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Repair of endogenous DNA base lesions modulate lifespan in mice

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Abstract

The accumulation of DNA damage is thought to contribute to the physiological decay associated with the aging process. Here, we report the results of a large-scale study examining longevity in various mouse models defective in the repair of DNA alkylation damage, or defective in the DNA damage response. We find that the repair of spontaneous DNA damage by alkyladenine DNA glycosylase (Aag/Mpg)-initiated base excision repair and O^6-methylguanine DNA methyltransferase (Mgmt)-mediated direct reversal contributes to maximum life span in the laboratory mouse. We also uncovered important genetic interactions between Aag, which excises a wide variety of damaged DNA bases, and the DNA damage sensor and signaling protein, Atm. We show that Atm plays a role in mediating survival in the face of both spontaneous and induced DNA damage, and that Aag deficiency not only promotes overall survival, but also alters the tumor spectrum in *Atm*$^{-/-}$ mice. Further, the reversal of spontaneous alkylation damage by Mgmt interacts with the DNA mismatch repair pathway to modulate survival and tumor spectrum. Since these aging studies were performed without treatment with DNA damaging agents, our results

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CONFLICT OF INTEREST STATEMENT
The authors declare that there are no conflicts of interest.

indicate that the DNA damage that is generated endogenously accumulates with age, and that DNA alkylation repair proteins play a role in influencing longevity.

Keywords

AAG/MPG; Mgmt; DNA adducts; DNA glycosylase; aging; base excision repair

1 INTRODUCTION

Aging can be thought of as a progressive decline in function at the cellular, tissue, and organismal level, possibly resulting from cumulative damage to important biomolecules [1]. The reasons why we age and modulators of the aging process have been intensively studied for decades (reviewed in [1]). One predominant but recently contested school of thought [2, 3], is described in the mitochondrial free radical theory of aging. In 1956, Harman first proposed that mitochondrially-generated reactive oxygen and nitrogen species (RONS), along with other harmful environmental physical and chemical agents, result in accumulating damage in numerous biomolecules critical for proper cell function [4]. Genetic experiments in various model organisms have pinpointed a variety of genes and pathways that influence how an organism ages (reviewed in [5–8]); however, it has become clear that additional random events also play an important role in the determination of longevity. In fact, the accumulation of unrepaired DNA damage causing decreased genomic integrity, has long been proposed as a major source of stochastic changes that can influence aging (reviewed in [9, 10]). Accordingly, animals with genetic deficiencies in double-strand-break repair or telomere maintenance have much shorter lifespans than wild-type (WT) mice [11, 12]. Further demonstrating the importance of unrepaired DNA damage in aging, mice or patients carrying mutations in the transcription-coupled branch of nucleotide excision repair (NER) suffer from a premature onset of aging-related symptoms and consequent shortening of lifespan, but interestingly, with the exception of skin cancers, the decreased longevity occurs in the absence of increased cancer development (reviewed in [13, 14][10]).

Inactivating mutations that disrupt the maintenance of genome stability can decrease longevity through either increasing cancer predisposition or causing more general premature aging and progeroid-like characteristics (reviewed in [10, 15, 16]). Indeed, multiple important DNA damage response proteins were originally identified through the investigation of cancer-prone patients. Cancer-prone Li-Fraumeni and ataxia telangiectasia (AT) patients exhibit germline mutations in two important DNA damage response proteins, namely p53 and ataxia telangiectasia mutated (ATM), respectively [17]. p53 (i.e., Trp53) is a stress sensor and transcription factor responsible for inducing cell cycle checkpoints, apoptosis or senescence upon exposure to DNA damage, hypoxia, and oncogene activation among other stimuli; p53 was originally identified as a tumor suppressor and is known as the “guardian” of the genome [17–19]. p53 also appears to have additional roles in modulating aging, independent of its role in tumor suppression, presumably related to its role in cellular senescence [17, 20]. ATM, an integral DNA damage signaling protein, is a serine/threonine protein kinase that is activated in response to double-strand DNA breaks; ATM’s activation

initiates important signaling pathways, some of which involve p53, responsible for cell cycle checkpoint activation, apoptosis and DNA repair (reviewed in [21]).

DNA is exposed to a wide-range of damaging agents, not only from exogenous, but also from endogenous sources. DNA base alkylation is one common consequence of multiple endogenous metabolic processes (reviewed in [22]). For example, alkylation can occur as a consequence of the non-enzymatic transfer of methyl groups to DNA from the universal methyl donor, S-adenosylmethionine. Additionally, RONS, inevitable byproducts of aerobic metabolism and also an important component of the innate immune response, are highly reactive chemical species that produce numerous types of DNA damage. Furthermore, RONS can indirectly induce alkylation DNA damage as a result of lipid peroxidation reactions that generate reactive alkylating agents that react to produce etheno (ε) and other DNA base adducts [23–27]. Livers of aged animals exhibit an accumulation of ε adducts, specifically εA, suggesting a possible role for these lipid peroxidation reactions in aging organisms [28]. DNA base lesions are also increased under conditions of chronic inflammation, and are believed to contribute to the increased risk of carcinogenesis observed in patients with chronic inflammation [29–32]. Therefore, various types of alkylation damage arise in cells as a function of normal metabolic functions, and the role of such endogenous DNA damage in influencing longevity has yet to be determined.

The pathways primarily responsible for the repair of alkylated DNA base lesions are base excision repair (BER) and direct reversal (reviewed in [33].) BER is initiated when a damaged DNA base is recognized and excised by a DNA glycosylase; alkyladenine DNA glycosylase (Aag, a.k.a Mpg) recognizes numerous alkylated DNA base lesions, including 3-methyladenine (3meA) and 7-methylguanine (7meG) in mammals. Aag also recognizes many lesions induced by RONS and lipid peroxidation products including hypoxanthine and εA respectively [34–36]. Other alkylated DNA bases are subject to direct reversal repair, either by oxidative demethylation catalyzed by the AlkB homolog (Alkbh) family of proteins (reviewed in [33]), or by the efficient transfer of the unwanted methyl group on O^6-methylguanine (O^6MeG) lesions to a cysteine residue in the O^6MeG DNA methyltransferase (Mgmt) in a suicide reaction [37]. Unrepaired O^6MeG lesions pair with thymine during replication. The mismatch repair (MMR) pathway recognizes O^6MeG:thymine (O^6MeG:T) mismatches and subsequent MMR processing plays an essential role in alkylation-induced cytotoxicity. MutSα, a heterodimer of Msh2 and Msh6 MMR proteins, recognizes O^6MeG:T mispairs, and this recognition is required for the induction of apoptosis [38–40]. The MMR pathway then engages in futile cycles of Exonuclease 1 (Exo1)-mediated excision and DNA polymerase resynthesis at O^6MeG:T mismatches, with excision and reinsertion of the thymine opposite O^6MeG; this results in persistent strand breaks that ultimately culminate in the formation of double-strand DNA breaks at collapsed replication forks (reviewed in [33, 37]). This alkylation hypersensitivity observed in the absence of Mgmt is dependent on the presence of a functional MMR pathway [40–43].

