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Prolonged lifespan with enhanced exploratory behavior in mice overexpressing the oxidized nucleoside triphosphatase hMTH1

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Summary

The contribution that oxidative damage to DNA and/or RNA makes to the aging process remains undefined. In this study, we used the hMTH1-Tg mouse model to investigate how oxidative damage to nucleic acids affects aging. hMTH1-Tg mice express high levels of the hMTH1 hydrolase that degrades 8-oxodGTP and 8-oxoGTP and excludes 8-oxoguanine from both DNA and RNA. Compared to wild-type animals, hMTH1-overexpressing mice have significantly lower steady-state levels of 8-oxoguanine in both nuclear and mitochondrial DNA of several organs, including the brain. hMTH1 overexpression prevents the age-dependent accumulation of DNA 8-oxoguanine that occurs in wild-type mice. These lower levels of oxidized guanines are associated with increased longevity and hMTH1-Tg animals live significantly longer than their wild-type littermates. Neither lipid oxidation nor overall antioxidant status is significantly affected by hMTH1 overexpression. At the cellular level, neurospheres derived from adult hMTH1-Tg neural progenitor cells display increased proliferative capacity and primary fibroblasts from hMTH1-Tg embryos do not undergo overt senescence *in vitro*. The significantly lower levels of oxidized DNA/RNA in transgenic animals are associated with behavioral changes. These mice show reduced anxiety and enhanced investigation of environmental and social cues. Longevity conferred by overexpression of a single nucleotide hydrolase in hMTH1-Tg animals is an example of lifespan extension associated with healthy aging. It provides a link between aging and oxidative damage to nucleic acids.

Key words: 8-oxoG; aging; oxidative stress; senescence; behavior.

Introduction

Oxidative stress, an imbalance in the production and detoxification of reactive oxygen and nitrogen species (ROS and RNS), is implicated in the pathogenesis of cancer and neurodegenerative disorders as well as in the aging process (Ames, 1983; Halliwell, 2007; Campisi & Yaswen, 2009; Hoeijmakers, 2009; Kenyon, 2010). As DNA alterations induced by ROS/RNS are often mutagenic, mammalian cells have evolved strategies to prevent the accumulation of oxidized DNA bases and preserve genome stability. DNA 8-oxoguanine (8-oxoG) has received most attention because of its ease of formation and miscoding properties. Together with direct oxidation of DNA, the nucleotide pool has been shown to be a significant target for oxidative stress (Haghdoost *et al.*, 2006). MTH1–a member of the family of hydrolases that eliminate oxidized precursors from the dNTP pool (Ishibashi *et al.*, 2003)–degrades both 8-oxodGTP and 8-oxoGTP and prevents 8-oxoG accumulation in DNA and RNA (Sakumi *et al.*, 1993; Hayakawa *et al.*, 1999). MTH1 inactivation is associated with a mutator phenotype in mouse cells (Hori *et al.*, 2010) and increased cancer incidence in mice (Tsuzuki *et al.*, 2001). Recent findings link MTH1 deficiency to neurodegeneration. Thus *Mth1*$^{-/-}$ mice have increased DNA 8-oxoG levels after kainate-induced excitotoxicity (Kajitani *et al.*, 2006) and loss of dopaminergic neurons in a Parkinson's disease mouse model (Yamaguchi *et al.*, 2006). In addition, human hMTH1 overexpression in hMTH1 transgenic (hMTH1-Tg) mice confers significant protection against neurodegeneration and motor impairment induced by the mitochondrial toxin 3-nitropropionic acid (3-NP) (De Luca *et al.*, 2008). Chronic accumulation of oxidative damage to macromolecules is linked to progressive decline in cellular functions and aging (Harman, 1956; Sohal & Weindruch, 1996). Oxidation of lipids, DNA, and proteins increases with age in a variety of tissues and animal models (Bokov *et al.*, 2004) although it remains unclear whether this is a major causal factor in aging (Salmon *et al.*, 2010). As hMTH1 overexpression in the hMTH1-Tg mouse model protects several organs against oxidative DNA damage (De Luca *et al.*, 2008), we have used these mice to examine the possible role of oxidative DNA damage in aging.

Results

hMTH1 overexpression in transgenic mice prolongs lifespan

hMTH1-Tg animals live longer. Their median lifespan was 4 months greater than that of their wild-type littermates (914 vs. 790 days) ($P < 0.0004$; Kaplan Meier test) (Fig. 1A). Differences in maximum

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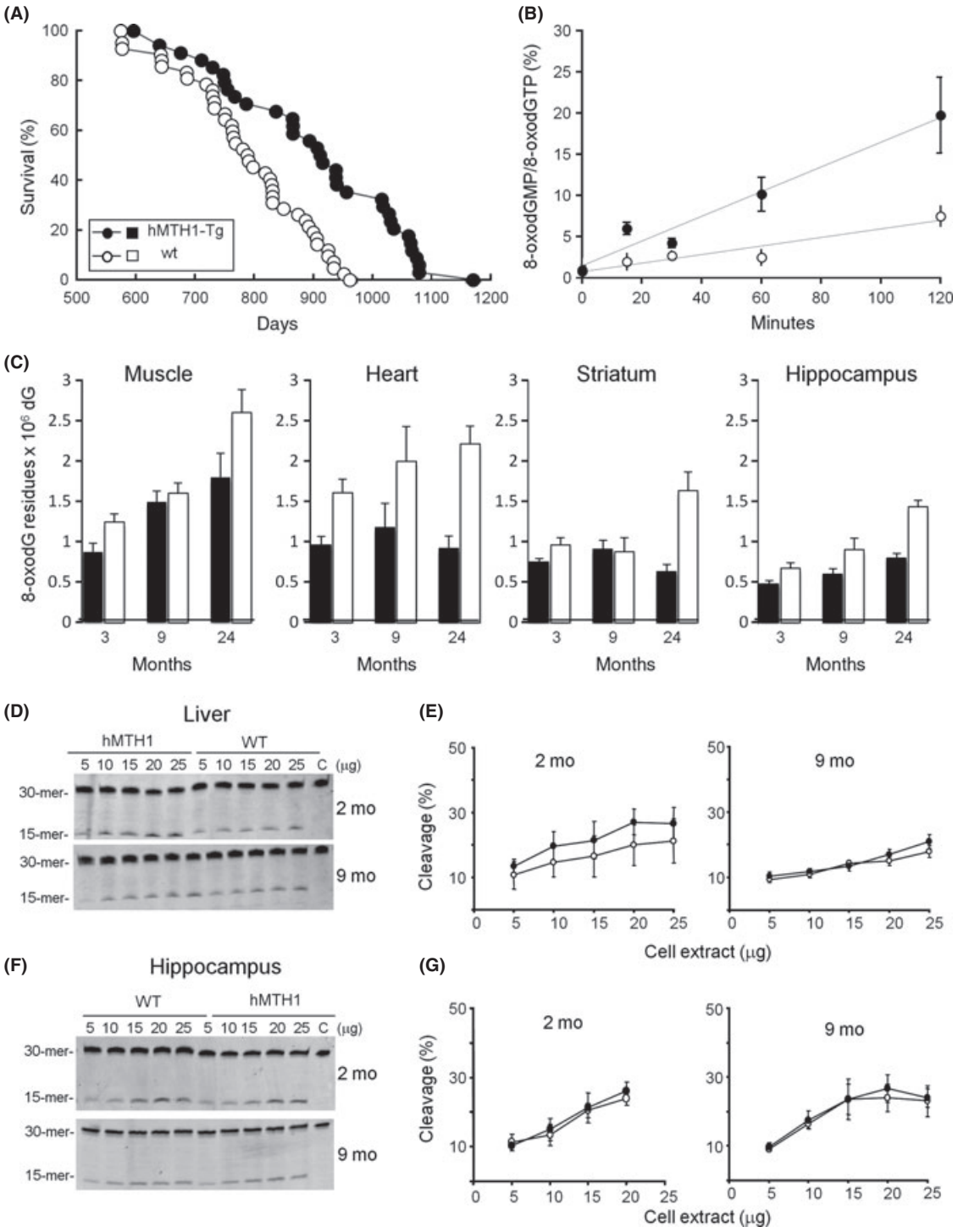

