACT OF THE DISSERTATION

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by

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An unsuspected finding in cancer research is that different cancers result from mutations in different genes. If cancer is a problem of multicellularity, why is there not a single set of genes regulating all cancers of multicellular animals? Cancer risk is expected to be higher in large, long-lived species since the risk of cancer-initiating somatic mutations increases with the number of lifetime cell divisions. However, this expected relationship is not observed, and has been termed Peto's Paradox. The evolution of additional mechanisms of cancer suppression in large/long-lived species can resolve this paradox and may explain the differences in the genetics of cancer between species and tissues. In a proof-of-concept experimental evolution study, fruit flies with hereditary tumors were selected to evolve suppression of these tumors. A significant reduction in the incidence of tumors was observed in the final populations. Next, I tested for evidence of the two forms of genetic changes that are likely to be involved in the evolution of cancer suppression. First, a cancer-suppression mechanism may be recruited by its up-regulation of gene expression in the target tissue. To address this, I compared the levels of gene

expression of 15 tumor suppressor genes and 8 proto-oncogenes across dozens of non-diseased human tissues. I found that 14 of the 23 genes have their highest level of expression in the tissue types where they are implicated in hereditary cancer, relative to those that are not. Second, additional mechanisms may arise by duplications of pre-existing tumor suppressor genes. The recent finding of additional retrogene copies of the critical tumor suppressor gene, *TP53*, in the African Elephant has been suggested to explain the low-rates of cancer in these species. However, in a phylogenetic comparison of the coding regions of these retrogene copies relative to the normal *TP53* copies in 24 mammals, I find that the additional copies in the elephant (and the long-lived bats) are truncated early by stop codons and are poorly conserved. Possible explanations for these findings are given. In conclusion, this work shows the importance of incorporating evolutionary theory into cancer biology for understanding the variation in cancer genetics.

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Introduction

In 1971, Richard Nixon declared the "War on Cancer." However, in reality, this war has been waging since the evolutionary transition to multicellularity. Cancer is a group of diseases unique to multicellular animals (Doonan & Sablowski, 2010) that is defined by unregulated cell division and the invasion of cells into other tissues (Hanahan & Weinberg, 2000). Despite the many advances of cancer research, preventing or treating the disease has proven difficult. Several unresolved questions raised by mechanistic approaches may benefit from the novel insights that evolutionary theory can offer (Greaves, 2001; Leroi et al., 2003; Crespi & Summers, 2005; Aktipis & Nesse, 2013).

One of the major goals of cancer research is to identify the nature and number of genetic alterations that initiate cancer. The mechanistic approach that pervades cancer research has yielded many successes on this front, such as the discovery of the tumor suppressor genes (TSGs) and proto-oncogenes (POGs) that initiate cancer when mutated, as well as unraveling the functions and molecular pathways of these genes (Vogelstein & Kinzler, 2004). However, an important question has been difficult to address under the mechanistic perspective: Why do the genetics of cancer differ between tissues and species? In other words, why do the genetic discoveries for one cancer type not necessarily apply to all others?

A number of observations in humans suggest that the nature and number of genes defending against cancer can differ across tissue types. It has been found that only a small subset of tissues are affected by inherited mutations in a given tumor suppressor or proto-oncogene, even though the mutation is present in all cells of the individual (Fearon, 1997).

For example, in humans, women that inherit mutations in *BRCA1* are at high risk of breast and ovarian cancer but have no added risk of cancer in many other tissue types (Montiero, 2003). This tissue-specificity of hereditary cancer suggests that the action of individual genes may be restricted to specific tissues. The number of genes involved in the suppression of cancer can also differ between tissues. For example, the initiation of retinoblastoma (cancer of retinal cells) requires two mutational "hits" (Knudson, 1971), leading to the loss of one TSG (*RB1*), while the simplest path to colorectal cancer requires seven "hits", involving the loss of three TSGs and the activation of a single POG (Fearon & Vogelstein, 1990). Understanding why these genetic differences exist can inform us as to why the applicability of a given gene-specific drug is limited to specific cancers.

The nature and number of genes controlling a specific cancer type have also been found to differ across species. For example, mice with inherited mutations of *Brca1* do not develop breast cancer, as do humans (Rangarajan & Weinberg, 2003). Similarly, relative to mouse fibroblast cells, human fibroblasts must incur mutations in four additional genes to be transformed in cell culture (Rangarajan et al., 2004). Our choice of animal model for studying specific cancers can be informed by understanding why these differences (and similarities) exist, and may lead to the discovery of novel therapeutic avenues in specific species (Tian et al., 2013). The application of evolutionary theory can help unify these observations and help formulate testable predictions.

Based on the earlier epidemiological findings that cancer initiates by somatic mutations in multiple loci (Armitage & Doll, 1954), Peto (1977) proposed that

vulnerability to cancer should increase with body size and/or lifespan across species. Since somatic mutations during cell division drive the initiation of cancer (Albanes & Winick, 1998), any trait that increases the number of lifetime cell divisions, such as increased body size (more cells; Savage et al., 2007) or longevity (more divisions/cell lineage, Lynch, 2010), should also increase the frequency of cancer (Peto, 1977). When viewed within a single species, it is evident that cancer incidence increases with lifespan and body size. Age-dependent cancer incidence curves show that the risk of cancer in humans increases exponentially with age (Nordling, 1953; Armitage & Doll, 1954). Furthermore, there is now strong support that cancer risk increases with body size (Nunney, 2013) based upon incidence data from dogs (Fleming et al., 2011) and humans (Green et al., 2011). However, when viewed across species, no correlation exists between body size, longevity, and cancer. This observation has become known as Peto's Paradox (Nunney, 1999). The paradox originated from the observation of a similar lifetime cancer risk in mice and humans (Peto, 1977), yet no correlation has been found with additional species added (Martineau et al., 2002; Nagy et al., 2007; Abegglen et al., 2015). On the basis of body size alone, if a species has 1,000 times as many cells (e.g. humans versus mice; Rangarajan & Weinberg, 2003) then a single somatic mutation should be 1,000 times more likely. The expected added risk in larger species is compounded further by the fact that generation time increases as $(\text{size})^{0.4}$ (Fenchel, 1974).

Nunney (1999) developed a dynamic model of anticancer adaptation that can resolve Peto's Paradox; this model also explains the discrepancy in the nature and number of genes suppressing cancer across tissue types and species. The model begins by

estimating the probability of developing cancer (p) based upon the average lifespan and body size of a given species:

$$p = 1 - [1 - \prod_{i=1\ldots M} (1 - \exp(-\ (1 + D_i)\mu_i K))\ ]^C$$

The parameters include cell number (C; measure of body size), cell divisions (K; measure of lifespan), somatic mutation rate (μ), the total number of mutational hits (M) needed, and whether a TSG that requires two-hits (D=1) or a POG that requires one-hit (D=0) is involved. The probability of cancer approximates the selection coefficient (s). If the strength of selection exceeds the effects of drift such that the inequality, $s > 1/Ne$, is satisfied then we expect that selection will act to minimize this cancer risk. The model shows that the number of TSG or POG loci suppressing cancer in a small tissue type of a small, short-lived species, such as one TSG locus (M=2) in a rat, is an inadequate level of control in the same tissue of a larger, longer-lived species such as humans. This predicts that larger, longer-lived species must evolve extra layers of TSGs or POGs (must increase M) to minimize their cancer risk.

An important corollary of the model is that, as size and/or longevity increases over evolutionary time, new cancer suppression mechanisms are expected to be recruited in an *ad hoc* fashion depending on the genetic variation available to minimize pre-reproductive cancer death. This suppression may involve either tissue-specific mechanisms that directly reduce the risk of the target cancer causing the greatest decline in fitness (e.g. a TSG up-regulated in a single tissue) or more general mechanisms affecting all tissues (e.g. global telomerase suppression) that would lower the risk of all

cancers. However, since the number of mechanisms is limited (e.g. the number of potential TSGs is relatively small), a gene that is functioning as a cancer suppressor in one tissue may be independently recruited in a second. This evolutionary process allows different genetic mechanisms to be recruited in different tissues and species independently over time.

However, does such genetic variation for the evolution of cancer suppression exist in natural populations? A direct proof-of-concept experiment is needed to address this question. In **Chapter 1**, I perform an experimental evolution study breeding *Drosophila melanogaster* stocks that possess hereditary melanotic tumors to evolve suppression of these tumors.

Nunney's (1999) model predicts that the typical mode of adaptive change, amino acid substitution, is less likely in the evolution of cancer suppression. Changes to a TSG's or POG's sequence do not circumvent the risk of gene knockouts resulting from the increased risk of somatic mutation. Instead the recruitment of additional genes in the target tissue is needed to minimize this risk.

Two possible mechanisms may give rise to the recruitment of additional genes in the target tissue. First, a TSG or POG that is acting to suppress cancer elsewhere, may be added to the target tissue by the upregulation of its gene expression level. This prediction may explain the cell-type specificity of hereditary cancer. For example, inherited mutations in *BRCA1* may pose a risk of cancer limited to breast and ovarian tissues because *BRCA1* has been recruited in these susceptible tissues to suppress cancer but has not been recruited for that role in non-susceptible tissues. This prediction is tested

empirically in **Chapter 2** by testing for higher expression levels of 15 TSGs and 8 POGs in the tissues susceptible to mutations of that gene relative to those that are not. Second, duplications of genes that are already acting to suppress cancer in the target tissue may be recruited. The genome of the large-bodied African Elephant has been found to contain 19 additional retrogene copies of the tumor suppressor *TP53* (Ableggan et al., 2015), which is involved in apoptosis and DNA repair. Up to five retrogene copies have also been found in the genomes of the long-lived microbats (Sulak et al., Unpublished), which can live up to to 41 years (Podlutsky et al., 2005). In **Chapter 3,** I test for signatures of functionality in the DNA sequences of these retrogene copies. We examine the retrogene copies for the presence of premature stop codons and test the rate of codon substitution in these copies relative to the normal *TP53* copies of 24 mammal species.

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Chapter 1

Experimental Evolution of Cancer Suppression in *Drosophila Melanogaster*

Abstract

An experimental evolution study was conducted in fruit flies, *Drosophila melanogaster*, to determine whether mechanisms that limit the occurrence of cancer can evolve in a laboratory population. Flies with hereditary melanotic tumors (Hop^{Tum} allele) were selected to suppress these tumors indirectly by selecting for longevity and late-life fecundity; the type of selection most likely to suppress cancer in the wild. At the start of the experiment, the fecundity of females and the lifespan of males were significantly lower in flies with tumors vs. those without tumors ($p < 0.05$), allowing an indirect selection protocol to be used. Flies were subsequently selected for longevity and late-life fecundity for between 20-30 generations. The fitness of flies significantly increased over the course of the experiment for lifetime fecundity ($p < 0.001$), lifespan ($p < 0.001$), and productivity ($p < 0.001$) measures. The selection achieved significant declines in the incidence of tumors ($p < 0.01$) and large tumors ($p < 0.001$) relative to flies that did not experience selection. We found that variation for cancer suppression was available in the initial stock population, such that this population responded to selection just as well as flies that had additional genetic variation added to them by outbreeding with wildtype populations and treatment with the mutagen EMS. This is the first study to demonstrate that cancer suppression can evolve in a natural population, and suggests that the evolution of cancer suppression is a dynamic process that may give rise to the differences in the genetics of cancer between tissues and species.

Introduction

Cancer suppression has been largely considered as a non-evolving, fixed property of the cells of multicellular animals. As such it is has been difficult to understand the finding that the genes involved in cancer can differ between species (Rangarajan & Weinberg, 2003) and tissue types (Bignold, 2004; Rangarajan et al., 2004). The multistage model predicts that cancer risk increases with both the number of cells in a tissue and the number of times they divide (Peto, 1977). Consistent with this, Albanes and Winnick (1988) argued that tall humans (with more dividing cells) showed elevated cancer rates. Recent data have established this as a reality (Green et al., 2011). However, there is a lack of such as a relationship among species varying in size and/or longevity, known as Peto's Paradox (Nunney, 1999). This is unsurprising since evolutionary theory predicts that additional mechanisms of cancer suppression are expected to evolve in large-bodied and long-lived organisms to lower the increase in cancer risk (Nunney, 1999, 2003, 2013). Support for this prediction has been found in a series of studies in rodents. In a phylogenetic analysis of 15 rodent species with variation in body size and lifespan, the additional mechanism of telomerase suppression was found to increase with body size in these species (Seluanov et al., 2007). In follow-up studies, the additional mechanisms of early-contact inhibition (Tian et al., 2013) and concerted necrotic cell death (Gorbonova et al., 2012) were found to have evolved in the long-lived naked mole rat and blind mole rat, respectively.

While such observations are compelling, a more direct "proof of concept" experiment is needed to establish evolutionary change directly. If a population is exposed

to an increased cancer risk that substantially reduces the average fitness of individuals, then natural selection will favor genetic variation that increases fitness by reducing the incidence of cancer. This outcome is inevitable provided that the appropriate genetic variation is present in the population. But does such variation typically exist in a population? If natural populations lack genetic variation for cancer suppression, this may pose constraints to the evolution of larger body sizes and longer lifespans (Galis & Metz, 2003).

To perform this experiment we used *Drosophila melanogaster* exhibiting melanotic tumors as our model system. Experimental evolution using *D. melanogaster* has been used successfully to investigate many complex traits. For example, Rose and Charlesworth (1981) showed that it was possible to substantially increase the lifespan of adult flies by artificial selection in the lab, and these selection lines are still being studied today (Burke et al., 2010).

D. melanogaster is too small and short-lived to be expected to be a natural victim of cancer. However, there has been a relatively recent realization that it has great potential for studying some of the fundamental problems of cancer biology (Vidal and Cagan, 2006). Specifically, there is a wide array of tools available for the study of this species, and many of the genes in *D. melanogaster* controlling the expression of tumors have homologs in mammals (see Halder & Mills, 2011). A variety of tumor types are observed in *D. melanogaster*, with the most common spontaneous neoplasm of adult flies being melanotic tumors of the lymph gland tissue (Harshbarger & Taylor, 1968). The lymph glands are one of the few tissue types that continue dividing in adult flies

(Pellettieri & Alvarado, 2007), which may explain their relatively high incidence. While invasive cancers can now be engineered in *Drosophila* (Rudrapatna et al., 2012), the occurrence of melanotic tumors due to single gene mutations has long been known. More than thirty genes can give rise to melanotic tumors if altered by germline mutations (Minakhina & Steward, 2006). This tumor class was selected for the study because the tumors are externally visible in adult flies as dark spots on the abdomen, head, and legs.

The aim of this study was to determine if genetic variation for tumor suppression existed in laboratory populations of *D. melanogaster* exhibiting melanotic tumors. The evolution of cancer suppression in nature would act through selection for increased fitness assuming that individuals with tumors have reduced survival and/or fecundity relative to those without tumors. Therefore, populations of flies exhibiting tumors were selected for lower cancer risk indirectly in this study by selecting for increased late-life fecundity, following the general approach adopted by Rose and Charlesworth (1981). Given the absence of prior information on the availability of genetic variation capable of suppressing tumors, the genetic background of some of the mutant populations was increased by outbreeding and/or by using a mutagen.

Materials and Methods

Overview

First, longevity and fecundity analyses were performed for three genetically different mutant stocks with hereditary melanotic tumors, to obtain a stock with a short lifespan and low fecundity. Second, within flies of the chosen stock, we assessed whether lower fecundity and a shorter lifespan were found in flies with tumors relative to flies

without tumors, to determine whether selection for late-life fecundity could indirectly select for lower tumor incidence. Third, populations fixed for a melanotic tumor inducing allele were subject to selection for late-life fecundity for ≥23 generations. Fourth, the effect of this selection on the fecundity and lifespan of flies and on tumor incidence was assessed. Finally, we investigated the possibility that there was genetic variation for cancer suppression present in the original mutant stock of flies that formed the basis of the selection experiment.

Preliminary Fecundity Tests used for Stock Selection

Three fly stocks with the melanotic tumor phenotype (all X-linked) were obtained from the Bloomington Stock Center: Hop^{Tum} (*Hopscotch* gene; Stock #8492), P{lacW}$Zfrp8^{k13705}$ (*Zfrp8* gene; Stock #12199), and $tu(1)Sz^{1}$ (*tu(1)Sz* gene; Stock #5834). The first two stocks were maintained as balanced lethals, with the FM7c and the CyO balancer X chromosomes, respectively. To determine if these mutations shortened lifespan and reduced fecundity, the age-specific fecundity of homozygous mutant females was assessed for each stock. Heterozygous Hop^{Tum} females were also assessed, since some tumors are expressed in heterozygotes. Fecundity was measured by pairing 4 mutant females with 4 hemizygous mutant males of the same stock in two replicate vials. To compare the fitness of the females while controlling for the fitness of males, a second set of 2 replicate vials were established, each with 4 homozygous mutant females of each stock paired with 4 wildtype males from a wild-caught population (Mayo, CA). Flies were transferred into new vials every 5 days to lay until all females were dead. Males were not replaced since each vial contained multiple males. Lifetime fecundity was

calculated as the number of adult offspring produced by all females in each vial over the course of their lifespan. The lifespan of females was scored in 5-day intervals, as their maximum possible age in the vial in which they died (e.g. flies that died in the first vial were scored as having a 5-day lifespan). The lifespan of each of the four females per vial were used as replicate measures.

Longevity and lifetime fecundity differences between each stock was examined. All statistical tests were performed in the statistical program R (R Development Core Team, 2008). An ANOVA model was used to determine the differences in lifespan between the stocks: Lifespan = Female Genotype (Homozygous mutants of each stock, plus Hop^{Tum} heterozygotes). A follow-up Tukey HSD post-hoc test was performed. An additional ANOVA model was used to determine the differences in lifetime fecundity between the female genotypes tested: Lifetime Fecundity = Female Genotype (4 genotypes) + Male Genotype (Mutant, Wildtype) + Female Genotype x Male Genotype. A follow-up Tukey HSD post-hoc test was performed to test for significant differences between the female genotypes.

Other stocks used in the experiment were populations maintained as large outbred bottle populations in the lab. Gala and Mayo were described in Enders and Nunney (2010). Riverside was an outbred population field collected in the UCR orange groves, Riverside, CA in spring 2013.

Effect of Tumors on Fitness

The stock with the shortest average lifespan was chosen for all further experiments. However, it was necessary to establish that the low fitness observed in the

chosen stock was linked to the presence of melanotic tumors, indicating that selection for longevity and late-life fecundity had the potential to indirectly select for tumor suppression. Using three separate lines of flies that were outbred (described later) to carry novel wildtype autosomal genetic variation, the effect of tumors on fitness was analyzed in age-specific fecundity tests performed separately for females and males (Table 1.1). Melanotic tumors are visible by eye as dark spots present on the abdomen, head, and legs allowing homozygous mutant females from each line to be subdivided into flies with and without tumors. These females were paired individually with a male from the wildtype Riverside stock. Similarly, hemizygous mutant males from each line were subdivided into flies with no tumors, small tumors (all tumors < 0.5mm), and large tumors (one or more tumors > 0.5mm), and paired individually with a Riverside female. Flies were transferred into new vials every 5 days to lay until the mutant fly died. Wildtype males that died were replaced each transfer. Wildtype females were not replaced. Female mutant flies that did not produce offspring in the first two vials were assumed to be unmated and were removed from the study. This criterion removed one female with tumors from lines 2 and 3 and had no material effect on the results (data not shown).

The effect of tumors on lifetime fecundity, and its component measures of lifespan and productivity, was analyzed in females and its effect on lifespan was analyzed in males. Lifetime fecundity was calculated as the number of adult offspring produced by each female over the course of their life. Lifetime fecundity was subdivided into independent productivity (average fecundity per 5-day vial) and lifespan measures. The lifespan of male and female flies was scored as their maximum possible age in the vial in

which they died (e.g. flies that died in the first vial were scored as a 5-day lifespan). A square root transformation of lifetime fecundity was used to improve normality (e.g. see Nunney & Cheung, 1996). For females, a one-tailed ANOVA model was performed for each of the three fitness measures: Fitness Measure = Tumor State (Presence vs. Absence) + Line (3 Lines) + Tumor State x Line. For males, an ANOVA model was performed for lifespan: Lifespan = Tumor State (No Tumors, Small Tumors, Large Tumors) + Line (3 Lines) + Tumor State x Line. The effect of line was treated as a random factor. We tested for significant differences between the tumor states in males using a Tukey HSD post-hoc test.

Selection Experimental Design

Four different treatment groups of mutant flies were established in a 2x2 design. In half of the treatments the mutant stock flies were outbred to add novel wildtype variation (vs. no outbreeding in the other half), and each of these groups was split so that half were treated with the mutagen ethyl methyl sulfonate (EMS) and half received no EMS. This created the four treatments fixed for the melanotic tumor allele: OUTBMUT (outbred background); OUTBMUT+EMS (outbred plus EMS exposure); STCKMUT (unmodified genetic background); and STCKMUT+EMS (stock background plus EMS exposure). Two treatment groups of wildtype flies using the Riverside stock were also used in the study: a LATEWILD treatment was selected for longevity and late-life fecundity in a way that matched the selection imposed on the mutant lines; and a CTRLWILD treatment, which did not experience selection for longevity and late-life fecundity. We used 5 replicate lines for the four mutant treatments and two lines for the

two wildtype treatments. All lines were maintained in two panmictic bottles to allow high population sizes at the start of each generation (>500).

In generations 1-4, to establish the populations, all flies were kept on 17 day generation cycles. Adults of each line were split equally into two bottles where they laid eggs for 3 days before being removed. Fourteen days later the next generation was started by mixing all flies from the two replicate bottles into one bottle. They were then anesthetized (with CO_2) prior to splitting them in half (based on weight) to establish two new laying bottles.

In later generations, flies still surviving and laying at a later age were favored. Thus, beginning in generation 5, adult flies of each mutant line were allowed to lay for 5 days in their two initial bottles, after which they were mixed, split into two roughly equal groups (based on weight), and transferred into two fresh laying bottles. After each 5 days of laying the procedure was repeated until the surviving population of flies was less than 100 flies, at which point the transfers were terminated to avoid inbreeding. The offspring from the final pair of bottles was used to initiate the next generation. This procedure selected for the approximately 20% of flies in each line that had the greatest late-life fecundity. The weight criterion for the mutant lines was based on 4 samples of 100 flies from the OUTBMUT flies (0.075gm ± 0.013 sd).

Also beginning generation 5, while the CTRLWILD populations continued on the 17-day generation cycle, the LATEWILD treatment was selected for longevity and late-life fecundity in step with the OUTBMUT or OUTBMUT+EMS line that had the greatest longevity (i.e. the final LATEWILD laying bottles rearing the next generation of

flies was the same age as the bottles from the OUTBMUT or OUTBMUT+EMS line that survived the longest with a population size >100). This was done to assess the effect of the selection regime on age-specific fecundity by comparing the results of the LATEWILD and CTRLWILD treatments (described later). The weight of wild-type flies was also measured each transfer as a proxy for body size to ensure that they were not experiencing inbreeding under their selection regimes. The weight of four samples of 100 CTRLWILD flies was calculated as 0.113gm ± 0.005.

The selection process was run for 23 generations in the OUTBMUT, OUTBMUT+EMS, and LATEWILD lines, and for 27 generations in the STCKMUT and STCKMUT+EMS lines. Since the STCKMUT and STCKMUT+EMS lines were not able to reach the same age thresholds as the other treatments they underwent more generations. Flies were maintained at 25°C for the first 10 generations (12 generations for STCKMUT/STCKMUT+EMS), but after that, because the lines of the STCKMUT and STCKMUT+EMS treatments were difficult to maintain at 25°C due to their low fitness, all lines were shifted to a lower (room) temperature of approximately 23.5°C.

Scheme of Genetic Crosses used to Add Genetic Variation

To increase the amount of background genetic variation associated with the mutant expression of tumors, additional autosomal variation was added to the mutant stock to establish the OUTBMUT and OUTBMUT+EMS treatments. Autosomal genetic variation was introgressed from three outbred wildtype populations, while the balancer X chromosome was used to keep the mutant-stock X chromosome intact. The introgression involved a series of four crosses resulting in experimental lines predicted to carry 56.25%

of the autosomal variation of the wildtype populations (Table 1.2). We used 100 male-female pairs each generation. This number of pairs met the criterion that given a hypothetical autosomal SNP at a frequency of 0.01 in the initial wildtype populations, the final outbred population would have a chance ≥99% of obtaining at least 1 copy of this SNP (assuming Hardy-Weinberg ratios). Five mutant male and five homozygous mutant female offspring were taken from each of the 100 vials of the final outbreeding cross to generate an initial pool of 500 males and 500 females.

Treatment with Ethyl Methanesulfonate (EMS)

Since the natural level of genetic variation capable of contributing to tumor suppression was unknown, we included EMS treatment to increase the chance of rare beneficial mutations. In generations 1 and 9, EMS was applied to the STCKMUT+EMS and the OUTBMUT+EMS treatments in the following way. The first 100 males that eclosed (approximately ~40% of all males used in the next generation) from the STCKMUT+EMS and OUTBMUT+EMS lines were treated with 2.5μM EMS (see Keightley & Ohnishi, 1998) using the protocol of Lewis and Bacher (1968). EMS has been demonstrated to alkylate guanine residues leading to G to A transitions (Sega, 1993), which is the most common mutation caused by EMS. At concentrations lower than 10μM of EMS deletions or translocations, which are often deleterious, have never been observed (Sega, 1993).

Age-Specific Fecundity Tests

At generations 1, 9, and 23, a sample of females from each of the lines was assessed for age-specific fecundity to quantify the response to the selection regime. To

control for the effect of larval density on the longevity and fecundity of the adult test flies, the females used were reared at constant densities of 50 larva/vial. To collect these larvae, adults of each line from the preceding generation were placed in bottles to lay eggs on food media within petri dishes. The larvae were subsequently transferred to vials.

In generations 9 and 23, all lines were tested (five replicate lines of the four mutant treatments, STCKMUT, STCKMUT+EMS, OUTBMUT, OUTBMUT+EMS, and two replicate lines of the two wildtype treatments, CTRLWILD, LATEWILD) using 10 females (generation 9) or 5 females (generation 23) per line. The females were collected as virgins and paired with a single Riverside male when <2 days old. The males were 2-4 days old. The pairs were placed in separate vials at 25°C, and transferred to new vials every 5 days. Males that died were replaced at the start of the next five-day period. Transfers continued until the female died.

The generation 1 test was set up differently because the parental generation 0 had not been split into STCKMUT vs. STCKMUT+EMS, OUTBMUT vs. OUTBMUT+EMS, and CTRLWILD vs. LATEWILD, nor were the replicate lines established. Thus 10 females from the offspring of generation 0 STCKMUT and OUTBMUT populations were randomly assigned to each of the 5 replicate lines of STCKMUT and STCKMUT+EMS, and of OUTBMUT vs. OUTBMUT+EMS, respectively. Similarly, 7 (replicate 1) or 8 (replicate 2) females were randomly assigned to CTRLWILD and LATEWILD. The test flies were reared, collected, and tested as for the later generations.

The response to the selection regime was assessed by comparing the fitness of flies across generations 1, 9, and 23 for the outbred mutant and wildtype treatments

(OUTBMUT, OUTBMUT+EMS, CTRLWILD, LATEWILD) and generation 1, 9 and 27 for the mutant treatments that were not outbred (STCKMUT, STCKMUT+EMS), noting that generation 27 of these stocks was contemporaneous with generation 23 of the others. The data collected on age-specific fecundity was subdivided into three measures of fitness: lifetime fecundity and its component measures, lifespan, and productivity (defined above).

The mutant treatments were examined to determine if the treatments had caused changes in any of the three fitness measures over time. The initial ANCOVA used the model: Fitness Measure = EMS (no-EMS, EMS) + Background (outbred, not outbred) + Generation (covariate: generation 1, 9, and 23 or 27) + Line(EMS x Background) + EMS x Background + EMS x Generation + Background x Generation + Line(EMS, Background) x Generation + EMS x Background x Generation. Line was treated as a random effect nested within EMS and Background.

The presence or absence of EMS treatment was involved in significant interactions with Background and Generation, complicating the interpretation of the main effects, so the analysis was split by EMS, testing the no EMS treatments (STCKMUT, OUTBMUT) and EMS treatments (STCKMUT+EMS, OUTBMUT+EMS) separately. The simplified model used was: Fitness Measure = Background (outbred, not outbred) + Generation (covariate: generation 1, 9, and 23) + Line(Background) + Background x Generation + Line(Background) x Generation. The significance level necessary was halved for these tests (e.g. 5% significance required $p < 0.025$) to adjust for multiple

testing. Tukey HSD post-hoc tests were performed to test for significant differences between each of the six (3x2) generation-by-treatment groupings

Comparing the two wildtype treatments, we tested whether the different treatment regimes affected fitness components. ANCOVA were performed for each of the three fitness measures using the model: Fitness Measure = Treatment (CTRLWILD, LATEWILD) + Generation (covariate: generation 1, 9, and 23 or 27) + Line(Treatment) + Treatment x Generation + Line(Treatment) x Generation. Line was treated as a random effect nested within treatment.

Effect of Selection on Tumor Incidence

The frequency of tumors in the two mutant populations (STCKMUT and OUTBMUT) at the start of the experiment was assessed from a sample of 126 females and 126 males from each. The OUTBMUT flies were obtained directly from vials as offspring of the final cross adding genetic variation (see Table 1.2). STCKMUT flies were obtained from the stock population (Stock #: 8492).

The effect of the selection regime on the incidence of tumors was examined by comparing the frequency of tumors in populations of flies that experienced selection relative to flies that had not. In order to compare the incidence of tumors under the same controlled conditions selected and unselected flies needed to be synchronized. For the selected lines, this involved comparing the STCKMUT and STCKMUT+EMS treatments of generation 24 to the OUTBMUT and OUTBMUT+EMS treatments of generation 20, and for the unselected reference lines, it involved using new populations of outbred (OUTBNEW) and stock mutant (STCKNEW) flies.

The STCKNEW treatment was established by expanding a population of stock mutants that had been maintained on constant generation cycles into five separate lines. The OUTBNEW treatment was re-created from the STCKNEW population using the same protocol for adding genetic variation performed for the OUTBMUT/OUTBMUT+EMS treatments in the beginning of the experiment (Table 1.2). To remove the effect of genetic drift experienced by the OUTBMUT and OUTBMUT+EMS treatments, which were in their 20^{th} generation of selection, in their comparison to the OUTBNEW treatment, the OUTBNEW treatment was inbred commensurately. Five separate lines were created for the OUTBNEW treatment by breeding three male-female pairs individually for each line and mixing the resulting offspring. This inbreeding scheme was commensurate with the inbreeding experienced by the OUTBMUT/OUTBMUT+EMS treatments, which had an approximate effective population size of ≥100 for 20 generations. The effective population size of the OUTBNEW ($Ne_{,0}$) required to replicate the effect of genetic drift experienced by the OUTBMUT and OUTBMUT+EMS treatments ($Ne_{,20}$) was calculated as: $1/(2Ne_{,0}) = 1-(1-1/(2Ne_{,20}))^{20}$. The effective population size to be applied to the OUTBNEW population ($Ne_{,0}$) was calculated as 5.24 flies, which was approximated by three male-female pairs. The OUTBNEW lines were expanded over the next three generations to establish a larger population size to begin the experiment.