The contribution of endogenous DNA alkylation damage to longevity has not been rigorously examined. It is well-documented that Mgmt expression protects mice from tumors induced by treatment with exogenous alkylating agents and therefore prolongs survival following treatment with alkylating agents [44–47]. Intriguingly, transgenic

C3HeB/FeJ male mice overexpressing Mgmt are protected against spontaneous hepatocellular carcinomas in a susceptible mouse strain [48], suggesting a role for O^6MeG lesions in spontaneous tumors in a predisposed background and perhaps enhanced overall survival. It has been more challenging to investigate the role for BER in longevity due to the embryonic lethality observed upon deletion of many BER proteins [49–51]. However, studies in heterozygous mice have provided insight into the importance of BER proteins in modulating survival; for example, *Polb*$^{+/-}$ mice exhibit an acceleration in the age-dependent mortality rate as well as increased tumorigenesis [52]. Finally, we and others have illustrated the importance of Mgmt- and Aag-initiated DNA repair in chronic inflammation-associated cancer, a frequent aging-associated phenomenon; *Aag*$^{-/-}$ and *Mgmt*$^{-/-}$ mice are more susceptible to chronic inflammation-associated colon carcinogenesis [31, 32, 53, 54]. However, the contribution of Aag and Mgmt to overall longevity was, heretofore, not specifically investigated.

Here we exploit mouse model systems to determine whether DNA alkylation repair proteins acting on spontaneous DNA damage contribute to aging and longevity. We performed a large-scale study to assess whether two major DNA alkylation repair pathways, namely Aag-initiated BER and Mgmt-mediated direct reversal, promote longevity. Supplemental Table 1 lists the mouse genotypes used in this study. We compared the longevity of *Aag*$^{-/-}$, *Mgmt*$^{-/-}$ and *Aag*$^{-/-}$/*Mgmt*$^{-/-}$ mice with that of WT mice. We also investigated putative genetic interactions that *Aag* and *Mgmt* might have with the DNA damage response pathways controlled by p53 and Atm. Finally, because the MMR pathway is an important modulator of cellular responses to *O* MeG, we investigated possible genetic interactions between Mgmt and MMR by examining *Mgmt* $^{-/-}$/*Msh6* $^{-/-}$ and *Mgmt* $^{-/-}$/*Exo1* $^{-/-}$ mice. Together, our comprehensive study illustrates that the repair of spontaneous DNA base damage, likely to be primarily alkylation damage, influences the longevity of mice, and provides information about potential interactions between DNA alkylation repair proteins and downstream DNA damage response mediators.

2 RESULTS

2.1 Deficiency in alkylation repair alters long-term survival

Given that the endogenous generation of DNA damage is ubiquitous and continuous, we determined whether repair of spontaneous DNA base damage, primarily alkylation damage, contributes to longevity in mammals by assessing the long-term survival of mice deficient in genes for the repair of alkylated DNA bases, i.e, *Aag*$^{-/-}$ and *Mgmt*$^{-/-}$ mice. Large cohorts of WT, *Aag*$^{-/-}$, *Mgmt*$^{-/-}$ and *Aag*$^{-/-}$/*Mgmt*$^{-/-}$ mice were established and carefully monitored for up to three years. Unlike mice deficient in NER [10, 14], none of the genotypes exhibited any signs of premature aging. As mice became moribund, survival and histological data were collected. Compared to WT mice, *Aag*$^{-/-}$ and *Mgmt*$^{-/-}$ mice exhibit a trend toward decreased longevity, which did not reach statistical significance (Figure 1A). However, the *Aag*$^{-/-}$/*Mgmt*$^{-/-}$ animals display a significantly shorter life-span compared to WT (p=0.04); the median survival of *Aag*$^{-/-}$/*Mgmt*$^{-/-}$mice was 89.5 weeks, more than 15 weeks shorter than the median survival observed in WT mice (Figure 1A). These data indicate the importance of repairing spontaneous DNA base lesions for attaining maximum longevity.

The large-scale aging studies included detailed histopathological examination to identify pathological features in the mice, including classification of any tumors, to determine whether modulating DNA repair altered tumor incidence and/or tumor spectrum. In our study, the most prevalent tumor type in WT, *Aag*$^{-/-}$, *Mgmt*$^{-/-}$, and *Aag*$^{-/-}$ /*Mgmt*$^{-/-}$ mice was histiocytic sarcoma, a macrophage neoplasm and the most common tumor classification in the C57Bl/6 strain [55]. We find that the absence of either Aag or Mgmt activity (or both) did not significantly alter the tumor incidence or spectrum when compared to the WT mice (Figure 1B); in other words, although the repair-deficient mice succumb earlier than WT, the spectrum of disease and cause of death remains similar.

2.1 Influence of DNA damage response proteins on responses to endogenous DNA alkylation damage

The p53 and Atm proteins are important stress mediators that respond to DNA damage. Mice deficient in these proteins exhibit drastically reduced longevity, developing thymic lymphoma within the first year of life [56, 57]. We sought to determine whether accumulating unrepaired spontaneous DNA base damage (again, primarily alkylation damage) may contribute to lymphomagenesis and diminished longevity in *Atm*$^{-/-}$ and *p53*$^{-/-}$ mice. Although it is well established that *Atm*$^{-/-}$ mice exhibit significantly shortened life spans [56, 58], detailed longevity, studies have not been reported for the C57Bl/6 strain background. Figure 2A shows Kaplan-Meier survival curves for *Atm*$^{-/-}$ mice, both alone and in combination with the *Aag* or *Mgm*t null alleles. *Atm*$^{-/-}$, *Aag*$^{-/-}$/*Atm*$^{-/-}$, and *Mgmt*$^{-/-}$/*Atm*$^{-/-}$ mice all exhibit decreased survival when compared to the WT mice (all pair-wise comparisons to wild type, $p<0.0001$). However, in contrast to previous studies in mixed background mice, we find that *Atm*$^{-/-}$ C57Bl/6 mice survive significantly longer than *Atm*$^{-/-}$ mixed background mice [56, 58]. In fact, in our aging study, 20% of *Atm*$^{-/-}$ C57Bl/6 mice survive longer than one year (Figure 2A), whereas most *Atm*$^{-/-}$ mice on a mixed background succumbed to thymic lymphoma by 4.5 months [56, 58]. We find that the addition of the *Mgmt* null allele does not significantly change the survival of *Atm*$^{-/-}$ animals ($p=0.3423$), suggesting that endogenously formed O^6MeG lesions are not determinants of survival in *Atm*$^{-/-}$ mice. Surprisingly, *Aag*$^{-/-}$/*Atm*$^{-/-}$ C57Bl/6 mice live significantly longer than *Atm* $^{-/-}$ C57Bl/6 mice (pair-wise comparison between *Atm*$^{-/-}$ and *Aag*$^{-/-}$/*Atm*$^{-/-}$, $p=0.0193$) (Figure 2A). These results indicate that, in contrast to Aag-mediated repair of endogenous DNA base damage extending longevity, Aag activity in *Atm*$^{-/-}$ mice actually decreases longevity. This counterintuitive finding is considered further in the discussion.

We also monitored disease incidence and tumor spectrum in the aged *Atm*$^{-/-}$, *Mgmt*$^{-/-}$/*Atm*$^{-/-}$ or *Aag*$^{-/-}$/*Atm*$^{-/-}$ mice; Figure 2B shows the incidence of spontaneous pathology. WT, *Mgmt*$^{-/-}$ and *Aag*$^{-/-}$ mice exhibit a remarkably similar spectrum of tumors, but this spectrum is significantly different from that in the *Atm* deficient genotypes (*Atm*$^{-/-}$, *Mgmt*$^{-/-}$/*Atm*$^{-/-}$, and *Aag*$^{-/-}$/*Atm*$^{-/-}$) that exhibit a predominance of lymphoma [56, 58]; 94% of the *Atm*$^{-/-}$ mice in our study presented with lymphomas at the time of death. *Aag*$^{-/-}$/*Atm*$^{-/-}$ animals exhibit a decreased incidence of lymphoma (70%), and the overall difference in tumor spectrum between *Atm*$^{-/-}$ and *Aag*$^{-/-}$/*Atm*$^{-/-}$ mice is statistically significant ($p<0.016$). This suggests that one mechanism by which Aag deficiency increases longevity in *Atm*$^{-/-}$ animals may be by decreasing the development of aggressive

lymphomas. Although *Mgmt*$^{-/-}$/*Atm*$^{-/-}$ also display a decrease in lymphoma incidence (71.5%), it did not alter the overall tumor spectrum (p=0.19) (Figure 2B) or longevity (Figure 1B), suggesting that the tumors that arose instead of lymphoma in *Mgmt*$^{-/-}$/*Atm*$^{-/-}$ mice were as aggressive as lymphoma.