Fig. 1 Lifespan and hMTH1 overexpression. (A) Survival of hMTH1-Tg (n = 34) and wild-type (n = 42) littermate pairs. Relative risk (RR) 3.19 (SE = 0.89), $P < 0.0004$; Kaplan Meier test. The effect of genotype on lifespan: $F = 13.13$, $P < 0.001$ by two-way analysis of variance. (B) hMTH1 enzymatic activity in cell-free extracts from the brain of hMTH1-Tg (n = 4) and wild-type animals (n = 4). (C) 8-oxodG levels in mtDNA of 3-, 9- and 24-month-old hMTH1-Tg (n = 21) and wild-type (n = 24) mice. Two-way analysis of variance: genotype effect: F (1,39) = 9.3, $P < 0.004$; F(1,39) = 20.3, $P < 0.001$; F(1,39) = 14.9, $P < 0.0005$; $F = 20$, $P < 0.0001$, skeletal muscle, heart, striatum and hippocampus, respectively; age effect: F (1,37) = 48.6, $P < 0.0001$ and F (1,36) = 44.6, $P < 0.0001$ for skeletal muscle and hippocampus; age × genotype: F (1,36) = 7.18, $P = 0.01$ and F(1,30)=11.2, $P = 0.002$ for hippocampus and striatum). Values are indicated as mean ± SE. Wild-type (open bar) and hMTH1-Tg mice (black bar). (D and F) Representative gels showing the reaction products of OGG1 activity on a duplex DNA substrate (100 fmol) containing a single 8-oxodG:C mismatch, following incubation with increasing amounts of nuclear extracts prepared from liver (E) and hippocampus (G) of 2- and 9-month-old mice (n = 3 for each genotype). Lane C in each gel is the control sample containing the DNA substrate without cell extracts. Panels E and G contain the plots of product formation shown in D and F. Data are indicated as mean ± SD.

lifespan (the frequency of mice living longer than the global 90% percentile of lifespan distribution) were also highly significant ($P < 0.001$, Fisher's exact test) (Wang *et al.*, 2004). Eight of 34 hMTH1-Tg mice survived beyond the 1036 day threshold, whereas none of the wild-type mice did. A two-way analysis of variance with survival as a dependent variable and genotype and sex, respectively, as sources of variation revealed that the effect of genotype on lifespan was highly significant ($P < 0.001$) and independent of gender; hMTH1 overexpression extended lifespan in both sexes independently of their differential mortality.

We previously reported high levels of the hMTH1 protein in the brain of hMTH1-Tg animals (De Luca *et al.*, 2008). Direct enzyme assays indicated that this protein was active, and hMTH1 activity was threefold higher in brains of transgenic animals compared to control mice (Fig. 1B). hMTH1 overexpression prevented the accumulation of oxidized guanine in nuclear DNA from young (2–3 months) and middle-aged (9–10 months) mice (Fig. S1A,B). DNA 8-oxodG levels in heart, brain and small intestine were significantly lower in hMTH1-Tg than in wild-type animals (two-way analysis of variance with genotype and age as sources of variation; genotype effect: $P = 0.001$). The only organ in which DNA 8-oxodG significantly accumulated with age was the liver (age effect: $P = 0.001$). In this case, the hMTH1-mediated protection was limited and did not reach statistical significance.

In addition, when DNA oxidation was investigated in brain areas of aged animals (age range: 26–30 months), significantly lower levels of DNA 8-oxodG (2.1-fold; $P < 0.05$) were observed in the striatum and hippocampus of hMTH1-Tg compared to wild-type mice of similar ages (Fig. S1C). A similar trend was also found in the cortex. RNA oxidation was also lower in the striatum of hMTH1-Tg mice (2.8-fold) (Fig. S1D).

MTH1 is localized in the cytosol and the mitochondrial matrix and contributes to the sanitization of both nuclear and mitochondrial dNTP pools (Kang *et al.*, 1995). As expected (de Souza-Pinto *et al.*, 2001), the steady-state level of oxidation in mitochondrial DNA (mtDNA) was almost twofold higher than that of nuclear DNA (Figs 1C and S1). High level hMTH1 expression was associated with significantly lower mtDNA 8-oxodG levels in skeletal muscles, heart, striatum and hippocampus of hMTH1-Tg animals in comparison with wild-type animals (genotype effect: $P \leq 0.004$ by two-way analysis of variance). mtDNA 8-oxoG levels increased linearly with age in skeletal muscle, hippocampus of 3-, 9- and 24-month-old mice (Fig. 1C) (age effect: $P < 0.0001$). In addition, in the hippocampus and striatum, there was a nonlinear interaction between age and genotype (age $\times$ genotype, $P \leq 0.01$) indicating that protection by hMTH1 was enhanced in old animals (Fig. 1C). In conclusion, although hMTH1 expression protects both nuclear and mtDNA, its defensive role in aging mice is more evident in the mitochondrial compartment.

The OGG1 glycosylase protects against direct DNA oxidation by removing 8-oxoG from DNA *via* the base excision repair (BER) pathway. OGG1 activity was comparable in liver and hippocampus of wild-type and transgenic animals at 2 and 9 months (Fig. 1D–G). We conclude that the contribution of OGG-1 is similar in hMTH1-Tg and wild-type mice and that their different DNA 8-oxodG levels reflect the differential activities of hMTH1.

To address the molecular basis of longevity in hMTH1-Tg mice, we examined several factors acknowledged to influence aging. Reduced levels of free radicals and/or increased antioxidant defenses are a common feature of mouse models of extended lifespan (Salmon *et al.*, 2010). Increased longevity in hMTH1-overexpressing mice was not associated with altered antioxidant defenses. In particular, peripheral antioxidant capacity (AOC) was comparable in hMTH1-Tg and wild-type mice at 3, 9, and 24–36 months, and the decreased AOC associated with aging (24–36 months vs. younger ages) was unaffected by hMTH1 genotype (Fig. 2A). In addition, the levels of F2-isoprostane (15-F_{2t}-IsoP), a marker for *in vivo* free radical generation, were comparable in different brain regions in old-wild-type and hMTH1-Tg mice (Fig. 2B), indicating that increased longevity in hMTH1 mice is not associated with changes in general oxidative status.