Tumor incidence was measured in the five lines of these six different treatments. Flies were reared at constant larval densities of 50 larva/vial using the same collection protocol performed for the age-specific fecundity tests. Two replicate vials of 50 larva

were reared at the two temperatures, 26.0°C and 28.5°C, for each of the 5 lines of the six treatments, since the frequency of tumors has been found to increase at higher temperatures (Hanratty & Dearolf, 1993). The number of flies per treatment scored for tumors ranged from 705-762 flies. Each fly was scored for tumors as: no tumors, small tumors (all tumors small, < 0.5mm), or large tumors (one or more large tumors, >0.5mm).

The incidence of tumors across treatments was analyzed in two ways, as the presence vs. absence of tumors (no tumors vs. small or large tumors), and as the presence vs. absence of large tumors (no tumors or small tumors vs. large tumors). The proportion of flies in each vial with tumors (or only large tumors) was arcsin square root transformed in all analyses to improve normality.

Since EMS was not applied to the two control treatments, the effect of EMS on tumor incidence was assessed separately in the four treatments that experienced selection. A three-way ANOVA was run to assess the effects of EMS, genomic background, and temperature: Incidence = EMS (no EMS, EMS) + Background (outbred, not outbred) + Temperature (26.0°C, 28.5°C) + Line(EMS, Background) + EMS x Background + EMS x Temperature + Background x Temperature + Line(EMS, Background) x Temperature + EMS x Background x Temperature. The effect of lines was treated as a random effect nested within EMS and Background.

EMS presence or absence was involved in a significant interaction with temperature making it difficult to interpret main effects. Therefore, in the comparisons of flies from lines that did and did not experience selection, the analysis was split by EMS. Separate ANOVA models were performed to compare incidence between the no-EMS

treatments (STCKMUT, OUTBMUT) and the EMS treatments (STCKMUT+EMS, OUTBMUT+EMS) that experienced selection relative to the treatments that did not experience selection (STCKNEW, OUTBNEW). The flies from each vial tested for the STCKNEW and OUTBNEW treatments were randomly assigned into two groups to allow independent comparisons to the flies that experienced selection in the separate ANOVAs. The ANOVA models used to assess the effect of selection, genomic background, temperature, and lines (nested with treatment) were: Incidence = Selection (selection, no selection) + Background (outbred, not outbred) + Temperature (26.0°C, 28.5°C) + Line(Selection x Background) (5 lines) + Selection x Background + Selection x Temperature + Background x Temperature + Line(Selection x Background) x Temperature + Selection x Background x Temperature. The effect of lines was treated as a random effect nested within EMS and Background. The significance level was adjusted for the double testing (see above).

The interaction between EMS and temperature was investigated further to determine if differential survival of larvae at the high and low temperature could account for the pattern. An ANCOVA model was run across treatments and temperatures on Fly Count, the number of the 50 larva/vial that survived to adulthood (and hence were scored for tumors). Tumor incidence was used as a covariate in the model to determine the relationship between fly count and tumor incidence: Fly Count = Incidence + Treatment (6 treatments) + Temperature (26.0°C, 28.5°C) + Line(Treatment) (5 lines) + Treatment x Temperature + Line(Treatment) x Temperature. Line was nested within treatment and was treated as a random effect.

To determine whether the incidence of tumors in the control populations used in the above experiment (OUTBNEW, STCKNEW) reflected the incidence of tumors at the start of the experiment, we compared their incidence to the initial populations of flies (G0 OUTBMUT, G0 STCKMUT). Two 2x2 chi-square tests were performed to compare the incidence of tumors between the OUTBNEW and G0 OUTBMUT flies and the STCKNEW and the G0 STCKMUT flies.

Effect of Adding Genetic Variation on Tumor Incidence

To test the hypothesis that the initial mutant stock had, over its history, evolved some degree of tumor suppression, crosses were performed to compare the incidence of tumors between flies that had the original genetic background and those that had different degrees of novel background derived from the Riverside outbred stock (Table 1.3). In addition, the crosses allowed a preliminary check of the inheritance of tumors (by comparing the offspring of partially outbred mutant males with or without large tumors) and an evaluation of the effect of maternal inheritance on tumor incidence. A new order of the same mutant line (#8492) was purchased from the stock center to begin this experiment.

In the parental crosses, 15 individual male-female pairs were established in vials for each of the three types of crosses (Table 1.3). The six vials that had the most similar density of offspring for each cross type were examined for tumor incidence. In the 2nd generation crosses, nine pairs of mutant flies (all offspring of the previous "Not Outbred" cross) were set up, as were five pairs of the backcross with the male parent having no

tumors (2 males) or small tumors (3 males), and six pairs of the backcross with the male parent having at least one large tumor.

Tumor incidence in offspring was assessed using the same protocol as described for the previous experiment (see above), and compared among crosses as defined in Table 1.3 using a chi-squared test based on both (1) presence vs. absence of tumors and (2) presence vs. absence of large tumors. Each comparison was assessed using an F ratio test computed from the ratio of the main effect chi-square (1df) to the residual chi-square due to the heterogeneity among vials, divided by its dfs. The main effect chi-square (X_M^2) was calculated from a 2x2 table of counts of flies with and without tumors (or large tumors) between the two comparison groups (e.g. outbred vs. not outbred). An overall chi-square (X_T^2), was obtained from the 2xN test of all N replicates (across both groups), and the residual chi-square was the difference between this value and the main effect value. The F-test was therefore: F = X_M^2 / (($X_T^2 - X_M^2$) / ($df_T - 1$)) with 1,(df_T-1) df.

Results

To obtain a stock with a low initial fitness suitable for selection for longevity and late-life fecundity, the age-specific fecundity and survival of females of three stocks with the melanotic tumor phenotype was measured at 25.0°C (Figure 1.1). The average lifespan and lifetime fecundity of all melanotic tumor stocks were lower than values typical of wildtype female flies (which are about 40 days and 300 offspring at 25°C; Nunney & Cheung, 1996). The average lifespan of females was found to be significantly different between the four female genotypes tested ($F_{(3,48)} = 22.00$; $p < 0.001$). A Tukey HSD post-hoc test found the average lifespan of Hop^{Tum} homozygous females (8.8 ± 0.9

(s.e) days) to be significantly lower than Hop^{Tum} heterozygous females (35.0 ± 3.1 days; $p < 0.001$), $tu(1)Sz^{1}$ homozygous females (35.6 ± 6.9 days; $p < 0.001$), and Zfrp8 homozygous females (31.3 ± 7.2 days ; $p < 0.001$). The same results were found for lifetime fecundity as with average lifespan ($F_{(3,8)} = 20.32$; $p < 0.001$). A Tukey HSD post-hoc test found the lifetime fecundity of a Hop^{Tum} homozygous female (10.6 ± 3.5 (s.e) days) to be significantly lower than a Hop^{Tum} heterozygous female (142.4 ± 16.0 days; $p < 0.001$), a $tu(1)Sz^{1}$ homozygous female (118.8 ± 23.9 days; $p < 0.005$), and a Zfrp8 homozygous female (78.0 ± 17.5 days ; $p < 0.01$). No significant effect of male genotype and no interaction between female and male genotype was found. The Hop^{Tum} stock was selected for the study.

Next, we examined whether the lower fitness of this stock was linked to the presence of tumors, which would facilitate the indirect selection protocol for longevity and late-life fecundity. The effect of tumors on fitness was examined separately for female and male flies from three lines of the OUTBMUT treatment. In females, lifetime fecundity and its component measures of productivity and lifespan were compared in separate one-tailed ANOVAs between females with tumors and those without. We found lifetime fecundity to be significantly lower in females with tumors, 229.4 ± 49.5 offspring, relative to those without tumors, 166.3 ± 13.3 offspring ($F_{(1,88)} = 2.85$; $p < 0.05$) (Figure 1.2). No significant effect of line and no significant interaction of tumor state and line were found. Productivity (per 5 day period) was found to be significantly lower in females with tumors, 35.7 ± 8.3 offspring, vs. those without tumors, 25.7 ± 3.9 offspring ($F_{(1,88)} = 2.99$; $p < 0.05$). A significant effect of line was found ($F_{(1,88)} = 9.83$; $p < 0.005$),

but there was no significant interaction between tumor state and line. However, the average lifespan of females with tumors, 32.8 ± 7.8 days, was not significantly different from flies without tumors, 33.6 ± 2.8 days ($F_{(1,88)} = 0.52$; $p = 0.47$). Again, a significant effect of line was found ($F_{(1,88)} = 18.8$; $p < 0.001$), with no significant interaction between tumor state and line.

In males, the average lifespan of flies with large tumors, small tumors, and no tumors was found to significantly differ between the three tumor states ($F_{(2,91)} = 3.79$; $p < 0.05$). A Tukey HSD post-hoc test found average lifespan to be significantly lower in flies with large tumors, 27.8 ± 1.1 days, relative to flies with small tumors, 36.5 ± 2.0 days ($p < 0.05$), and no tumors, 36.8 ± 3.0 days ($p < 0.05$) (Figure 1.3). No significant difference in average lifespan was found between flies with small tumors and no tumors.

The Hop^{Tum} populations were subjected to a selection protocol for late-life fecundity for 23 (OUTBMUT/OUTBMUT+EMS) or 27 generations (STCKMUT/STCKMUT+EMS). Since the STCKMUT/STCKMUT+EMS lines were less fit than the OUTBMUT/OUTBMUT+EMS lines, they had a shorter generation time and experienced a greater number of generations. A shift towards greater longevity was observed over the course of the experiment, with an average shift of approximately 3.5 days every four generations in the OUTBMUT and OUTBMUT+EMS lines up to generation 20, and of approximately 1.8 days every four generations in the STCKMUT and STCKMUT+EMS lines (Figure 1.4). A decline in the age at laying was observed for generations 21-23 in the OUTBMUT and OUTBMUT+EMS lines after our tests for the effect of selection on tumor incidence.

The effect of selection for longevity and late-life fecundity was measured more accurately using age-specific fecundity tests during generation 1, generation 9, and generation 23 (or generation 27 for STCKMUT/STCKMUT+EMS lines) of the experiment. The effect of selection on lifetime fecundity, and its component measures, lifespan and productivity, were examined in three ANCOVA models that were performed separately for the mutant (Table 1.4) and wildtype treatments (Table 1.5). No significant effects were found in the comparison of the wildtype controls (CTRLWILD) with the wildtype lines that were matched to the selection imposed on the mutant lines (Table 1.5; Figure 1.5).

In mutant flies, no significant effect of EMS (no EMS, EMS treated) was found for all three fitness measures, yet significant EMS x Background (not outbred, outbred) and EMS x Background x Generation (covariate: generation 1, 9, 23 or 27) interactions were found (data not shown). The analysis for mutant flies was subsequently divided into separate ANCOVA models for no-EMS and EMS mutant flies. The results of these models are shown in Table 1.4A for the two no-EMS treatments and in Table 1.4B for the two EMS treatments.

All fitness measures were found to significantly increase with generation for the no-EMS and EMS treatments (Table 1.4). All treatments had significantly higher fitness for all measures in the last generation tested (generation 23 or 27) relative to generation 1 and 9, while the outbred treatments also achieved significant increases between generation 1 and 9 (Figure 1.5). In the no-EMS treatments, lifetime fecundity was found to be significantly higher in the STCKMUT treatment vs. the OUTBMUT treatment

(main effect: Background), 74.4 ± 13.2 vs. 64.4 ± 6.9 offspring per female ($p < 0.025$), respectively (Table 1.4A). However, lifespan was significantly higher in the OUTBMUT vs. STCKMUT treatment, 15.9 ± 0.8 vs. 12.9 ± 0.9 days ($p < 0.001$), respectively. In the EMS treatments, a significant effect of Background was found for all three measures. Fitness was significantly higher in OUTBMUT+EMS flies relative to STCKMUT+EMS flies for lifetime fecundity, 103.3 ± 6.8 vs. 55.1 ± 6.3 offspring per female ($p < 0.001$), lifespan, 19.6 ± 1.4 vs. 11.9 ± 0.6 days ($p < 0.001$), and productivity measures, 19.4 ± 0.4 vs. 12.8 ± 0.8 offspring per 5 days ($p < 0.001$), respectively (Table 1.4B). Significant Generation x Background interactions were found for lifetime fecundity and lifespan. These interactions were due to the OUTBMUT+EMS treatment having a greater increase in fitness than the STCKMUT+EMS treatment in the last generation tested relative to generation 9, despite undergoing fewer generations of selection (generation 23 vs. 27, respectively) (Figure 1.5).

The effect of selection for longevity and late-life fecundity on the incidence of tumors and large tumors was analyzed in the four mutant treatments relative to two control treatments that never experienced selection (STCKNEW, OUTBNEW). An initial ANOVA model testing the effect of EMS on tumor incidence in the flies that experienced selection found no main effect of EMS, yet a significant EMS x Temperature interaction ($F_{(1,40)} = 7.35$, $p < 0.01$) was found. This interaction was driven by the EMS treatments (STCKMUT+EMS, OUTBMUT+EMS), which, unlike the no-EMS treatments and the control treatments (not examined in the above ANOVA), incidence was found to be higher at 26.0°C relative to 28.5°C (see Figure 1.6). Therefore, the analysis was split by

EMS to compare the incidence of tumors in no-EMS treatments (STCKMUT, OUTBMUT) and EMS treatments (STCKMUT+EMS, OUTBMUT+EMS) separately to the two control populations (STCKNEW, OUTBNEW).

The incidence of tumors and large tumors in the no-EMS lines (Figure 1.7A, B) and the EMS treated lines (Figure 1.7C, D) was found to be significantly lower in the flies that experienced selection for all models tested (see Table 1.6). The EMS treated lines were again found to respond differently to temperature (see Figure 1.6) as indicated by a significant Selection x Temperature interaction for tumors ($p < 0.05$) and large tumors ($p < 0.05$) (see Table 1.6). Additionally, a significant Selection x Background interaction was found for the incidence of large tumors ($p < 0.05$; see Table 1.6), which was driven by a higher tumor incidence in the OUTBNEW vs. STCKNEW treatment and a lower tumor incidence in the OUTBMUT+EMS vs. STCKMUT+EMS treatment (see Figure 1.7D). No large tumors were found in 2 of the 5 lines of the OUTBMUT and OUTBMUT+EMS treatments, and in 3 of the 5 lines of the STCKMUT treatment.

We examined whether the reversed temperature effect of tumor incidence observed in EMS treated lines could have been due differential effects of larval survival across treatments and temperatures, which in turn could influence tumor frequencies if larval fitness and adult tumor incidence were linked. The results of the ANOVA model comparing the fly count (number of the 50 larvae per vial that survived to adulthood and were scored for tumors) between the six treatments and two temperatures is shown in Table 1.7. No significant relationship between the incidence of tumors and fly count was found. However, fly count was significantly lower at 28.5°C vs. 26.0°C, 34.0 ± 1.0 vs.

38.8 ± 0.8 flies, respectively ($p < 0.001$; see Table 1.7), and did significantly differ among treatments ($p < 0.05$). A significant Treatment x Temperature effect was also found ($p < 0.005$), which is shown in Figure 1.8. This interaction was driven primarily by the drop in fly count at 28.5°C for the STCKNEW treatment.

The contemporaneous comparison of STCKNEW and OUTBNEW with the selected lines controlled for environmental factors influencing tumor expression in the tested flies; however, it is possible that uncontrolled genetic differences could have influenced the result (since the selected and "new" lines were created at different times. To address this issue, we compared the incidence of tumors found in the contemporaneous experiment to the incidence of tumors obtained at the beginning of the experiment (Figure 1.9). The incidence of tumors in OUTBMUT and STCKMUT flies at the start of the experiment was significantly higher than in the newly generated NEW-OUTBNEW and STCKNEW control lines used in the final experiment. Tumor incidence was 83.3% vs. 11.7% ($X_1^2 = 464.18$, $p < 0.001$) for OUTBMUT flies at the start of the experiment relative to OUTBNEW flies, and 57.9% vs. 8.9% ($X_1^2 = 260.82$, $p < 0.001$) for STCKMUT flies at the start of the experiment relative STCKNEW flies, respectively.

Finally, the possibility that some genetic suppression was already present in the stock flies at the start of the experiment was tested by examining the effect of adding novel genetic variation on the incidence of tumors. The preliminary incidence data collected before selection was applied suggested that adding genetic variation increased the incidence of tumors. The incidence of tumors was 83.3% in OUTBMUT flies vs. 57.9% in STCKMUT flies at the start of the experiment and 11.7% in OUTBNEW vs.

8.9% in STCKNEW (see Figure 1.9). To this end the crosses and comparisons defined in Table 1.3 were performed. In the first generation of crosses, it was found that the incidence of tumors, and most especially of large tumors, was higher in F1 males that were 50% outbred relative to males derived from mutant stock parents and hence 0% outbred ($p < 0.001$ for both; Table 1.8). Relative to the stock flies, the incidence of tumors increased in the outbred flies from 79.7% to 97.7% and of large tumors from 28.7% to 85.7% (Figure 1.10). The incidence of tumors and large tumors in 0% outbred females, which were not included in the analysis, was very similar to the 0% outbred males (79.5%, and 79.7%, respectively). In the second generation of crosses, no difference was found in the backcross males and females that were 25% outbred relative to flies that were not outbred for the incidence of tumors ($p = 0.93$) and large tumors ($p = 0.29$). Additionally, there was no evidence of a maternal effect, or that males with large tumors (vs. none or small tumors) passed that characteristic to their offspring (Table 1.8; Figure 1.10).

Discussion

In this study, lines homozygous for the mutant allele Hop^{Tum}, which dramatically increases the incidence of melanotic tumors in adult flies, were selected for late-life fecundity for ≥ 23 generations. The four different types of line were examined (STCKMUT, STCKMUT+EMS, OUTBMUT, OUTBMUT+EMS) and all responded to this selection with increased longevity and increased productivity (Figure 1.5). Furthermore, the selected lines showed a significant reduction in the expression of melanotic tumors (Figure 1.7), consistent with the finding that the presence of tumors,

especially large tumors, generally reduced fitness (Figures 1.2 & 1.3). These observations strongly support the hypothesis that genetic variation enabling tumor suppression exists and was present in all lines.

Over the course of the selection experiment, a significant increase in lifetime fecundity, lifespan, and productivity was found for all four mutant treatments. However, no difference in these measures was found for wildtype flies, demonstrating that the selection did not automatically increase fitness, as measured by lifetime fecundity and its components of longevity and productivity. Increases in these three fitness measures were found to differ between flies that were outbred and those that were not. Outbred mutant flies (OUTBMUT, OUTBMUT+EMS) achieved an increase in fitness in generation 9 (vs. generation 1) and in generation 23 (vs. generation 9). However, an increase in fitness for the flies that were not outbred (STCKMUT, STCKMUT+EMS) was not achieved until generation 27. This result was supported by the finding that the age at which flies were able to lay in the transfer bottle (with a population size >100) that was used to generate the next generation, was shifted to later ages at a greater rate in flies that were outbred. An average shift of 3.5 days every four generations was achieved in outbred flies (OUTBMUT and OUTBMUT + EMS) and 1.8 days every four generations in flies that were not outbred (STCKMUT and STKMUT+EMS). These findings support the expectation that additional variation for fitness was present in the outbred compared to the stock flies. No significant effect of EMS on fitness was found indicating that the two treatments in generation 1 and 9 did not markedly increase the relevant genetic variation.

For the selection regime to influence tumor suppression, it was necessary that the presence of tumors reduced fitness. We found the lifetime fecundity of females with tumors to be significantly lower than females without tumors, an effect driven by significant differences in productivity (adult offspring per 5 days) (Figure 1.2). No effect of tumors on average lifespan was found in female flies, but males flies with large tumors had a significantly shorter average lifespan (Figure 1.3). This effect of large tumors may explain the observation that 7 of the 20 lines of flies that experienced selection had no large tumors while all lines of the control populations retained large tumors.

The incidence of tumors was found to be significantly lower at the end of the experiment for the no-EMS ($p < 0.01$) and EMS treatments ($p < 0.01$) that experienced selection for longevity and late-life fecundity relative to control populations of flies that did not experience selection. The difference in tumor incidence between the selected and control populations was more significant when the incidence of large tumors was examined alone ($p < 0.001$ for both).

The true magnitude by which selection lowered tumor incidence may not have been captured in the above experiment, which compared incidence between flies that experienced selection relative to newly generated control populations under the same conditions. The incidence of tumors in STCKMUT and OUTBMUT treatments at the start of the experiment was much higher than in the STCKNEW (83.3% vs. 11.7%, $p < 0.001$) and the OUTBNEW (57.9% vs. 8.9%, $p < 0.001$) treatments used in the above experiment.

Contrary to our expectation, we did not find a significant effect of genomic background (outbred vs. not outbred) on tumor incidence following more than 20 generations of selection for late-life fecundity. This result suggests that variation for tumor suppression was already present in the initial population of stock mutants (STCKMUT), a view supported by the finding that the incidence of tumors was found to increase upon adding new genetic variation from wildtype populations to the stock population. At the beginning of the experiment the incidence of tumors was higher in OUTBMUT flies that were 56% outbred relative to the 0% outbred STCKMUT population (83% vs. 58% tumorous, respectively). This effect was examined in more detail in experimental crosses. F1 males that were 50% outbred had a significantly higher incidence relative to comparably reared males of the mutant stock in both the incidence of tumors (98% vs. 80%) and of large tumors (86% vs. 29%). The stock females, which were not included in the analysis, had an incidence of tumors identical to the males. Furthermore, the backcross of the F1 to the mutant stock resulted in offspring with a level of tumors equal to the mutant stock (Figure 1.10). The F1 result is consistent with the presence of one or more recessive suppressor alleles, and the backcross suggests the involvement of multiple loci. For example, a single locus fixed for a recessive suppressor allele in the stock population would result in a halving of the average level of suppression in the backcross, since half of the offspring would be heterozygotes (and show more tumors), but half would by homozygotes and exhibit the stock level of suppression.

Evidence that the variation revealed in these crosses was fixed in the parental stock was provided by a comparison of the offspring of the two types of backcross (Table

1.3). The male F1 parents sired offspring with the same incidence of tumors regardless of whether they had no/small tumors or had large tumors. This result indicated that all F1 males were genetically identical with respect to alleles affecting the expression of the melanotic tumors.

The incidence of tumors in Hop^{Tum} mutants has been found to be ameliorated by nine recessive alleles of seven different genes (Bina et al., 2010; Bausek & Zeidler, 2014). Alleles at these loci may have been fixed in the initial stock population (STCKMUT), favored by their effect of increasing individual fitness of the stock flies. An alternative possibility is that the wildtype populations were fixed for dominant alleles that enhance the expression of melanotic tumors. Dominant alleles at five different genes (Bina et al., 2010; Bausek & Zeidler, 2014) have been shown to exacerbate the incidence of tumors. However, there is no apparent reason why such alleles would have become fixed in these recently wild caught stocks.

The effect of EMS (no-EMS vs. EMS treatment) on tumor incidence was found to depend on temperature (Figure 1.6). For all treatments that were not treated with EMS, we found the incidence of large tumors increased with temperature, as has been found previously for this melanotic tumor stock (Hanratty and Dearolf, 1993; Luo et al., 1995). However, in flies treated with EMS, the incidence of tumors was lower at 28.5°C than at 26.0°C. This relationship between EMS and temperature was not found to be driven by the EMS treatment having fewer larva survive to adulthood and scored for tumors at 28.5°C relative to the other treatments. The reversed pattern seen in the temperature

response of the EMS treated lines (Figure 1.6) could have been the result of EMS-induced genetic variation interacting with temperature.

The flies of the Hop^{Tum} melanotic tumor stock that were used in the study were found to have significantly shorter lifespans and lower lifetime fecundity than melanotic tumor stocks with mutations of the Zfrp8 and tu(1)Sz genes. The hopscotch (Hop) gene is an ortholog of the human developmental gene, Janus Kinase (JNK), and is classified as a semi-dominant proto-oncogene since the tumor phenotype is expressed to a lesser degree in heterozygous females (Sorrentino et al., 2002). The Hop^{Tum} allele was created by EMS (Hanratty & Dearolf, 1993) and contains a single amino acid substitution caused by a G to A transition (Harrison et al., 1995; Luo et al., 1995). The resulting neoplasms have been characterized as having increased numbers of hemolymph cells known as lamellocytes.

In conclusion, this is the first experimental evolution study to test whether a population could be selected to suppress the incidence of tumors. This was achieved by selecting indirectly on tumor incidence via selection for longevity and late-life fecundity; the type of selection that is most likely to act on tumor incidence in the wild. We found that variation for cancer suppression was available in the initial stock population, such that this population responded to selection just as well as flies that had additional genetic variation added to them by outbreeding with wildtype populations and treatment with the mutagen EMS. Our results suggest that cancer suppression should not be considered as a fixed property of the cells of multicellular animals. Instead, the results support the view that cancer suppression is a dynamic evolutionary process that may occur at different

times and in different lineages if the incidence of tumors poses a significant decline in pre-reproductive fitness (Nunney 1999, 2003, 2013). Additional work is needed to identify the mechanisms of suppression that evolved to counteract the risk of melanotic tumors in this model system. More generally, understanding the mechanisms that have evolved to suppress cancer in lineages where expansions in body size and lifespan is expected to result in elevated cancer rates are likely to significantly increase our understanding of how cancer can be prevented.

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Tables and Figures

Table 1.1: **Experimental set-up for testing the effect of tumors on fly fitness.** The age-specific fecundity of females with tumors and without tumors, and males with no tumors, small tumors, and large tumors, was assessed for three lines of the OUTBMUT treatment. The number of individual flies assessed for age-specific fecundity is shown.

		Females		**Males**		
Treatment	*Line*	*# with Tumors*	*# without Tumors*	*# with Large Tumors*	*# with Small Tumors*	*# without Tumors*
OUTBMUT	1	20	10	9	10	8
OUTBMUT	2	19	20	10	9	20
OUTBMUT	3	12	12	9	6	16

Table 1.2: **Scheme of genetic crosses used to create the OUTBMUT/OUTBMUT+EMS treatments.** Four generations of crosses were performed to add autosomal genetic variation from wild-type males to a lineage of females from the mutant stock. Two loci are represented: the tumor gene of interest with "-" for the mutant allele and "+" for the wild-type and the Bar gene, where "B" represents the dominant Bar mutation marking the balancer X chromosome (with b representing the wild type allele). The parental cross was repeated 3 times with males from: (1) Riverside; (2) Mayo; and (3) Gala outbred populations, with female parents from the mutant stock. The resulting daughters (cross 1) or sons (crosses 2 & 3) were then used as parents as shown. In each subsequent cross, the female parents were daughters of the previous generation cross (shown in light gray). The proportion of genetic variation that is outbred on the maternal and paternal chromosomes (along with a total percent outbred value) is given for all of the progeny genotypes that are of interest for each cross. The offspring from the fourth generation used in the selection experiment are outlined with a thick black border. The balancer chromosome suppressed X recombination in females.

Cross Type	Female parent	Male parent	Offspring	Maternal Chromo-somes	Paternal Chromo-somes	Percent Outbred
Parental Cross (Gen 1)	+B/-b	+b/Y 1. Riv 2. Mayo 3. Gala	+B/+b	+B (0%)	+b (100%)	50%
			-b/Y	-b (0%)	Y (100%)	50%
			-b/+b			
			+B/Y			
Gen 2 Cross	+B/+b (from 1)	+b/Y (Riv)	+B/+b	+B (50%)	+b (100%)	75%
			+b/+b			
			+b/Y			
			+B/Y			
Gen 3 Cross	+B/+b	-b/Y (from 2)	+B/-b	+B (75%)	-b (50%)	62.5%
			+b/Y			
			-b/+b			
			+B/Y			
Gen 4 Cross	+B/-b	-b/Y (from 3)	-b/-b	-b (62.5%)	-b (50%)	56.25%
			-b/Y	-b (62.5%)	Y (50%)	56.25%
			-b/+B			
			+B/Y			

Table 1.3: **Experimental procedure for testing the effect of novel genetic variation on the incidence of tumors.** The incidence of tumors in the Hop^{Tum} tumor stock (m/m, m/Y) was compared to F1 and backcross flies from two generations of crosses involving the wildtype Riverside stock (+/+, +/Y). The relevant offspring of the crosses are letter-coded to define the comparisons of tumor incidence performed in each generation, and, in the parental cross, to show which male offspring were used to set up the backcrosses. In the backcrosses, the inheritance of tumors was assessed by comparing the incidence of tumors in offspring (labeled as: (2) and (3)) resulting from crosses where the parental males (C) were subdivided into flies with no or small tumors versus large tumors.

Parental Cross

F1: Not Outbred	F1: 50% Outbred; Maternal Effect	F1: 50% Outbred; No Maternal Effect
$\frac{m}{m} \; x \; \frac{m}{Y}$	$\frac{m}{m} \; x \; \frac{+}{Y}$	$\frac{+}{+} \; x \; \frac{m}{Y}$
↓	↓	↓
$\frac{m}{m}, \frac{m}{Y}$ (A)	(B) $\frac{m}{+}, \frac{m}{Y}$ (C)	(D) $\frac{m}{+}, \frac{+}{Y}$

Effect of Outbreeding: m/Y (A) vs. m/Y (C)
Maternal Effect (in heterozygotes): +/m (B) vs. +/m (D)

2nd Generation

F2: Not Outbred	Backcross: 25% Outbred None or Small Tumors in Male Parents	Backcross: 25% Outbred Large Tumors in Male Parents
$\frac{m}{m} \; x \; \frac{m}{Y}$ (A)	$\frac{m}{m} \; x \; \frac{m}{Y}$ (C)	$\frac{m}{m} \; x \; \frac{m}{Y}$ (C)
↓	↓	↓
(1) $\frac{m}{m}, \frac{m}{Y}$ (1)	(2) $\frac{m}{m}, \frac{m}{Y}$ (2)	(3) $\frac{m}{m}, \frac{m}{Y}$ (3)

Effect of Outbreeding: (1) vs. (2) and (3)
Inheritance of Tumors: (2) vs. (3)

Table 1.4: **The effect of generation and genomic background on the fitness of mutant flies.** Three separate ANCOVA models were performed for three measures of fitness: lifetime fecundity, and its component measures of lifespan and productivity. The analysis was split by EMS: (A) the no-EMS treatments (STCKMUT, OUTBMUT); and (B) the EMS treatments (STCKMUT+EMS, OUTBMUT+EMS). Fitness measures were examined across generation (covariate: generation 1, 9, 23 or 27), by background (not outbred, outbred), and by line nested within background (5 lines). Significance levels (* 5%; ** 1%; *** 0.1%) were corrected for double testing across the no-EMS and EMS treatments.

A) no-EMS Treatments

		Lifetime Fecundity		Lifespan		Productivity	
	Df	*F*	*p*	*F*	*p*	*F*	*p*
Generation	1	407.69	2.2E-16 ***	197.49	2.2E-16 ***	382.14	2.2E-16 ***
Background	1	5.59	0.019 *	16.26	7.5E-5 ***	1.07	0.30
Line(Background)	8	1.65	0.11	2.03	0.044	0.95	0.48
Generation x Background	1	1.08	0.30	2.22	0.14	2.34	0.13
Generation x Line(Background)	8	2.19	0.029	1.68	0.10	2.50	0.013 *

B) EMS Treatments

		Lifetime Fecundity		Lifespan		Productivity	
	Df	*F*	*p*	*F*	*p*	*F*	*p*
Generation	1	327.13	2.2E-16 ***	157.68	2.2E-16 ***	245.37	2.2E-16 ***
Background	1	47.51	5.2E-11 ***	67.00	1.9E-14 ***	17.97	3.2E-5 ***
Line(Background)	8	0.75	0.65	2.42	0.016 *	0.25	0.98
Generation x Background	1	8.71	0.0035 **	5.39	0.021 *	3.12	0.079
Generation x Line(Background)	8	1.09	0.37	1.33	0.23	1.16	0.32

Table 1.5: **The effect of generation and treatment on the fitness of wildtype flies.** Three separate ANCOVA models were performed for three measures of fitness: lifetime fecundity, and its component measures of lifespan and productivity. Fitness measures were examined across generation (covariate: generation 1, 9, 23 or 27) for the two wildtype treatments (CTRLWILD, LATEWILD). The effect of line nested within treatment (2 lines) was also examined.

