The age of heterozygosity

Audrey Carrière · Xingxing Liu · Siegfried Hekimi

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Abstract Two mutant mouse models of longevity in which the loss of only one copy of the gene leads to a significantly increased lifespan have recently been described: *Igf1r*^{+/-} and *mclk1*^{+/-}. *Igf1r* encodes a transmembrane receptor kinase for the insulin-like growth factor-1, and *mclk1* encodes a hydroxylase that is necessary for the biosynthesis of ubiquinone. Interestingly, the motivation for testing the longevity of both of these mutants came from observations in the nematode *Caenorhabditis elegans*. IGF-1R protein is homologous to DAF-2 and mCLK1 is the mouse orthologue of the *C. elegans* enzyme CLK-1. In worms, the homozygous inactivation of both of these longevity genes is viable and no dominant mutations are known. In addition to aging slowly, old *mclk1*^{+/-} mice were found to undergo loss-of-heterozygosity at the *mclk1* locus, which results in clones of *mclk1*^{-/-} cells in the liver, presumably because *mclk1*^{-/-} cells can outcompete *mclk1*^{+/-} cells under certain conditions. We will discuss how these observations suggest novel directions of research, but also call for some caution in the interpretation of past and future results.

Key words aging · evolutionary conservation · loss-of-heterozygosity · *mclk1* · mouse models of longevity · ubiquinone

Abbreviations

IGF-1 insulin-like growth factor-1
LOH loss-of-heterozygosity
ES embryonic stem

Introduction

Although aging is a multifactorial process, it should be examined like any other phenotype: as the product of the interaction of the genotype with the environment. When this view was pursued in a variety of invertebrate models and in mammals, the surprising discovery was made that mutations in single genes are able to induce a reliable and marked increase in lifespan. In this review, we will focus principally on two mutant mouse models of increased lifespan in which inactivation of a single copy of the gene is sufficient for a significant effect (*Igf1r* and *mclk1*). Other models will be mentioned when appropriate to the discussion.

Igf1r^{+/-} mice

The first two mutants that were found to display an increased lifespan in *Caenorhabditis elegans* (*age-1* and *daf-2*) participate in the same signal transduction pathway in worms (Dorman et al. 1995), a pathway that regulates dauer formation, an alternative developmental stage in many nematodes. The *age-1* locus encodes the worm PI3 kinase (Morris et al. 1996), and *daf-2* encodes

A. Carrière · X. Liu · S. Hekimi (✉)
Department of Biology, McGill University,
1205 avenue Docteur Penfield,
H3A 1B1 Montreal, Quebec, Canada
e-mail: siegfried.hekimi@mcgill.ca

a transmembrane receptor that strongly resembles the insulin/insulin-like growth factor receptor family in vertebrates, including the insulin-like growth factor-1 receptor (IGF-1R) (Kimura et al. 1997). *daf-2* mutants have a dramatically increased lifespan (>2 fold) (Kenyon et al. 1993), and are resistant to a variety of adverse conditions, including oxidative stress (Honda and Honda 1999; Barsyte et al. 2001; Murakami and Johnson 1996). *daf-2* mutants are temperature-sensitive and enter the dauer stage when placed at 25°C, which is irreversible in these mutants unless the temperature is lowered. Some *daf-2* mutations also appear to interfere with development, which results in non-negligible lethality during early larval stages (Gems et al. 1998). Moreover, it is believed that *daf-2* mutations described to date are not complete loss-of-function mutations. Thus, what made it possible to observe the effect of a severe inactivation of this gene in worms is linked to its particular function in this organism (the alternative, temperature-sensitive dauer pathway), and to the fact that, in an invertebrate genetic model system like *C. elegans*, a spectrum of mutations (including conditional mutations) can readily be obtained and studied. However, the fact that the model used has specific advantages does not change the conclusion that interfering with the function of DAF-2 has a major effect on longevity.