The IGF-1/insulin signaling pathway that is less active in several models of extended lifespan (Holzenberger *et al.*, 2003; Liang *et al.*, 2003; Kenyon, 2010; Selman & Withers, 2011) was unaffected by hMTH1 overexpression. Serum IGF-1 levels were comparable in wild-type and hMTH1-Tg mice of 3–36 months (Fig. 2C). In agreement with their similar IGF-1 levels, blood glucose levels were also unaffected by hMTH1 expression (Fig. 2D), indicating that lifespan extension associated with hMTH1 is independent of the IGF-1-insulin axis.

In some mouse models, delayed aging is associated with reduced body mass. hMTH1 expression did not affect body weight gain from neonatal up to 12 months (Fig. 3A,B). At 28 months, however, hMTH1-tg mice were significantly heavier than wild-type (genotype effect: $P < 0.05$; age $\times$ genotype: $P = 0.069$; $P \leq 0.05$ after *post hoc* comparison) (Fig. 3B). To investigate whether the differences in body weight reflected different adipose tissue burdens, we measured the levels of circulating leptin. Serum leptin concentrations were comparable in the two genotypes at both young (9 months) and old ages (24–36 months) (Fig. 3C), suggesting that the weight increase is related to general body mass.

Increased hMTH1 expression enhances exploration of environmental and social cues

To investigate the effect of hMTH1 overexpression on behavior, we examined male hMTH1-Tg and wild-type mice in several behavioral tasks, from the early postnatal phase to old age. As aging can influence social and exploratory behavioral patterns in rodents (Torras-Garcia *et al.*, 2005; Berry *et al.*, 2007), we selected tasks involving affective and cognitive responses to environmental or social novelty. Moreover, we chose to assess learning and memory functions by means of tasks (i.e. object and social recognition) generally less affected by diminished motor performances possibly associated with aging in 2-year-old mice.

Achievement of early developmental milestones was similar in the two genotypes, and at 11 days, there were no apparent differences in motor behavior and recognition of nest-related olfactory cues (Table S1).

Exploratory behavior and anxiety levels in the open-field arena and elevated zero-maze, social motivation and memory, and

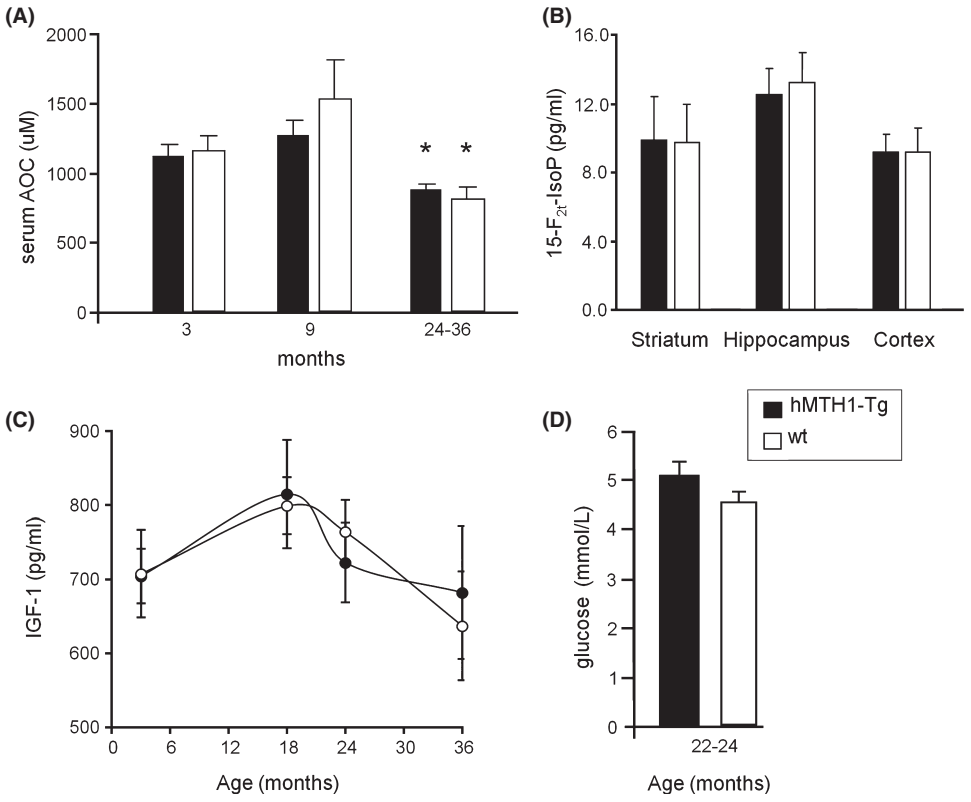

Fig. 2 Antioxidant capacity, F2-isoprostane, IGF-1 and glucose levels in hMTH1-Tg mice. AOC levels were measured by colorimetric assay in serum from 3-month-old hMTH1-Tg (n = 11) and wild-type (n = 11) mice, 9-month-old hMTH1-Tg (n = 10) and wild-type (n = 4) mice and 24- to 36-month-old hMTH1-Tg (n = 16) and wild-type (n = 6) mice. *P < 0.05 vs. 9 or 3 months. (B) 15-F_{2t}-IsoP levels were determined in tissue extracts from brain areas in 18-month-old hMTH1-Tg (n = 8) and wild-type (n = 5) mice; (C) IGF-I levels were estimated by an immunoassay in serum from hMTH1-Tg (n = 23) and wild-type (n = 16) animals of the indicated age. (D) Glucose levels were measured by an enzymatic assay in the blood of aged (22-24 months) hMTH1-Tg (n = 11) and wild-type (n = 9) animals. Data are indicated as mean ± SE. Wild-type (open bar) and hMTH1-Tg mice (black bar).

capacity to discriminate between a familiar and a novel object were assessed in two different groups of mice at 2 months and 2 years of age. In the open-field test spontaneous motor activity and exploratory behaviors were scored. Levels of crossing, grooming and immobility, and time spent in the open and peripheral area of the arena were comparable in hMTH1-Tg and wild-type mice at both ages. Rearing behavior (including open rearing and wall rearing)–an important component of the exploratory motor pattern – was significantly more pronounced in old hMTH1-Tg mice compared to old wild-type animals (Table 1). hMTH1-Tg mice exhibited less anxiety than wild-type controls in the elevated zero-maze (Fig. 4A). They spent significantly more time in the open sectors of the maze (genotype effect: $F(1,29) = 4.39$, $P < 0.05$), an effect largely attributable to the old-age group (hMTH1-Tg vs. wild-type $P < 0.05$). At 2 years, hMTH1-Tg mice displayed a higher frequency of head-dipping behavior, an index of active exploration, than wild-type mice of the same age (genotype effect: $F(1,29) = 33.35$, $P < 0.005$; age × genotype: $F(1,29) = 14.69$, $P < 0.005$).