		Lifetime Fecundity		**Lifespan**		**Productivity**	
	Df	*F*	*p*	*F*	*p*	*F*	*p*
Generation	1	1.71	0.19	0.89	0.35	0.72	0.40
Treatment	1	0.53	0.47	0.77	0.38	0.039	0.84
Line(Treatment)	2	0.53	0.59	2.09	2.09	0.089	0.91
Generation x Treatment	1	0.0037	0.95	0.48	0.48	0.060	0.81
Generation x Line(Treatment)	1	0.55	0.58	0.059	0.56	2.55	0.084

Table 1.6: Effect of treatment and temperature on the incidence of tumors and large tumors. The incidence of flies that experienced selection relative to the control treatments (STCKNEW, OUTBNEW) was compared separately for the two no-EMS treatments (STCKMUT, OUTBMUT) in A) and for the two EMS treatments (STCKMUT+EMS, OUTBMUT+EMS) in B). ANOVA models were run separately on the arcsin-sqrt transformed incidence of tumors and large tumors. The effect of temperature (26.0°C, 28.5°C) and line nested within treatment (5 lines) was examined. Significance levels (* 5%; ** 1%; *** 0.1%) were corrected for double testing across the no-EMS and EMS treatments.

1.6A) ANOVA model of no-EMS Treatments

		Tumors		Large Tumors	
	Df	*F*	*p*	*F*	*p*
Selection	1	12.12	0.0012 **	35.22	5.8E-7 ***
Background	1	3.27	0.078	5.03	0.031
Temperature	1	2.10	0.16	2.65	0.11
Line(Selection x Background)	16	0.28	0.99	0.80	0.68
Selection x Background	1	2.35	0.13	1.18	0.28
Selection x Temperature	1	0.72	0.40	0.11	0.74
Background x Temperature	1	0.15	0.70	0.01	0.99
Line(Selection x Background) x Temperature	16	0.40	0.97	0.72	0.76
Selection x Background x Temperature	1	0.11	0.74	0.02	0.89

1.6B) ANOVA model of EMS Treatments

		Tumors		Large Tumors	
	Df	*F*	*p*	*F*	*p*
Selection	1	8.31	0.0063 *	30.73	2.1E-6 ***
Background	1	0.01	0.94	0.01	0.96
Temperature	1	0.82	0.37	0.05	0.83
Line(Selection x Background)	16	2.28	0.018 *	1.90	0.051
Selection x Background	1	0.53	0.47	6.30	0.016 *
Selection x Temperature	1	5.49	0.024 *	6.35	0.016 *
Background x Temperature	1	0.38	0.54	1.39	0.25
Line(Selection x Background) x Temperature	16	2.08	0.031	1.61	0.11
Selection x Background x Temperature	1	1.17	0.29	0.87	0.36

Table 1.7: **The effect of treatment and temperature on larval survival.** Larval survival was the number of the 50 larva per vial (2 vials per Treatment-Temperature combination) that survived to adulthood and were scored for tumors. An ANCOVA model was run using tumor incidence as a covariate. The effect of treatment (6 Treatments), temperature (26.0°C, 28.5°C), and line nested within treatment (5 lines) was examined.

	Df	*F*	*p*
Incidence	1	1.26	0.27
Treatment	5	2.47	0.042 *
Temperature	1	47.91	3.7E-9 ***
Line(Treatment)	24	1.96	0.019 *
Treatment x Temperature	5	4.03	0.0033 **
Temperature x Line(Treatment)	24	0.56	0.94

Table 1.8: **The effect of adding new genetic variation on the incidence of tumors.** Likelihood ratio tests were performed separately for the incidence of tumors and large tumors. The effect of outbreeding was compared in male offspring (0% vs. 50% outbred); A vs. C, Table 1.3) and in male and female offspring (25% outbred vs. 0% outbred). A maternal effect on tumor incidence was examined in heterozygous females (B vs. D, Table 1.3) and the inheritance of tumors was examined in male and female offspring of backcrosses.

	Tumors			**Large Tumors**		
	Df	*F*	*p*	*Df*	*F*	*p*
Effect of 50% Outbreeding (F1 males)	1, 10	32.54	0.0002 ***	1, 10	30.49	0.0003 ***
Effect of 25% Outbreeding	1, 18	0.0090	0.93	1, 18	1.21	0.29
Maternal Effect (on heterozygous females)	1, 10	0.94	0.36	1, 10	0.0040	0.95
Inheritance	1, 9	0.026	0.88	1, 9	0.55	0.38

Figure 1.1: **Age-specific fecundity of mutant females from three melanotic tumor stocks.** The average fecundity of four females is shown at five day intervals of their lifespan. Three stocks with mutant alleles were investigated: Hop^{Tum}, $tu(1)Sz^{1}$, and $P\{lacW\}Zfrp8^{k13705}$ (labeled as: Zfrp8). Homozygous (labeled as: Hom) and heterozygous (labeled as: Het) females were assessed for the Hop^{Tum} stock, and only homozygous mutant females were investigated for the other two stocks. Females from each stock were paired with mutant males or with wildtype males (labeled as: x Wildtype).

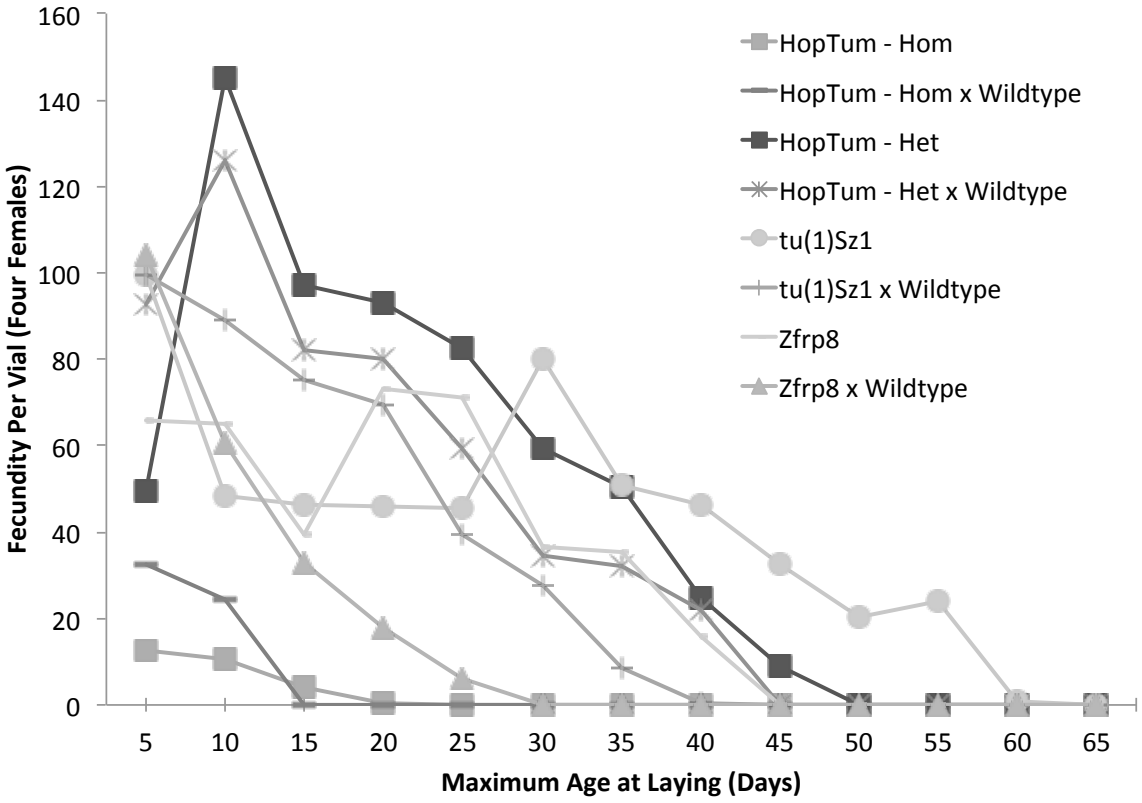

160
140
120
100
80
60
40
20
0
Fecundity Per Vial (Four Females)
5
10
15
20
25
30
35
40
45
50
55
60
65
Maximum Age at Laying (Days)
HopTum - Hom
HopTum - Hom x Wildtype
HopTum - Het
HopTum - Het x Wildtype
tu(1)Sz1
tu(1)Sz1 x Wildtype
Zfrp8
Zfrp8 x Wildtype

Figure 1.2: **Summary of the effect of tumors on the lifetime fecundity of mutant females.** The lifetime fecundity (± 1 s.e.) is shown for the three lines of OUTBMUT females for flies without tumors (black) vs. flies with tumors (gray). Females were paired with wildtype males.

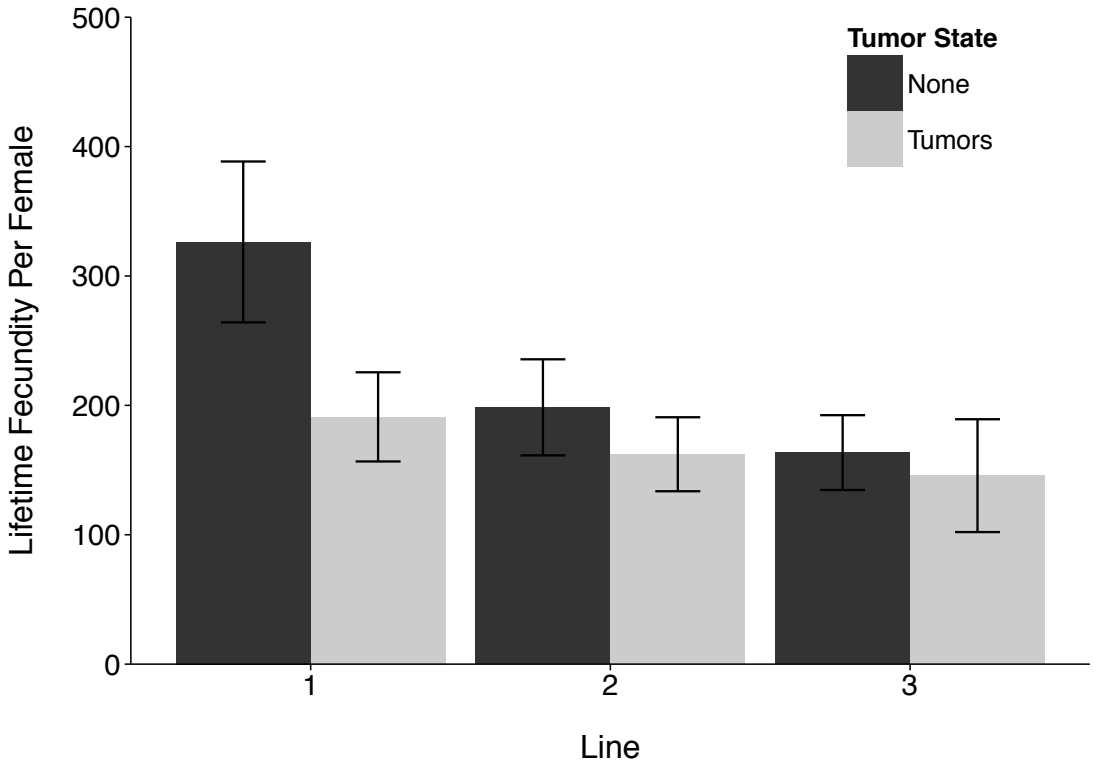

Tumor State
None
Tumors
Lifetime Fecundity Per Female
500
400
300
200
100
0
1
2
3
Line

Figure 1.3: **Summary of the effect of tumors on the average lifespan of mutant males.** The average lifespan (± 1 s.e.) is shown for the three lines of OUTBMUT males for flies without tumors (black), with small tumors (dark gray), and with large tumors (light gray).

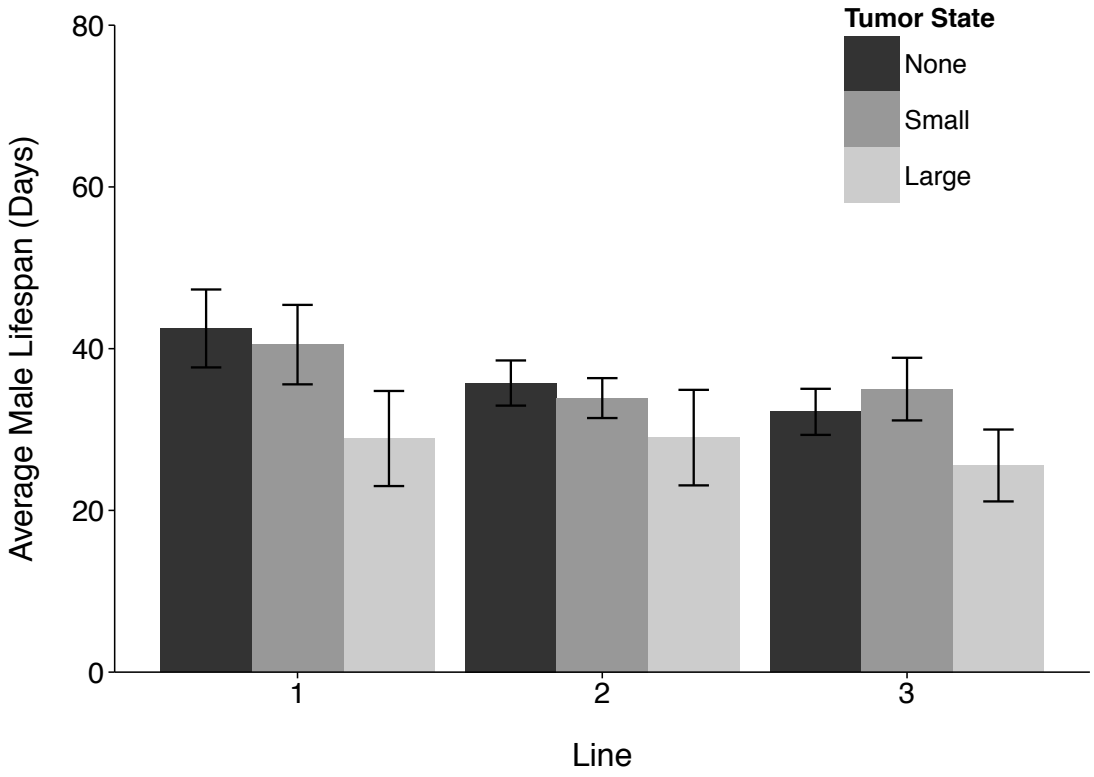

Tumor State
None
Small
Large
Average Male Lifespan (Days)
80
60
40
20
0
1
2
3
Line

Figure 1.4: **The effect of selection for increased longevity and late-life fecundity by generation.** The age of flies (± 1 s.d.) used to produce the next generation is shown and corresponds to the maximum age of flies in the transfer bottle in which fly numbers were reduced to ~100 (from ~500). Data for the OUTBMUT/OUTBMUT+EMS lines (combined) and STCKMUT/STCKMUT+EMS (combined) are shown. The "OUTBMUTs" were not selected beyond generation 23. Flies were transferred to new bottles every 4-6 days.

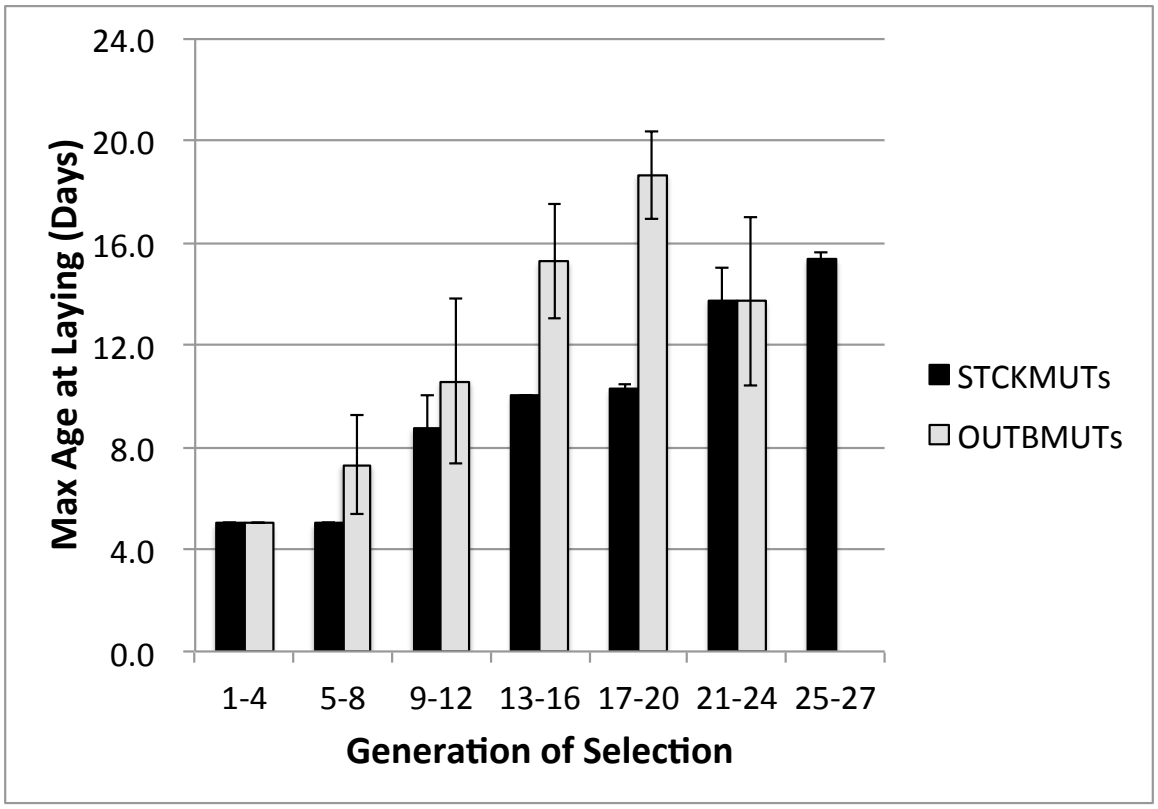
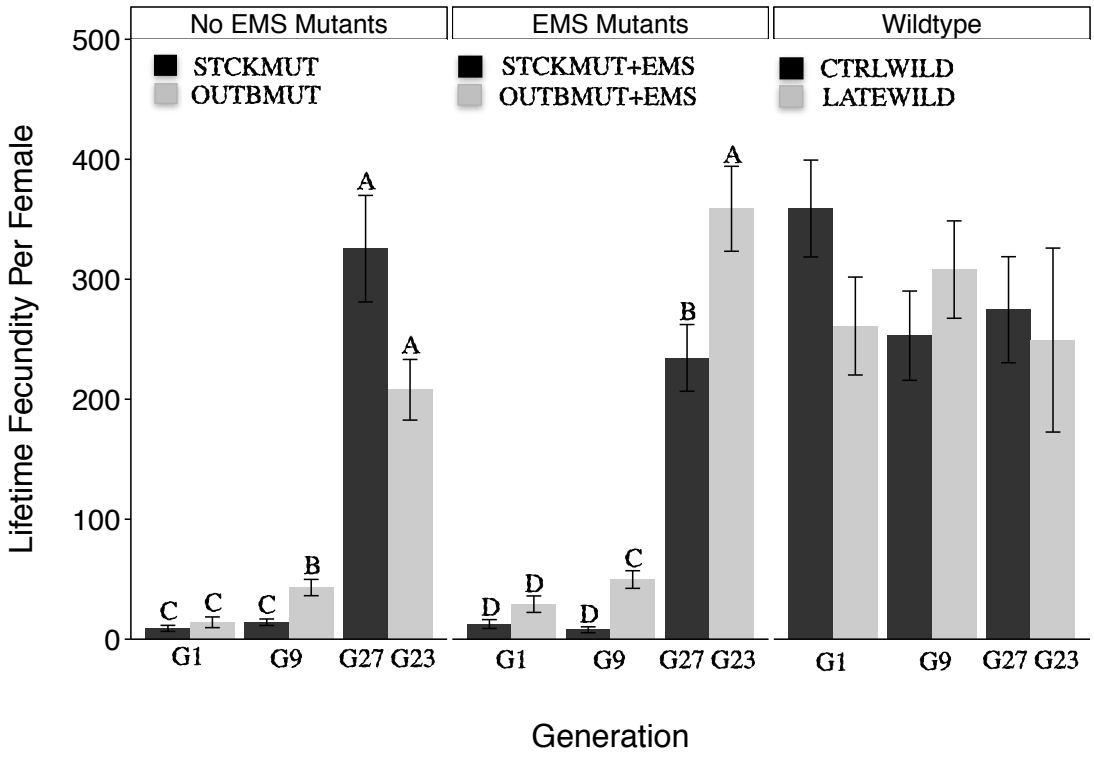
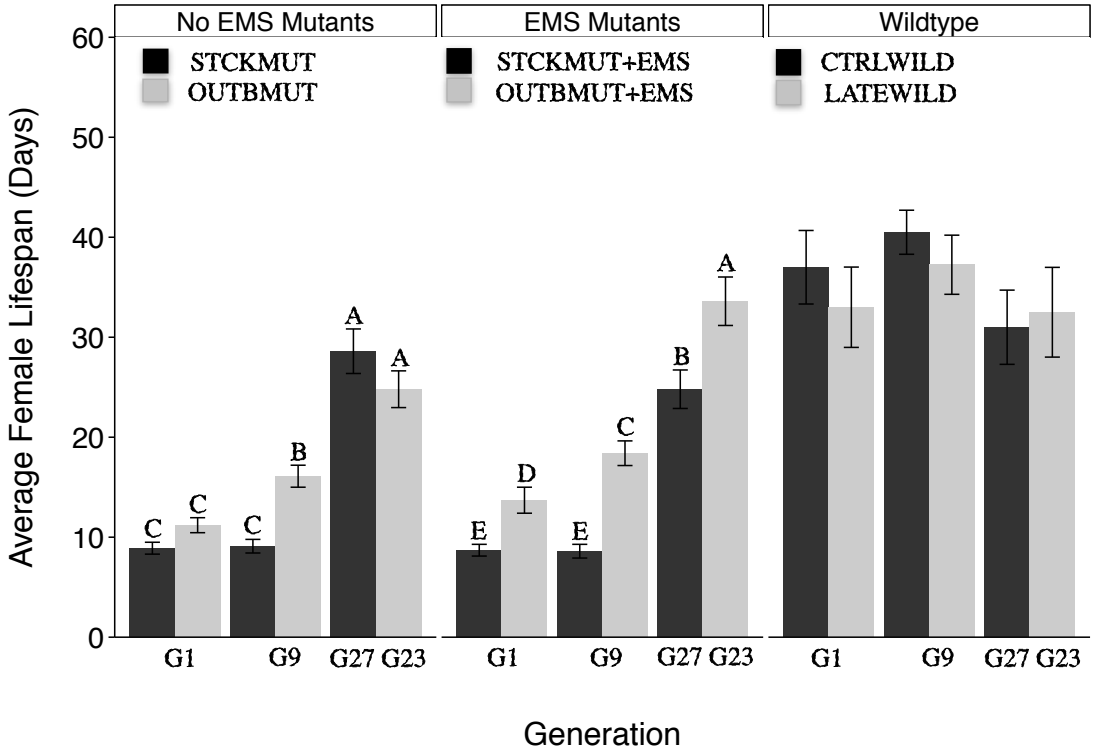

24.0
20.0
16.0
12.0
8.0
4.0
0.0
Max Age at Laying (Days)
1-4
5-8
9-12
13-16
17-20
21-24
25-27
Generation of Selection
STCKMUTs
OUTBMUTs

Figure 1.5: **The effect of selection for longevity and late-life fecundity on fitness by generation.** The effect of selection on (A) lifetime fecundity, and its components (B) lifespan, and (C) productivity (per 5 days) (all ± 1 s.e. of line means). "No EMS mutants" (STCKMUT, OUTBMUT), "EMS mutants" (STCKMUT+EMS, OUTBMUT+EMS), and "Wildtype" (CTRLWILD, LATEWILD) were analyzed separately. The change in fitness across generations 1, 9, and 23 (or 27) is shown. The significance categories of the Tukey HSD post-hoc tests are shown for significant ANOVA results.

A) Lifetime Fecundity



B) Lifespan


No EMS Mutants
EMS Mutants
Wildtype
STCKMUT
OUTBMUT
STCKMUT+EMS
OUTBMUT+EMS
CTRLWILD
LATEWILD
Average Female Lifespan (Days)
0
10
20
30
40
50
60
C
C
C
B
A
A
E
D
E
C
B
A
G1
G9
G27 G23
G1
G9
G27 G23
G1
G9
G27 G23
Generation

C) Productivity

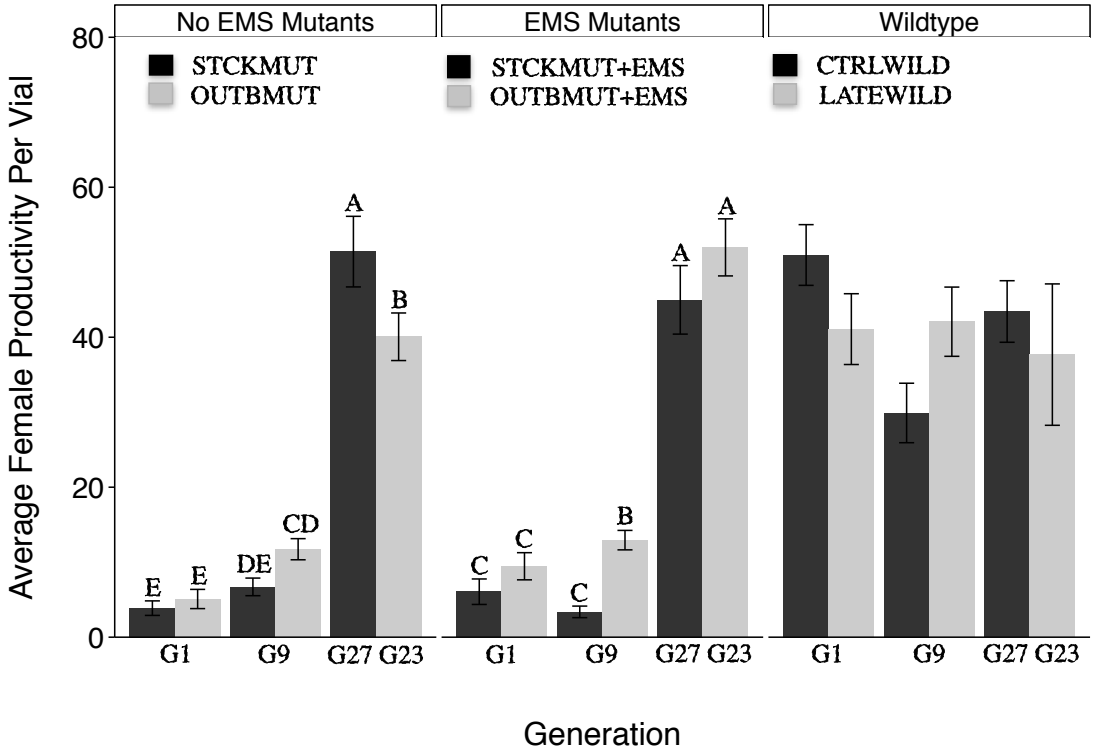


Figure 1.6: Interaction of temperature and treatment on tumor incidence. The Treatment (6 Treatments) x Temperature (26.0°C, 28.5°C) interaction is shown for the incidence of tumors (± 1 s.e.). The treatments in the legend are ordered by the incidence values at 28.5°C (Highest to Lowest).

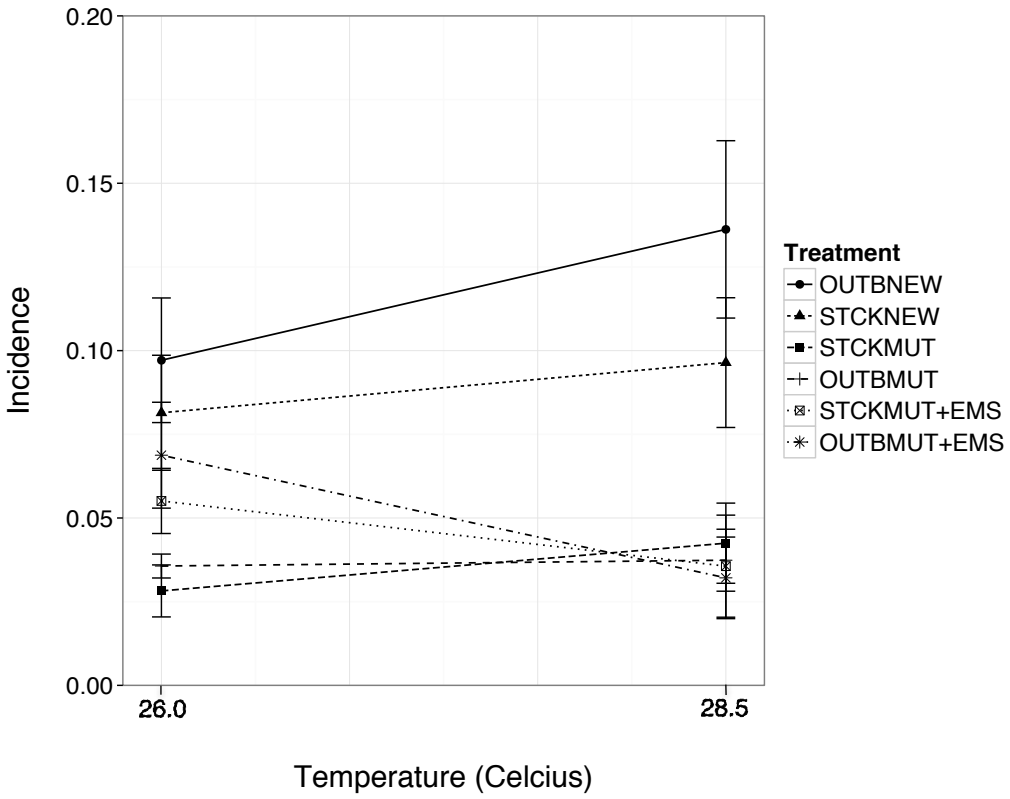

0.20
0.15
0.10
0.05
0.00
Incidence
26.0
28.5
Temperature (Celcius)
Treatment
OUTBNEW
STCKNEW
STCKMUT
OUTBMUT
STCKMUT+EMS
OUTBMUT+EMS

Figure 1.7: **The effect of selection and genomic background on the incidence of tumors and large tumors.** The incidence of tumors and large tumors (± 1 s.e. of line means) in flies that experienced selection relative to the control treatments (STCKNEW, OUTBNEW) is shown for the two no-EMS treatments (STCKMUT, OUTBMUT) in A) and B), respectively, and for the two EMS treatments (STCKMUT+EMS, OUTBMUT+EMS) in C) and D), respectively.