Detailed survival studies have been published for *p53*$^{-/-}$ mice [57], and here we set out to determine whether decreased repair of primarily alkylated DNA bases would affect longevity in *p53*$^{-/-}$ mice. In stark contrast to the effect of combining *Aag*$^{-/-}$ or *Mgmt*$^{-/-}$ with the *Atm*$^{-/-}$ genotype, we observe virtually identical survival in *p53*$^{-/-}$, *Aag*$^{-/-}$/*p53*$^{-/-}$, and *Mgmt*$^{-/-}$/*p53*$^{-/-}$ mice; all three genotypes exhibited significant and similarly-decreased survival compared to WT mice (p<0.0001) (Figure 3A). All *p53* deficient genotypes exhibited an altered tumor spectrum compared to WT mice, but there was no difference in tumor spectrum between *p53*$^{-/-}$, *Aag*$^{-/-}$/*p53*$^{-/-}$, and *Mgmt*$^{-/-}$/*p53*$^{-/-}$ mice (Figure 3B).

2.3 Genetic interaction between Mgmt and the MMR pathway

Given the established link between Mgmt and MMR in modulating alkylation-induced cytotoxicity [37, 40, 41, 59, 60], we investigated whether deficiency of both Mgmt and MMR proteins may cooperate to alter longevity. The effect of eliminating mismatch recognition and excisions steps of MMR in combination with Mgmt was investigated. As described, *Mgmt* deficiency does not significantly alter long-term survival *in vivo* (Figure 1A). Figure 4A presents survival data for WT, *Mgmt*$^{-/-}$, *Msh6*$^{-/-}$, *Exo1*$^{-/-}$, *Mgmt*$^{-/-}$/*Msh6*$^{-/-}$ and *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ mice. Similar to previous reports, we observe significantly decreased survival in *Msh6*$^{-/-}$ and *Exo1*$^{-/-}$mice compared to WT mice (p<0.0001) [61, 62]. We observed a trend toward increased longevity in *Mgmt*$^{-/-}$/*Msh6*$^{-/-}$, which did not reach statistical significance (pairwise comparison between *Msh6*$^{-/-}$and *Mgmt*$^{-/-}$ *Msh6*$^{-/-}$, p<0.3388). Similarly, the trend toward increased survival in *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ versus *Exo1*$^{-/-}$ mice did not reach statistical significance (pairwise comparison between *Exo1*$^{-/-}$and *Mgmt*$^{-/-}$ *Exo1*$^{-/-}$, p=0.1352) (Figure 4A). We infer that Mgmt substrates do not significantly impact whole-animal survival, even in the absence of functional MMR. Although a genetic interaction was observed between Mgmt and Msh6 or Exo1 in terms of mediating alkylation cytotoxicity upon treatment with exogenous alkylating agents *in vivo* [63], this does not appear to translate to effects from endogenous alkylation arising *in vivo*.

Detailed histological examination of the aged animals showed that the trends toward increased survival were accompanied by differences in pathology. Figure 4B shows the incidence of spontaneous pathology in animals with combinations of the *Mgmt* null allele with either *Msh6* or *Exo1* null alleles, namely *Mgmt*$^{-/-}$, *Msh6*$^{-/-}$, *Exo1*$^{-/-}$, *Mgmt*$^{-/-}$/*Msh6*$^{-/-}$, and *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ mice. The majority of the MMR defective animals exhibit lymphomas at the time of death; 70% of *Msh6*$^{-/-}$animals and 70.5% of *Exo1*$^{-/-}$ animals present with lymphoma, consistent with the published literature (Figure 4B) [61, 62]. The additional inactivation of the *Mgmt* gene does not significantly alter the tumor spectrum in *Msh6* mutant background; 73% of *Mgmt*$^{-/-}$/*Msh6*$^{-/-}$ mice develop lymphoma (Figure 4B). Remarkably, *Mgmt* deficiency results in a greater than 50% reduction in the incidence of lymphoma in *Exo1*$^{-/-}$ mice; 70.5% in *Exo1*$^{-/-}$ mice develop lymphoma whereas only 31% of *Mgmt*$^{-/-}$ /*Exo1*$^{-/-}$ mice present with this pathology. The reduction of lymphoma in

Mgmt$^{-/-}$ /*Exo1*$^{-/-}$ mice coincides with a two-fold increase in histiocytic sarcoma, the predominant pathology observed in *Mgmt*$^{-/-}$ mice. The change in tumor spectrum between the *Exo1*$^{-/-}$ and the *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ is significant (p=0.03).

2.4 The contribution of *Atm* to cellular responses following exogenous DNA alkylation damage

Aag deficiency resulted in a counter-intuitive increase in longevity in *Atm*$^{-/-}$ deficient mice, accompanied by alterations in the tumor spectrum (Figures 2A and 2B). To further examine this genetic interaction between Atm and Aag, we used the tractable bone marrow (BM) *ex vivo* clonogenic survival assay to determine whether, as seems to be the case for endogenous DNA damage, Atm modulates Aag-mediated alkylation-induced cytotoxicity. BM cells were treated *ex vivo* with the alkylating agent methyl methane sulfonate (MMS) and then plated on semisolid media to allow formation of hematopoietic myeloid progenitor colonies. MMS is an S_N2 alkylating agent that induces predominantly 7MeG and 3MeA DNA lesions, known Aag substrates [34]. Consistent with a previous report [64], we show here that *Aag*$^{-/-}$ BM cells are resistant to MMS (Figure 5). This is consistent with multiple recent reports showing that initiation of BER by DNA glycosylases generates repair intermediates (AP-sites, and single-strand breaks (SSBs)) that, if accumulate due to downstream BER enzymes being limited, are more toxic than the original DNA base lesions (reviewed in [33]). This is supported by evidence indicating that alkylation sensitivity is dependent on Aag-initiated BER both in cultured cells and in animals [65–67]. Interestingly, *Atm*$^{-/-}$ BM cells display increased MMS sensitivity in comparison to all other genotypes, indicating that Atm signaling is an important mediator of MMS-mediated toxicity. The increased sensitivity observed in *Atm*$^{-/-}$ BM cells is almost totally suppressed in *Aag*$^{-/-}$/*Atm*$^{-/-}$ BM cells, suggesting that much of the alkylation sensitivity observed in the *Atm*$^{-/-}$ cells is due to Aag-initiated BER of MMS-induced base damage followed by the accumulation of toxic BER intermediates that are ultimately sensed by Atm (Figure 5A). Together, these *ex vivo* assays illustrate that Atm is an integral modulator of toxicity induced by Aag-initiated BER, and pinpoints a role for the Atm DNA damage response protein in signaling downstream of toxic BER intermediates.