In both learning and memory paradigms performed, hMTH1-Tg mice displayed enhanced curiosity toward either nonsocial or social cues. In the object recognition task, mice were exposed to two identical objects for 15 min (T1, familiarization) and then subsequently presented at 30 min (trial 2, T2) and 24 h (trial 3, T3) with two objects, one familiar and one novel (Fig. 4B). Overall, mice of both genotypes spent less time investigating the familiar object than the novel one at both T2 (short-term memory) and T3 (long-term memory) trials (time on novel object in T2: $F(1,21) = 102.43$, $P < 0.005$; in T3: $F(1,21) = 20.66$, $P < 0.005$). hMTH1-Tg mice were, however, more likely than wild-type mice to explore either object, during the familiarization (T1), and in T2 and T3 trials (main effect of genotype: $F(1,23)$ T1 = 6.19, T2 = 6.34, Ps < 0.05, and T3 = 3.65, $P = 0.06$).

In T2, the difference between investigation of the novel vs. the familiar object was significantly greater in hMTH1-Tg than in wild-type mice (object × genotype: $F(1,23) = 6.35$, $P < 0.05$) (Fig. 4B). In T3, the difference between exploration of the novel vs. the familiar object was highly significant in hMTH1 mice only (Fig. 4B).

In the social memory task (Fig. 4C), the time spent in social investigation after repeated presentations of the same partner in the home cage decreased in both genotypes (repeated trials: $F(3.87) = 7.98$, $P < 0.005$). On presentation of a novel partner (T5), 2-year wild-type mice failed to respond, whereas hMTH1-Tg mice showed significant renewal of investigation. The behavior of these older transgenic mice was similar to that of younger wild-type and hMTH1-Tg mice (genotype × age: $F(1,29) = 4.17$, $P < 0.05$; Ps < 0.05 after comparison hMTH1 vs. wild-type in the 2-year group, and wild-type 2-month vs. wild-type 2-year). We did not observe significant differences in motor activity between hMTH1-Tg and wild-type mice either in object recognition or in social memory task.

hMTH1 overexpression increases the proliferative capacity of adult neural stem/progenitor cells

As BER-defective mice ($Pol\beta^{-/-}$ and $Neil3^{-/-}$ mice) may show altered neurogenesis (Sugo *et al.*, 2000; Regnell *et al.*, 2012), we investigated whether hMTH1 overexpression provided some neurogenic advantage. To this end, we assessed the formation of neurospheres generated by adult neural stem/progenitor cells

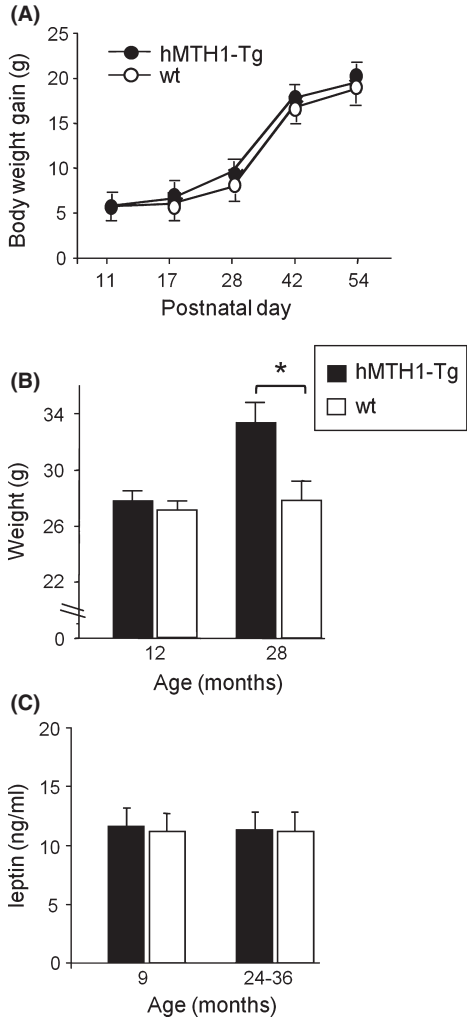

Fig. 3 Body growth of hMTH1-Tg. (A) Body weight (mean ± SE) of developing wild-type ($n = 9$) and hMTH1-Tg ($n = 6$) mice and (B) 12- and 28-month (mo) hMTH1-Tg ($n = 4$) and wild-type ($n = 8$) mice. Genotype effect: $F(1,20) = 4,77$, $P < 0.05$; age × genotype interaction: $F(1,40) = 3,47$ $P = 0.069$; $P \leq 0.05$ after *post hoc* comparison. (C) Leptin was measured by a specific enzyme immunoassay in sera from hMTH1-Tg ($n = 5$) and wild-type ($n = 5$) mice, at two different ages (9 and 24–36 months). Data were compared by two-way ANOVA. * $P < 0.05$ after *post hoc* comparison performed on genotype × age interaction. Wild-type (open bar) and hMTH1-Tg mice (black bar).

(NSPC) from the subventricular zone (SVZ) of 3-month-old mice. We did not observe any differences in number of neurospheres generated from wild-type or hMTH1 mice. The mean size of and total cell content of neurospheres from hMTH1-Tg animals were significantly higher in both secondary and tertiary cultures (Fig. 5), however. We conclude that the proliferative capacity of NSPC from hMTH1-tg mice is higher than that of wild-type NSPC.

hMTH1 overexpression confers growth advantage and prevents cellular senescence *in vitro*

Cellular senescence *in vitro* may recapitulate the loss of regenerative capacity of cells *in vivo* (Campisi & d'Adda di Fagagna, 2007). We investigated whether hMTH1 overexpression delayed cellular senescence *in vitro*. There was a noticeable difference in the proliferation of hMTH1-Tg and wild-type MEFs prepared from pooled littermate embryos. The difference was most evident as cells cultured at high density approached the presenescence crisis (passages 7–10, Fig. 6A). As low-density cultures are less permissive for growth (Todaro & Green, 1963), we investigated whether hMTH1 overexpression also conferred a growth advantage at low density. Low-density, hMTH1-expressing MEFs (hMTH1-1, hMTH1-7) showed no evidence of a senescence crisis. There was little expression of senescence-associated beta-galactosidase activity (Fig. 6B,D) or of the p16INK4a cell cycle inhibitor (Fig. 6E) (Rai *et al.*, 2009). In contrast, two independent cultures of wild-type MEFs (wt-1, wt-2) underwent replicative senescence after a few passages (Fig. 6B and E). The levels of intracellular ROS and 15-F2t-IsoP were higher in proliferating hMTH1-overexpressing MEFs than in quiescent wt-1 cultures (Fig. S2A–C). The escape from replicative senescence in hMTH1 overexpressing MEFs is therefore not the result of reduced ROS levels.