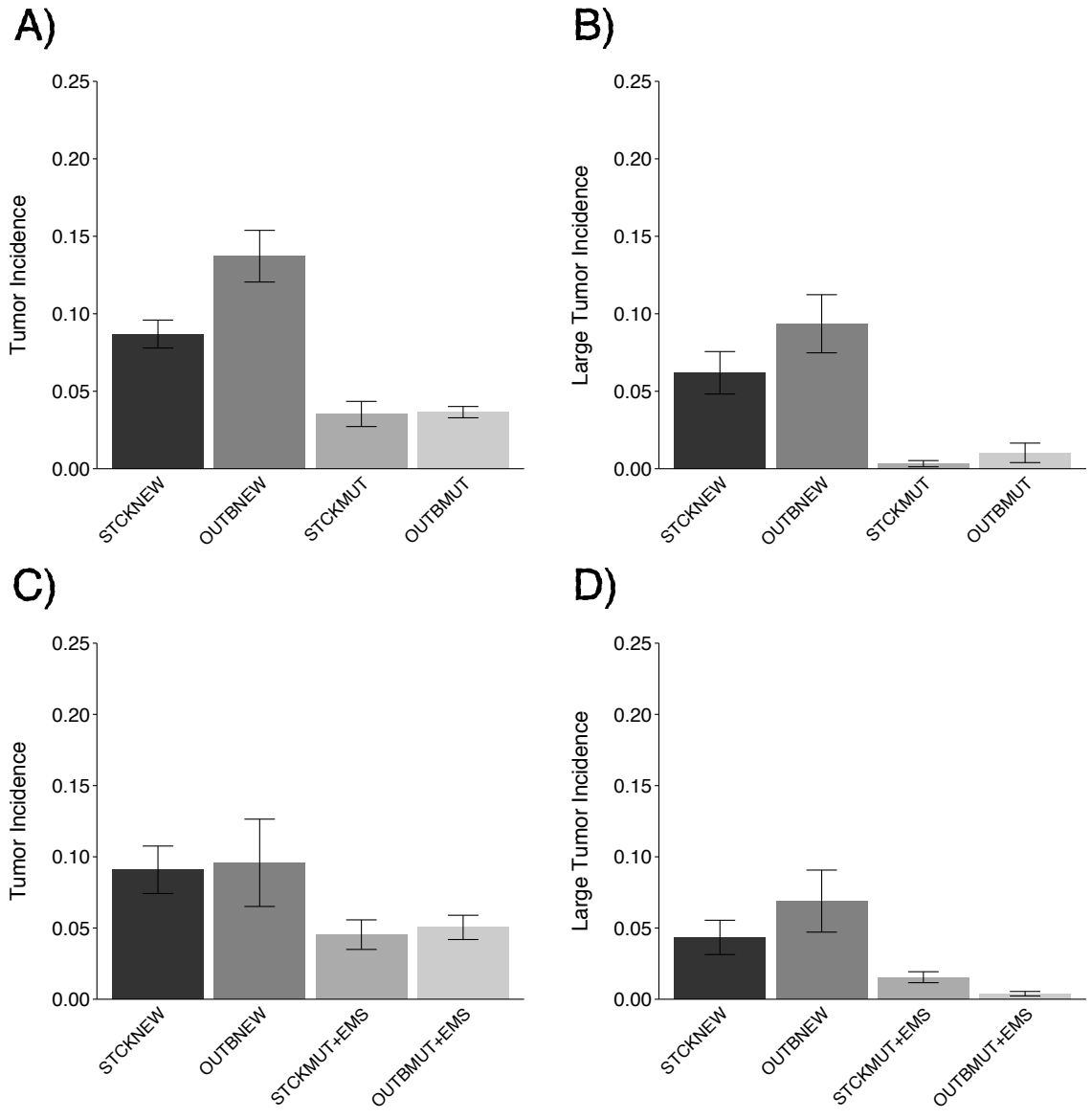

A)
B)
C)
D)
Tumor Incidence
Large Tumor Incidence
0.25
0.20
0.15
0.10
0.05
0.00
STCKNEW
OUTBNEW
STCKMUT
OUTBMUT
STCKMUT+EMS
OUTBMUT+EMS

Figure 1.8: **Interaction of temperature and treatment on the average number of adult flies scored per vial.** The average number of adult flies (± 1 s.e.) scored per vial (Fly Count) refers to how many of the 50 larva per vial (2 vials per Treatment-Temperature combination) survived to adulthood and were scored for tumors. The Treatment (6 Treatments) x Temperature (26.0°C, 28.5°C) interaction is shown. The treatments in the legend are ordered by the incidence values at 28.5°C (Highest to Lowest).

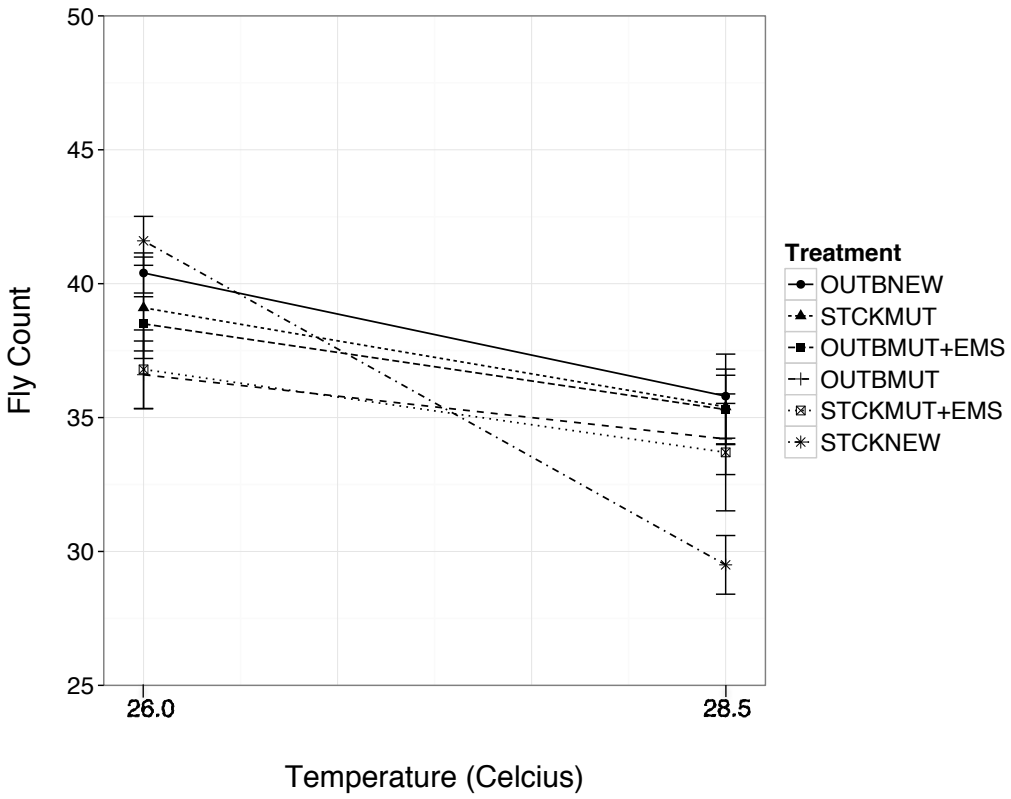

50
45
40
35
30
25
Fly Count
26.0
28.5
Temperature (Celcius)
Treatment
OUTBNEW
STCKMUT
OUTBMUT+EMS
OUTBMUT
STCKMUT+EMS
STCKNEW

Figure 1.9: **Summary of tumor incidence at the start and end of the selection experiment.** The incidence of tumors is shown for OUTBMUT and STCKMUT flies at the start of the experiment (February 2014) and in the final incidence experiment (December 2015). The incidence of flies that did not experience selection for longevity and late-life fecundity are shown on the left (No Selection), and those that did are shown on the right (Selection). The incidence of tumors during generation 0 (labeled as: G0) is shown for flies that were outbred (G0 OUTBMUT) and not outbred (G0 STCKMUT). The incidence of tumors in the newly generated control populations used in the final experiment is represented as NEW-OUTBMUT and NEW-STCKMUT. The incidence of tumors in flies that experienced selection is shown for OUTBMUT flies of generation 20 and in STCKMUT flies of generation 24. The EMS and no-EMS treatments were pooled for these flies (*). The number of flies assessed for tumor incidence is shown.

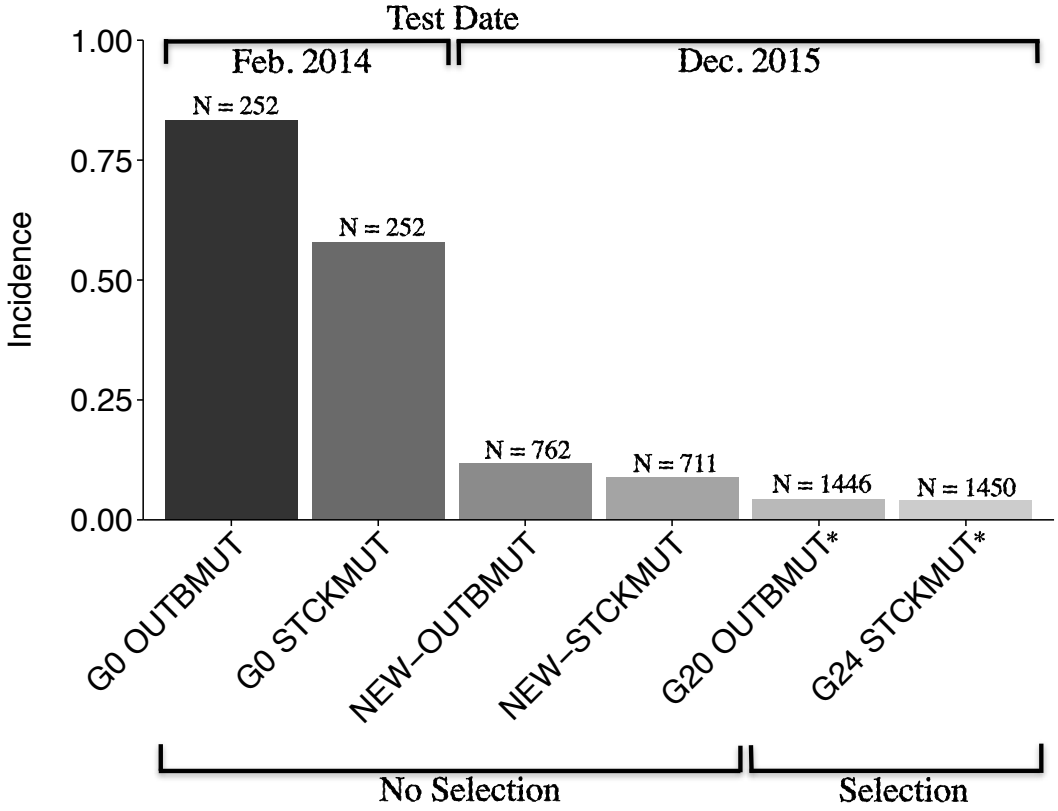
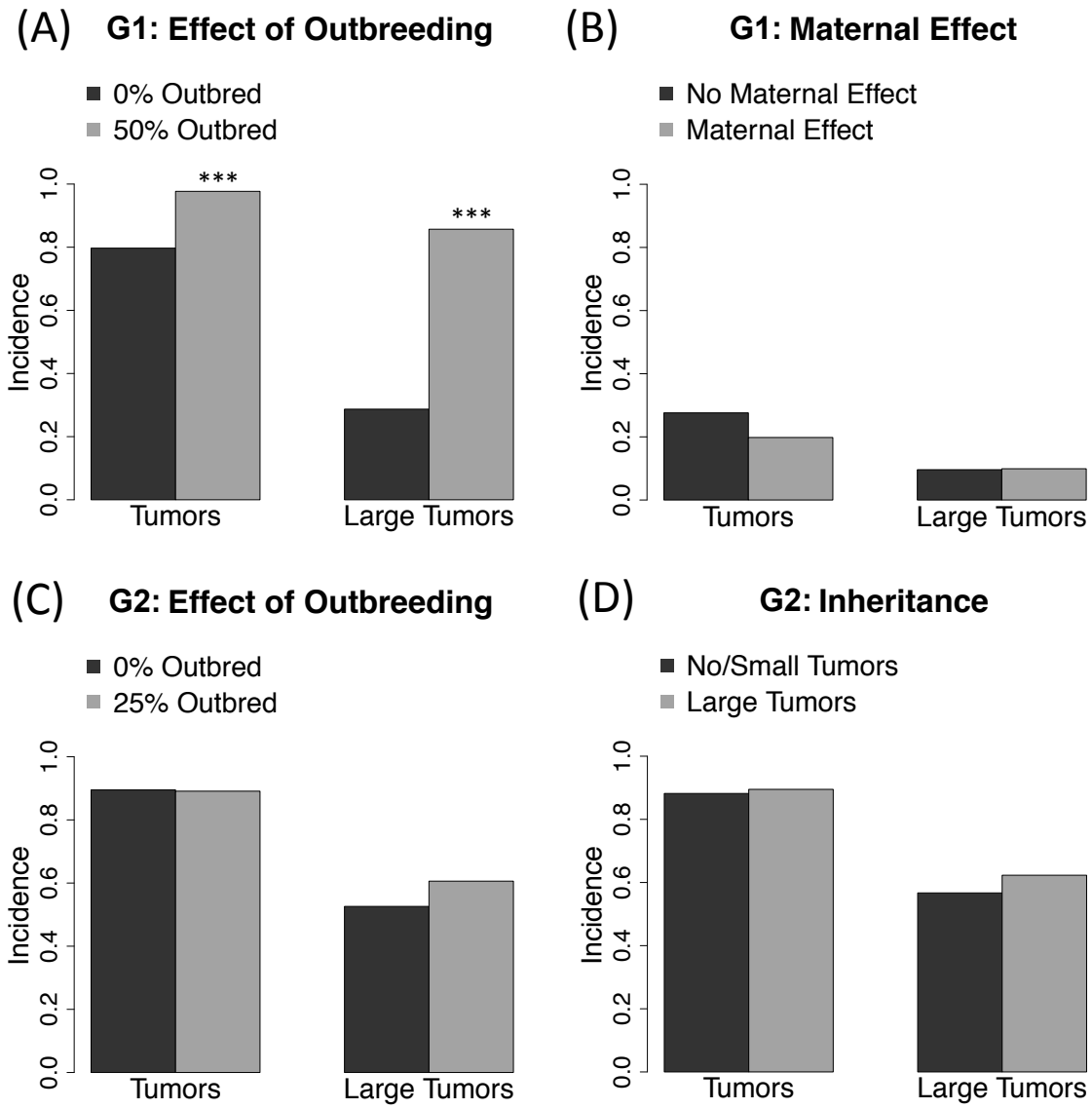

Test Date
Feb. 2014
Dec. 2015
1.00
0.75
0.50
0.25
0.00
Incidence
N = 252
N = 252
N = 762
N = 711
N = 1446
N = 1450
G0 OUTBMUT
G0 STCKMUT
NEW–OUTBMUT
NEW–STCKMUT
G20 OUTBMUT*
G24 STCKMUT*
No Selection
Selection

Figure 1.10: **Effect of adding new genetic variation on the incidence of tumors.** The incidence of tumors and large tumors is shown for the four comparisons analyzed in the study in two generations of crosses (G1 and G2) (see Table 1.3). The effect of outbreeding is shown in (A) for F1 males (50% outbred) and in (B) for male and female offspring of a backcross (25% outbred). Flies that were not outbred are shown in black and outbred flies are shown in gray. The effect of maternal inheritance on tumor incidence, shown in (C), was examined in F1 females with those that paternally inherited the mutant allele shown in black and those that maternally inherited it shown in gray. The inheritance of tumors is shown in (D) for male and female backcross offspring with those that had male parents with no tumors or small tumors shown in black and those with male parents with large tumors shown in gray. The significance levels found in the likelihood-ratio tests are shown for the significant comparisons.


(A)
G1: Effect of Outbreeding
0% Outbred
50% Outbred
***
***
Incidence
0.0
0.2
0.4
0.6
0.8
1.0
Tumors
Large Tumors
(B)
G1: Maternal Effect
No Maternal Effect
Maternal Effect
Incidence
0.0
0.2
0.4
0.6
0.8
1.0
Tumors
Large Tumors
(C)
G2: Effect of Outbreeding
0% Outbred
25% Outbred
Incidence
0.0
0.2
0.4
0.6
0.8
1.0
Tumors
Large Tumors
(D)
G2: Inheritance
No/Small Tumors
Large Tumors
Incidence
0.0
0.2
0.4
0.6
0.8
1.0
Tumors
Large Tumors

Chapter 2

The Expression of Tumour Suppressors and Proto-Oncogenes in Tissues Susceptible to Their Hereditary Cancers

Abstract

Studies of familial cancers have found that only a small subset of tissues are affected by inherited mutations in a given tumor suppressor (TSG) or proto-oncogene (POG), even though the mutation is present in all tissues. Previous tests have shown that tissue specificity is not due to a presence vs. absence of gene expression, since TSGs and POGs are expressed in nearly every type of normal human tissue. Using published microarray expression data we tested the related hypothesis that tissue-specific expression of a TSG or POG is highest in tissue where it is of oncogenic importance. We tested this hypothesis by examining whether individual TSGs and POGs had higher expression in the normal (non-cancerous) tissues where they are implicated in familial cancers relative to those tissues where they are not. We examined data for 15 TSGs and 8 POGs implicated in familial cancer across 12 human tissue types. We found a significant difference between expression levels in susceptible vs. non-susceptible tissues. It was found that 9 (60%, $p<0.001$) of the TSGs and 5 (63%, $p<0.001$) of the POGs had their highest expression level in the tissue type susceptible to their oncogenic effect. This highly significant association supports the hypothesis that mutation of a specific TSG or POG is likely to be most oncogenic in the tissue where the gene has its highest level of expression. This suggests that high expression in normal tissues is a potential marker for linking cancer-related genes with their susceptible tissues.

Introduction

The tissue-specificity of an inherited predisposition to cancer has been known of and speculated upon for a long time (Morgan, 1922; Little, 1923). It is a pattern seen in both tumor suppressor genes (TSGs) and proto-oncogenes (POGs). For example, inherited mutations in the TSG *BRCA1* severely increase the risk of cancer in breast and ovarian tissues, but cause no added risk of cancer in many other tissue types (Welcsh & King, 2001). Similarly, inherited mutations in the *KIT* proto-oncogene specifically increase the risk of gastrointestinal stromal tumors (Lindor et al., 2008).

Attempts to explain these phenomena have largely focused on tumor suppressor genes (Bignold, 2004), with the usual assumption being that a given TSG acts as a critical cancer suppressor in the predisposed tissues, but not in those tissues with no elevated risk of hereditary cancer (Horowitz et al., 1990). For example, Weinberg (Horowitz et al., 1990; see Weinberg, 1988) proposed that *RB1* had been recruited by evolutionary processes for the regulation of cell division in retinal and bone tissues, given that *RB1* germline mutations were associated with high lifetime risks of retinoblastoma (Knudson, 1971) and osteosarcoma (Stratton et al., 1989).

This explanation of tissue-specificity was formalized into an evolutionary model of cancer suppression by Nunney (1999). The model is based on multistage carcinogenesis and quantifies how cancer risk increases with a larger body size (more cells) and a longer lifespan (more cell divisions) (a relationship strongly supported by data from humans and dogs (Nunney, 2013), and how increased pre-reproductive risk drives natural selection for tissue-specific increases in cancer suppression. This

recognition that cancer suppression is an evolving trait resolves the paradox first noted by Peto (1977): why don't large long-lived humans have much higher rates of cancer than small short-lived mice? This suppression is predicted to involve either tissue-specific mechanisms that directly reduce the risk of the target cancer (e.g., a TSG up-regulated in a single tissue) or more general mechanisms affecting all tissues that would also lower the risk of all cancers. Examples of general mechanisms (global telomerase suppression, and early contact inhibition) have already been identified in large and long-lived rodents (Seluanov et al., 2007, Seluanov et al., 2009). Tissue-specific mechanisms are harder to detect, but the serendipitous availability of tissue-specific mutations would predict that different TSGs may be recruited independently over time in different tissues within a species, and that different TSGs may be recruited in the same tissue in different species clades.

One potential consequence of this evolutionary process would be that TSGs are only expressed in tissues where they actively suppress carcinogenesis. However, most TSGs and POGs are expressed in every type of normal human tissue. This was first investigated using *RB1*, which was found to be expressed in every tissue tested (Friend et al., 1986; Fung et al., 1987; Lee et al., 1988). Studies of other genes have found the same pattern in gene expression (Fearon, 1997; Vogelstein & Kinzler, 2004) and protein data (Plevová et al., 2005).

The ubiquitous expression of TSGs and POGs has thus been recognized as an enigma of cancer research (Weinberg, 1989; Knudson, 1989; Eng & Ponder, 1993; Fearon, 1997; Brown & Solomon, 1997; Vogelstein & Kinzler, 2004; Bignold, 2004;

Weinberg, 2007), prompting alternative explanations that do not predict differences in expression across tissue types (see Bignold, 2004; Friedenson, 2010). For example, it has been proposed that the specificity of *BRCA1* to breast and ovarian cancers might result from *BRCA1* mutations being less likely to induce apoptosis in breast and ovarian tissues than in other tissues (Elledge & Amon, 2002; see also Monteiro, 2003). However, such *ad hoc* hypotheses lack generality because they fail to explain why such tissue specificity is the rule rather than the exception.

Recent work on TSGs suggests that their level of expression, rather than presence or absence, may be an important indicator of the cancer suppressing activity. TSGs have multiple functions affecting tissues generally, in addition to their roles in cancer suppression (Venkitaraman, 2002; Silver & Livingston, 2012); however, a higher tissue-specific level of expression may indicate a role in suppressing cancer in the target tissue. Berger et al. (2011) recently argued that higher levels of TSG expression correspond to lower cancer risk. For example, mice with slightly reduced expression levels (80%) of the TSG *Pten* have intermediate mammary tumor incidence compared to *Pten*+/+ (100%) and *Pten*+/- (50%) mice (Alimonti et al., 2010). In humans, haploinsufficiency of the TSGs *PTEN* (Marsh et al., 1998) and *TP53* (Varley et al., 1997) has been linked to cancer, which runs counter to the expectation that a single copy of a TSG is sufficient to suppress cancer. In such cases, loss-of-heterozygosity events of the remaining wild-type allele were not found. This suggests that, at least in some cases, a 50% reduction in expression substantially reduces a TSGs protective effect.

Overall, these findings suggest that TSGs may be expressed at constitutively higher levels in the tissues where they are recruited to suppress cancer relative to those in which they are not. This hypothesis has been tested by two studies to date; however, support was weak or absent. Plevová et al. (2005) examined the protein expression levels of the DNA-mismatch-repair tumor suppressors, MLH1 and MSH2, commonly associated with colorectal and uterine cancers. They found that both proteins had significantly higher expression in the susceptible tissues, but only if non-susceptible testes tissue that showed the highest expression level for both proteins, was excluded. Lage et al. (2008) used microarray data from non-cancerous samples of 73 tissues to examine the relative expression of 51 genes linked to hereditary cancer and did not find the predicted pattern in proto-oncogenes or tumor suppressor genes.

The 2-hit role of TSGs in cancer suppression is well established; however, it is also possible for POGs to be recruited to provide 1-hit protection (Nunney, 1999). The role of POGs in transmitting growth signals (Bunz, 2008) appears to make them less likely candidates for cancer suppression, since it requires that each added POG defines a new necessary signal for tissue growth. However, POGs are implicated in some familial cancers (the first was *RET*; Mulligan et al., 1993) and their possible role in the evolution of cancer suppression needs to be investigated. To this end, we were interested in testing the same hypothesis in POGs that has been proposed for TSGs: that a POG has higher expression in the susceptible tissue type(s) assuming that a susceptible tissue is where the POG provides a required signal for tissue growth.

The goal of this study was to test the prediction that TSGs and POGs implicated in familial cancer in a specific tissue are expected to have a high level of expression in that tissue. Using 15 TSGs and 8 POGs implicated in various tissue-specific familial cancers, we tested for higher expression in the affected tissue across 12 tissue types (plus some additional subtypes) using published mRNA expression data from normal (non-cancerous) tissues.

Materials and Methods

Gene Expression Dataset

We used data from 35 independently published microarray datasets documenting gene expression for a range of human tissues (see Table 2.1 and Supplementary Table 2.1) using the HG-U133A Affymetrix platform. These data were compiled and normalized by Zheng-Bradley et al. (2010), and the dataset is publicly available online at ArrayExpress (Accession#: E-MATB-27). The multi-study dataset was downloaded using the Bioconductor software (Gentleman et al., 2004) in R (R Development Core Team, 2008). All probesets were retained in our subset. Most cancer-related genes were covered by a single probeset. In those cases where two probesets were present (*RB1* and *PTCH1*), these were included as replicate measures of expression.

In our analysis we used all of the broad tissue categories used to group the biological samples in the dataset except for cell line and liver, because cell line samples may not accurately reflect expression at the tissue level *in vivo*, and the liver tissue category contained only a single sample. We also removed tissues annotated as "disease"

or "neoplasia" since only normal tissue was being analyzed. The final 12 tissue categories used in the analysis and their corresponding sample sizes are represented in Table 2.1.

Criteria for selecting genes.

Our initial screen required that a TSG or POG could be included in the analysis only if it was implicated in cancer via both germline and somatic mutations (using Table S1 in Futreal *et al*, 2004). Both types of mutational effect were included in the screen in order to select only those TSGs and POGs likely to increase susceptibility across a range of developmental conditions in which the genes are mutated. For example, if a gene had a germline effect, but no known somatic effect, then this could indicate that the oncogenic activity of the mutant gene was restricted to very early development, a possibility our analysis of adult tissue could not detect.

In our second screen, we identified tissues significantly at risk from germline mutation using the criterion that lifetime cancer risk (by age 70) for individuals carrying a mutation in the target gene had to be ≥25% using median values from Lindor et al. (2008). When relative risk (RR) scores were reported, the RR was converted using baseline risk data (x) from Surveillance Epidemiology and End Results (SEER) (Ries et al., 2008), i.e. $(x)(\mathrm{RR}) \geq 25$. For some genes, the 25% criterion excluded all tissue types. For these genes alone, a single tissue type that most exceeded a lower threshold of 15% lifetime risk was retained.

In applying this second screen it was found that of the POGs identified in the first screen only *RET* (Multiple Endocrine Neoplasia Type II) was included in Lindor et al. (2008). *RET* passed the second screen; however, since the absence of the other POGs was

probably due to their relatively recent discovery as hereditary cancer genes we used other sources to assess risk. Each one of these genes was associated with only one major cancer type, as reported in the Cancer Gene Census (Futreal et al., 2004, Table S1), and all of these cancer types were found to be above the 25% lifetime risk threshold using data from OMIM (Online Mendelian Inheritance in Man; see Hamosh et al., 2005).

Cancers affecting a tissue not represented in the 12 tissue types were not considered (e.g. colorectal cancer). Also excluded were (a) cancer types with diverse tissues of origin such as harmartomas and desmoid tumors, and (b) cancer types with unknown tissues of origin, such as rhabdoid tumors.

Statistical Tests

All statistical tests were performed in the statistical program R (R Development Core Team, 2008), with the exception of the nested ANOVA model for which Minitab 16 (Minitab Inc, Pennsylvania, PA, USA) was used. The normality and equal variance assumptions of the $\log_2$(expression) data for each gene were tested using the Jarque-Bera test and the Bartlett test. Since the data for several genes were found to be non-normal, in most cases nonparametric statistical tests were performed.

Tissue and Subtissue Categories.

All statistical testing for higher expression in susceptible tissues relative to non-susceptible tissues were performed initially using the 12 broad tissue categories with (usually) one tissue identified as "susceptible" for each gene and the remainder grouped as "non-susceptible". For some genes it was possible to use subtissue data (given an adequate sample size >2; see Table 2.1) to refine the initial analysis by identifying the

susceptible subtissue within the susceptible tissue using OMIM (Hamosh et al., 2005), so that the remaining subtissue samples of that tissue type could be grouped as "non-susceptible subtissues", creating a 13th category.

Comparing gene expression in susceptible vs. non-susceptible tissue groups.

The grand mean expression values of each gene in its susceptible vs. non-susceptible tissues/subtissues were compared using a one-tailed Wilcoxon signed rank test applied separately to TSGs and POGs. A one-tailed test was performed, given the directional alternate hypothesis of higher expression in susceptible tissue. For each gene, the grand means were calculated as the unweighted average of the means of the relevant tissue/subtissue groups.

In a second analysis, each gene was tested individually. First, for each gene, we tested for significant differences in expression levels among the tissue/subtissue groups using a Kruskal-Wallis test. If significant expression level differences between tissues were established ($p<0.05$ after Bonferroni correction for testing multiple genes), the Kruskal-Wallis multiple comparison test was performed across the tissue types. In this *a posteriori* test, which controlled the family-wise error rate at 0.05, the tissue means were ranked and grouped by significance, with the “A” group corresponding to the highest expression level. Tissues could be assigned to more than one group, e.g. expression in a tissue classified as “AB” would only be significantly higher than tissues not in groups “A” or “B”. Only those tissue types marked as “A” alone (i.e. not “AB” etc.) were considered most highly expressed (see Figure 2.1).

The number of "A" classifications assigned to the susceptible tissue/subtissue of TSGs or POGs relative to the total number of "A" classifications was used to determine if there was a non-random association. The statistical significance of the relationship was determined by simulated resampling of the data to determine the distribution of the number of "A"s expected to be assigned to the susceptible group under the null hypothesis. Ten thousand trials were run and each trial involved cycling through each of the TSGs (or POGs) in turn and taking a random sample of size 1 or 2 (corresponding to the number of susceptible tissues for that gene) from the observed distribution of letter groupings for that gene. The number of "A"s chosen across all TSGs (or POGs) was the score for that trial. The statistical significance of the observed score was evaluated based on its position relative to the null distribution.

Tests of Lab Effect

We examined the effect of using data from different laboratories, since these effects are known to be strong (Zilliox & Irizarry, 2007). Most labs tested only one tissue type, so to avoid subtissue variation we only compared labs that used the same subtissue type to represent that tissue. The resulting dataset was analyzed using a nested ANOVA model (Model: Expression = Subtissue + Gene + Lab(Subtissue) + Gene x Subtissue + Gene x Lab(Subtissue)), with Gene and Subtissue as fixed effects, and Lab as a random effect. Of interest is the Gene x Lab(Subtissue) interaction where the null expectation is that the ordering of expression across genes is constant for a given subtissue. We also calculated the (Pearson) correlation for gene expression levels both within and between labs. Within labs the multiple correlation coefficients were averaged using the Fisher z-

transform ($z=(1/2)\ln[(1+r)/(1-r)]$), and between labs the correlation was based on the average expression levels of each lab.

Results

We identified 36 genes that had both hereditary and somatic mutations linked to cancer, of which 28 were represented by the probesets within the expression array. Of these 36 genes, 23 (15 TSGs and 8 POGs) satisfied our criteria for a high risk familial effect, with each affecting a single susceptible tissue, with the exception of *PRKAR1A*, a TSG that defined two susceptible tissues (Table 2.2). Within this group, it was possible to identify susceptible vs. non-susceptible subtissues within the susceptible tissue category in 11 TSGs and 2 POGs (Table 2.2).