Insulin and insulin-like growth factors are very important mediators of cell growth, physiology and differentiation, and also contribute crucially to whole-body homeostasis. IGF-1 exerts all its effects via IGF-1R (Delafontaine 1995). Given the importance of this signaling system in vertebrates, perhaps it is not surprising that *Igf1r*^{-/-} mice are not viable and die at birth—likely because of respiratory failure (Liu et al. 1993)—while heterozygotes have only some mild physiological impairments. Male *Igf1r*^{+/-} mice have normal serum insulin but are glucose intolerant and show slightly elevated plasma glucose levels whereas females do not show any changes in these parameters (Holzenberger et al. 2003). Both male and female *Igf1r*^{+/-} mice grow normally until they are weaned, at which time a slight decrease in body weight (6–8%) is observed and maintained throughout life. The

metabolic rate, food intake and fertility of *Igf1r*^{+/-} mice appear similar to those of wild-type mice (Holzenberger et al. 2003). In addition, female *Igf1r*^{+/-} mice seem to be protected against oxidative stress as indicated by one particular measure: survival after a challenge with intraperitoneally injected paraquat, a reactive oxygen species generator. *Igf1r*^{+/-} female mice had a 33% increase in mean lifespan compared to female wild-type mice, whereas the 16% increase in mean lifespan that was reported in male *Igf1r*^{+/-} did not reach statistical significance (Holzenberger et al. 2003). This does not mean that male lifespan is not increased in these mutants, as is sometimes suggested, but only that the jury is still out. The glucose intolerance of the males might be responsible for their reduced lifespan compared to the glucose tolerant females.

One obvious interpretation of the increased lifespan of the *Igf1r*^{+/-} mutants is that a lowering of the activities associated with the encoded protein is beneficial, while a total ablation of the gene is nonviable, obviously hiding any beneficial effect. Another way to get around the lethality and be able to study the effect of gene loss is to knock out the gene in only one tissue. This was accomplished for the insulin receptor, another DAF-2 homologue. While mice null for the insulin receptor gene die within a few days after birth because of diabetic ketoacidosis (Accili et al. 1996; Joshi et al. 1996), FIRKO mice (fat-specific insulin receptor knockout), lacking the insulin receptor only in the adipose tissue, are fully viable (Bluher et al. 2003). Creation of this model led to the exciting discovery that deletion of the insulin receptor gene only in adipose tissue was correlated with a substantial increase in lifespan in both male and female FIRKO mice (Bluher et al. 2003). Explanations for this extension of longevity are still debated, but one hypothesis is that it is due mainly to the reductions in body weight and fat mass in FIRKO mice (Bluher et al. 2003).

Environmental conditions play a determining role in lifespan and can significantly affect the consequences of genetic mutations on phenotypes. In dwarf mice (e.g., Ames and Snell dwarf mice), congenital deficiencies in pituitary function that ultimately also impinge on insulin

signaling are associated with a remarkable increase in lifespan. This increase in longevity is associated with several phenotypic characteristics indicating a reduced metabolism, as well as reductions in body core temperature, metabolic rate, growth, maturation and fertility (Brown-Borg et al. 1996). Strikingly, the Snell and Ames dwarf mice require special housing or husbandry conditions: first, they do not thrive if weaned at the age of 21 days (the usual age of weaning normal mice) and must remain with their mother for a longer period, even for a second lactation. Second, likely because of their low body core temperature, after weaning they need to be placed with normal female animals or at least with other dwarfs (Bartke et al. 2001).

In conclusion, although the insulin signaling pathway (and pituitary hormone signaling) clearly affects lifespan, particular conditions of partial inactivation (partial loss-of-function mutations, heterozygosity, tissue-specific inactivation) or environmental mitigation (control of temperature, nutrition, housing) have to be present in mice, as well as in worms, to be able to observe effects on aging without the ultimate confounding effect: early death.

mclk1 mice

The *clk-1* gene encodes a hydroxylase necessary for the biosynthesis of ubiquinone (Stenmark et al. 2001). In *C. elegans*, the *clk-1* gene affects many physiological rates: *clk-1* mutant worms display an average slowing of embryonic and postembryonic development, adult rhythmic behaviors, reproduction and aging (Wong et al. 1995; Branicky et al. 2000). *clk-1* mutants do not synthesize ubiquinone but accumulate demethoxyubiquinone, a ubiquinone synthesis intermediate that is able to partially sustain mitochondrial respiration in worms as well as in mammals (Levavasseur et al. 2001; Miyadera et al. 2001). However, despite the presence of functional mitochondria, *clk-1* mutant worms fail to complete development when feeding on bacteria that do not produce ubiquinone (Jonassen et al. 2001; Hihi et al. 2003).