Table 1 Behavioral items recorded during a 15-min open-field test by wild-type and hMTH1-Tg mice

Genotype and age	Crossing frequency *	Duration of grooming	Rearing frequency†
Wild-type, 2 months	208.67 ± 15.84	41.50 ± 15.02	91.5 ± 14.11
Wild-type, 2 years	294.75 ± 33.90	35.47 ± 5.75	99.5 ± 12.95
hMTH1-Tg, 2 months	185.55 ± 16.30	38.94 ± 9.40	69.89 ± 8.4
hMTH1-Tg, 2 years	320.75 ± 20.08	35.9 ± 9.75	129.75 ± 8.36‡

Data are means ± SE.
*Significant main effect of age; ANOVA $P < 0.05$.
†Significant interaction between age and genotype; ANOVA $P < 0.05$.
‡Significant difference 2-year hMTH1-Tg vs. 2-month hMTH1-Tg and 2-year wild-type.
$P < 0.05$ after *post hoc* comparisons.

Discussion

The only known activity of MTH1 is cleansing of the oxidized nucleotide pools. We have previously shown that adult differentiated neurons in hMTH1-Tg mice are markedly protected from neurotoxicity (De Luca *et al.*, 2008; Ventura *et al.*, 2012). Here, we report that hMTH1 overexpression also significantly prolongs lifespan.

The increased longevity is associated with a lower steady-state level of DNA 8-oxoG in several organs. In aged mice, oxidative damage clearly increased in mtDNA of every organ we examined (skeletal muscles, heart and brain). hMTH1 overexpression always reduced 8-oxoG levels in mitochondria, and this protection is maintained from adulthood to old age (24 months). It is noticeable that mtDNA of some brain areas (hippocampus and striatum) is particularly defended by hMTH1 in the old age. Although hMTH1 overexpression is associated with decreased levels of 8-oxoG also in nuclear DNA, there is a limited accumulation of the oxidized purine as a function of age in this mouse strain (Russo *et al.*, 2004), the

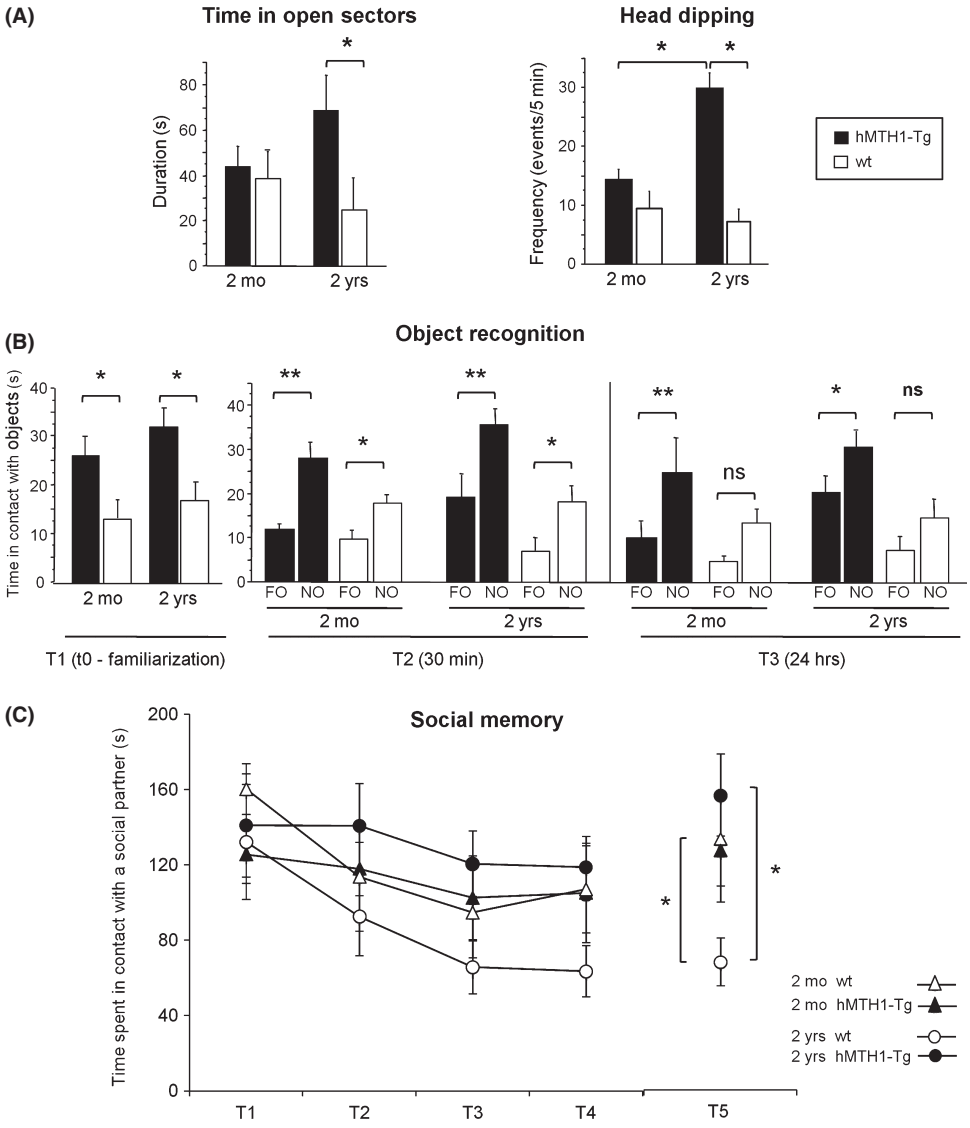

Fig. 4 Behavioral phenotype of hMTH1-Tg. (A) Behavioral items recorded during a 5-min test in the elevated zero-maze (2-month hMTH1-Tg $n = 9$ and wild-type $n = 6$; 2-year hMTH1-Tg $n = 8$ and wild-type $n = 10$): time spent in the open sectors of the maze and head-dipping behavior * $P < 0.05$ indicates significant difference after *post hoc* comparisons. (B) Memory performance in the object recognition test (2-month hMTH1-Tg $n = 9$ and wild-type = 6; 2-year hMTH1-Tg $n = 8$ and wild-type $n = 4$). Each trial lasted 15 min. Both hMTH1-Tg and wild-type mice discriminated the novel (NO) from the familiar (FO) object either 30 min or 24 h after the familiarization session (T1). A significant effect of the genotype was found in T1 and T2 trials (Ps < 0.05) and very close to significance ($P = 0.06$) in T3, hMTH1-Tg mice exploring more than wild-type both objects. In T2, there was a significant interaction genotype × object ($P < 0.05$) as the preference for the novel object was more marked in hMTH1-Tg than in wild-type mice. This effect was confirmed in T3, where recognition memory (preference for the novel object after 24 h) was significant only in hMTH1-Tg mice either young adult or old, just missed significance in 2-month wild-type, and it was far from significance in 2-year wild-type mice (*$P < 0.05$; ** $P < 0.01$; ns, not significant) (C) Mean duration of social investigation (2-month hMTH1-Tg $n = 8$ and wild-type = 8; 2-year hMTH1-Tg $n = 10$ and wild-type = 7). Each trial lasted 5 min. The habituation profile from Trial 1 to Trial 4, where mice were repeatedly presented with the same individual (an unfamiliar male of the same strain and age), was comparable in the four experimental groups. Significant renewal of investigation toward the new intruder in Trial 5 was found in all groups except in 2-yr wild-type mice (significant interaction genotype × age, * $P < 0.05$ after *post hoc* comparison old hMTH1-Tg vs. old wild-type mice and 2-month wild-type vs. 2-year wild-type mice). Data are mean ± SE. Wild-type (open bar) and hMTH1-Tg mice (black bar).

only exception being the liver. In addition, hMTH1 expression protects brain against RNA oxidation. This hMTH1-mediated elimination of oxidized RNA precursors might reduce translational errors associated with mRNA oxidation. Although hMTH1 protects multiple targets against accumulation of oxidized bases, our data suggest that mitochondria preservation might be particularly important in the aging process.