Analysis of the average expression of the 15 TSGs and 8 POGs showed that both groups had significantly higher expression in susceptible tissue categories, where mutations result in familial cancer, relative to non-susceptible tissue categories. The mean $\log_2$ expression levels (± 1 se) of TSGs in susceptible and non-susceptible tissue were 0.368±0.254 and -0.130±0.082, respectively, and in POGs they were 2.24±1.06 and 0.225±0.209, respectively (Table 2.2) (Wilcoxon signed rank test: TSGs, V=89, p=0.05, df=14; POGs,V=35, p=0.008, df=7). This reflected the finding that 10 of 15 TSGs and 7 of 8 POGs had a higher expression level in the susceptible tissue categories relative to the mean of the non-susceptible tissue categories (Table 2.2). The same trend was also found when the susceptible tissue was more narrowly defined as a subtissue, (Wilcoxon signed rank test: TSGs, V=91, p=0.04, df=14; POGs,V=33, p=0.02, df=7), again with 10 of 15

TSGs and 7 of 8 POGs having a higher expression level in the susceptible subtissue relative to the mean of the non-susceptible tissues (Table 2.2).

We next examined the more stringent hypothesis that in its susceptible tissue or subtissue the expression level of a TSG or POG is in the statistically most highly expressed tissue (or group of tissues) for that gene. We first established that there were highly significant differences in expression among tissues for all 23 genes (Kruskal-Wallis test; $p < 0.01$ in all cases, after Bonferroni correction; see Table 2.2). *A posteriori* testing to assign a significance category to each susceptible tissues showed that 33% (5/15) of TSGs and 63% (5/8) of POGs were most highly expressed in their susceptible tissue (Table 2.2). Data re-sampling tests identified these patterns to be significant for both TSGs ($p = 0.02$) and POGs ($p=0.0003$). The pattern for TSGs became much stronger when we refined the analysis using, where possible, expression data from susceptible subtissue rather than from the broad tissue category. Of the 11 TSGs where a susceptible subtissue was identified, 4 out of the 8 that were not characterized by significance category “A” in the initial tissue-level analysis moved into significance category “A” in the subtissue analysis. The 3 TSGs significant at the tissue category level remained so in the subtissue analysis (Table 2.2), so that 9 out of 15 TSGs (60%; $p = 0.0001$) had their significantly highest expression in their susceptible tissue (Figure 2.2a). There was no difference in the number of "A"s between the subtissue and the broad tissue category score for POGs (Table 2.2), so their significance was unchanged ($p=0.0003$). The overall pattern in POGs is shown in Figure 2.2b.

We examined whether using data from different laboratories added significant variation within our tissue groupings using the cases where it was possible to test for heterogeneity in the ranking of the expression values of all genes within the same subtissue. There were 6 types of subtissue that were used in more than one lab, with 2-3 labs per subtissue (Table 2.3). With lab nested within subtissue, ANOVA revealed a highly significant Gene by Lab(Subtissue) interaction ($p < 0.001$; Table 2.3), indicating that, for at least some genes, the ranking of the expression levels across subtissues varied among labs. This laboratory effect would lessen the chance of the data revealing a consistent pattern.

Despite this heterogeneity, the correlation in gene expression across labs was generally quite high. Of the 10 possible pair wise correlations, 5 explained more than 50% of the variance in the ranking (i.e. $r^2 > 0.5$), although 4 explained less than 33% (Table 2.3).

Another potential source of variation is between replicate samples within labs. Using the same set of labs, the correlation in the gene expression values within labs was the same, averaging 0.705 (vs. 0.704 between labs; Table 2.3), with all values explaining >33% of the variance (and 5/9 explaining >50% of the variance). However, it is clear that the consistency of replication within labs is relatively poor.

Discussion

We examined the expression levels of 15 TSGs and 8 POGs implicated in a high risk of familial cancer and found that a gene's highest expression was typically found in the tissue susceptible to the cancer with which it was associated. Specifically, the overall

expression level was generally higher in susceptible tissue (or subtissue) compared to the average of non-susceptible tissue for both TSGs (10/15 TSGs, $p<0.05$) and POGs (7/8 POGs, $p<0.05$), and, more importantly, that gene expression in the susceptible tissue (or subtissue) was within the highest expression group observed in 9/15 TSGs ($p<0.001$) and in 5/8 POGs ($p<0.001$). Despite the high level of variability in the data (see below), 3 TSGs and 3 POGs showed a level of expression significantly higher in their susceptible tissue (or subtissue) than in all 11 (or 12) other types (see Table 2.2). The remainder showed high expression in their susceptible tissue (or subtissue) that could not be statistically distinguished from levels in some other tissues; however our statistical testing (via data resampling) incorporated this ambiguity in establishing the highly significant relationship between high expression and the susceptible tissue.

This relationship was predicted by Weinberg's (Weinberg, 1988; Horowitz et al., 1990) hypothesis that TSGs have been evolutionarily recruited in the susceptible tissues to suppress cancer and that this has occurred via up-regulation of the expression of different genes in different tissues. This is a basic assumption of Nunney's (1999) model for the evolution of cancer suppression: if a given cancer results in a significant loss of fitness, then natural selection will act on any genetic variation for tissue specific expression of TSGs and POGs that results in a decrease in the occurrence of that cancer during the pre-reproductive and reproductive period. POGs were found to show the same pattern as TSGs. This result could be due to POGs being recruited as additional cancer suppressors in a fashion analogous to TSGs. This would require that each added POG adds one more necessary tissue-specific signaling pathway and hence one more "hit" in

the progression of multistage carcinogenesis. An alternative possibility is that POGs generally have constitutively higher expression in the tissues in which they have a critical role in tissue development and maintenance (Sharma & Sen, 2013). If so, hereditary cancers arising from mutations in a specific POG occur because the tissue is especially sensitive to growth signals sent by that POG, or because such mutations have a bigger effect of perturbing some other potentially oncogenic cell function than mutations in a POG that is expressed at lower levels. The link between high expression and an important role in cell signaling is appealing, and may be a very general pattern; however understanding the potential role of POGs in the evolution of cancer suppression requires a comparative approach to determine if larger, longer lived organisms tend to have additional layers of highly expressed POGs associated with a given tissue.

A previous study (Lage et al., 2008) failed to find a consistent relationship between the expression of TSGs and POGs in susceptible relative to non-susceptible tissues. They did find a trend towards overexpression of POGs in susceptible tissues consistent with our results; however they also found a trend for the under expression of TSGs in susceptible tissues. This difference is likely due to differences in methodology. For each gene-disease combination, the authors ranked the tissues by their degree of susceptibility based upon the number of times a tissue was co-mentioned in the PubMed literature with a disease of the given gene. The most highly mentioned 25 tissues were considered as the susceptible tissues for the given disease-gene combination in their statistical test. The use of 25 susceptible tissues for a given disease-gene combination are

expected to mask the pattern that we were looking for: the evolutionary recruitment of cancer suppression in a very tissue-specific manner.

Our finding that the highest level of expression of a TSG or a POG generally occurs in the tissue susceptible to the oncogenic effects of that gene was highly statistically significant, but it was only established for 14 of the 23 genes examined. However, it is likely that our results are conservative given a range of biological and experimental factors that could act to mask the relationship between gene expression and cancer suppression activity. A number of experimental factors can cause unpredictable variation in the measures of gene expression. One important source of variation in expression that our study has highlighted is the precise nature of the tissue samples. The results of subtissue tests showed that in 8 tests on TSGs where the susceptible tissue was not in the most highly expressed group the outcome was reversed for 4 of the TSGs when the susceptible subtissue was used (Table 2.2). This result shows that reliable results can only be obtained when the specific cell type(s) that are susceptible to the cancer are examined.

Other important sources of experimental variation acting to mask general patterns were between-sample (within-lab) variation and between-lab variation. Within labs using the same subtissue, the average correlation in the expression of the 23 genes was $r = 0.705$. This leaves 50% of the variance unexplained due to between-sample effects. When all within-lab expression values were averaged to minimize the between-sample variance and these averages were compared between labs, the correlation was not improved ($r = 0.704$), highlighting substantial between-lab variation. This high level of between-lab

variation (vs. within-lab, between sample variation) was substantiated using ANOVA, which revealed a highly significant lab(subtissue) x gene interaction ($p < 0.001$; see Table 2.3). The same Affymetrix array was used by all labs so this was not the source of the variation. Many factors could contribute to both the between-sample and between-lab variation including the nature and physiological state of the patients sampled (gender, age, ethnicity, general health).

It may be possible to control for many of these factors in future studies by standardizing tissue collection protocols, and by using either protein levels directly or higher quality gene expression data such as RNAseq. However, other biological factors may still act to mask the association between expression and tissue susceptibility. For example, some of these genes may only act as critical cancer suppressors, and thus be highly expressed, during certain periods of development. This possibility has been proposed (but not supported) in the relationship between the *RB1* gene and retinoblastoma (Lee et al., 1988). In our study, two DNA-repair TSGs, *MSH2* and *BRCA1*, that both predispose to ovarian cancer, were found to have low expression in the ovaries, but extremely high expression in testes tissue. Since ovarian tissues stop dividing before adulthood, but adult testes tissues continue to proliferate, the expression of these genes in ovarian tissues may be highest during early development. The small sample size of pre-adult tissues in our dataset precluded the testing of this hypothesis. It would be useful to include tissue from different developmental stages (e.g. infant, child, adult) in future analyses. Alternatively, the failure to find elevated expression in the ovaries may

be due to choosing an inappropriate susceptible tissue, as many high-grade serous ovarian tumors have been found to originate in the fallopian tube (Kurman & Shih, 2010).

In conclusion, our findings support the hypothesis that a high level of expression is a marker of oncogenic importance both in TSGs and POGs, as predicted under Nunney's (1999) model for the evolution of cancer suppression. This approach has potential clinical applications. First, cancer-related genes showing unusually high expression in normal tissue can be examined further for a possible role in the sporadic cancers of that tissue. Second, findings of high expression of a known TSG or POG in a particular cell type is a potentially useful tool for identifying candidate genes in our search for causes of familial cancers. Third, high expression can be used to determine the specific cell type involved in cancers that have unknown origin yet the gene is known (such as rhabdoid tumors and *SMARCB1*). And beyond the basic task of linking genes to cancers, mimicking or generating the signals from these genes may ultimately provide methods of cancer prevention for the susceptible tissues.

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Tables and Figures

Table 2.1: **The 12 broad tissue categories and the 38 subtissue types contained in the analyzed multi-study dataset, which was compiled from 35 independent studies.** The total number of biological samples being tested for each broad tissue category and its corresponding subtissues is shown. Each of the 35 independent studies are listed in numerical code next to the tissue categories for which they contributed data. See Supplemental Table 2.1 for the complete reference corresponding to each code number. For additional details, see (see Zheng-Bradley et al., 2010).

Tissue Category	Sample Size	Original Data Source
Adipocyte	**14**	1, 2
adipose tissue normal	8	
adipose-derived adult stem cells	6	
Bone	**7**	3
bone	7	
Brain + Nerve	**150**	4, 5, 6, 7, 8, 9
amygdala	1	
brain	39	
caudate nucleus	30	
cerebellum	26	
frontal cortex	27	
hippocampus CA1	5	
hypothalamus	20	
prefrontal cortex	2	
Endocrine Organs	**7**	7, 10
thyrocyte	6	
thyroid gland	1	
Epithelium	**33**	11
bronchial epithelium	33	
Gastrointestinal Organs	**13**	12, 13
esophagus epithelium	7	
small intestines	6	
Head + Neck	**33**	14, 15, 16, 17, 18
conjuctiva	4	
hypopharynx	3	
oropharynx	1	
T cell	9	
tonsil	10	
trabecular meshwork cell	6	
Heart + Muscle	**74**	19, 20, 21, 22,23, 24, 25, 26

heart	36	
myometrium	12	
skeletal muscle	17	
smooth muscle	9	
Immune System	**25**	15, 27, 28
CD34+ blood cell thymus	1	
lymph node	10	
thymocyte	14	
Female Reproductive	**34**	26, 29, 30, 31
ovary	4	
placenta basal plate	21	
smooth muscle	1	
theca	8	
Male Reproductive	**12**	32, 33
prostate gland	11	
testis	1	
Skin	**25**	26, 34, 35
endothelial cells	8	
epidermis	5	
keratinocyte	8	
skin	4	
Total	**427**	

Table 2.2: **The susceptible tissue/subtissue and cancer type associated with the TSGs and POGs identified for analysis, plus the expression of these genes in susceptible vs. non-susceptible tissues.** For each gene, results are shown for a Kruskal-Wallis test across the 12 broad tissue categories (p value = significance after Bonferroni correction) and the significance categories assigned to the susceptible tissue in the subsequent Kruskal-Wallis multiple comparison test. For genes in significance category "A", the total number of tissues tied in that category is given in parentheses. Also shown are the $\log_2$ expression (± 1 se) of each gene in the susceptible tissue type, the mean for the 11 other tissues, and the ratio of these expression levels. For those genes that could also be analyzed at the level of subtissue, the results from the subtissue analysis are also shown.

Gene Type	Gene	High-Penetrance Cancer	Susceptible Tissue Type	Kruskal-Wallis Test P Value	Expression (Susc. Tissue)	Expression (Other Tissues)	Significance Category	Relative Expression	Susceptible Subtissue	Rel. Expr. in Susc. Subtissue	Subtissue Significance Category
Tumor Suppressor Genes	ATM	leukemia, lymphoma	Immune System	5.06E-15	2.886 ± 0.472	-0.028 ± 0.206	A (2)	7.54	lymph node	11.68	A (1)
	STK11	gastrointestinal polyps	Gastrointestinal	5.06E-15	1.814 ± 0.173	0.108 ± 0.224	A (2)	3.26	small intestine	3.34	A (3)
	NF1	glioma, other CNS cancers	Brain and Nerve	5.06E-15	0.645 ± 0.076	-0.432 ± 0.205	A (1)	2.11	N/A		
	RB1	osteosarcoma	Bone	5.06E-15	0.62 ± 0.203	-0.060 ± 0.123	A (2)	1.60	N/A		
	SMAD4	gastrointestinal polyps	Gastrointestinal	1.29E-03	0.878 ± 0.324	0.260 ± 0.106	A (2)	1.53	small intestine	2.21	A (1)
	CDKN2A	melanoma	Skin	2.99E-07	0.904 ± 0.510	0.386 ± 0.389	B	1.43	epidermis	0.41	E
	MEN1	parathyroid adenoma	Endocrine	5.06E-15	-0.246 ± 0.139	-0.758 ± 0.249	B	1.43	thyrocyte	1.26	A (7)
	BRCA2	prostate	Male Reproductive	1.17E-10	0.106 ± 0.190	-0.237 ± 0.194	BC	1.27	prostate gland	1.01	A (6)
	PRKAR1A	myxoma	Heart and Muscle	5.06E-15	0.655 ± 0.099	0.015 ± 0.331	BC	1.56	heart	2.36	A (2)
	PHOX2B	neuroblastoma	Brain and Nerve	2.02E-12	-0.066 ± 0.067	0.134 ± 0.203	BC	0.87	N/A		
	PTCH1	skin basal cell	Skin	5.06E-15	-0.069 ± 0.116	0.136 ± 0.156	BC	0.69	keratinocyte	0.86	BC
	MLH1	endometrial	Female Reproductive	5.06E-15	-0.211 ± 0.088	-0.387 ± 0.190	CD	1.13	placenta basal plate	1.19	CD
	BRCA1	ovarian	Female Reproductive	5.06E-15	-0.779 ± 0.070	-0.394 ± 0.173	CDE	0.77	ovary	0.77	DE
	TP53	osteosarcoma	Bone	5.06E-15	-0.378 ± 0.045	-0.222 ± 0.172	D	0.90	N/A		
	MSH2	ovarian	Female Reproductive	5.06E-15	-0.807 ± 0.081	-0.487 ± 0.157	DE	0.80	ovary	1.67	A (3)
	PRKAR1A	testicular	Male Reproductive	5.06E-15	-0.198 ± 0.260	0.093 ± 0.335	EF	0.82	N/A		
				TSG Average:	0.368 ± 0.254	-0.130 ± 0.082					
Proto-Oncogenes	RET	medullary thyroid	Endocrine	1.40E-11	7.097 ± 2.437	0.158 ± 0.164	A (3)	122.77	N/A		
	TSHR	thyroid adenoma	Endocrine	7.13E-03	6.588 ± 4.292	0.868 ± 0.399	A (1)	52.72	N/A		
	MPL	myeloproliferative disorder	Bone	1.08E-12	2.594 ± 0.598	0.519 ± 0.211	A (1)	4.21	N/A		
	ALK	neuroblastoma	Brain and Nerve	5.06E-15	0.697 ± 0.065	-0.261 ± 0.108	A (1)	1.94	N/A		
	EGFR	non small cell lung cancer	Epithelium	5.06E-15	0.473 ± 0.056	0.112 ± 0.186	A (5)	1.28	N/A		
	LMO1	neuroblastoma	Brain and Nerve	7.59E-07	0.179 ± 0.076	0.106 ± 0.140	ABCD	1.05	N/A		
	KIT	gastrointestinal stromal tumor	Gastrointestinal	5.06E-15	1.053 ± 0.255	1.062 ± 0.138	CD	0.99	small intestine	0.79	CD
	HRAS	rhabdomyosarcoma	Heart and Muscle	5.06E-15	-0.743 ± 0.089	-0.76 ± 0.329	DE	1.01	skeletal muscle	1.6	C
				POG Average:	2.24 ± 1.06	0.225 ± 0.209					

Table 2.3: **Variation in gene expression levels within and between laboratories testing the same subtissue.** The subtissues and their corresponding laboratories (represented by an ArrayExpress experiment ID) are given along with the number of tissue samples that were tested in each lab. For each lab (with sample size >1) an average within-lab correlation was calculated using the expression values across all 23 genes in each sample. For each subtissue, the pairwise correlation between all labs is given (in the order lab 1 vs. 2, 1 vs. 3, and 2 vs. 3) using the mean expression values across all 23 genes for each lab. The ANOVA model and results of the data analysis are shown at the bottom of the table.

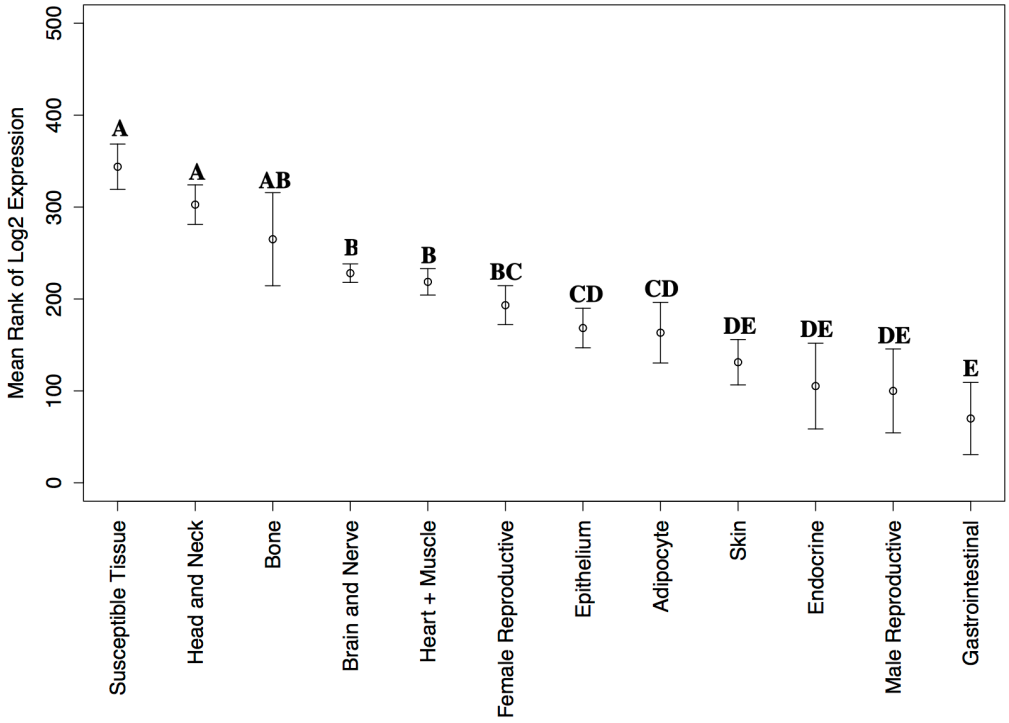
Tissue Category	Subtissue	Lab ID	Sample Size	Within Lab Correlation (r)	Between Lab Correlation (r)
brain + nerve	caudate nucleus	E-AFMX-6	29	0.715	0.732
		GSE3790	1	N/A	
heart + muscle	heart	GSE2240	23	0.722	0.880
		GSE974	13	0.606	
heart + muscle	smooth muscle	E-MEXP-569	8	0.749	0.570
		E-MEXP-66	1	N/A	
endocrine organs	thymocyte	E-MEXP-337	13	0.623	0.853
		GSE1460	1	N/A	
brain + nerve	brain	GSE5392	23	0.582	0.662
		E-LGCL-5	15	0.679	0.301
		E-TABM-145	1	N/A	0.598
heart + muscle	skeletal muscle	GSE3307	9	0.797	0.822
		GSE6011	7	0.803	0.793
		GSE1786	1	N/A	0.449

Expression = Subtissue + Gene + Lab(Subtissue) + Subtissue x Gene + Lab(Subtissue) x Gene

Effect	df	SS	F	p
Subtissue	5	155.5	6.97	0.003
Gene	22	1003.7	11.86	<0.001
Lab(Subtissue)	8	22.8	1.1	0.362
Subtissue*Gene	110	2510.6	6.14	<0.001
Lab(Subtissue)*Gene	176	554.2	4.18	<0.001
Error	3303	2489.0		

Figure 2.1: **The results of the Kruskal-Wallis Multiple Comparison Test are diagrammed for the *ATM* tumor suppressor gene for its initial test based on (a) tissue category and (b) the final test using the susceptible subtissue.** The figure shows the significance categories (A, AB, etc) separating the samples. In the subtissue test, the susceptible tissue category (Immune System) was divided into a susceptible subtissue (lymph node) and a non-susceptible subtissue (two subtissues) grouping (see Table 2.1).

(a) Susceptible Tissue Level Test



(b) Susceptible Subtissue Level Test

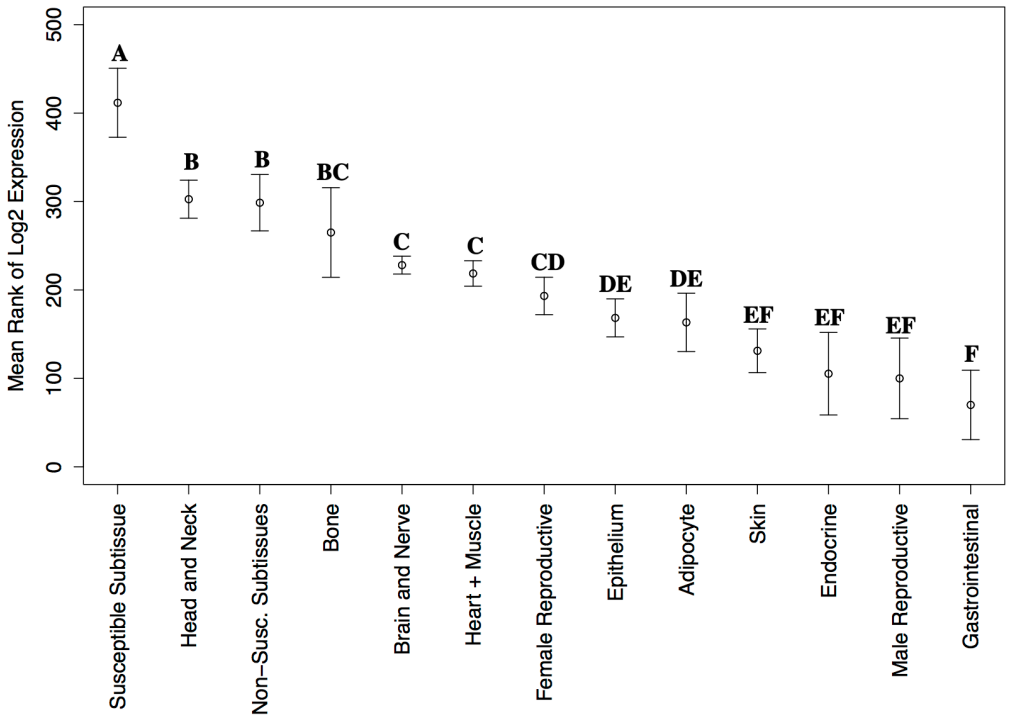
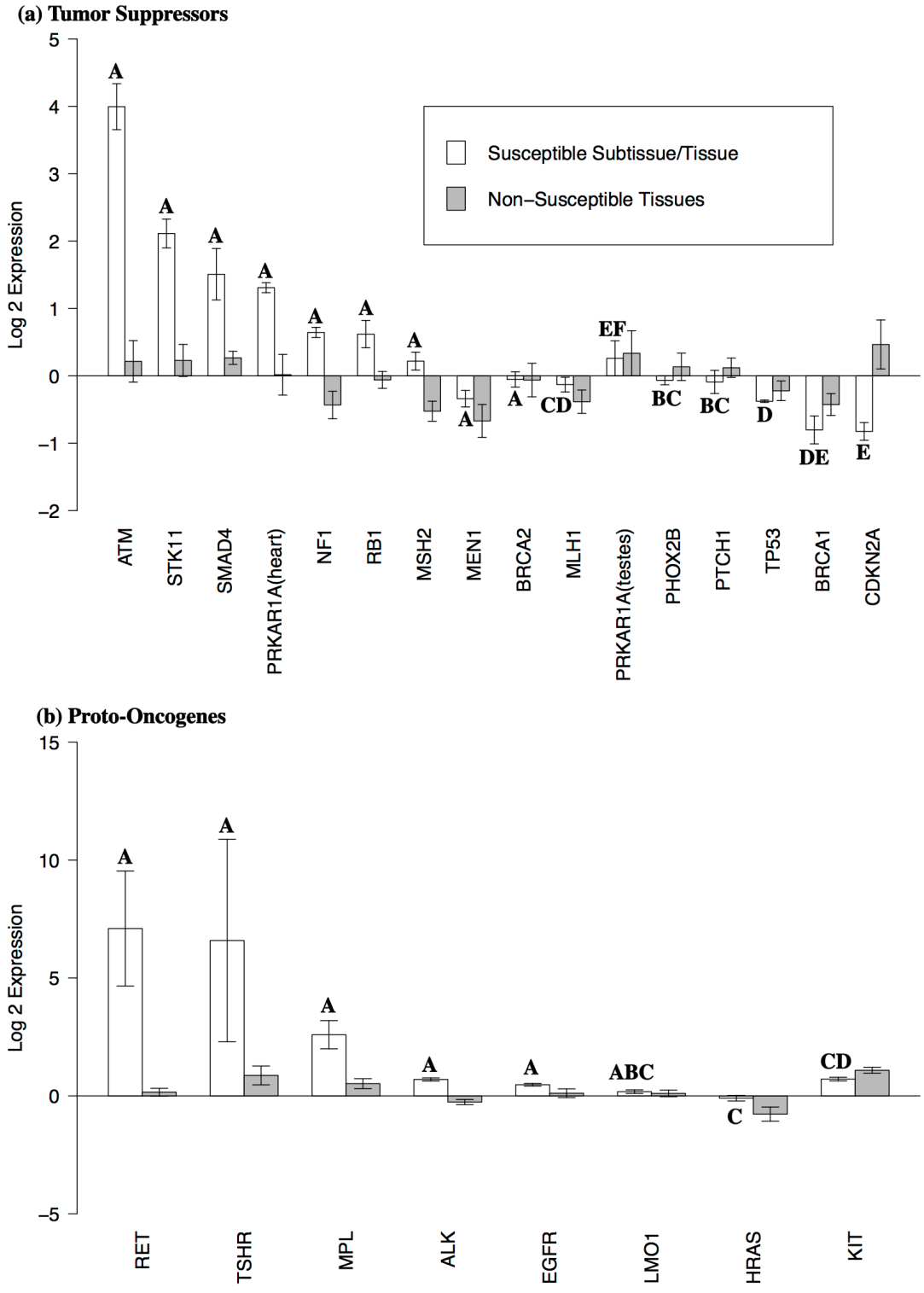


Figure 2.2: **Expression levels (± 1 se) in susceptible tissues relative to non-susceptible tissues for (a) TSGs and (b) POGs.** The “Susceptible Subtissue/Tissue” bars show the mean of log expression for the susceptible subtissue (where possible) or of the susceptible tissue. The “Non-Susceptible Tissue” bars show the average of the mean values for the non-susceptible tissues. The letters above each "Susceptible Subtissue/Tissue" bar show its relevant significance category. The abbreviation for each gene is given on the x-axis.


(a) Tumor Suppressors
Log 2 Expression
5
4
3
2
1
0
−1
−2
Susceptible Subtissue/Tissue
Non−Susceptible Tissues
A
A
A
A
A
A
A
A
CD
EF
BC
BC
D
DE
E
ATM
STK11
SMAD4
PRKAR1A(heart)
NF1
RB1
MSH2
MEN1
BRCA2
MLH1
PRKAR1A(testes)
PHOX2B
PTCH1
TP53
BRCA1
CDKN2A
(b) Proto-Oncogenes
Log 2 Expression
15
10
5
0
−5
A
A
A
A
A
ABC
C
CD
RET
TSHR
MPL
ALK
EGFR
LMO1
HRAS
KIT

Supplementary Materials

Supplementary Table 2.1: **The references for the 35 independent studies composing the analyzed dataset.** The reference numbers correspond to the citation numbers given in Table 2.1. The ArrayExpress experiment IDs are also provided for each study.

Code # of Reference	ArrayExpress ID	Citation
1	E-GEOD-5090	Cortón M, Botella-Carretero JI, Benguria A, Villuendas G, Zaballos A, San Millán JL, Escobar-Morreale HF, Peral B (2007) Differential gene expression profile in omental adipose tissue in women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology & Metabolism* **92**(1): 328-337.
2	E-MEXP-167	Boquest AC, Shahdadfar A, Frønsdal K, Sigurjonsson O, Tunheim SH, Collas P, Brinchmann JE (2005) Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression after in vitro cell culture. *Molecular biology of the cell* **16**(3): 1131-1141.
3	E-MEXP-847	Reppe S, Stilgren L, Abrahamsen B, Olstad OK, Cero F, Brixen K, Nissen-Meyer LS, Gautvik, KM (2007) Abnormal muscle and hematopoietic gene expression may be important for clinical morbidity in primary hyperparathyroidism. *American Journal of Physiology-Endocrinology and Metabolism* **292**(5): E1465-E1473.
4	E-LGCL-5	no citation found
5	E-AFMX-6 E-GEOD-3790	Hodges A, Strand AD, Aragaki AK, Kuhn A, Sengstag T, Hughes G, Elliston LA, Hartog C, Goldstein DR, Thu D, Hollingsworth ZR, Collin F, Synek B, Holmans PA, Young AB, Wexler NS, Delorenzi M, Kooperberg C, Augood SJ, Faull RLM, Olson JM, Jones L, Luthi-Carter R (2006) Regional and cellular gene expression changes in human Huntington's disease brain. *Human molecular genetics* **15**(6): 965-977.
6	E-MEXP-114	Rinn, JL, Rozowsky JS, Laurenzi IJ, Petersen PH, Zou K, Zhong W, Gerstein M, Snyder M (2004) Major molecular differences between mammalian sexes are involved in drug metabolism and renal function. *Developmental cell* **6**(6): 791-800.
7	E-TABM-145	Vaquerizas JM, Kummerfeld SK, Teichmann SA, Luscombe NM (2009) A census of human transcription factors: function, expression and evolution. *Nature Reviews Genetics* **10**(4): 252-263.
8	E-GEOD-5392	Ryan MM, Lockstone HE, Huffaker SJ, Wayland MT, Webster MJ, Bahn S (2006) Gene expression analysis of bipolar disorder reveals downregulation of the ubiquitin cycle and alterations in synaptic genes. *Molecular psychiatry* **11**(10): 965-978.
9	E-GEOD-1297	Blalock EM, Geddes JW, Chen KC, Porter NM, Markesbery WR, Landfield PW (2004) Incipient Alzheimer's disease: microarray correlation analyses reveal major transcriptional and tumor suppressor responses. *Proceedings of the National Academy of Sciences* **101**(7): 2173-2178.
10	E-MEXP-429	Borrello MG, Alberti L, Fischer A, Degl'Innocenti D, Ferrario C, Gariboldi M, Marchesi F, Allavena P, Greco A, Collini P, Pilotti S, Cassinelli G, Bressan P, Fugazzola L, Mantovani A, Pierotti MA (2005) Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. *Proceedings of the National Academy of Sciences* **102**(41): 14825-14830.