We also assessed the contribution of Atm to O^6meG-mediated cytotoxicity following exposure to the S_N1 alkylating agent, N-methyl-N-nitrosourea (MNU), which generates toxic and mutagenic O^6MeG, in addition to 7meG and 3meA DNA base lesions. *Ex vivo* clonogenic survival assays were performed with BM from WT, *Mgmt*$^{-/-}$, *Atm*$^{-/-}$ and *Mgmt*$^{-/-}$/*Atm*$^{-/-}$mice. In contrast to MMS, *Atm*$^{-/-}$ BM cells exhibit no difference in MNU sensitivity compared to WT cells at the doses used (Figure 5B), presumably because both WT and *Atm*$^{-/-}$ cells express Mgmt to reverse the toxic

O^6meG lesions. Accordingly, *Mgmt*$^{-/-}$ cells exhibit increased sensitivity to MNU compared to both WT and *Atm*$^{-/-}$ cells. Strikingly, we observe a massively synergistic interaction between Mgmt and Atm; *Mgmt*$^{-/-}$/*Atm*$^{-/-}$ cells exhibit dramatically increased sensitivity to MNU when compared to *Mgmt*$^{-/-}$ or *Atm*$^{-/-}$ cells (Figure 5B). We infer that when O^6MeG base lesions are unrepaired (as in the *Mgmt*$^{-/-}$ cells), Atm plays a pivotal role in modulating the toxicity induced by MMR processing of DNA containing O^6MeG DNA lesions.

3 DISCUSSION

Here we describe a large-scale aging study of numerous mouse models defective in several DNA repair genes and DNA damage response genes (Supplemental Table 1). Essential for a study of this magnitude, all animals were backcrossed to the C57Bl/6 genetic background for at least 10 generations to ensure that any differences observed could not be attributed to differences in strain background.

Accumulating toxic and mutagenic damage in mitochondrial and nuclear DNA is known to affect the aging process in model organisms [68–71]. We show here that endogenously damaged DNA bases that are substrates for two DNA alkylation repair pathways contribute to long-term survival; mice deficient in both Aag and Mgmt activity exhibit decreased life-span that is statistically significant. The accumulation of unrepaired DNA damage and mutations associated with aging may simply arise due to long-term exposure to endogenous metabolites that damage DNA, and may be exacerbated by an age-related decline in the DNA repair capacity. The capacity to perform BER, NER and double-strand break (DSB) repair have, in fact, all been shown to decline with age (reviewed in [72–74]). Further, studies in mice indicate that certain tissues or anatomical sites may be more susceptible to such age-related DNA repair decline [69] perhaps due to differing exposure to RONS [75, 76]. All of these possibilities are not necessarily mutually exclusive and likely cooperate as contributing factors in influencing longevity. Indeed, in the worst case scenario, aging tissues could have both decreased DNA repair and DNA damage responses accompanied by increased levels of endogenous metabolites that damage DNA.

We and others have demonstrated that for certain cell types Aag-mediated initiation of BER can lead to cell death, and that Aag deficiency can actually be protective [65, 66]. Here, we find that Aag deficiency protects $Atm^{-/-}$ mice both in terms of increasing overall longevity and in reducing the development of lymphoma; this protection is consistent with a role for Aag in generating toxic BER intermediates that trigger the DNA damage response orchestrated by Atm. Further, Aag deficiency provided protection against MMS-induced toxicity in $Atm^{-/-}$ BM cells, *ex vivo*. Together, this indicates that Atm is required for protection against Aag-mediated alkylation-induced toxicity, and that endogenously-generated Aag substrates can influence organismal longevity. This may not be surprising given that Aag acts on a wide range of endogenously-generated base lesions including 7meG, 3meA, deaminated adenine, oxidized guanine and etheno-base lesions [26, 34, 77, 78]. A link between Atm and BER has been implicated in numerous reports [79–81], but the data here provide *in vivo* evidence that Atm plays a key role in protecting against the detrimental effects of Aag-mediated BER intermediate formation at sites of spontaneous DNA base damage.

Interestingly, although ATM is known to phosphorylate, stabilize and activate p53 [82], there is no change in survival in $Aag^{-/-}/p53^{-/-}$ mice, in contrast to enhanced survival in $Aag^{-/-}/Atm^{-/-}$ mice. The protection in $Aag^{-/-}/Atm^{-/-}$ mice compared to $Aag^{-/-}/p53^{-/-}$ mice may be explained by the numerous p53-independent functions of Atm [82]. Alternatively, it has been shown previously that Aag physically interacts with and represses p53 [83];

therefore genetic deletion of both Aag and p53 would be epistatic and not alter overall survival compared to *p53*$^{-/-}$ mice.

Mgmt deficiency does not affect survival or tumor spectrum in *Atm*$^{-/-}$ mice. However, a clear genetic interaction between Mgmt and Atm was observed in the *ex vivo* BM clonogenic survival assays. Predictably, *Mgmt*$^{-/-}$ BM cells exhibit alkylation hypersensitivity but surprisingly, *Mgmt*$^{-/-}$/*Atm*$^{-/-}$ BM cells exhibit a synergistic increase in alkylation sensitivity. We propose that in the absence of Mgmt, futile cycling of MMR at O^6meG:T mispairs results in the generation of DSBs that activate Atm [83–86]. Without Atm and Mgmt, MMR-mediated futile cycling continues without the Atm-mediated signaling pathways, further exacerbating cell death. Together, this illustrates that Atm contributes to the cellular response to O^6meG induced by exogenous alkylating agents, but implies that spontaneous O^6meG lesions are not relevant in the development of morbidity in *Atm*$^{-/-}$/*Mgmt*$^{-/-}$ mice, although it is possible that the decreased lifespan of *Atm*$^{-/-}$ mice precludes any potential cumulative detrimental effects of endogenous O^6meG lesions in *Atm*$^{-/-}$/*Mgmt*$^{-/-}$ mice.

It is intriguing that Mgmt deficiency protected *Exo1*$^{-/-}$ mice against the development of lymphoma; instead *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ mice developed histiocytic sarcoma, the prevalent disease in C57Bl/6 mice. Although *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ mice exhibited decreased incidence of lymphoma, there was only a trend toward increased longevity, indicating that the protection against lymphoma and the overall shift in tumor spectrum did not prolong lifespan. *Mgmt*$^{-/-}$ mice develop histiocytic sarcoma at an average latency of 25.5 months, whereas in *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ mice, the onset of histiocytic sarcoma is significantly earlier (p=0.0035), with an average latency of 21.7 months. Although *Mgmt* deficiency altered tumor penetrance in *Exo1*$^{-/-}$ mice, this was not observed in *Msh6*$^{-/-}$ mice. Msh6 deficiency results in a strong predisposition to lymphomagenesis, which occurs significantly earlier than in *Exo1*$^{-/-}$ mice (p=0.0002). The constitutive MMR deficiency (CMMRD) cancer syndrome in humans substantiates the role for Msh6 in preventing hematological malignancies and other cancers [87–89] and reinforces the finding that Msh6 deficiency is a strong inducer of lymphoma in mice [61]. *EXO1* deficiency is not causative of CMMRD, but *EXO1* mutations have been found in diffuse B-cell lymphoma [87]. The strong association between *MSH6* mutations and lymphoma may explain why Mgmt deficiency was insufficient to change tumor spectrum in *Msh6*$^{-/-}$ mice, whereas *Mgmt*$^{-/-}$/*Exo1*$^{-/-}$ mice exhibited a shift in tumor spectrum towards histiocytic sarcoma.