Our findings indicate that hMTH1 overexpression preserves neuronal functions in brain areas involved in the control of the complex behaviors. Increased longevity was associated with a typical behavioral profile characterized by enhanced investigative behavior in different contexts. This was seen as increased exploration of the environment, reduced anxiety and neophobia and higher sociability. In rodents, these behavioral traits reliably predict increased lifespan

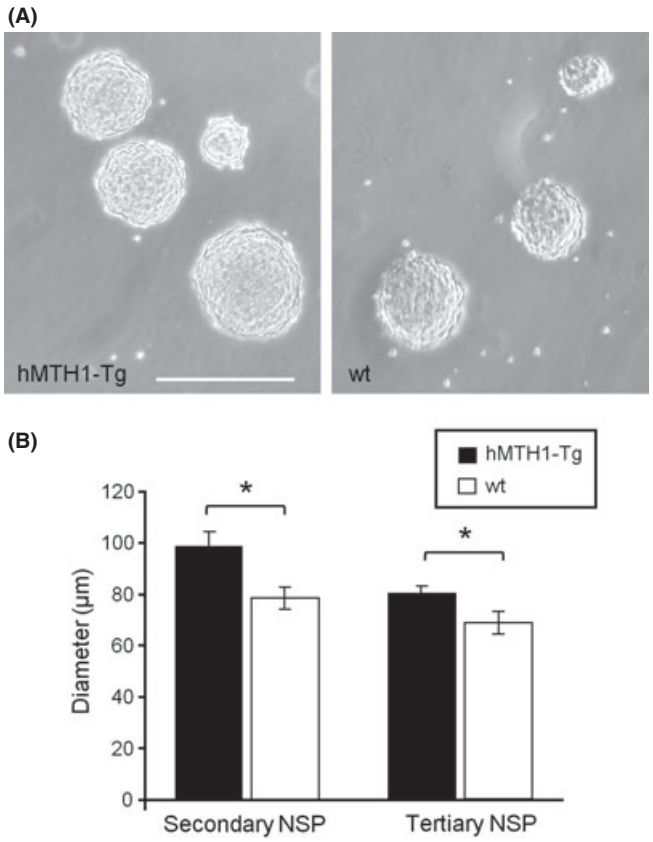

Fig. 5 Generation of neurospheres from SVZ. (A) Spheres were generated from SVZ from 3-month-old hMTH1-Tg ($n = 8$) and wild-type ($n = 8$) mice following dissociation and plating at a density of 10 cells μL^{-1}. (B) Diameters of neurospheres in secondary and tertiary cultures. Data are mean ± SE from three experiments. Scale bar = 200 μm. Wild-type (open bar) and hMTH1-Tg mice (black bar).

and this has been linked to an improved ability to cope with environmental stressors and an enhanced immune response (Perez-Alvarez *et al.*, 2005; Yee *et al.*, 2008; Cavigelli *et al.*, 2010; Aguilera, 2011). Decreased anxiety, in association however with learning impairment and enhanced locomotor activity, has been reported in a mouse model of defective mitochondrial DNA repair expressing a mutant version of the mitochondrial *UNG* gene (Lauritzen *et al.*, 2010). The extended behavioral analysis performed in our study clearly indicates that the baseline motor activity of hMTH1 mice is unaltered. In addition, both hMTH1 and wild-type mice spent more time in the peripheral than in the central area of the open field, showing comparable levels of ambulation close to the walls. Noticeably, hMTH1 mice displayed proper risk assessment responses, as indicated by frequency of stretched-attend postures in the elevated zero-maze. In addition, the increase in time spent in the open areas was accompanied by a characteristic increase in head-dipping behavior, which, in agreement with the enhanced object investigation, indicates higher curiosity and exploration of the environment rather than a nonspecific decrease of anxiety producing disinhibited and perseverative behavior. The consistent findings obtained in the different behavioral tasks performed in our study suggest that the characteristic behavioral phenotype of hMTH1 mice possibly enables them with more efficient processing of environmental and social cues, forming the basis for cognitive enhancement.

The impact of hMTH1 on behavior is particularly evident in aging mice. Older hMTH1-Tg mice show more efficient recognition of spatial environmental features and a more intact social memory. Their levels of social investigation toward a novel conspecific were comparable to those of younger wild-type mice. Although our behavioral findings do not identify how hMTH1 expression prevents age-associated changes in the brain function, we suggest that the distinctive behavior of these mice reflects a more efficient functioning of neuroendocrine brain circuits that integrate sensory information and responses to novelty.

hMTH1-Tg mice differ from other transgenic models of extended lifespan in several ways. hMTH1 protection is independent of the levels of ROS and antioxidant defenses (Migliaccio *et al.*, 1999; Schriner *et al.*, 2005). In addition, hMTH1-Tg mice have no apparent gross alterations in glucose metabolism or IGF-1-insulin axis and gain weight normally as they age. This distinguishes hMTH1-Tg mice from other long-lived mice with slow metabolic rates or small body size (Migliaccio *et al.*, 1999; Schriner *et al.*, 2005). The reasons for the increased weight of older transgenic mice remain unclear. They do not involve gross alterations in the adipose tissue burden, their plasma levels of leptin are normal and there is no evidence of macroscopic fat deposition (data not shown).

At the cellular level, loss of BER of oxidative lesions is associated with in impaired proliferation of neurospheres from *Polβ*$^{-/-}$ and *Neil3*$^{-/-}$ mice (Sugo *et al.*, 2000; Regnell *et al.*, 2012). In our experiments, NSPC cultures derived from hMTH1-Tg mice proliferated better than those from wild-type animals, suggesting that enhanced protection against oxidative damage provides a proliferative advantage. We found that hMTH1 overexpression also abolished senescence of cultured MEFs. These findings are consistent with the rapid induction of senescence in hMTH1-knockdown early-passage human fibroblasts (Rai *et al.*, 2009). These observations are also consistent with an intriguing link between organismal aging and cellular senescence (Campisi & d'Adda di Fagagna, 2007).

The life shortening associated with defective DNA repair in human progeroid syndromes and their mouse models suggests that the accumulation of DNA damage plays a major role in the aging process (Niedernhofer *et al.*, 2006; van der Pluijm *et al.*, 2007; Schumacher *et al.*, 2008; Garinis *et al.*, 2009). Although premature aging phenotype has not been reported in *Mth1*$^{-/-}$ mice, their cancer proneness may represent an alternative age-related outcome. We propose that by decreasing the burden of oxidative damage to nucleic acids, MTH1 overexpression represents a previously unrecognized mechanism by which healthy longevity can be attained.