11	E-GEOD-994	Spira A, Beane J, Shah V, Liu G, Schembri F, Yang X, Palma J, Brody JS (2004) Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proceedings of the National Academy of Sciences* **101**(27): 10143-10148.
12	E-GEOD-1420	Kimchi ET, Posner MC, Park JO, Darga TE, Kocherginsky M, Karrison T, Hart H, Smith KD, Mezhir JJ, Weichselbaum RR, Khodarev NN (2005) Progression of Barrett's metaplasia to adenocarcinoma is associated with the suppression of the transcriptional programs of epidermal differentiation. *Cancer research* **65**(8): 3146-3154.
13	E-MEXP-325	Troost FJ, Brummer RJM, Haenen GR, Bast A, Van Haaften RI, Evelo CT, Saris WH (2006) Gene expression in human small intestinal mucosa in vivo is mediated by iron-induced oxidative stress. *Physiological genomics* **25**(2): 242-249.
14	E-MEXP-750	Rasheed AU, Rahn HP, Sallusto F, Lipp M, Müller G (2006) Follicular B helper T cell activity is confined to CXCR5hiICOShi CD4 T cells and is independent of CD57 expression. *European journal of immunology* **36**(7): 1892-1903.
15	E-GEOD-2665	Martens JH, Kzhyshkowska J, Falkowski-Hansen M, Schledzewski K, Gratchev A, Mansmann U, Schmuttermaier C, Dippel E, Koenen W, Riedel F, Sankala M, Tryggvason K, Kobzik L, Moldenhauer G, Arnold B, Goerdt S (2006) Differential expression of a gene signature for scavenger/lectin receptors by endothelial cells and macrophages in human lymph node sinuses, the primary sites of regional metastasis. *The Journal of pathology* **208**(4): 574-589.
16	E-GEOD-7144	no citation found
17	E-GEOD-1722	no citation found
18	E-GEOD-2513	Wong YW, Chew J, Yang H, Tan DTH, Beuerman R (2006) Expression of insulin-like growth factor binding protein-3 in pterygium tissue. *British journal of ophthalmology* **90**(6): 769-772.
19	E-GEOD-2724	Vanharanta S, Pollard PJ, Lehtonen HJ, Laiho P, Sjöberg J, Leminen A, Aittomäki K, Arola J, Kruhoffer M, Ørntoft TF, Tomlinson IP, Kiuru M, Arango D, Aaltonen LA (2006) Distinct expression profile in fumarate-hydratase-deficient uterine fibroids. *Human molecular genetics* **15**(1): 97-103.
	E-GEOD-2725	
20	E-GEOD-3307	Bakay M, Wang Z, Melcon G, Schiltz L, Xuan J, Zhao P, Sartorelli V, Seo J, Pegoraro E, Angelini C, Shneiderman B, Escolar D, Chen Y, Winokur ST, Pachman LM, Fan C, Mandler R, Nevo Y, Gordon E, Zhu Y, Dong Y, Wang Y, Hoffman EP (2006) Nuclear envelope dystrophies show a transcriptional fingerprint suggesting disruption of Rb–MyoD pathways in muscle regeneration. *Brain* **129**(4): 996-1013.
21	E-GEOD-2240	Barth AS, Merk S, Arnoldi E, Zwermann L, Kloos P, Gebauer M, Steinmeyer K, Bleich M, Kääb, S, Pfeufer A, Űberfuhr P, Dugas M, Steinbeck G, Nabauer M (2005) Functional profiling of human atrial and ventricular gene expression. *Pflügers Archiv* **450**(4): 201-208.
22	E-MEXP-569	Gallego-Delgado J, Connolly SB, Lázaro A, Sadlier D, Kieran NE, Sugrue DD, Doran P, Brady HR, Osende J, Egido J (2009) Transcriptome of hypertension-induced left ventricular hypertrophy and its regression by antihypertensive therapies. *Hypertension Research* **32**(5): 347-357.

23	E-GEOD-6011	Pescatori M, Broccolini A, Minetti C, Bertini E, Bruno C, D'amico A, Bernardini C, Mirabella M, Silvestri G, Giglio V, Modoni A, Pedemonte M, Tasca G, Galluzzi G, Mercuri E, Tonali PA, Ricci E (2007) Gene expression profiling in the early phases of DMD: a constant molecular signature characterizes DMD muscle from early postnatal life throughout disease progression. *The FASEB Journal* **21**(4): 1210-1226.
24	E-GEOD-974	Hall JL, Grindle S, Han X, Fermin D, Park S, Chen Y, Bache RJ, Mariash A, Guan Z, Ormaza S, Thompson J, Graziano J, de Sam Lazaro SE, Pan S, Simari RD, Miller LW (2004) Genomic profiling of the human heart before and after mechanical support with a ventricular assist device reveals alterations in vascular signaling networks. *Physiological genomics* **17**(3): 283-291.
25	E-GEOD-1786	Radom-Aizik S, Hayek S, Shahar I, Rechavi G, Kaminski N, Ben-Dov I (2005) Effects of aerobic training on gene expression in skeletal muscle of elderly men. *Medicine and science in sports and exercise* **37**(10): 1680-1696.
26	E-MEXP-66	Wang HW, Trotter MW, Lagos D, Bourboulia D, Henderson S, Mäkinen T, Elliman S, Flanagan AM, Alitalo K, Boshoff C (2004) Kaposi sarcoma herpesvirus–induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma. *Nature genetics* **36**(7), 687-693.
27	E-MEXP-337	Dik WA, Pike-Overzet K, Weerkamp F, de Ridder D, de Haas EF, Baert MR, van der Spek P, Koster EEL, Reinders MJT, van Dongen JJM, Langerak AW, Staal FJ (2005) New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *The Journal of experimental medicine* **201**(11): 1715-1723.
28	E-GEOD-1460	Lee MS, Hanspers K, Barker CS, Korn AP, McCune JM (2004) Gene expression profiles during human CD4+ T cell differentiation. *International immunology* **16**(8): 1109-1124.
29	E-GEOD-5999	Winn VD, Haimov-Kochman R, Paquet AC, Yang YJ, Madhusudhan MS, Gormley M, Feng KV, Bernlohr DA, McDonagh S, Pereira L, Sali A, Fisher SJ (2007) Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term. *Endocrinology* **148**(3): 1059-1079
30	E-GEOD-1615	Wood JR, Nelson-Degrave VL, Jansen E, McAllister JM, Mosselman S, Strauss JF (2005) Valproate-induced alterations in human theca cell gene expression: clues to the association between valproate use and metabolic side effects. *Physiological genomics* **20**(3): 233-243.
31	E-GEOD-6008	Hendrix ND, Wu R, Kuick R, Schwartz DR, Fearon ER, Cho KR (2006) Fibroblast growth factor 9 has oncogenic activity and is a downstream target of Wnt signaling in ovarian endometrioid adenocarcinomas. *Cancer research* **66**(3): 1354-1362.
32	E-TABM-26	Liu P, Ramachandran S, Seyed MA, Scharer CD, Laycock N, Dalton WB, Williams H, Karanam S, Datta MW, Jaye DL, Moreno CS (2006) Sex-determining region Y box 4 is a transforming oncogene in human prostate cancer cells. *Cancer research* **66**(8): 4011-4019.

33	E-GEOD-3218	Korkola JE, Houldsworth J, Chadalavada RS, Olshen AB, Dobrzynski D, Reuter VE, Bosl GJ, Chaganti RSK (2006) Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13. 31, is associated with in vivo differentiation of human male germ cell tumors. *Cancer research* **66**(2): 820-827.
34	E-GEOD-5667	Plager DA, Leontovich AA, Henke SA, Davis MD, McEvoy MT, Sciallis GF, Pittelkow MR (2007) Early cutaneous gene transcription changes in adult atopic dermatitis and potential clinical implications. Experimental dermatology 16(1): 28-36.
35	E-GEOD-6932	Hong J, Lee J, Min KH, Walker JR, Peters EC, Gray NS, Cho CY, Schultz PG (2007) Identification and characterization of small-molecule inducers of epidermal keratinocyte differentiation. *ACS chemical biology* **2**(3): 171-175.

Chapter 3

Duplicate Retrogene Copies of *TP53* in the African Elephant and Bats: Additional Cancer Suppression Mechanisms or Pseudo-Retrogenes?

Abstract

The lack of correlation between body size, longevity, and cancer across species, known as Peto's Paradox, can be resolved by the evolution of additional mechanisms of cancer suppression in large, long-lived species. The finding of 19 additional retrogene copies of the tumor suppressor *TP53* in the large-bodied African elephant has been suggested to explain the low cancer rate in this species. Two studies have shown that elephant cells display an enhanced TP53 DNA-damage and apoptotic response, which was indirectly attributed to the retrogene copies. Up to five *TP53* retrogenes are also found in the long-lived microbats. To determine whether these retrogene copies in the African elephant and microbats are functional, we examine the DNA sequences of these copies for stop codons and compare their rates of codon substitution to the normal *TP53* copies of 24 mammal species. Our analysis of stop codons shows that all retrogene copies possess truncated reading frames. The retrogene copies in the African elephant were less conserved ($dN/dS = 0.808$) relative to the normal TP53 copies ($dN/dS = 0.276$) ($p < 0.001$), yet their rate of codon substitution was significantly slower than the rate of neutral evolution ($dN/dS = 1$) ($p < 0.01$). We found no evidence for differences in rates of codon substitution between the elephant retrogene copies. In the bats, the rates of codon substitution in their retrogene copies were not significantly different from neutrality, which is indicative of pseudogenization. Possible explanations for our findings in the African elephant are discussed.

Introduction

If all else is equal then large, long-lived organisms, such as elephants, are expected to have higher rates of cancer under the multistage model of carcinogenesis (Peto 1977). Since each cell division poses a risk of somatic mutations during DNA replication (Albanes & Winick, 1988), organisms that have additional cells (larger body size) or that undergo more lifetime cell divisions (long lifespan) should have a higher risk of cancer. However, the expected relationship is not found (Caulin and Maley, 2010; Abegglen et al., 2015). This lack of correlation between body size, lifespan, and cancer across species has become known as Peto's Paradox (Nunney, 1999).

Nunney (1999, 2003) proposed an evolutionary model that can explain this paradox: following expansions in body size and lifespan, natural selection acts to recruit additional mechanisms to reduce the loss of fitness caused by any significant increase in pre-reproductive cancer risk. Such mechanisms could act in two different ways, by more effectively eliminating cancer cells, via immunological policing, or by acting to suppress the occurrence of cancer cells. Evolutionary arguments combined with data from immunosuppressed individuals indicate that enhanced suppression is likely to be the usual response (Nunney, 2017), and many of the genes associated with this role are classified as tumor suppressor genes (Weinberg, 1991; Knudson, 1993). These genes are involved in cell cycle control, apoptosis, and DNA repair, and typically require both copies of the gene to be knocked out by mutation for their function to be lost. Each additional tumor suppressor gene that is recruited would therefore require a cell to acquire two additional mutational "hits" for cancer to initiate.

Three mechanisms could allow a cell to acquire an additional tumor suppressor are: (1) the duplication of a tumor suppressor gene currently suppressing cancer in the at risk tissue; (2) the upregulation within the target tissue of a tumor suppressor gene that is already acting elsewhere; or (3) the evolution of a novel mechanism. Support for these possibilities has been found, despite there being only a limited number of studies. Seluanov et al. (2007) found that increased telomerase suppression, an anti-cancer mechanism, correlates with increasing body size in rodents. Additionally, long-lived rodents, the naked mole rat and the blind mole rat, were found to have additional anti-cancer mechanisms, early contact inhibition (Seluanov et al., 2009; Tian et al., 2013) and concerted necrotic cell death (Gorbunova et al., 2012), respectively. These results appear to provide examples of mechanisms (2) and (3), but not of mechanism (1), gene duplication. Support for this possibility has come from the analysis of the genome of the African elephant (Loxdonta africana), which contains 19 retrogene copies of the tumor suppressor *TP53*. The *TP53* gene has been called the "Guardian of the Genome" (Lane, 1992) because it is arguably the most vital tumor suppressor gene, having a critical role in both DNA damage surveillance and apoptosis, and exemplified by the observation that it is mutated in the majority of human cancers. For this reason it has been proposed that these additional copies of the *TP53* gene are responsible for the low cancer rate in this large-bodied species (a lifetime risk of 4.81%; Abegglen et al., 2015).

Additional retrogene copies of TP53 are also found in the close relative of the African elephant, the Asian elephant, *Elephas maximus*, which has an estimated 12-20 retrogene copies in its draft genome (Abegglen et al., 2015; Sulak et al., Unpublished).

Comparing these species with other members of the paenungulata, the Cape rock hyrax (*Procavia capensis*), the Florida manatee (*Trichechus manatus latirostris*) and three extinct Elephantid species, revealed a positive relationship between the copy number of *TP53* retrogenes and body size (Sulak et al., Unpublished). All copies in the paenungulata appear to be derived from a single ancestral event (Sulak et al., Unpublished).

TP53 retrogene copies have also been found in numerous other lineages of mammals, with two groups of species having high copy numbers (Sulak et al., Unpublished): the long-lived microbats, which can live up to 41 years (Podlutsky et al., 2005), have been found to possess up to five *TP53* retrogene copies; and five copies are also present in the rat genome, *Rattus norvegicus*.

Two studies have found that African elephant cells show an enhanced *TP53*-pathway response relative to other species (Abegglen et al., 2015; Sulak et al., Unpublished). Abegglen et al. (2015) found that the p53-mediated apoptotic response to DNA damage increased with *TP53* copy number in the lymphocyte cells of humans with Li-Fraumeni syndrome (1 normal copy), human controls (2 normal copies), and in the African elephant (1 normal copy, 19 retrogene copies). Sulak et al. (2015) showed that dermal fibroblast cells of the African and Asian elephants had up-regulated *TP53* signaling in response to lower doses of DNA damage relative to closely related species without an expansion in *TP53* copy number.

Although these studies showed that African elephants have an enhanced TP53-pathway response, whether this is due to the *TP53* retrogene copies is unclear. Sulak et al.

(Unpublished) found that all 19 copies are truncated prematurely by stop codons, with a maximum size of 210 amino acids relative to 393 amino acids coded by the normal *TP53* gene. Experimental studies by Sulak et al. (Unpublished) suggest that only one retrogene copy in the African elephant, TP53RTG12, is transcribed and translated. This copy is also limited in function since the TP53RTG12 protein is truncated before the critical DNA-binding domain, the region commonly mutated in human cancers. Sulak et al. (Unpublished) tested whether an anti-cancer function is retained within the intact MDM2-binding domain and the dimerization domain of TP53RTG12. MDM2 binds p53 and ubiquinates it for degradation. It has been suggested that retrogene copies with intact MDM2-binding sites may upregulate normal p53 by acting as decoys disrupting MDM2-mediated degradation (Abegglen et al., 2015; Sulak et al., Unpublished). Similarly, Sulak et al. (Unpublished) proposed that dimerization of normal p53 to the retrogene protein copies may block MDM2 from gaining access to the ubiquitinization sites of normal p53. Sulak et al. (Unpublished) found support for the latter hypothesis: TP53RTG12 is capable of dimerizing normal p53, but is not capable of binding with MDM2.

In summary, data from the African elephant suggest that of the 19 retrogene copies of TP53, only one is still active (TP53RTG12) and that it acts by protecting the "canonical" form of TP53 from degradation. Complicating this interpretation, with TP53RTG12 alone playing the key role, is the apparent absence of this homologue in the Asian elephant (Sulak et al. Unpublished), plus the association between body size and the number of retrogene copies within the paenungulata.

The purpose of this study was to determine whether individual retrogene copies in the African elephant retain functionality in specific regions of their DNA sequence or are evolving neutrally as pseudogenes. First, we tested whether, along the entire length of sequence, the pattern of codon substitution, as measured by the relative rate of nonsynonymous to synonymous changes (dN/dS), was different in the African elephant retrogene copies relative to the normal copies of 24 mammal species. We also examined the same question in the bats, which given their long lifespans represent an independent test of the hypothesis that retrogene duplication of TP53 may play an important role in cancer suppression following the evolution of increased body size and/or longevity. Pseudogenes, as they decay, are expected to develop a value of dN/dS ≈1. On the other hand, protein sequences conserved by selection exhibit dN/dS <1. Second, we examined whether there was evidence for differences in dN/dS amongst the retrogene copies of the African elephant and bats, to examine whether some copies are more conserved (e.g. TP53RTG12). In the case of the African elephnant retrogenes we adopted a hierarchical approach, first testing the whole gene, regardless of premature stop codons, then testing the region prior to the premature stop codons, and finally testing the dN/dS of the individual codon sites of the dimerization domain.

Materials and Methods

Selection of Normal TP53 Mammal Sequences

We included the normal *TP53* gene sequences of 24 mammal species in our analysis for comparison to the retrogene sequences. All seven Afrotheria with published genome sequences were used in this study: African elephant (*Loxondonta africana*;

Loxafr3.0), Cape rock hyrax (*Procavia capensis*; Pcap_2.0), Florida manatee (*Trichechus manatus latirostris*; TriManLat1.0), lesser hedgehog tenrec (*Echinops telfairi*; EchTel2.0), aardvark (*Orycteropus afer*; OryAfe1.0), Cape golden mole (*Chrysochloris asiatica*; ChrAsi1.0), and the elephant shrew (*Elephantulus edwardii*; EleEdw1.0). All 10 bats species with published genome sequences were used: Brandt's bat (*Myotis brandtii*; ASM41265v1); David's Myotis (*Myotis davidii*; ASM32734v1), little brown bat (*Myotis lucifugus*; Myoluc2.0), big brown bat (*Eptesicus fuscus*; EptFus1.0), Parnell's mustached bat (*Pteronotus parnelli*; ASM46540v1), greater horseshoe bat (*Rhinolophus ferrumequinum*; ASM46549v1), Indian false vampire (*Megaderma lyra*; ASM46534v1), straw-colored fruit bat (*Eidolon helvum*; ASM46528v1), black flying fox (*Pteropus alecto*; ASM32557v1), and the large flying fox (*Pteropus vampyrus*; ASM32557v1). Two species from each of the remaining eutherian superorders (Xenarthra, Laurasiatheria, Euarchontoglires) were included for even taxonomic sampling. The Hoffman's two-toed sloth (*Choloepus hoffmanni*; C_hoffmanni-2.0.1) and nine-banded armadillo (*Dasypus novemcinctus*; Dasnov3.0) were selected from Xenarthra, the only two published genomes of this group. The dog (*Canis lupis familiaris*; CanFam3.1) and cow (*Bos Taurus*; Bos_taurus_UMD_3.1.1) were selected from Laurasiatheria, to represent the carnivores and ungulates of this group, respectively. Finally, the human (*Homo sapiens*; GRCh38.p8) and Norway rat (*Rattus norvegicus*; Rnor_6.0)) were selected from Euarchontoglires, to represent the primates and rodents of this group. These last four species were selected because of their position within each super-family, as well as their availability of published RNA sequences. The marsupial, Tasmanian devil (*Sarcophilus*

harrisii; Devil_ref v7.0), was used as the outgroup. The phylogenetic relationships of these mammal species (Meredith et al., 2011) was used. The species used in the study are referred to throughout by common names that are unambiguous relative to each other (e.g. African elephant as elephant in the phylogenetic analyses since the Asian elephant was not included).

TP53 is composed of 11 exons and 10 introns with translation beginning in exon 2 (Zakut-Houri et al., 1985). The coding-regions of the *TP53* gene (exons 2-11) were obtained by performing BLASTn searches on the whole genome contig libraries of the 24 mammal species. For this purpose, we used the following procedure. TP53 sequences for the human, rat, cow, and dog were obtained by performing a BLASTn search on the corresponding whole genome contig libraries using the whole of the sequenced RNA for each species obtained from NCBI's Nucleotide database as the queries. Each RNA query was manually aligned to the matching contig(s) in SeaView (version 5.0; Galtier et al., 1996). The coding regions of the matching contig(s) were obtained by excising the unaligned intron sequences. For the remaining species, sequenced RNA was not available; therefore, RNA sequences predicted by Gnomon (Souvorov et al., 2010) were obtained from NCBI's Nucleotide database for use as queries. The predicted RNA sequence of the African elephant was used for the Afrotheria and Xenarthra, the Brandt's bat predicted RNA was used for all bat species, and the Tasmanian devil predicted RNA was used for this species. The exon sequences of all 24 species were flanked by AT/GT splice-sites in all 10 transcribed introns, providing good support for a conserved exon structure.

Selection of TP53 Retrogene Sequences

We searched for additional *TP53* retrogene copies in the Afrotheria, to which the African elephant belongs, and in the bats using BLASTn searches on the whole genome contig libraries. The same sequences were used as queries in the BLASTn searches described for the normal *TP53* copies. To identify retrogenes, we used the criteria that the BLASTn alignment score was >80, the sequence aligned to at least 50% of the query, and that no introns were present. The contig sequence(s) containing the retrogene was manually aligned in Seaview to the query sequence. The portions of the contigs that flanked the retrogene sequences were excised.

Retrogene Stop Codons

The retrogene sequences were examined for stop codons to identify the length of their open reading frames. Sequences that were missing information at the beginning 5' sequence had their first available 5' base pair aligned to the corresponding normal *TP53* sequence before examining it for stop codons. Although some of the retrogene sequences did not begin with the start codon "ATG" and may be altogether non-coding, the position of the first stop codon was used for these sequences to determine their open reading frame length.

Alignment and Phylogenetic Tree

All alignments were performed manually using SeaView, with exon/intron boundaries marked by inserting three ambiguity characters. The retrogene sequences often had indels that caused them to become out of frame relative to the normal *TP53* sequences. The effect of these indels was removed as a conservative approach to keep all

sequences in frame. Each deleted base in a retrogene sequence was represented as a gap in the alignment. For insertions, if they were not multiples of three, one or two gaps were added 3' of the insertion to put the retrogene back in frame and sequences not possessing the insertion had the appropriate multiple of three gaps added to keep all of the sequences aligned.

A phylogenetic tree of all sequences was created from the alignment using PhyML (version 3.0; Guindon & Gascuel, 2003). We created our tree using a general time reversal model (GTR) with aLRT (SH-like) branch supports. The same parameters used by Sulak et al. (Unpublished) were used. Although the retrogenes contained internal stop codons, we used the full length of these sequences. The resulting relationships of the retrogene sequences were used in the final tree. However, the relationships of the normal *TP53* sequences were constrained to reflect supported mammal relationships (Meredith et al., 2011) (see Figure 3.1). The phylogenetic tree of the normal and retrogene *TP53* copies was used in subsequent analyses.

Elephant *TP53* retrogene sequences were annotated with the same numbering system (ElephantRTG1, ElephantRTG2, etc.) used by Sulak et al. (Unpublished), which numbered the retrogenes in ascending order beginning with the most distantly related copy. We used the same procedure to number the bat retrogene copies for each species in top-to-bottom phylogenetic order.

Separate Origins and Locations of Bat Retrogenes

The phylogenetic relationships found amongst the bat retrogene sequences and their locations in the tree relative to the normal *TP53* sequences were further investigated

by a comparison of their genomic backgrounds. First, we investigated the question of whether there was more than one retrogene origin in the bats. The bat genomes are resolved to the level of contigs; therefore, the contig sequences flanking each retrogene were compared. All contigs were first manually aligned along their retrogene sequence, and then the retrogene sequences were excised. The first 1000 base pairs upstream and downstream of the excised retrogene were extracted and then manually aligned in Seaview. The phylogenetic relationships of the genomic backgrounds were assessed in PhyML using the previous parameters (see above).

We also examined whether the genomes of the bat species that were missing retrogene sequences despite diverging after the proposed origin of a retrogene, showed evidence of a degraded retrogene sequence at the same genomic location. Highly degraded retrogenes were unlikely to meet our BLASTn criteria, and thus would not have been included in the phylogenetic analyses. The genomes of each species were queried using the genomic neighborhood of the retrogene copy (1kb 5' and 3' of the copies) from the closest relative in the investigated retrogene clade. The matching contig(s) was investigated for the presence of a retrogene copy by performing a second BLASTn search on this contig; this time using the retrogene sequence of the species used in the previous query, instead of its genomic neighborhood. Degraded retrogene copies (or copies that fell in regions with no coverage) were identified by the presence of regions with BLASTn alignment scores that were >80, yet aligned to less than 50% of the sequence.

Codeml Models

The phylogenetic tree was used in the codeml program of PAML (Phylogenetic Analysis by Maximum Likelihood, version 4; Yang, 2007) to assess the average dN/dS ratio (rate of non-synonomous mutations per non-synonomous site/ rate of synonomous mutations per synonomous site) across the sequence alignment. The branch model of codeml was used for all analyses (except where stated), which computes the average dN/dS ratio per codon across the full length of the sequence(s) in the specified branch or branches. Since stop codons are not handled by codeml, the stop codons of all sequences were removed. The internal stop codons of the retrogene sequences were modified by replacing the third base pair of each stop codon with the ambiguity character, "N" (e.g. TGA became TGN).

The rate of codon substitution was first examined using only the tree of normal *TP53* copies. The retrogene copies were removed for this analysis as to not skew estimates of certain parameters for the normal copies, such as the transition-transversion ratio or the base-frequency at codon positions. The branch model was applied to the normal *TP53* copies using the hierarchical method of Nunney and Schuenzel (2006) to characterize the level of heterogeneity in dN/dS across branches. In this hierarchical "bottom-up" approach, the tree was systematically divided into clades and tested for heterogeneity in dN/dS values. For example, the test begins by testing for heterogeneity in the outgroup, the Tasmanian devil, by comparing the likelihood of the null model, which fits a single dN/dS ratio to the entire tree, relative to a two-dN/dS model that gives a separate dN/dS to the outgroup. The significance of the dN/dS value added to the outgroup is determined by comparing the log-likelihood of the two models in a one

degree of freedom chi-square test ($X_1^2 = 2$ x $(\text{LogLike}_2 - \text{LogLike}_1)$. This method avoids multiple testing by testing for any residual variation in the remaining branches before testing the next clade. In the example above, the two-dN/dS model of the outgroup was then compared to a model with a separate dN/dS value for each branch of the tree. A chi-square test is used to test for significance with a degree of freedom equal to the difference in the number of dN/dS values used for each model. These tests are performed regardless of whether the added dN/dS value was significant. This "bottom-up" approach is continued until no significant heterogeneity remains in the tree. The Afroinsectivora shared branch had a dN/dS value equal to 0, due to its short branch length. Therefore, this branch could not be tested and was ignored in the calculation of degrees of freedom for all relevant tests.

Additional tests were needed to finalize the model for the normal copies in which a separate dN/dS value is given to all significantly heterogeneous branches. The "bottom-up" approach evaluates whether both sister-clades are significant or non-significant; therefore, for all significant tests we evaluated whether this was due to one or both sister-clades being significantly different from the proceeding branch. Beginning at the bottom of the tree, significant sister-clades were assessed by comparing the final model to a revised model with the last dN/dS value only given to the sister clade with the least similar dN/dS value relative to the proceeding branch. If no significant effect of removing the dN/dS of the specified branch was found, the final model was updated. We then performed an additional test to confirm that no residual heterogeneity remained in the tree after removing a dN/dS value before moving on to the next pair of sister clades.

Clades that had significant findings for their shared branch (common ancestor) were also investigated in this manner relative to the descendants of this shared branch to determine if the dN/dS value of the shared branch could be removed from the model. The final version of the dN/dS tree of the normal copies of TP53 assigned a separate dN/dS for each normal-copy branch that was found to be significantly different from the preceding branch.

The relative rate of evolution of nonsynonymous and synonymous sites was examined separately for the retrogenes of the elephant and its relatives and of the bats by adding their sequences back to the alignment and the final version of the normal TP53 phylogenetic tree. In the analysis of each retrogene clade, a null model giving that clade the same dN/dS as the preceding branch of the normal tree was compared to a model giving a separate dN/dS for the retrogene clade. To evaluate whether there was heterogeneous values of dN/dS amongst the retrogenes, the above model was then compared to a modified model with a separate dN/dS given to each retrogene branch. Significantly different dN/dS ratios were added to branches as needed. The final model that included all of the retrogenes was tested by comparing the final model to a model with one dN/dS value for the entire tree. We also tested this model to confirm that no residual heterogeneity remained.

We examined whether the dN/dS values observed for the retrogenes were significantly different from neutral evolution (dN/dS = 1). We performed separate tests for the Afrotherian and bat retrogene clades, by comparing the best-fit model to one where the retrogenes were given a fixed value of dN/dS = 1.

Amino Acid Conservation of Dimerization Domain

We compared the level of amino acid conservation in the dimerization domain of the African elephant retrogenes relative to the normal *TP53* copies of the 24 mammal species. The dimerization domain of p53 in mice is formed by five amino acids: Pro^{174}, His^{175}, Glu^{177}, Arg^{178}, and Met^{240} (Ho et al., 2006). Amino acid sequences were manually aligned in Seaview. The retrogene sequences, which contained stops codons prior to the dimerization domain, were kept in frame relative to the normal copies in this alignment, as in the previous analyses. In the normal copies, the level of conservation in these five residues was examined by quantifying the number of amino acid substitutions across all 24 species. In the African elephant retrogene copies, the number of amino acid substitutions relative to the normal copy in this species was quantified.

Codeml Models Testing the Dimerization Hypothesis

The previous tests for heterogeneous rates of dN/dS amongst the African elephant retrogenes calculated an average dN/dS across the length of the sequences, regardless of whether any of these sequences could be fully translated given presence of premature stop codons. We next determined whether specific retrogenes had lower values of dN/dS in the regions prior to the first four amino acids of the dimerization domain, since that domain has been proposed to be functionally important in one retrogene (TP53RTG12). We retested for heterogeneous rates of dN/dS in the African elephant retrogenes up to exon 4 of their sequences (180aa), which is the 5' region that includes the first four amino acids of the dimerization domain. The hyrax and manatee retrogenes were used as the outgroup. We used the hierarchical "bottom-up" method of Nunney & Schuenzel

(2006) described above, beginning with a model giving separate dN/dS values to each of the outgroup species.