It appears therefore that the *clk-1* gene is essential for viability but that, under certain condi-

tions that allow for survival (here, supplementation with ubiquinone), its absence can actually lead to an increase in lifespan.

mclk1^{-/-} mice are not viable and die at midgestation. However, *mclk1*^{+/-} mice develop normally. Interestingly, similarly to *Igf1r* heterozygous mice, *mclk1*^{+/-} mice displayed a substantial increase in lifespan in three different genetic backgrounds (Liu et al. 2005). Figure 1 shows an update of the study in the C57BL/6J background, which is now complete and shows a robust increase in both mean and maximal lifespan. This is an interesting observation because the previously published data were less robust in demonstrating an increased lifespan for males than for females (Liu et al. 2005). So far, no obvious differences in phenotypes such as growth curve, body weight and fertility have been detected in *mclk1*^{+/-} mice compared to their wild-type littermates. One obvious possibility to explain the increased lifespan of *mclk1*^{+/-} mice is that it is the consequence of the cumulative effect of a moderate physiological change occurring throughout the life of the animals. To explore this possibility, we are currently scrutinizing young *mclk1*^{+/-} animals, in the hope of discovering mutant phenotypes, with particular attention to mitochondrial and oxidative stress parameters.

As no reduction in ubiquinone levels was detected between newborn *mclk1*^{+/-} animals and their wild-type littermates (Levavasseur

Figure 1 Extended lifespan in *mclk1*^{+/-} mice. Kaplan-Meier survival curves are shown. The median lifespan of *mclk1*^{+/-} mice (green line, both males and females) is 19% greater than that of their wild-type (WT) littermates (red line). An increase of lifespan was also demonstrated in two other genetic backgrounds: 129Sv/J and 129Sv/J × Balb/c background (Liu et al. 2005). WT *n* = 5, mutants *n* = 8. Mantel-Haenszel one-tailed logrank test: *P* = 0.0035

et al. 2001), the magnitude of the effect of *mclkl* heterozygosity on lifespan was surprising. However, analysis of livers from old *mclkl*^{+/-} mice showed the presence of large groups of cells that do not express *mclkl* at either the mRNA or protein levels (Liu et al. 2005). We demonstrated that the loss of expression of *mclkl* appears to be linked to the specific loss of the wild-type allele of *mclkl*: the phenomenon of loss-of-heterozygosity (LOH). This is associated with a decrease in ubiquinone content in the livers of old *mclkl*^{+/-} mice. LOH is a phenomenon that has been studied almost exclusively in the context of tumor suppressor genes (Devilee et al. 2001). Such genes are usually recessive or partially haploinsufficient (Sherr 2004). However, when both copies of the genes are lost, the resultant mutant cells have a strongly increased predisposition to develop into a tumor (Devilee et al. 2001; Suzuki et al. 1998; Nakau et al. 2002). Therefore, such tumors develop much more readily in a heterozygous animal, in which a single event—the loss of the wild-type allele—is sufficient to create a cell in which the gene is inactivated on both homologous chromosomes. We have reason to believe that the properties of *mclkl*^{-/-} cells might indeed allow them to multiply more readily than phenotypically wild-type cells in an aged organism. Indeed, *mclkl*^{-/-} embryonic stem (ES) cells have been shown to have a reduction in reactive oxygen species levels and oxidative damage compared to wild-type ES cells (Liu et al. 2005). Furthermore, *mclkl*^{-/-} ES cells are protected against apoptosis mediated by the oxidative stress-inducing agent menadione. Livers of *mclkl*^{+/-} mice also show low levels of oxidative stress damage. We proposed that *mclkl*^{-/-} hepatic cells, similarly to *mclkl*^{-/-} ES cells, could be resistant against oxidative stress occurring in old livers, providing them with a growth advantage and thus rendering them able to out-compete *mclkl*^{+/-} hepatocytes.