Experimental procedures

Animal care and genotyping

All animal procedures were carried out according to EU Directive 86/609/EEC and to Italian legislation on animal experimentation.

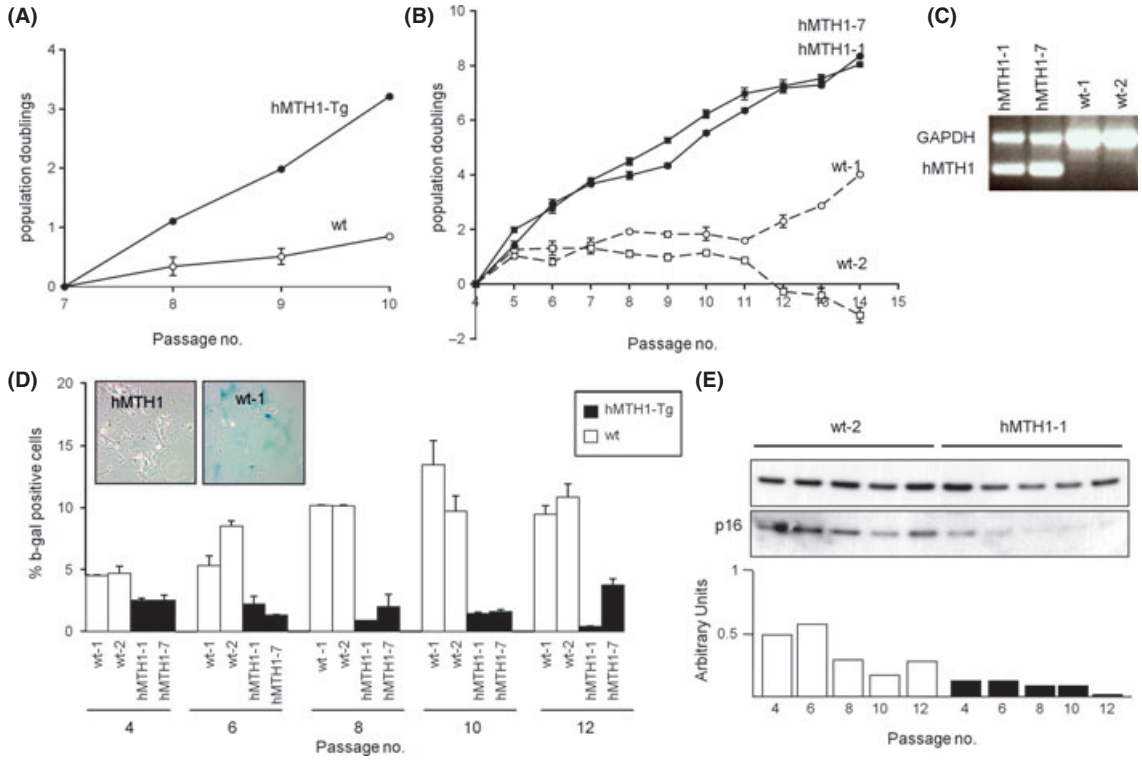

Fig. 6 Proliferation rates and senescence of hMTH1-overexpressing MEFs. (A) Population doublings of MEFs derived from pooled hMTH1-Tg and wild-type embryos (passage 7–10). (B) Population doublings of MEF cultures derived from independent hMTH1-Tg and wild-type embryos (passage 4–14). (C) Expression of hMTH1 in clones hMTH1-1, hMTH1-7 and wt-1 and wt-2 by RT-PCR at passage 10. (D) wild-type and transgenic MEFs stained for SA b-gal activity at the indicated passages. (E) Levels of p16 detected by immunoblotting at different passages in wild-type and transgenic MEFs. Wild-type (open bar) and hMTH1-Tg mice (black bar).

To ensure that mice were on a pure C57BL/6 genetic background, a previously described hMTH1-Tg mice generated at our Institute (De Luca *et al.*, 2008) was crossed with wild-type C57BL/6 partners for more than 12 generations. To maintain the colony hemizygous, hMTH1-expressing male mice were routinely cross-bred with hemizygous and wild-type female mice. Hemizygous × hemizygous crosses were used to generate homozygous and wild-type mice used in the experiments reported in this manuscript. hMTH1-Tg animals were genotyped as reported previously. All animals were housed under standardized temperature, humidity and on a 12-h light- to 12-h dark cycle with free access to water and food. All tests were performed from 9:00 a.m. to 2:00 p.m. Differences due to the sex factor were always calculated and reported when present.

Quantification and analysis of the 8-oxo-dGTPase activity

8-oxodGTPase activity was determined essentially as previously reported (Bialkowski *et al.*, 2009). The method to measure OGG1 activity is described in Supplementary Materials.

8-OxodG and 8-oxoG determinations

8-oxodG and 8-oxoG were measured by high performance liquid chromatography/electrochemical detection as described previously (De Luca *et al.*, 2008). Details of the methods are provided in Supplementary materials. The PromoKine Mitochondrial DNA isolation kit (PromoCell GmbH, Heidelberg, Germany) was used to isolate mitochondrial DNA from mouse tissues.

Measurement of antioxidant capacity

The quantification of AOC in serum samples was determined using an assay, based on the reduction in Cu++ to Cu+ by the activity of all antioxidant species present in the sample as previously described (Minghetti *et al.*, 2006). The reduced copper (Cu+) forms a stable complex with bathocuproine that shows maximal absorbance at 490 nm. A standard curve of uric acid as typical reducing agent is used to calculate the antioxidant activity present in the sample. Data are expressed as μmoles L^{-1} of reducing power. The sensitivity was 22 μM of reductive capacity; intra- and inter-assay variability showed a coefficient of variance CV that was lower than 4%. Serial dilutions of each sample were analyzed in duplicate.

Measurement of IGF-1, glucose and leptin levels

IGF-1 levels were measured in mouse sera using the Quantikine immunoassay (R&D Systems, Minneapolis, MN, USA) according to manufacturer' instructions. Following reaction sample, values are read off the standard curve at 450 nm. Glucose levels were measured in total blood of using the Glucocard G sensor assay (ARKRAY) according to manufacturer' instructions. Leptin levels were measured in mouse sera using a specific Enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) according

to manufacturer' instructions. Following reaction sample, values are read off the standard curve (0.03–300 ng mL^{-1}) at 450 nm. All measurements were performed in overnight-fasted animals.

Spontaneous activity (open-field test)

At 2 months or 2 years, mice underwent a single 15-min session of open-field testing. The behavior of each animal was observed in a black open-field arena (35 × 35 × 36 cm) and videotaped for 15 min under red light. The following behavioral categories were scored: frequency of crossing (crossing the square limits with both forepaws), frequency and duration of rearing, wall rearing, grooming and immobility (Ricceri *et al.*, 2006).