Although the link between accumulating DNA damage and aging has been clearly established, the consequences of lifestyle interventions that increase longevity and their role on altering DNA repair capacity remain unresolved. One proven strategy demonstrated to enhance longevity is calorie restriction (CR); the consequence of CR on DNA repair remains controversial. CR has been shown to reduce the age-dependent decline in non-homologous end joining activity [90], whereas other studies show a decrease in DNA repair transcript levels following CR [91]. Additionally, it is well-known that habitual endurance exercise improves health-span [92, 93], and although endurance exercise is associated with an increase in oxidative DNA damage [94], exercise-induced RONS are thought to induce DNA repair and other molecular systems to cope with increased RONS damage [95–98].

Finally, resveratrol, a polyphenol found in red wine and an activator of the NAD-dependent deacetylase sirtuin-1 (Sirt1), has been hypothesised to increase longevity. Resveratrol increases the formation of APE/XRCC1 complex during BER [99], but also reduces the activity or expression of other DNA repair proteins [100, 101]. These few examples underscore the fact that much remains to be learned regarding the relationship between DNA repair and lifestyle interventions that may modulate longevity.

Significant progress has been made regarding the pathways and factors that modulate longevity [1, 102, 103], and yet many questions remain unanswered. Several theories of aging have been proposed including: the mitochondrial free radical theory of aging, telomere attrition, mitochondrial dysfunction, and more recently, the functional decline of stem cells (aging theories reviewed in [104–106]). It is likely that many of the proposed mechanisms of aging interact with each other to influence the longevity of an organism. Here, using long-term lifespan studies in DNA repair- and DNA damage-response deficient mouse models, we establish that the repair of DNA base alkylation damage arising from endogenous sources is at least one contributing factor to longevity.

4 METHODS

4.1 Mice

The *Aag*$^{-/-}$ mice [107] and *Mgmt*$^{-/-}$ mice [108] have been described. *Trp53*$^{-/-}$ mice (B6.129S2-Trp53^{tm1Tyj}, former name C57BL/6J-Trp53^{tm1Tyj}) and *Atm*$^{-/-}$ mice (129S6/SvEvTac-Atm$^{tm1Awb/J}$) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). *Exo1*$^{-/-}$ and *Msh6*$^{-/-}$ mice have been described previously [61, 62]. All mice were backcrossed at least 10 times to the C57BL/6 background. Mice were fed standard diet *ad libitum* and housed in an AAALAC accredited facility. Animals were sacrificed by CO_2 asphyxiation. All animal procedures were approved by the MIT Committee on Animal Care.

4.2 Longevity studies

Mice were allowed to age and observed for development of disease and subject to full necropsy when diseased or deceased. Tissues were fixed in Bouin's fixative, paraffin embedded, sectioned at 5 μm and stained with haematoxylin and eosin (H&E). Tissues harvested include: brain, eyes, salivary gland, thymus, heart, lung, liver, kidney, spleen, intestine, reproductive organs, and femur. All H&E stained slides were analyzed blind by a pathologist (R.T.B) for the cause of death as well as for identification of any tumors/lesions. Examples of lesions classified as other include: dermatitis, cystic endometrium/uterus, emphysema, kidney disease and osteoarthritis.

4.3 Bone marrow clonogenic survival assay

BM clonogenic survival assays were performed as described in [64]. Briefly, cells were harvested from the femurs of mice, treated *ex vivo* with MMS (Sigma-Aldrich Co, St. Louis, MO) or MNU (Sigma-Aldrich Co, St. Louis, MO) and plated in methylcellulose-containing media (Stem Cell Technologies, Vancouver, BC, Canada), and plated in duplicate. After approximately 2 weeks, colonies containing > 50 cells were scored. Experiments were performed at least three times.

4.4 Statistical analysis

GraphPad Prism was used to generate Kaplan-Meier plots for survival and to calculate significance using Log-rank (Mantel-Cox) test. Fisher's exact, programmed in R, was used to establish whether the differences in tumor spectra between genotypes were significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3meA	3-methyladenine
Aag/Mpg	alkyladenine glycosylase
AP	abasic
Atm	ataxia telangiectasia mutated
BER	base excision repair
BM	bone marrow
CR	calorie restriction
Exo1	exonuclease 1
Mgmt	O^6MeG DNA methyltransferase
MMR	mismatch repair
MMS	methyl methanesulfonate
MNU	N-methyl-N-nitrosourea
Msh6	MutS homolog 6
NER	nucleotide excision repair
***O*^{6}MeG**	O^6-methylguanine
RONS	reactive oxygen nitrogen species
WT	wildtype

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Highlights

- Large-scale mouse aging study examines role for DNA repair and DNA damage response.
- Repair of endogenous alkylation damage plays a role in determining longevity.
- Atm plays a key role in protecting against detrimental effects of Aag-mediated BER.