We extended this analysis of the dimerization domain by using the sites model in PAML to estimate the average values of dN/dS across all of the individual codons of the Afrotherian retrogenes, to determine whether the first four codons involved in the dimerization domain were more conserved (had lower dN/dS) in at least some of the retrogene copies. We implemented the site model M8 of codeml (Yang et al., 2007), which allows the dN/dS ratio to vary among codon sites in the sequence alignment. We subdivided the analysis into two groups: sequences related to TP53RTG12, and those that were not. The two analyses were run on using the elephant RTGs of each group over the first 180aa of sequence (included first four AAs of dimerization domain), as was done in the previous test. The estimated values of dN/dS for each codon were extracted from the Bayes Empirical Bayes (BEB, Yang et al., 2005) output and compared. We calculated the average ranking of dN/dS for these four codons relative to the other 173 (or 177) amino acids that were present in all sequences (i.e. no sequences had deletions of these AAs). We also calculated the average dN/dS in the codons outside of the dimerization domain for each retrogene clade, which could be statistically compared using a two-tailed Paired T-Test between the dN/dS values of the shared amino acids of RTGs 1-7 and RTGs 8-19.

Results

The relationships among the 24 mammalian taxa included in the study are shown in Figure 3.1. A single non-retrogene TP53 copy was found in all taxa, with the exception

of the little brown bat, which had two copies. The two copies differed by one base pair in exon 7, two base pairs in exon 8, and at 52 base pairs in the intron sequences.

In the Afrotheria, retrogene copies were found in the African elephant (19 copies), Cape Rock Hyrax (1 copy), Florida Manatee (1 copy), Lesser Hedgehog Tenrec (1 copy), and the Cape Golden Mole (3 copies), as reported previously (Sulak et al., Unpublished). The phylogenetic analysis grouped the retrogene copies of elephant *TP53* retrogene sequences with the retrogene copies found in the manatee (1 copy) and hyrax (1 copy) with high (97%) bootstrap, supporting a single origin predating the split of these species (Figure 3.2). Therefore, the retrogene copies of manatee and hyrax were retained in addition to the elephants. All other Afrotheria retrogene sequences had support for being of separate origin and were omitted from the analysis.

In the bats, we included all retrogene sequences that were identified: Little Brown Bat (5 copies), Brandt's Bat (4 copies), David's Myotis (4 copies), Big Brown Bat (1 copy), and the Indian False Vampire (1 copy). The relationships of the retrogene and normal copies strongly support exactly three separate retrogene origins: a single origin in the Yinpterochiroptera (Origin #1), and an earlier (Origin #2) and later (Origin #3) origin in the Yangochiroptera (see Figure 3.3). The clades developing from origins #2 and #3 are well supported (100% bootstrap support), so there is no indication of additional origins, and the three origins are separated by well-supported nodes within the phylogeny.

This finding of separate origins in the bats was examined further by comparing the genomic neighborhoods of the retrogene copies (1kb 5' and 3' of the copies). We found that the retrogene copies from the two apparent origins in the Yangochiroptera

(Origin #2 vs. #3; see Figure 3.3) reside within different genomic backgrounds, each with 100% bootstrap support. The contig containing FalseVampireBatRTG1 of Origin #1 did not cover any of the surrounding genomic background; therefore, Origin #1 in the Yinpterochiroptera was not examined.

The genomes of the bat species that did not possess a given retrogene copy despite diverging after the proposed origin of the retrogene were investigated for the expected presence of highly degraded retrogene copies that did not meet our BLASTn criteria for inclusion in the study. Thus, the Origin #3 RTGs did not include the expected copy in Brandt's bat (group A in Figure 3.3). However, we found evidence of a degraded retrogene copy in the Brandt's Bat in the genomic location of Origin #3. Specifically, the contig matching the genomic location of Origin #3 was found to align to the LittleBrownBatRTG1 in a follow-up BLASTn search along base pairs 1007-1153 (95% identity; E score = 1e-57). Furthermore, no retrogene copy was present in the genome of the Mustached Bat at this genomic location, which was evidenced by there being no match in a BLASTn search (using BigBrownBatRTG1 as the query) in the contig covering the genomic location of Origin #3. In support of Origin #2 in the bats, the mustached bat genome contained a degraded retrogene in this genomic location (group B in Figure 3.3). Less than 50% of the query (LittledBrownBatRTG2) was found to align with BLASTn alignment scores > 80 to the contig covering this genomic location, but sequence matches were found at base pairs 338-532 (83% identity, E score = 3e-46), 642-751 (86% identity, E score = 3e-26), 753-886 (85% identity, E score = 2e-34), and 888-962 (95% identity, E score = 5e-24). Additionally, the big brown bat was found to have a

retrogene copy in the genomic location of origin #2 (group B in Figure 3.3), although the majority of the retrogene sequence fell in a region with no coverage. The contig matching the upstream portion of the genomic location of origin #2 had the first 226 base pairs of a retrogene copy align to the LittleBrownBat2 query (89% identity, E score = 2e-73), and the contig matching the downstream portion had the last 153 base pairs of a retrogene copy align to the query (86% identity, E score = 2e-47). Similarly, the relationships of the bat retrogenes derived from Origin #3 are difficult to explain without the presence of a Little Brown Bat retrogene copy in the subclade represented by group (C) in Figure 3.3. Evidence for a retrogene copy was found in this genomic location of the little brown bat, which had 100% identity to the first 17 bases of the query (Brandt'sBatRTG2), but had the remaining retrogene sequence fall in a region without coverage.

We next examined the premature stop codons within the retrogene sequences. All retrogene sequences from the elephant, hyrax, and manatee (Figure 3.4), and from the bats (Figure 3.5) had reading frames truncated by stop codons. Reading frame lengths were typically longer in the elephant retrogene copies ranging from 79-210aa in 18 of the 19 copies (plus one much shorter), whereas only one of the bat RTGs was longer than 79aa, with the remaining 14 in the range of 13-66aas. We found that 9 of the 19 elephant retrogene copies retained a portion of the dimerization domain (4 of 5 amino acids), while the reading frame in the bat, manatee, and hyrax copies were all truncated 5' of this domain.

The relative rate of non-synonymous to synonymous substitutions (dN/dS) amongst the normal and retrogene copies across the phylogeny was highly heterogeneous

($X_{13}^2 = 406.22$; p < 0.001). All significantly different dN/dS ratios across the branches of the phylogeny are shown in Figure 3.6, leaving no residual heterogeneity in dN/dS ($X_{105}^2 = 116.12$; p = 0.22). For more detail see Supplementary Tables 3.1 and 3.2.

In the normal *TP53* copies, the background rate of codon substitution (dN/dS = 0.276) was indicative of a fairly typical level of sequence conservation. Nine clades were found to have dN/dS ratios that were significantly different from the proceeding rates, ranging from 0.097 in the outgroup (the Tasmanian devil; p<0.001) to 0.503 in the hyrax (p<0.05). The Yangochiroptera also had a notably high rate of non-synonymous substitution (dN/dS = 0.489; p < 0.001). The rate of codon substitution was not significantly different from the background rate in the normal copies of the elephant, manatee, and Yinpterochiroptera.

All retrogene sequences were found to have significantly higher values of dN/dS relative to their preceding branches, and were less conserved than all normal *TP53* copies: elephant/hyrax/manatee RTG (dN/dS = 0.801; $X_1^2 = 106.28$; p < 0.001), FalseVampireBatRTG1 (dN/dS = 0.892; $X_1^2 = 27.55$; p < 0.001), Yangochiroptera Clade 1 (dN/dS = 1.012; $X_1^2 = 9.73$; p < 0.005), and Yangochiroptera Clade 2 (dN/dS = 0.994; $X_1^2 = 19.21$; p < 0.001). Furthermore, these values were consistent within each clade, with no evidence for heterogeneity in the rate of codon substitution amongst the retrogenes of each clade (elephant, hyrax, and manatee: $X_{41}^2 = 42.63$, p = 0.40; Yangochiroptera Origin #2: $X_{20}^2 = 17.47$, p = 0.62; and Origin #3: $X_4^2 = 3.20$, p = 0.53). We then tested whether the retrogenes are evolving significantly different than neutrality (dN/dS = 1), which is suggestive of pseudogenization. The bat retrogene clades, which

were tested as a group, were consistent with neutrality ($X_3^2 = 0.27$; p = 0.97); however, the pattern of codon substitution in the elephant, hyrax, and manatee retrogenes showed significant evidence of selective constraint ($X_1^2 = 7.12$; p < 0.01).

We examined whether there was evidence for differences in the rate of dN/dS amongst the African elephant retrogenes along the first 180 amino acids of sequence, which contained the first four amino acids of the dimerization domain, and occurred before the first stop codon in 9 of the 19 retrogenes (Figure 3.4). However, no heterogeneity in the rate of codon substitution was found amongst the African elephant retrogenes in this region (dN/dS = 0.9789, $X_{36}^2 = 34.77$; p = 0.53).

Next we focused the analysis on the dimerization domain. The level of amino acid conservation in the dimerization domain was compared between the normal *TP53* copies (Figure 3.7A) and the retrogene copies of the African elephant (Figure 3.7B). All five amino acids of the dimerization domain were conserved across the normal copies of all 24 mammals, including the marsupial Tasmanian devil. The dimerization domain was less conserved across the elephant retrogenes, and they all included a three base insert between amino-acid positions 178 and 179 in the dimerization domain (Figure 3.7B). Except for this insert, all of the retrogenes from the closely related clade of predominantly long retrogenes (RTG8-RTG19; see Figure 3.4) retained the ancestral set of amino acids, except for RTGs 8 and 15 (Figure 3.7B), even though all retrogene sequences had a stop codon before the 5th dimerization residue at position 240, and RTGs 7, 10, 17, and 18 had a stop codon before the beginning of the dimerization domain (see Figure 3.4). Of the remaining elephant retrogenes (RTG1-RTG7), all of the

sequences included a stop codon before the beginning of the dimerization domain. RTG7 matched the ancestral residues, while RTGs1-5 each differing from the ancestral dimerization domain by one amino acid, and RTG6 differed by two.

The dN/dS estimated for each of the first four codons of the dimerization domain were compared to the dN/dS values estimated for the other codons in the first 180aa of the African elephant retrogenes. This was done separately for RTGs 8-19, 9 out of 12 of which have their first stop codon after the dimerization domain, and for RTGs 1-7, all of which have a stop codon before this domain (see Figure 3.4). In RTGs 8-19, three of the four amino acids in the domain (H^{175}, E^{177}, and R^{178}), as well as the inserted codon (C^{insert}) present in these copies, had dN/dS values that were <0.4, i.e. <33% of the average dN/dS outside of the domain (Table 3.1). In RTGs 1-7, none of the amino acids were <0.65, i.e. <65% of the average dN/dS outside of the domain (Table 3.1). However, these differences are not statistically significant. For the codons that were outside of the domain, and also present in both retrogene clades, a two-tailed Paired T-test found no significant difference ($t = 1.72$, $df = 166$, $p = 0.087$) between these dN/dS values (Table 1.1).

Discussion

The inclusion of the single retrogene copies of the hyrax and manatee in our analysis, in addition to the African elephant copies, showed high bootstrap support for a single origin of these retrogene copies occurring before the split of the three species. These results supported the previous findings of Sulak et al. (Unpublished), and suggest

an earlier origin than indicated by Abegglen et al. (2015), who did not include the hyrax and manatee copies in their phylogeny.

Sulak et al. (Unpublished) summarized the occurrence of multiple copies of TP53 in mammals, noting that, besides the 20 copies in the African elephant, 6 copies occur in the rat and 7 in the little brown bat. It appears that the frequency with which new copies of *TP53* arise is unusually high; however, it is not clear if these are being selectively retained due to the role of this gene as a tumor suppressor. We examined in detail the distribution of copies of TP53 in the bats, and found that there have been two origins of TP53 retrogenes in the microbat species of the Yangochiroptera and a third origin in the Yinpterochiropteran microbats. The finding of separate origins in the Yangochiroptera was supported by distinct genomic backgrounds being found around the retrogenes of each origin.

Our finding of an additional normal copy of *TP53* in the genome of the little brown bat may help explain the long lifespan of this species (up to 37 years; Austad & Fischer, 1991). The two copies differed by only three base pairs and one amino acid in their coding regions and had intact reading frames, suggesting they are both functional. This is the only known example of TP53 duplication in mammals that is not an example of intronless retrotransposition. Belyi et al (2010) suggested that there was an additional copy in the tenrec; however, upon re-examination we found it to be a retrogene copy.

In the normal *TP53* copies, the background pattern of codon substitution (dN/dS = 0.276) applied to the elephant, the Yinpterochiroptera, humans and rats (Figure 3.6) and is in line with previous estimates for the average level of dN/dS in the protein-coding

genes of primates (dN/dS = 0.28; Yang & Nielsen, 2000) The dN/dS for the other clade of bats, the Yangochiroptera, was higher at 0.489. The retrogene copies were expected to have a similar rate of codon substitution if the retrogenes retain the function of the normal *TP53* copies. However, all retrogene copies were found to be less conserved than the normal *TP53* copies. The rate of codon substitution in the retrogene copies of the African elephant, hyrax, and manatee, dN/dS = 0.801, was significantly less conserved than the preceding (background-level) branch ($p < 0.001$). In the bat retrogene copies, which formed three distinct clades, all retrogene clades were significantly less conserved than the preceding non-retrogene branches: FalseVampireBatRTG1 (dN/dS = 0.892), Yangochiroptera Clade 1 (dN/dS = 1.012), and Yangochiroptera Clade 2 (dN/dS = 0.994). In addition, all retrogene copies were found to contain premature stop codons, which together with the high dN/dS indicates a dramatic loss of functional constraint.

The retrogene copies of the elephant, hyrax, and manatee are more conserved than the retrogene copies of the bats. The pattern of base substitution in the three retrogene clades of the bats was not significantly different from neutral evolution (dN/dS = 1), while the retrogenes of African elephant, hyrax, and manatee clade have a dN/dS (= 0.801) that was significantly less than one ($p < 0.01$). In addition, the average open reading frame length was substantially longer in the African elephant retrogene copies (152 amino acids) than in the bat copies (32 amino acids).

One possible explanation for the differences observed between the bat and Afrotherian retrogenes, is that one or more specific regions of the Afrotherian retrogenes may be functional. Sulak et al. (Unpublished) suggested that the dimerization of

retrogene p53 with normal p53 might inhibit the degradation of the normal copy by blocking MDM2-mediated degradation. The bat retrogenes were all truncated by premature stop codons prior to the dimerization domain of *TP53*. However, 9 of the 19 copies in the African elephant were truncated after the first four of the five amino acids of the dimerization domain. Seven of these nine retrogene copies retained the first three amino acids of the dimerization domain. We found a three base pair insert in all 19 retrogene copies, which shifted the arginine residue (R^{178}) one position downstream. The last amino acid of the domain (M^{240}) was lost due to a premature stop codon, although it was retained in the DNA sequence (Figure 3.7). The dimerization domain of the African elephant retrogene copies were, however, less conserved than the normal *TP53* copies, which had all five amino acids conserved across the 24 mammal species.

Sulak et al. (Unpublished) found that the only retrogene copy that is transcribed and translated (ElephantRTG12) is capable of binding to normal p53. Given this observation, the rate of codon substitution among the retrogene copies was examined, to determine if this copy, or the other 8 copies that retained the first four amino acids of the dimerization domain, were more conserved. The first 180aa of sequence in the African elephants retrogenes was found to be poorly conserved (dN/dS = 0.979), with no evidence for heterogeneity in the rate of codon substitution amongst the retrogene copies. This region occurred before the first stop codon in the 9 retrogenes that retained the dimerization domain (RTGs 8-19), which shows that the coding portions of these copies also have poor sequence conservation.

We next examined whether the dimerization domain alone may have lower rates of dN/dS than the amino acids outside of this domain, and whether this effect may be greater in the African elephant RTGs 8-19, that retained the dimerization domain in their reading frame. There was only weak support for this expectation: three of the four amino acids of the domain in RTGs 8-19, and the inserted codon (C^{insert}), were <33% of the average dN/dS outside of this domain (although the first codon of the domain had a very high value), while none of the amino acids in RTGs 1-7 were lower than 65% of the average dN/dS outside of the domain. The average dN/dS outside of the domain was high for both retrogene clades examined (Table 3.1), but were not significantly different between RTGs 1-7 vs. RTGs 8-19, suggesting that the regions outside of the dimerization domain are poorly conserved.

An alternative explanation for our findings is that the elephant, hyrax, and manatee retrogene copies have become more recently pseudogenized than the bats, and are now no longer functional. However, the data do not support this possibility. Rough divergence estimates of the species involved (Hedges et al. 2006), suggests an older origin of the retrogenes in the elephants: Afrotherian RTGs (65.3 MYA), Yangochiropteran Clade 1 RTGs (52.7 MYA), Yangochiropteran Clade 2 RTGs (25.0 MYA), and FalseVampireBatRTG1 (52.5 MYA). However, all of the retrogene copies do appear to have become pseudo-retrogenes or very nearly so with only one elephant retrogene apparently having any potential function. The presence of 19 retrogene copies in the elephant, all of which are prematurely terminated, suggests an alternative hypothesis: The retrogene copies in the African elephant may have been functional earlier

on allowing the expansion in body size, but then became non-functional following the evolution of one or more different mechanisms that is under tighter regulatory control and/or less costly. Super-p53 mice engineered to have an extra two copies of the *TP53* gene have been shown to have drastically reduced rates of cancer (García-Cao et al., 2002), yet this added suppression trades off with senescence if not under tight regulatory control (Nakamura et al., 1995; Godley et al., 1996; Allemand et al., 1999). Therefore, a different mechanism of suppression may have replaced the function of the retrogene copies, which could explain the previous findings in the African elephant, which found an enhanced *TP53*-pathway response in this species. This same explanation may also apply to bats. It is certainly intriguing that the large elephant and the long-lived microbats both have multiple copies of TP53 retrogenes, but that in both cases these copies are largely or completely pseudogenized.

In conclusion, this is the first study to estimate the rate of codon substitution of the *TP53* retrogene copies in the African elephant and the bats. In the bats, our work suggests that the additional copies are non-functional pseudo-retrogenes. Our findings, in the African elephant, do not provide support for the assertion that these copies are currently responsible for their enhanced *TP53*-pathway response. Instead, these findings suggest that additional mechanisms of suppression may yet to be uncovered in the large-bodied elephant and the long-lived bats.

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Tables and Figures

Table 3.1: **dN/dS of individual codon sites inside and outside of the dimerization domain of the African elephant RTGs.** The dN/dS (± 1 s.d.) of the first four codons involved in the dimerization domain are shown for RTGs 8-19, and for RTGs 1-7, which all had stop codons occur before the domain, relative to the average dN/dS (± 1 s.e.) in the codons outside of this domain. The dN/dS of the inserted amino acid (C^{insert}, see Figure 7B) is also given. The rank of the dN/dS values for each codon involved in the dimerization domain across the codons analyzed is also given.

	Elephant RTGs 8-19		Elephant RTGs 1-7	
Dimerization AAs	dN/dS	Rank (Across 173 AAs)	dN/dS	Rank (Across 177 AAs)
P^{174}	4.261 ± 1.938	156	0.656 ± 0.387	8
H^{175}	0.367 ± 0.315	37	0.692 ± 0.415	22
E^{177}	0.315 ± 0.730	17	0.976 ± 0.735	116
C^{insert}	0.373 ± 0.875	45	0.997 ± 0.736	122
R^{178}	0.382 ± 0.894	49	2.038 ± 1.852	170
Outside Domain	1.217 ± 0.113		0.986 ± 0.046	

Figure 3.1: The phylogenetic relationships of the 24 mammal species. The placental mammal species are color-coded by their superorder classifications. The Tasmanian devil was used as an outgroup in the study. The bats are nested within the superorder Laurasiatheria, and divided into yangochiroptera and yinpterochiroptera groups. The microbats, indicated by asterisks, form a paraphyletic group. A second normal *TP53* copy was found in the little brown bat, which was incorporated into the study.

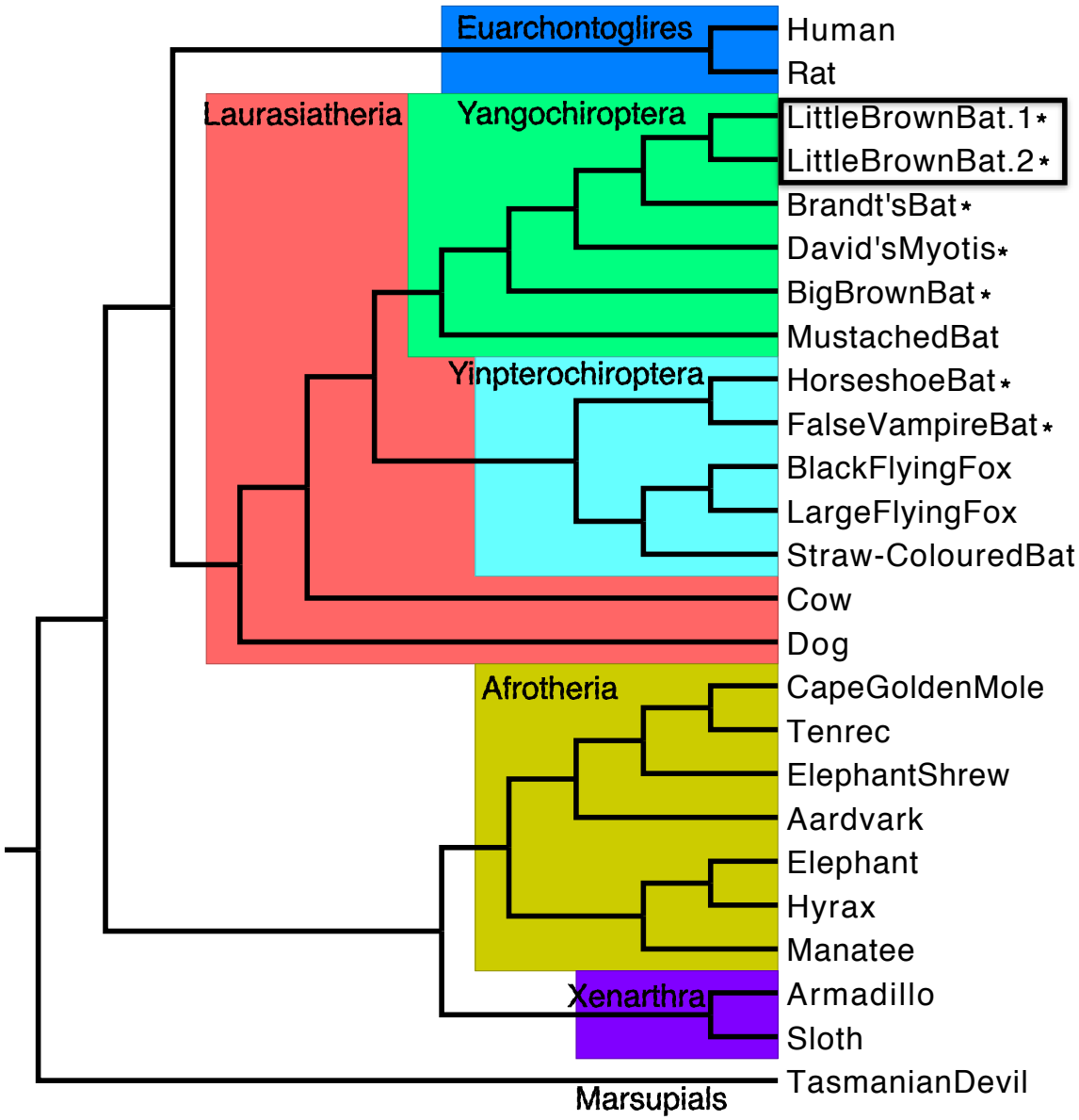

Euarchontoglires
Laurasiatheria
Yangochiroptera
Yinpterochiroptera
Afrotheria
Xenarthra
Marsupials
Human
Rat
LittleBrownBat.1*
LittleBrownBat.2*
Brandt'sBat*
David'sMyotis*
BigBrownBat*
MustachedBat
HorseshoeBat*
FalseVampireBat*
BlackFlyingFox
LargeFlyingFox
Straw-ColouredBat
Cow
Dog
CapeGoldenMole
Tenrec
ElephantShrew
Aardvark
Elephant
Hyrax
Manatee
Armadillo
Sloth
TasmanianDevil

Figure 3.2: A maximum likelihood tree showing the phylogenetic relationships of the normal and retrogene copies in the elephant, hyrax and manatee. The clustering of the retrogene copies in these species suggests a single retrogene origin that is supported by a high bootstrap value. The retrogene copies are denoted by "RTG" and were given the same labels as Sulak et al. (Unpublished). All bootstrap values are shown.

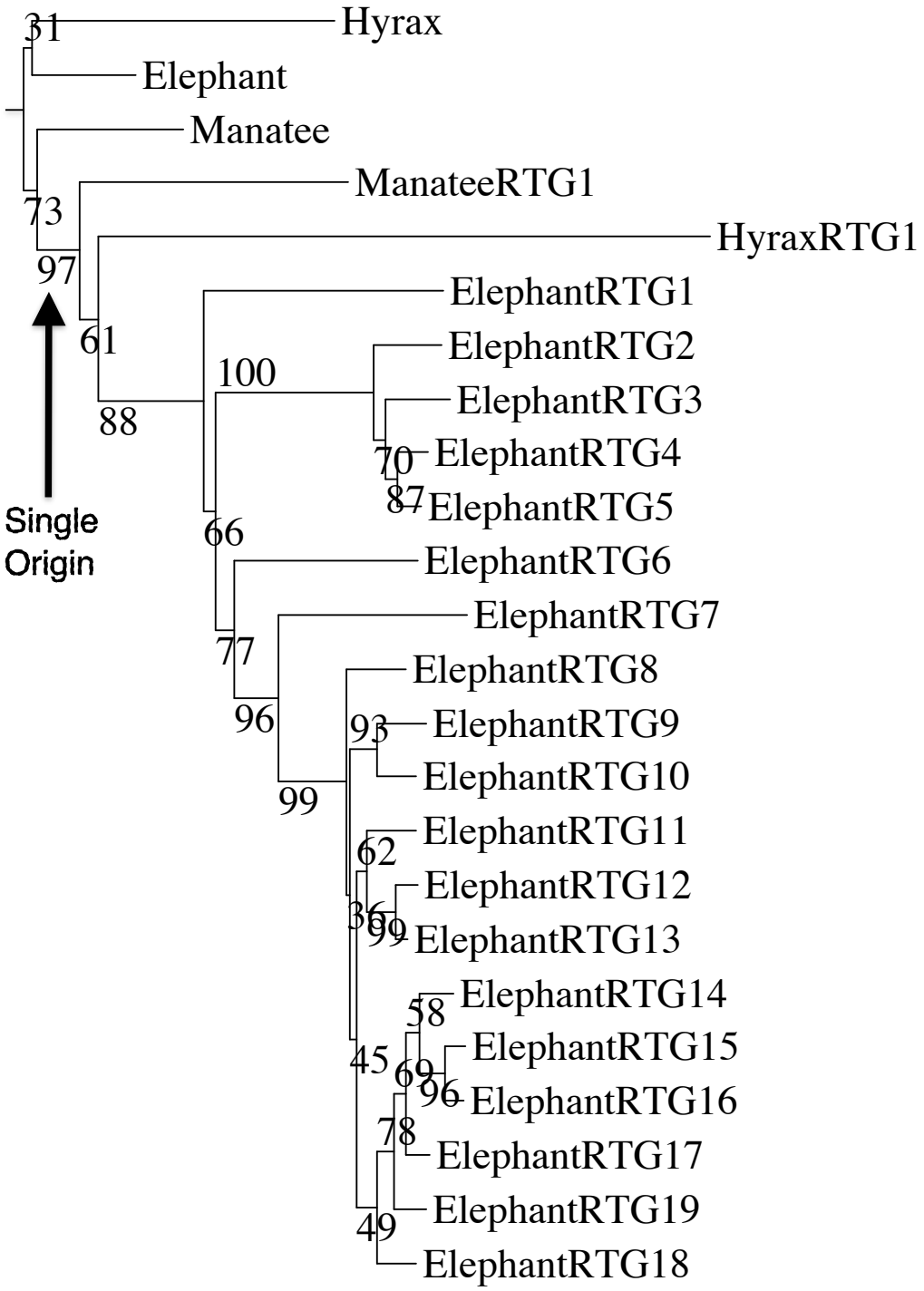

Hyrax
Elephant
Manatee
ManateeRTG1
HyraxRTG1
ElephantRTG1
ElephantRTG2
ElephantRTG3
ElephantRTG4
ElephantRTG5
ElephantRTG6
ElephantRTG7
ElephantRTG8
ElephantRTG9
ElephantRTG10
ElephantRTG11
ElephantRTG12
ElephantRTG13
ElephantRTG14
ElephantRTG15
ElephantRTG16
ElephantRTG17
ElephantRTG19
ElephantRTG18
31
73
97
61
88
100
70
87
66
77
96
93
99
62
36
99
58
45
69
96
78
49
Single
Origin

Figure 3.3: **A maximum likelihood tree showing the phylogenetic relationships of the normal and retrogene copies in the bats.** The clustering of the retrogene copies in these species suggests three separate retrogene origins that are supported by high bootstrap values. The retrogene copies are denoted by "RTG." All bootstrap values are shown. Further evidence was obtained by looking for degraded retrogenes. Group A: Found in Brandt's bat (but not in the mustached bat) supporting Origin #3. Group B: Found in the big brown bat and the mustached bat supporting Origin #2. Group C: Found in the little brown bat supporting this subclade of Origin #2.