Exploratory behavior and anxiety levels (elevated Zero-maze)

At 2 months or 2 years, mice were assessed in the zero-maze apparatus, a plastic annular runway (width 5.5 cm, outer diameter 46 cm, 40 cm above ground level) with two opposing 90° sectors protected by 10-cm-high walls (closed sectors) and the other two unprotected (open sectors). Each animal was placed in one of the closed sectors and observed for 5 min. The following parameters were scored using an event recording software: time and number of entries in open sectors, frequency of stretched-attend postures from closed to open sectors (elongated body posture stretched forward with at least the snout passing over the open/closed divide), frequency and duration of wall rearing and grooming.

Object recognition task

At 2 months or 2 years, mice were assessed in an object recognition task in a black plexiglas box (40 cm wide × 40 cm deep × 40 cm high). Objects to be discriminated were a small black glass cylinder (familiar object), a white cylinder and a half white-half black cylinder. The task assessed both short-term (STM) and long-term memory (LTM). Mice were habituated to the apparatus for 3 days. Familiarization phase (day 4th) consisted of a one-trial session of 15 min with two identical objects (two black cylinders) positioned in the back corners of the box, and mice were allowed to freely explore. Latency to approach the objects and time spent in contact with them were recorded. Retention test took place either 30 min or 24 h following familiarization, respectively, for the STM (day 4) and the LTM (day 5) and consisted of one-trial session of 15 min during which one black cylinder was replaced by a new object (unfamiliar), either a white or a half-black/half-white cylinder. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preference for particular locations or objects. The time spent exploring each object and the total time exploring both objects were recorded during sessions.

Social recognition test

At 2 months or 2 years, mice were tested individually in a social recognition task. A stimulus mouse (unfamiliar conspecific of the same sex and age enclosed in a small, round wire cage) was introduced inside the home cage for four consecutive trials (T1-T4) of 5 min each, separated by intertrial intervals of 15 min. The stimulus mouse was the same from T1-T4, whereas in T5 a novel stimulus mouse was used. The response variables recorded were the latency to the first snout contact with the stimulus mouse, the total duration of social investigation of the stimulus mouse and general motor activity not in relation with social investigation. The method for homing test and further references for behavioral studies are in Supporting Information.

15-F_{2t}-IsoP extraction and assay

A detailed procedure for 15-F2t-IsoP extraction has been described elsewhere (Minghetti *et al.*, 2000). Briefly, 200 μL of ice-cold Tris-HCl buffer pH 7.5 containing 10 μg mL^{-1} of the COX inhibitor indomethacin (stock solution 100× in ethanol) to avoid *ex vivo* PGE2 synthesis and 10 μM of the radical scavenger butylated hydroxytoluene (stock solution 100× in ethanol) to avoid auto-oxidation are added to each frozen sample, which is quickly thawed, homogenized with a Teflon pestle (Sigma, Milan, Italy)–20 cycles in an ice bath – vigorously vortexed and centrifuged at 20 817 *g* for 45 min at 4 °C. Supernatants are collected and stored at −80 °C until analysis. Measurement of 15-F2t-IsoP is performed in tissue extracts by high sensitivity colorimetric enzyme immunoassay (EIA kit; Cayman Chemical, Ann Arbor, MI, USA). According to the manufacturers, the cross-reactivity of anti-15-F2t-IsoP antibody for other prostaglandins was <1% (0.02% for PGE2). All measurements were run at least in duplicate for each sample. Results were expressed as pg per mg proteins.

Neurosphere generation

Neural stem/progenitor cells were obtained from the SVZ of 3 months old wild-type and hMTH1 male mice according to a previously described protocol (see ref. in Supporting Information). To generate spheres, living cells, counted by trypan blue exclusion, were plated in uncoated plastic flasks at a density of 50 cells μL^{-1}. Cells were grown at 37 °C in a 5% CO2 atmosphere in DMEM/F12 containing 1% penicillin/streptomycin, 0.1 M L-glutamine (Gibco), 23.8 mg per 100 mL Hepes, 7.5% NaHCO3, 0.6% glucose, 20 ng mL^{-1} of human recombinant epidermal growth factor (EGF; R&D), 10 ng mL^{-1} of basic fibroblast growth factor (bFGF; R&D) and B27 supplement. Cells were daily fed with fresh medium and passaged after 9 days using accutase (Sigma). For neurosphere assays, single cell dissociated spheres were plated at 10 cells μL^{-1} density in uncoated cell culture flasks for 9 days. To assess neurosphere number and size, the spheres were transferred to 48-well plates with grids. Neurosphere size was determined by measuring the diameters of individual neurospheres under light microscopy. The total number of viable cells after neurosphere dissociation was determined by the trypan blue exclusion method.

Preparation of primary mouse fibroblasts

MEFs were prepared from embryos derived from wild-type or hMTH1-Tg pregnant females sacrificed after 13 days of gestation. Tissue suspensions were incubated in a solution of 0.25% (w/v)

trypsin/1 mM EDTA at 37 °C for 10–15 min with vigorous stirring. Cells were expanded in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin (100 U mL^{-1}) and streptomycin (100 mg mL^{-1}) in a 5% CO2 atmosphere (90% nominal humidity). Transgene expression was analyzed by RT-PCR using specific primers and by Western blotting as previously described.

Measurements of proliferation rates

To determine the average rate of population doubling (PD), MEFs derived from pooled embryos were plated in duplicate at a density of 2×10^5 per 60-mm plate (high density) and the number of cells was counted every 3–4 days, with 2×10^5 cells being replated for the next count. For single embryo analyses, MEFs derived from single embryos were expanded for characterization and starting from passage four were plated in duplicate at a density of 5×10^4 cells per 60-mm plate (low density) and the number of cells was counted every 3–4 days, with 5×10^4 cells being replated for the next count. The numbers were converted into PDs, using the following formula: log (no. of cells counted)-log (no. of cells plated)/ log (2), as described in Rai *et al.*, 2009.

Senescence-associated β-galactosidase (SA-β gal) and ROS measurements

Detection of SA b-gal activity was performed as described in Rai *et al.*, 2009. Intracellular ROS accumulation was monitored using the chloromethyl derivative of dihydrofluorescein diacetate (CM-H2DCFDA, Invitrogen). Cell cultures ($2–4 \times 10^5$ cells) were washed with PBS, incubated for 30 min in the dark at room temperature with 5 μM CM-H2DCFDAs and suspended in PBS+EDTA. Fluorescence was measured using excitation and emission wavelengths of 485 and 535 nm, respectively, on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The values were expressed as mean fluorescence of the cell population.

Statistical analysis

A mixed-model analysis of variance (ANOVAs) for repeated measures was used to analyze body weight and behavioral data. *Post hoc* comparisons were performed using Tukey's test with Bonferroni's correction, which can be used in the absence of significant ANOVA results.

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Author contribution

G.D.L., I.V., V.S., MT.R., M.A.A., E.C., A.M., G.F., F.M., P.D. performed research; M.B., G.C., L.M., M.C., MM and P.P. designed and analyzed research; M.B. and G.C. wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 8-oxodG and 8-oxoG levels and hMTH1 overexpression

Fig. S2 ROS and F2-isoprostane levels in MEFs.

Table S1 Behavioral items recorded during a 3-min homing test on postnatal day 11.

Data S1 Supplementary experimental procedures