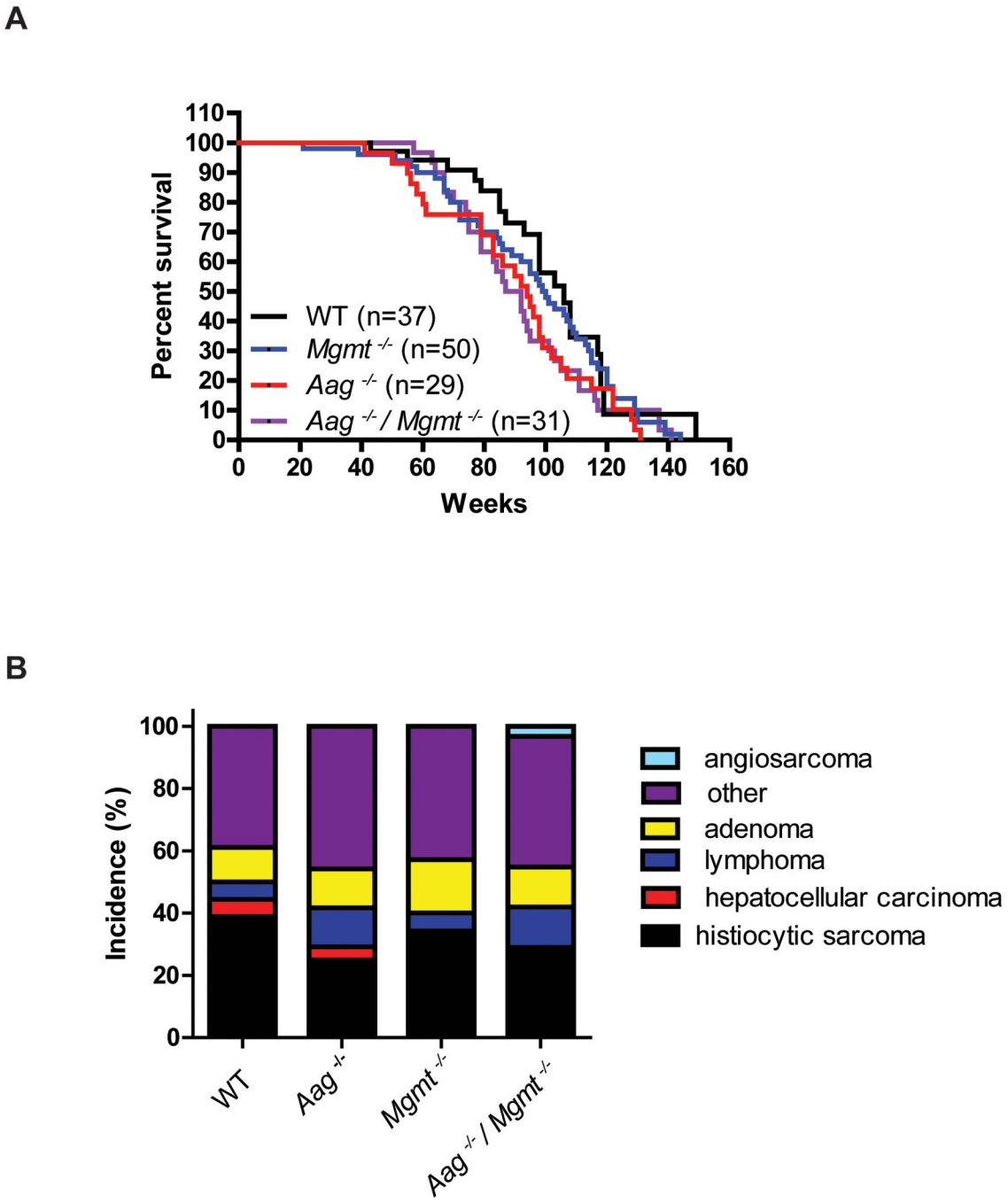

Figure 1. DNA alkylation repair contributes to longevity

A) Survival curves of wild type (black lines, n=37), *Aag*$^{-/-}$ (red line, n=29), *Mgmt*$^{-/-}$ (blue line, n=50) and *Aag*$^{-/-}$ *Mgmt*$^{-/-}$ (purple line, n=31). Pair-wise comparisons: *Aag*$^{-/-}$ to wild type, p=0.1003; *Mgmt*$^{-/-}$ to wild type, p=0.4752; *Aag*$^{-/-}$ *Mgmt*$^{-/-}$ to wild type, p=0.04, all Log-rank (Mantel-Cox) test. **B)** Histopathological classification of pathologies found in WT (n=18), *Aag*$^{-/-}$ (n=24), *Mgmt*$^{-/-}$ (n=35), and *Aag*$^{-/-}$ *Mgmt*$^{-/-}$ (n=31) mice.

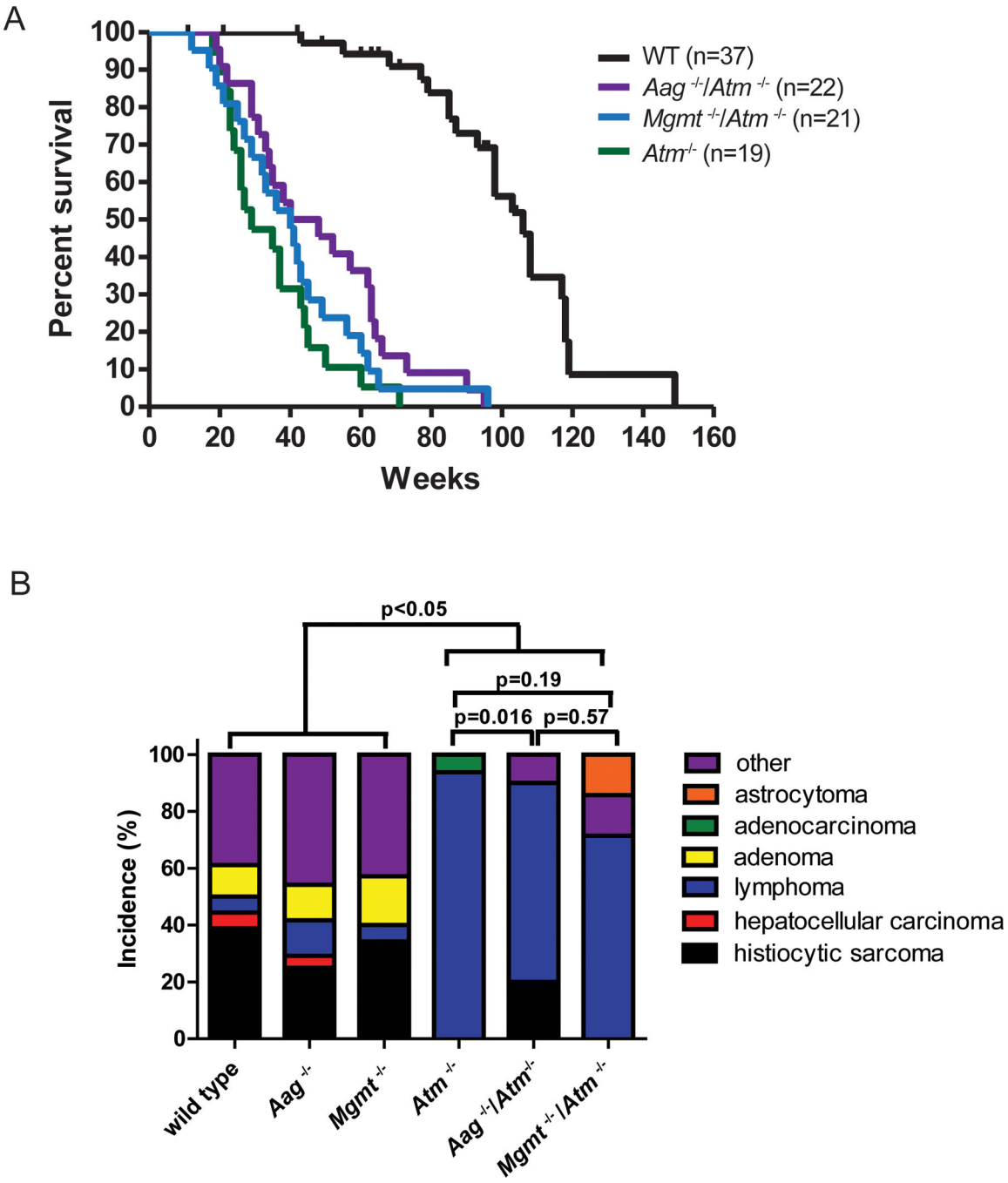

Figure 2. Atm plays a role in the response to endogenous/spontaneous DNA alkylation damage

A) Survival curves of wild type (black lines, n=37), *Atm*$^{-/-}$ (green line, n=19), *Aag*$^{-/-}$ *Atm*$^{-/-}$ (green/red line, n=22) and *Mgmt*$^{-/-}$ *Atm*$^{-/-}$ (cyan line, n=21). All pair-wise comparisons to wild type, p<0.0001. Pair-wise comparison between *Atm*$^{-/-}$ and *Aag*$^{-/-}$ *Atm*$^{-/-}$, p=0.0193, and between *Atm*$^{-/-}$ and *Mgmt*$^{-/-}$ *Atm*$^{-/-}$, p=0.3423, all comparisons Log-rank (Mantel-Cox) test. **B)** *Aag* deficiency protects against lymphoma in *Atm*$^{-/-}$ animals. Graph shows the incidence of age-related pathologies observed in Aag and Atm

genotypic combinations. Wild-type (n=18); *Aag*$^{-/-}$ (n=24); *Mgmt*−/− (n=35); *Atm*$^{-/-}$ (n=15); *Mgmt*$^{-/-}$ *Atm*$^{-/-}$ (n=7); *Aag*$^{-/-}$ *Atm*$^{-/-}$ (n=10).