These findings suggest a link between resistance to oxidative stress and apoptosis, and the longer lifespan of *mclkl*^{+/-} mice. Such a link also emerges from studies of the knockout of the mitochondrial protein p66^{SHC}. Indeed, p66^{Shc}^{-/-} mice, which are developmentally normal, have a 30% increase in median lifespan compared to

their wild-type counterparts, although the observed differences did not reach significance. Like *mclkl*^{-/-} ES cells, p66^{Shc}^{-/-} mouse embryonic fibroblasts are resistant to apoptosis induced by several agents, including H₂O₂ and ultraviolet light (Migliaccio et al. 1999). Resembling the findings in *mclkl*^{+/-} mice, this increase in longevity seems to be free of severe cost, as no significant differences were found in either body weight or food consumption, and no major side effects were observed.

Future investigations

During the past 15 years, the scientific community has tested and verified a surprising finding: the loss of function of a single gene could result in major changes in such a complex and multifactorial process as aging and longevity. The *Igflr*^{+/-} and *mclkl*^{+/-} mice now teach us that deletion of a single copy of a gene is also able to induce an important increase in longevity. These findings suggest that testing the effects of heterozygosity could be a general approach to studying the phenotypes associated with a partial reduction of function of a variety of genes whose full deletion would be too severe to permit positive effects on longevity. Indeed, it seems reasonable to believe that most genes will be slightly haploinsufficient, as we do not expect organisms to have evolved mechanisms of gene expression that compensate for the loss of a copy of the gene. Possibly, many heterozygous genotypes would live long, but they have not been tested because of the difficulties and the costs associated with aging studies.

There are some interesting candidate genes whose heterozygous disruption would be informative to test for the possible impact on aging and longevity (for example, genes involved in the inflammation process). Their complete inactivation is likely to be life-shortening, yet the finding of the study of “inflamm-aging,” i.e., the chronic inflammation status that characterizes aging (De Martinis et al. 2005; Franceschi et al. 2005), suggests that a mild reduction in pro-inflammatory gene activities could prolong lifespan, at least under experimental conditions relatively

free from infection. In fact, a wide range of age-related diseases, such as diabetes, cardiovascular diseases and cancer, share an inflammatory pathogenesis. Inflammation and the continuous production of reactive oxygen species exert a lifelong evolutionary pressure on the immune system, which undergoes a gradual remodeling in the attempt to re-establish a new balance that assures survival (De Martinis et al. 2005; Franceschi et al. 2005). It is reasonable to think that slight modifications in the activity of genes involved in immunity and regulation of inflammatory processes might have an impact on the emergence of age-associated disorders throughout life which, in the long term, could have appreciable consequences for lifespan.

Caveat 1

One caveat in the attempt to interpret the lifespan studies that looked at heterozygotes and found an increase in lifespan is that, as with the study of $p66^{\text{Shc}}$, these studies have been carried out only once, and with a limited sample size. Thus, there is a risk that such studies represent fortuitous findings that could not be easily replicated. Indeed, although the statistical analyses of these data indicate that the findings are robust, replication is an important aspect of demonstrating validity. In the $mcl1^{+/-}$ study, an increased lifespan was found in three different genetic backgrounds, which are reputed to predispose to different age-related pathologies (Frith et al. 1983; Smith et al. 1973). This may be considered a partial replication. Studies that replicate our initial experiments using larger samples are currently underway.