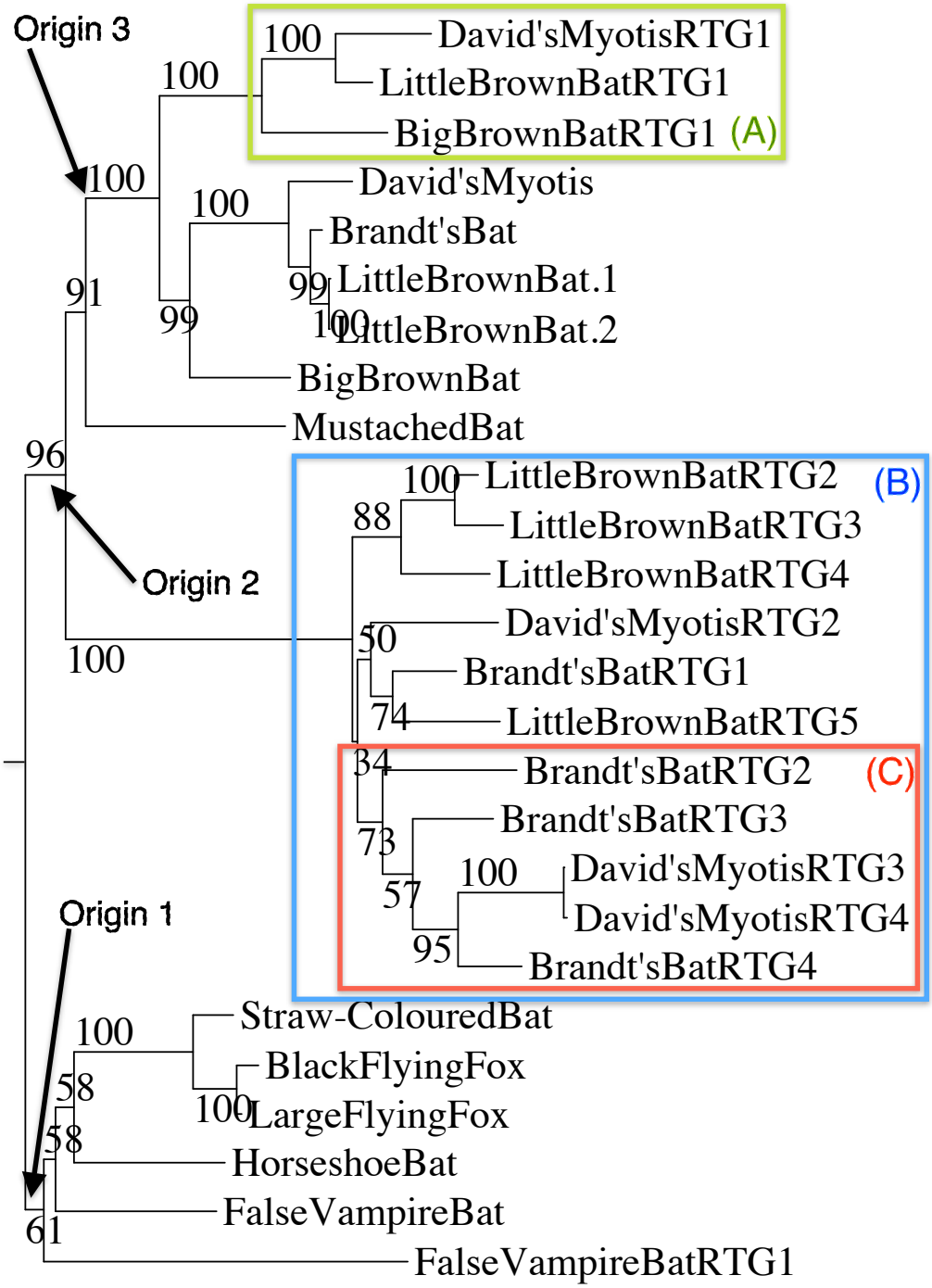

Origin 3
100
David'sMyotisRTG1
LittleBrownBatRTG1
BigBrownBatRTG1
(A)
100
100
David'sMyotis
100
Brandt'sBat
99
LittleBrownBat.1
91
99
100
LittleBrownBat.2
BigBrownBat
MustachedBat
96
100
LittleBrownBatRTG2
(B)
88
LittleBrownBatRTG3
LittleBrownBatRTG4
Origin 2
David'sMyotisRTG2
50
100
Brandt'sBatRTG1
74
LittleBrownBatRTG5
34
Brandt'sBatRTG2
(C)
Brandt'sBatRTG3
73
100
David'sMyotisRTG3
57
David'sMyotisRTG4
95
Brandt'sBatRTG4
Origin 1
100
Straw-ColouredBat
58
BlackFlyingFox
100
LargeFlyingFox
58
HorseshoeBat
FalseVampireBat
61
FalseVampireBatRTG1

Figure 3.4: **Open reading frame lengths of the retrogene copies in the elephant, hyrax, and manatee.** The reading frame lengths (amino acids) of the retrogene copies are shown in comparison to the normal TP53 copy in elephants. The retrogene copies are truncated at the position of their first stop codon. The phylogenetic relationships of the retrogene sequences are given and are color-coded by species. The positions of the upstream MDM2-binding and dimerization domains are shown.

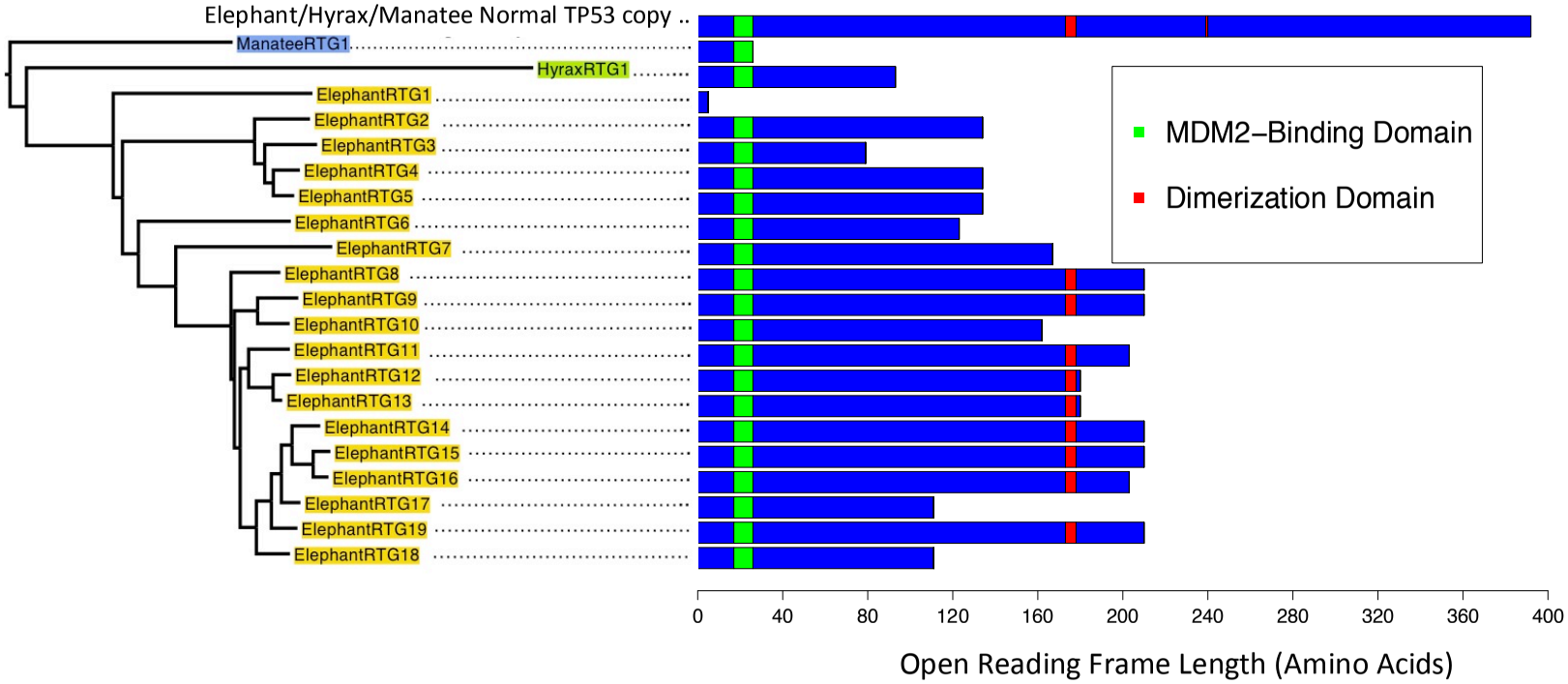

Elephant/Hyrax/Manatee Normal TP53 copy ..
ManateeRTG1
HyraxRTG1
ElephantRTG1
ElephantRTG2
ElephantRTG3
ElephantRTG4
ElephantRTG5
ElephantRTG6
ElephantRTG7
ElephantRTG8
ElephantRTG9
ElephantRTG10
ElephantRTG11
ElephantRTG12
ElephantRTG13
ElephantRTG14
ElephantRTG15
ElephantRTG16
ElephantRTG17
ElephantRTG19
ElephantRTG18
MDM2–Binding Domain
Dimerization Domain
0
40
80
120
160
200
240
280
320
360
400
Open Reading Frame Length (Amino Acids)

Figure 3.5: **Open reading frame lengths of the retrogene copies in the bats.** The reading frame lengths (amino acids) of the retrogene copies are shown in comparison to the normal TP53 copy in bats. The retrogene copies are truncated at the position of their first stop codon. The phylogenetic relationships of the retrogene sequences are given and are color-coded by species. The positions of the upstream MDM2-binding and dimerization domains are shown. The LittleBrownBatRTG1 was missing coverage at its 5' end, which is represented in white.

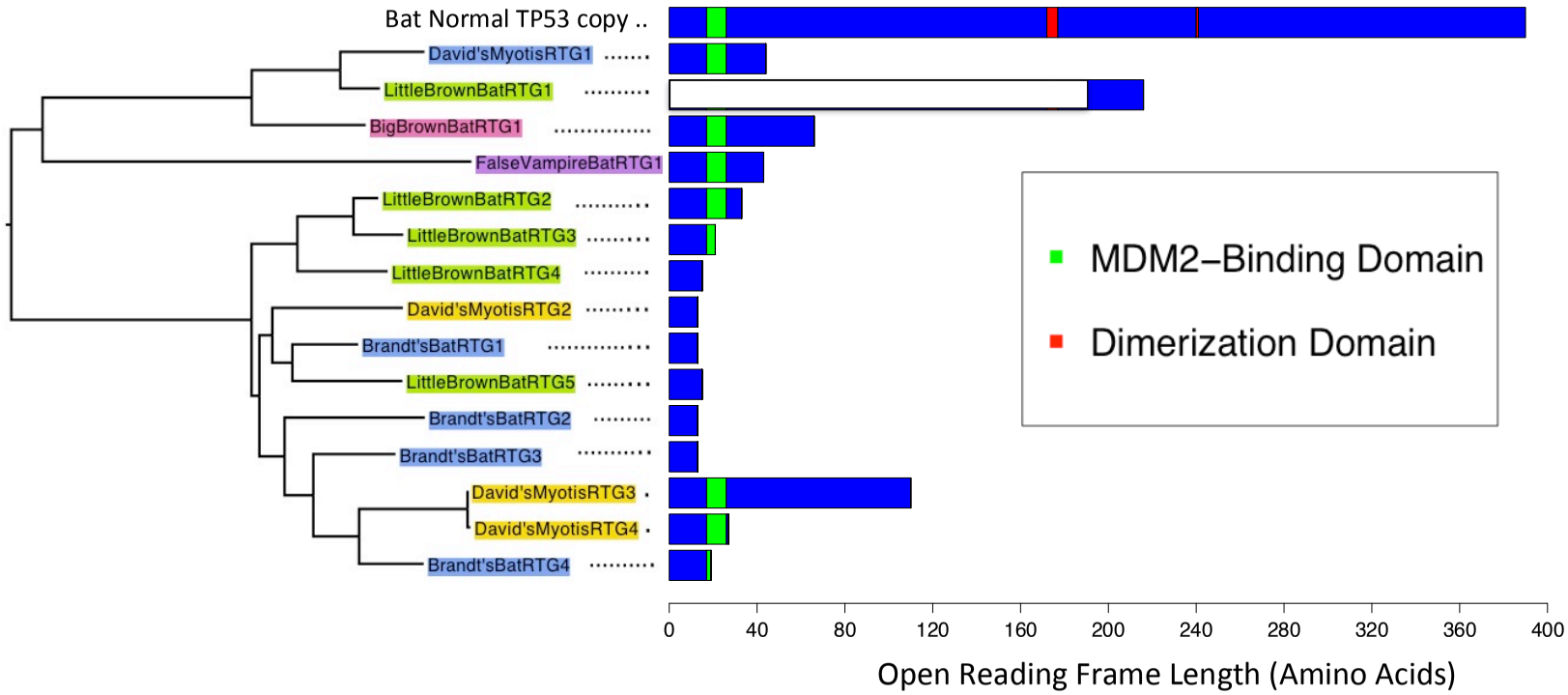

Bat Normal TP53 copy ..
David'sMyotisRTG1
LittleBrownBatRTG1
BigBrownBatRTG1
FalseVampireBatRTG1
LittleBrownBatRTG2
LittleBrownBatRTG3
LittleBrownBatRTG4
David'sMyotisRTG2
Brandt'sBatRTG1
LittleBrownBatRTG5
Brandt'sBatRTG2
Brandt'sBatRTG3
David'sMyotisRTG3
David'sMyotisRTG4
Brandt'sBatRTG4
MDM2–Binding Domain
Dimerization Domain
0
40
80
120
160
200
240
280
320
360
400
Open Reading Frame Length (Amino Acids)

Figure 3.6: The relative rate of non-synonymous to synonymous substitutions (dN/dS) in normal and retrogene TP53 sequences. The dN/dS ratios are shown for all branches (color-coded) that were significantly different. The branches shown in black represent the background rate of codon substitution found for the normal copies. The dN/dS ratios observed in the retrogene clades are labeled in red. The relationships of the retrogene clades with more than one copy are collapsed. The significance level of this final model (relative to a single dN/dS value) is shown.

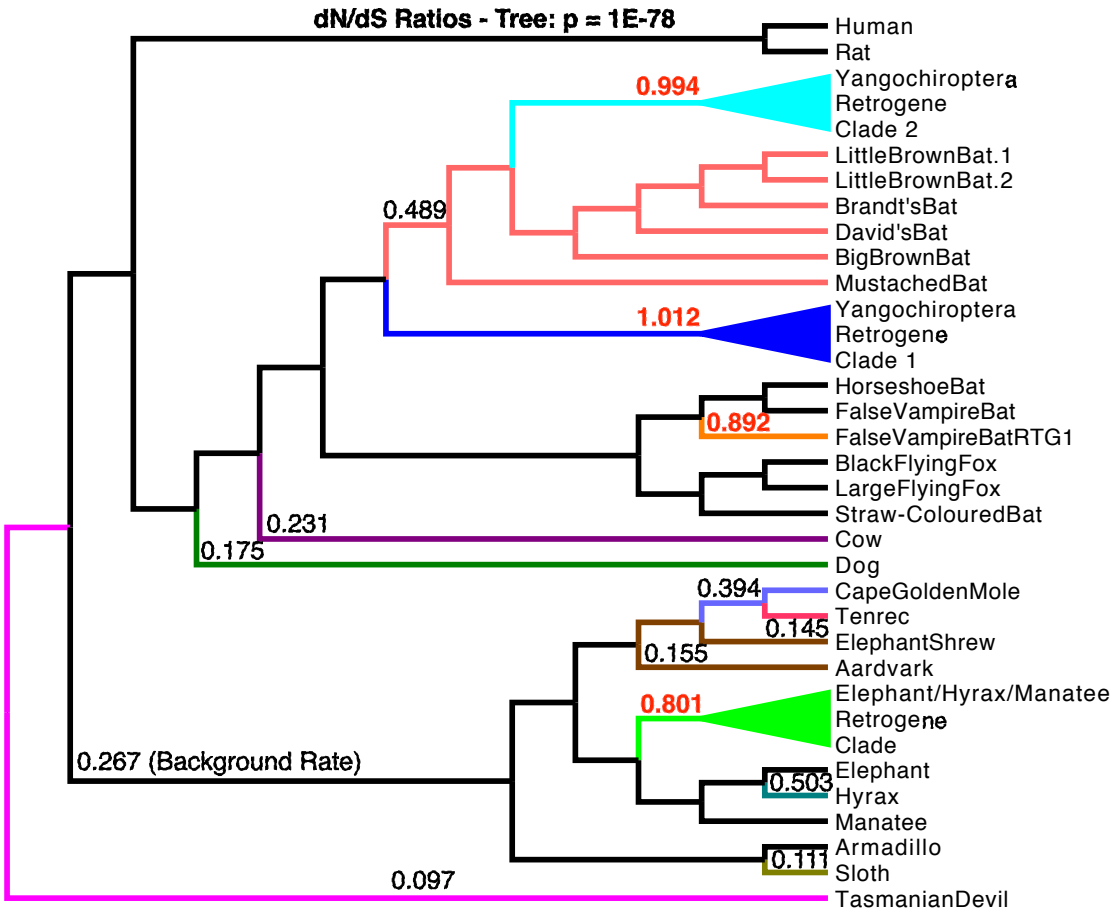
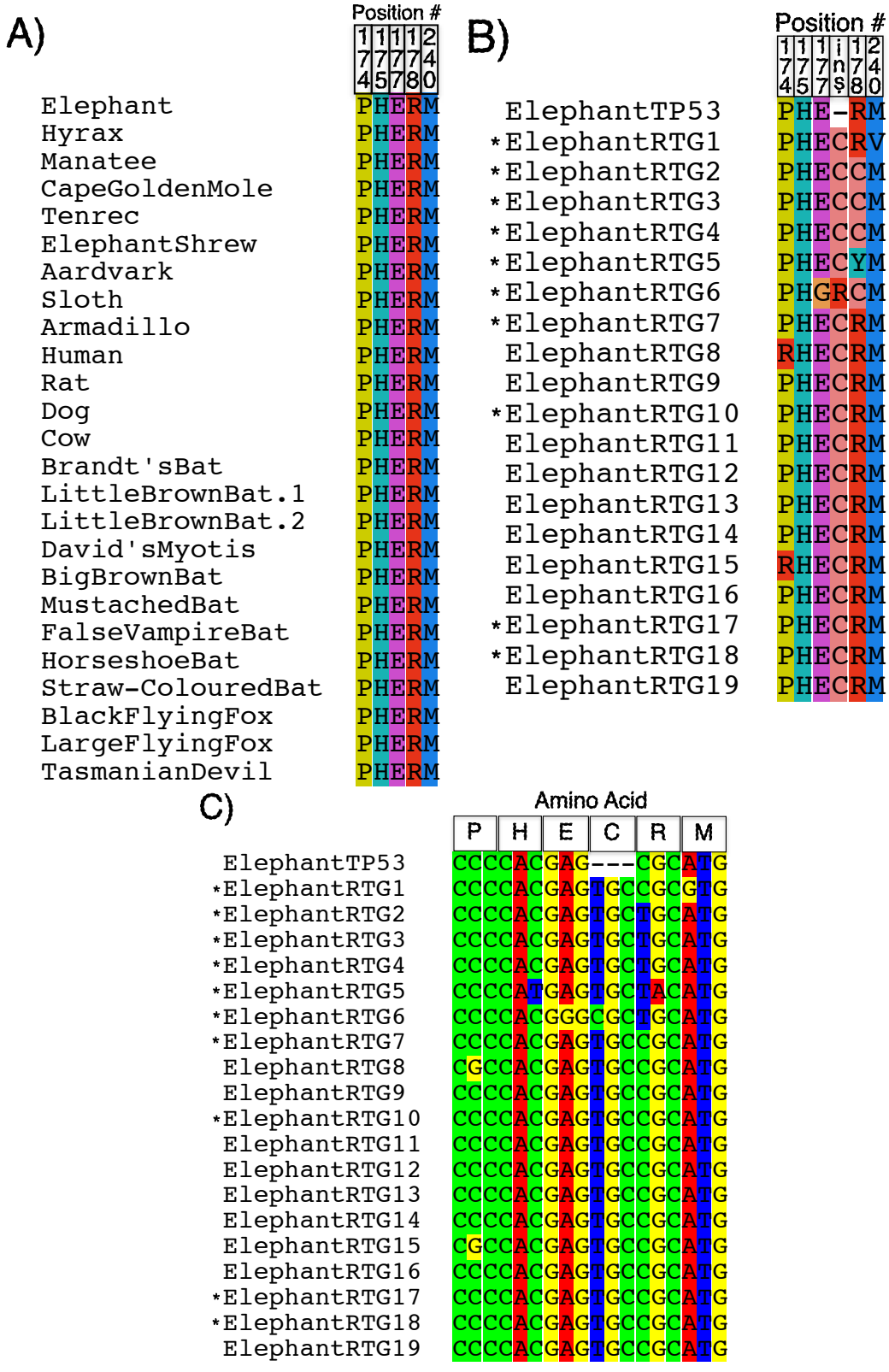

dN/dS Ratios - Tree: p = 1E-78
Human
Rat
0.994
Yangochiroptera
Retrogene
Clade 2
LittleBrownBat.1
LittleBrownBat.2
0.489
Brandt'sBat
David'sBat
BigBrownBat
MustachedBat
1.012
Yangochiroptera
Retrogene
Clade 1
HorseshoeBat
FalseVampireBat
0.892
FalseVampireBatRTG1
BlackFlyingFox
LargeFlyingFox
Straw-ColouredBat
0.231
Cow
0.175
Dog
0.394
CapeGoldenMole
Tenrec
0.145
ElephantShrew
0.155
Aardvark
0.801
Elephant/Hyrax/Manatee
Retrogene
Clade
0.267 (Background Rate)
Elephant
0.503
Hyrax
Manatee
Armadillo
0.111
Sloth
0.097
TasmanianDevil

Figure 3.7: **Amino acid and DNA conservation of the dimerization domain.** The level of conservation in the five amino acids of the dimerization domain are shown in (A) for the normal *TP53* copies of 24 mammal species and in (B) for the retrogene copies of the African elephant. The level of DNA sequence is shown in (C) for the African elephant retrogene copies. Note that position 176 and positions 179-239 are not part of the domain. A three base pair insertion ("ins.") shifted the Arginine residue (R) one position downstream of its position in the normal copies within 18 of the 19 retrogenes. *: The retrogene copies that have their first stop codon occurring before the domain, but were put in frame with the normal TP53 copy. The only retrogene copy known to be transcribed and translated is TP53RTG12.


A)
Position #
174 175 178 240
Elephant PHERM
Hyrax PHERM
Manatee PHERM
CapeGoldenMole PHERM
Tenrec PHERM
ElephantShrew PHERM
Aardvark PHERM
Sloth PHERM
Armadillo PHERM
Human PHERM
Rat PHERM
Dog PHERM
Cow PHERM
Brandt'sBat PHERM
LittleBrownBat.1 PHERM
LittleBrownBat.2 PHERM
David'sMyotis PHERM
BigBrownBat PHERM
MustachedBat PHERM
FalseVampireBat PHERM
HorseshoeBat PHERM
Straw-ColouredBat PHERM
BlackFlyingFox PHERM
LargeFlyingFox PHERM
TasmanianDevil PHERM
B)
Position #
174 175 177 ins 178 240
ElephantTP53 PHE-RM
*ElephantRTG1 PHECRV
*ElephantRTG2 PHECCM
*ElephantRTG3 PHECCM
*ElephantRTG4 PHECCM
*ElephantRTG5 PHECYM
*ElephantRTG6 PHGRCM
*ElephantRTG7 PHECRM
ElephantRTG8 RHECRM
ElephantRTG9 PHECRM
*ElephantRTG10 PHECRM
ElephantRTG11 PHECRM
ElephantRTG12 PHECRM
ElephantRTG13 PHECRM
ElephantRTG14 PHECRM
ElephantRTG15 RHECRM
ElephantRTG16 PHECRM
*ElephantRTG17 PHECRM
*ElephantRTG18 PHECRM
ElephantRTG19 PHECRM
C)
Amino Acid
P H E C R M
ElephantTP53 CCCCACGAG---CGCATG
*ElephantRTG1 CCCCACGAGTGCCGCGTG
*ElephantRTG2 CCCCACGAGTGCTGCATG
*ElephantRTG3 CCCCACGAGTGCTGCATG
*ElephantRTG4 CCCCACGAGTGCTGCATG
*ElephantRTG5 CCCCATGAGTGCTACATG
*ElephantRTG6 CCCCACGGGCGCTGCATG
*ElephantRTG7 CCCCACGAGTGCCGCATG
ElephantRTG8 CGCCACGAGTGCCGCATG
ElephantRTG9 CCCCACGAGTGCCGCATG
*ElephantRTG10 CCCCACGAGTGCCGCATG
ElephantRTG11 CCCCACGAGTGCCGCATG
ElephantRTG12 CCCCACGAGTGCCGCATG
ElephantRTG13 CCCCACGAGTGCCGCATG
ElephantRTG14 CCCCACGAGTGCCGCATG
ElephantRTG15 CGCCACGAGTGCCGCATG
ElephantRTG16 CCCCACGAGTGCCGCATG
*ElephantRTG17 CCCCACGAGTGCCGCATG
*ElephantRTG18 CCCCACGAGTGCCGCATG
ElephantRTG19 CCCCACGAGTGCCGCATG

Supplementary Materials

Supplementary Table 3.1: **Statistical results for the "bottom-up" tests in the normal *TP53* copies.** Individual tests were performed hierarchically to identify branches with significantly different dN/dS ratios than the proceeding branch. The first test compared a two-dN/dS model for the outgroup branch relative to a one-dN/dS model for the entire tree. Each test was followed by a test examining whether significant heterogeneity remained in the tree before moving on to the next branch. The process was discontinued if no significant heterogeneity remained. The branch highlighted in gray had a dN/dS ratio of 0 and was not included in the analysis or the subsequent calculations of degrees of freedom. The branches highlighted in red had significant findings that were examined in Supplementary Table 3.2 to determine, which of the branches tested were significantly different from the proceeding branch.

		Comparison to Previous Branch			Test for Residual Heterogeneity		
Branches Tested	*# of dN/dS*	*Chi-Square*	*Df*	*P Value*	*Chi-Square*	*Df*	*P Value*
Outgroup	2	15.02	1	0.0001 ***	116.35	45	3.12E-08 ***
Afrotheria/Xenarthra or Euarchontoglires/Laurasiatheria	3	3.67	1	0.0554	112.68	44	6.12E-08 ***
Afrotheria/Xenarthra Shared Branch	4	0.01	1	0.9951	112.68	43	3.71E-08 ***
Xenarthra or Afrotheria	5	2.63	1	0.1045	110.04	42	5.23E-08 ***
Xenarthra Shared Branch	6	1.42	1	0.2341	108.63	41	4.97E-08 ***
Armadillo or Sloth	7	5.01	1	0.0253 *	103.62	40	1.51E-07 ***
Afrotheria shared	8	5.63	1	0.0176 *	97.99	39	5.56E-07 ***
Paenugulata or Afroinsectophilia	9	4.31	1	0.0378 *	93.68	38	1.33E-06 ***
Paeungulata Shared Branch	10	0.05	1	0.8149	93.62	37	8.34E-07 ***
Manatee	11	0.30	1	0.5855	93.32	36	5.56E-07 ***
Hyrax/Elephant Shared Branch	12	0.45	1	0.5031	92.88	35	3.86E-07 ***
Hyrax or Elephant	13	6.51	1	0.0107 *	86.37	34	1.92E-06 ***
Afroinsectophilia Shared Branch	14	6.83	1	0.0090 **	79.53	33	1.03E-05 ***
Aardvark	15	0.95	1	0.3305	78.59	32	8.56E-06 ***
Afroinsectovore Shared Branch	15	NA	0	NA	NA	32	NA
Elephant Shrew	16	1.91	1	0.1668	76.15	31	1.14E-05 ***
Afrosoricida Shared Branch	17	4.61	1	0.0318 *	71.54	30	3.02E-05 ***
Tenrec or Cape Golden Mole	18	8.01	1	0.0047 **	63.53	29	2.20E-04 ***
Euarchontoglires/Laurasiatheria Shared Branch	19	0.16	1	0.6909	63.37	28	1.49E-04 ***
Laurasiatheria or Euarchontoglires	20	3.08	1	0.0791	60.29	27	2.42E-04 ***
Laurasiatheria Shared Branch	21	0.69	1	0.4069	59.60	26	1.90E-04 ***
Dog	22	9.21	1	0.0024 **	50.39	25	1.91E-03 **
Bats/Cow Shared Branch	23	1.29	1	0.2562	49.10	24	1.84E-03 **
Cow	24	4.00	1	0.0456 *	45.10	23	3.86E-03 **
Bats Shared Branch	25	1.00	1	0.3164	44.10	22	3.45E-03 **
Yinpterochiroptera or Yangochiroptera	26	13.09	1	0.0003 ***	31.01	21	7.34E-02
ANALYSIS STOPPED. NO HETEROGENEITY REMAINING							

Supplementary Table 3.2: **Post-hoc tests resolving ambiguities in "bottom-up" test results.** The branches highlighted in red in Supplementary Table 3.1 were found to have significantly different dN/dS ratios than their proceeding branch. However, post-hoc tests are needed to determine which of the sister clades examined were significantly different from the proceeding branch to finalize a model in which all significant branches are given a separate dN/dS ratio. Beginning at the bottom of the tree, the sister clade of each pair that had a dN/dS value most similar to the proceeding branch had their dN/dS value added back to the proceeding branch. This revised final model was then compared to the current final model to determine if removing this dN/dS value had no significant effect. Each test was followed by a test to confirm that removing the dN/dS value did not produce significant heterogeneity in the tree.

Sister Clades Tested		Test for No Effect of Removing Clade from Model			Test for No Residual Heterogeneity After Removing Clade		
Clade Added Back to Previous Branch	*Clade Given Separate dN/dS*	*Chi Square*	*Df*	*P Value*	*Chi Square*	*Df*	*P Value*
Armadillo	Sloth	0.01	1	0.988	39.07	31	0.151
Afrotheria Shared Branch	Afrotheria	3.40	1	0.065	42.46	32	0.102
Paenungulata	Afroinsectophilia	0.05	1	0.826	42.51	33	0.124
Elephant	Hyrax	3.08	1	0.079	45.59	34	0.088
Afroinsectophilia Shared Branch	Afroinsectophilia	3.59	1	0.058	49.18	35	0.056
Afrosoricida Shared Branch	Afrosoricida	5.89	1	0.015 *	Not Added Back		
Cape Golden Mole	Tenrec	1.37	1	0.242	50.55	36	0.055
Yinpterochiroptera	Yangochiroptera	1.08	1	0.298	51.63	37	0.056

Concluding Remarks

A number of disparate observations in cancer biology have found that the nature and number of genetic mechanisms suppressing cancer can differ across tissues and species (Rangarajan & Weinberg, 2003; Rangarajan et al., 2004; Bignold, 2004). These observations have been difficult to address under the mechanistic perspective that pervades cancer biology. Nunney's (1999) evolutionary model, however, provides an explanation for these differences: the independent evolution of larger body size and longer lifespan, and the resulting increased cancer risk, has selected for additional TSGs or POGs in the tissue(s) causing the greatest decline in fitness. This leads to a different nature and number of genes recruited in different species following their divergence, with more controls in larger, longer-lived species, as well as different genes being recruited in the different tissues of the same species. The dissertation work presented here showed support for this evolutionary model.

In **Chaper 1**, fruit flies (*Drosophila melanogaster*) with hereditary melanotic tumors were shown to evolve lower incidence rates of these tumors following selection for longevity and late-life fecundity. This proof-of-concept experiment demonstrated that genetic variation for cancer suppression does exist in natural populations, and that cancer suppression is an evolving trait.

Work in **Chapter 2** showed that hereditary mutations of a specific tumor suppressor or proto-oncogene is likely to be most oncogenic in the tissue where the gene has its highest level of expression. This work suggests that high expression in normal (non-diseased) tissues is a potential marker for linking cancer-related genes with their

susceptible tissues and can help explain the tissue-specificity of hereditary cancer (Fearon, 1997; Bignold, 2004).

Finally, in **Chapter 3**, we did not find support for the assertion that additional retrogene copies of the tumor suppressor, *TP53,* in the African elephant are responsible for the enhanced *TP53*-pathway response in this species (Abegglen et al., 2015; Sulak et al., Unpublished). The rate of codon substitution in the retrogene copies of the African elephant and the long-lived bats showed that these copies are significantly less conserved than the normal *TP53* copies of 24 mammals species, and are truncated by premature stop codons. Instead, our work suggests that different mechanisms, yet to be uncovered, may be responsible for the low cancer rates in these species.

In conclusion, this work illustrates the importance of incorporating evolutionary biology into the study of cancer genetics. By examining close-relatives with similar lifespans and body sizes to humans, we can confirm the involvement of specific TSGs or POGs in a particular human cancer. Furthermore, we can look to large and/or long-lived species, such as the naked mole rat, to identify novel mechanisms for cancer treatment and prevention (Tian et al., 2013).

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