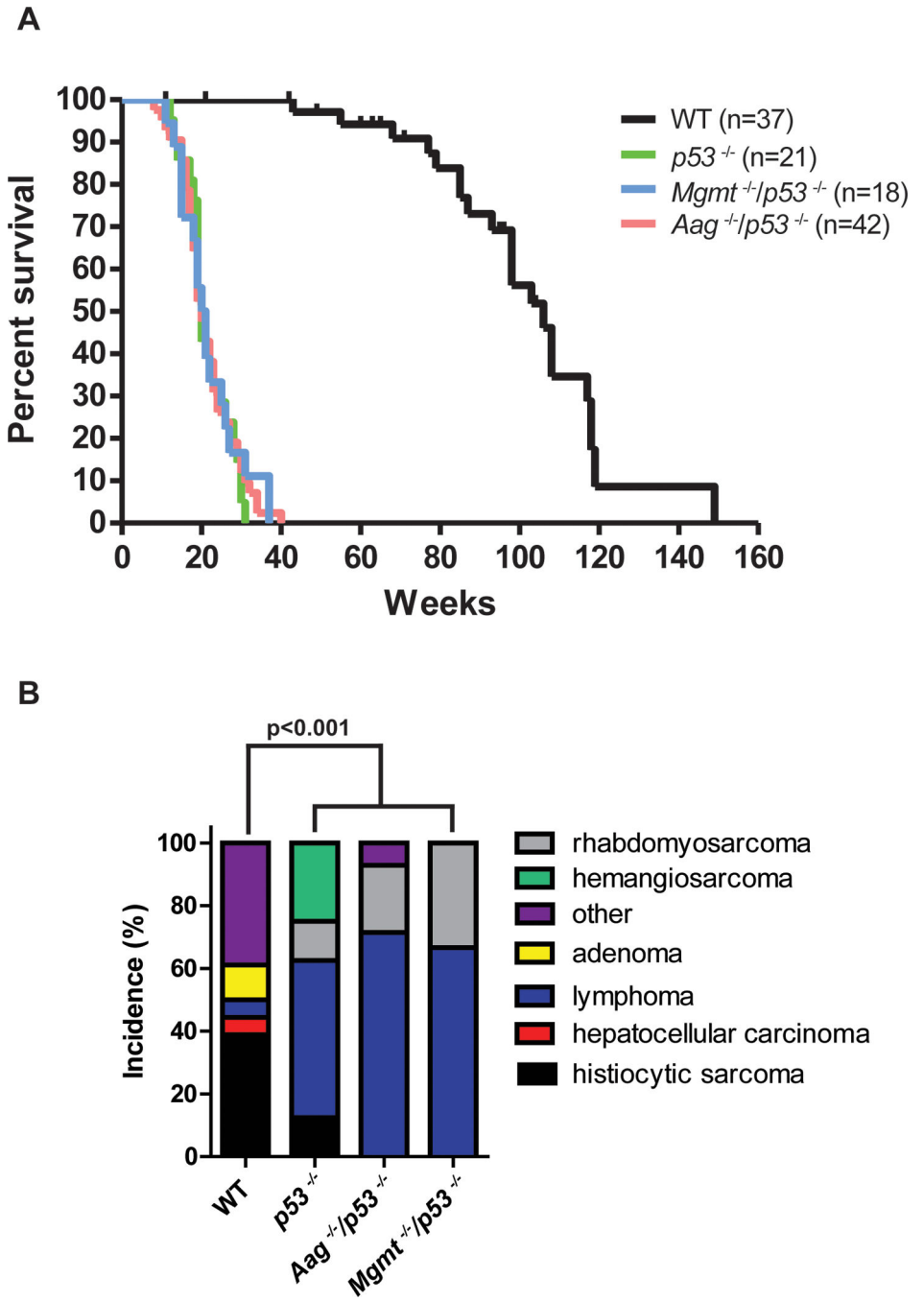

Figure 3. Aag and Mgmt mutations do not affect longevity of p53 mutant animals

A) Survival curves of wild type (black lines, n=37), $p53^{-/-}$ (light green line, n=21), $Aag^{-/-}$ $p53^{-/-}$ (light red line, n=42), and $Mgmt^{-/-}$ $p53^{-/-}$ (light blue line, n=18). All pair-wise comparisons to wild type, p<0.0001, Log-rank (Mantel-Cox) test. **B)** *Aag* or *Mgmt* deficiency does not shift tumor spectrum in $p53^{-/-}$ mice. Graph shows the incidence of age-related pathologies observed in Aag and p53 genotypic combinations. Wild-type (n=18); $p53^{-/-}$ (n=8); $Aag^{-/-}$ $p53^{-/-}$ (n=14); $Mgmt^{-/-}$ $p53^{-/-}$ (n=9).