Caveat 2

The true explanation for the increase in longevity in heterozygotes might not be as specific as is suggested by the rationale behind the studies that have successfully been carried out so far, where it was hoped that the gene products being studied directly impinge on fundamental, conserved and specific mechanisms of aging. In fact, different

explanations for the increase in longevity of the heterozygous mice in these studies can be proposed (Figure 2). One such explanation is based on the notion that lifespan is limited by the molecular damage that accumulates as a normal consequence of metabolism (energy production and consumption). In the heterozygotes of many gene knockouts, one can expect a small decline in the quality of cellular and organismal homeostasis resulting from a less-than-perfect level of the affected gene product. This might result in a minor slow-down of overall physiological or metabolic rates, which might be hard to detect through a limited analysis of the phenotype. Yet, such a slow-down might have non-negligible cumulative effects on lifespan, especially under laboratory conditions, where the survival of an animal is only rarely limited by energy expenditure. The point here is that the gene under study might not have to be specifically involved in any particular aspect of energy metabolism, damage production or repair to increase lifespan. It might be sufficient that the reduction in the level of its product prevents the cell or the animal from functioning optimally or “smoothly.” Such an explanation for the increased lifespan of heterozygotes is supported by a number of studies in invertebrates that have shown that almost any overall metabolic slowdown obtained by genetic, chemical or physical means (temperature, desiccation) generally results in an increase in lifespan (Hekimi et al. 2001; Jazwinski 1998). Another possibility is that mild perturbations in homeostasis induce a weak stress response, which in turn can increase lifespan, perhaps via a well-described phenomenon called hormesis (Masoro 2000). These possibilities are also supported by the findings that an ever-increasing, large and disparate collection of gene mutations slightly increase lifespan in *C. elegans* (Johnson et al. 2002). Future research should reveal whether mice heterozygous for mutations in other essential genes show an increased lifespan.

Caveat 3

Another possible mechanism by which loss-of-function (including knockout) heterozygotes,

Figure 2 How can heterozygosity lead to an increase in lifespan? Heterozygosity-induced increases in lifespan could be the consequence of either non-specific or specific effects of the gene of interest on the aging process. Mild lowering of the activity of a gene could induce a general and moderate slowdown of physiological rates and metabolism, which could, in turn, result in less damage and indirectly lead to an increase in lifespan. Conversely, a reduction in

the activity of a gene could have a specific impact on some processes involved in aging and thereby lead to an increase in lifespan. A final possibility is that the remaining wild-type allele could be progressively lost through loss-of-heterozygosity (LOH), resulting in cells that are completely devoid of the gene product, and whose altered properties impact the aging process. Filled denotes the wild type allele and open denotes a mutant allele

might live long is LOH and the creation of fully mutant ($-/-$) populations of cells by this mechanism. In fact, we have found that this process appears to occur in the liver of *mcl1* heterozygotes. At the present time, we do not know whether such clones arise in other tissues in addition to the liver, nor do we know in what way these *mcl1*^{-/-} clones contribute to the longevity of the *mcl1*^{+/-} animals. However, the liver is functionally crucial in digestion and detoxification, as well as in lipid and glucose metabolism. Thus, it is not too difficult to imagine that an alteration of the function of the liver could contribute to an increased lifespan. We are not aware whether the possibility of *Igf1r*^{-/-} clones has been examined in *Igf1r* heterozygotes, or, for that matter, in many other

heterozygotes, except for tumor suppressor genes.

Conclusions

The identification of single loss-of-function mutations that positively affect lifespan in mice is only just beginning. Very few homozygotes or heterozygotes have been studied for lifespan, and even fewer have been found to have an increased lifespan (Liang et al. 2003; de Magalhaes et al. 2005). The future will tell if the heterozygous approach can reveal many or only a few more such genes. This will likely depend on the underlying mechanism of the effect (Figure 2). The rationale for the two studies that have found such an effect so far was rather specific: the

studies were seeking to demonstrate an evolutionary conservation in mice of the effect of mutations in genes whose effect on aging had been evidenced in *C. elegans*. However, if a mild loss of homeostasis can result in increased lifespan under laboratory conditions (**Caveat 2**), then there will be many mutations that can increase lifespan when one wild-type allele is still present. If LOH is necessary for such an effect (**Caveat 3**), then, we believe, increased heterozygous lifespan is likely to be rare, because relatively few genes will have the property that their loss produces a growth advantage over their phenotypically wild-type neighbors.

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