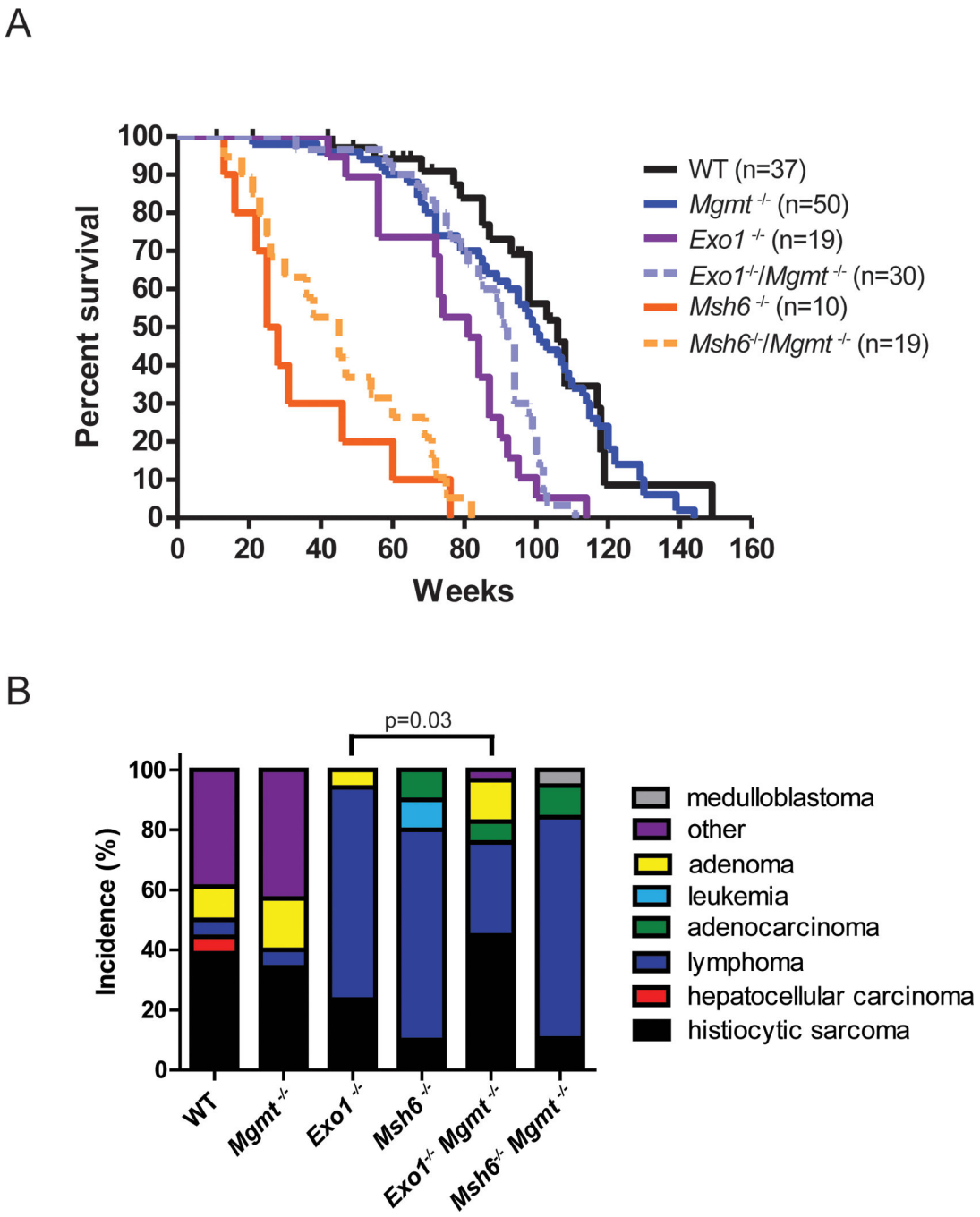

Figure 4. Interaction between Mgmt deficiency and the mismatch repair pathway

Survival curves of wild type (black lines, n=37), *Mgmt*$^{-/-}$ (blue line, n=50), *Exo1*$^{-/-}$ (purple line, n=19), *Mgmt*$^{-/-}$ *Exo1*$^{-/-}$ (dashed lilac line, n=30), *Msh6*$^{-/-}$ (orange line, n=10), and *Mgmt*$^{-/-}$ *Msh6*$^{-/-}$ (dashed light orange line, n=19). Pair-wise comparison between *Mgmt*$^{-/-}$ and *Mgmt*$^{-/-}$ *Exo1*$^{-/-}$, p=0.0008 and between *Mgmt*$^{-/-}$ and *Mgmt*$^{-/-}$ *Msh6*$^{-/-}$, p<0.0001, Log-rank (Mantel-Cox) test. **B)** The *Mgmt* null mutation leads to a decrease in the incidence of lymphomas in *Exo1*$^{-/-}$ animals but not in *Msh6*$^{-/-}$ animals. Wild-type (n=18); *Mgmt*$^{-/-}$

(n=33), *Exo1*$^{-/-}$ (n=17), *Msh6*$^{-/-}$ (n=10), *Mgmt*$^{-/-}$ *Msh6*$^{-/-}$ (n=19), *Mgmt*$^{-/-}$ *Exo1*$^{-/-}$ (n=29).

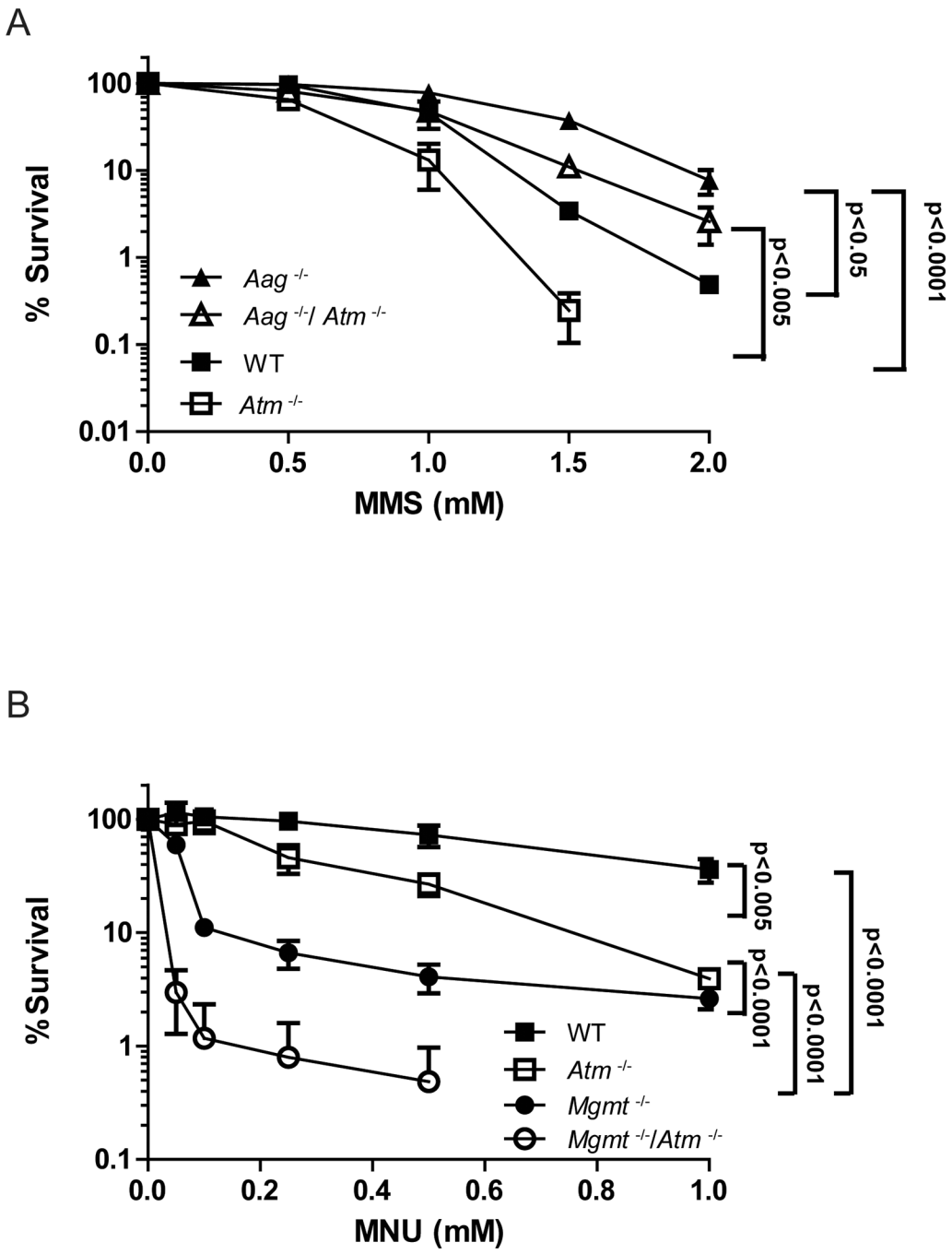

Figure 5. Atm and Aag interact in response to induced alkylation damage

A) *Ex vivo* alkylation sensitivity of BM cells to methyl methanesulfonate (MMS). BM cells were derived from wild type (closed squares), $Aag^{-/-}$ (closed triangle), $Atm^{-/-}$ (open squares) and $Aag^{-/-}$ $Atm^{-/-}$ (open triangle) mice. Experiments were done a minimum of three times each, data are mean ± SEM.. **B)** Synergistic interaction between Mgmt and Atm in response to MNU treatment. *Ex vivo* alkylation sensitivity of BM cells to methyl nitrosourea (MNU). BM cells were derived from wild type (closed squares), $Mgmt^{-/-}$

(closed circles), *Atm*$^{-/-}$ (open squares) and *Mgmt*$^{-/-}$ *Atm*$^{-/-}$ (open circles) mice. Experiments were done a minimum of three times each, data are mean ± SEM.

Table 1

Median survival of all mice, median survival of mice with tumors, and tumor penetrance in aging study.

Genotype	Median Survival, weeks	Median Survival (tumor), weeks	% tumor at termination
Wildtype	106	98	61.1
Aag$^{-/-}$	94	99	54.2
Mgmt$^{-/-}$	99.5	103.5	57.1
Atm$^{-/-}$	29	35	100
p53$^{-/-}$	20	21.5	100
Exo1$^{-/-}$	81	82.3	94.1
Msh6$^{-/-}$	26.5	25	100
Aag$^{-/-}$*Mgmt*$^{-/-}$	89.5	94.5	58
Aag$^{-/-}$*Atm*$^{-/-}$	44	63	90
Aag$^{-/-}$*p53*$^{-/-}$	20	22	92.8
Mgmt$^{-/-}$*Atm*$^{-/-}$	40	49	100
Mgmt$^{-/-}$*p53*$^{-/-}$	20.5	22	100
Mgmt$^{-/-}$*Exo1*$^{-/-}$	91.5	92	96.5
Mgmt$^{-/-}$*Msh6*$^{-/-}$	45	45	100