Free Radicals in Aging – An Evolutionary Perspective

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Abstract

Evidence supporting the role of oxidative stress in the aging process is presented in detail in this chapter. We also provide an overview of recent studies using novel animal models of successful aging, which have provided valuable additional information to support or counter the oxidative stress hypothesis of aging. We focus on comparative studies using three long-lived but mouse-sized mammalian species, the naked mole rat (*Heterocephalus glaber*), the white-footed mouse (*Peromyscus leucopus*), and the little brown bat (*Myotis lucifugus*), cells of long-lived and short-lived primates, and bivalve models of exceptional longevity to test predictions of the oxidative stress theory of aging. It is concluded that studies examining cellular and mitochondrial free radical generation and oxidative stress resistance in a broad array of successfully aging species seem to concur in general with predictions based upon the oxidative stress theory

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of aging. Further studies are evidently needed to investigate mechanisms related to ROS homeostasis, protein recycling, and DNA repair that might be informative about successful aging of these species.

Introduction

Harman originally proposed the free radical theory of aging half a century ago (Harman 1956). This original working concept invoked accumulation of such oxidative damage of proteins, lipids, and DNA as the primary causal factor in the aging process (Harman 1972). The mitochondrial theory of aging, put forward by Harman in the 1970s (Harman 1972), postulates that mitochondria are the main source of ROS in aged cells. Mitochondria-derived ROS, in particular, can cause mitochondrial DNA mutations and oxidative damage to respiratory enzymes, which in turn would further increase mitochondrial production of ROS, exacerbating mitochondrial damage and dysfunction. This vicious cycle leads to further decline in cellular function that can eventually progress to organ failure and death (Balaban et al. 2005). Antioxidants and antioxidant enzymes may neutralize ROS, thereby attenuating accrual of oxidative macromolecular damage. A large body of evidence has been published both in support of and against the free radical theory of aging (Sohal et al. 2002). As predicted by the theory, in lower organisms genetic manipulations that alter cellular redox homeostasis and cellular stress resistance pathways and/or treatment with antioxidants seem to extend life span (Wheeler et al. 1995; Honda and Honda 1999; Kops et al. 2002; Lithgow and Walker 2002; Sampayo et al. 2003; Tullet et al. 2008; Yang and Tower 2009; Pickering et al. 2012). There is also overwhelming evidence that mammalian aging is associated with oxidative stress and that significant oxidative macromolecular damage accrues with age in virtually every cell type and tissues studied (Guo et al. 2001; Hamilton et al. 2001b; Van Remmen and Richardson 2001; Van Remmen et al. 2003a; Bokov et al. 2004; Salmon et al. 2010). Also, the general concept that oxidative stress has a key role in the development of many age-related diseases, including cancer, atherosclerosis, hypertension, complications of diabetes mellitus, and Alzheimer's disease, appears robust. There is also an emerging view that ROS, in addition to causing oxidative macromolecular damage and cellular injury, play important signaling roles as well, which likely play a role in the development of aging phenotypes.

Although the oxidative stress theory of aging continues to be among the most commonly adduced mechanistic hypotheses to explain variation in aging rate, it is also a subject of ongoing debate due to recent findings inconsistent with it in genetically manipulated laboratory mice (Ran et al. 2007; Jang et al. 2009; Perez et al. 2009a, b; Van Remmen and Jones 2009; Zhang et al. 2009). Specifically, mouse models with genetic deficiency of major cellular antioxidant enzymes exhibit a relatively mild phenotype despite the considerable increases in cellular ROS levels and significantly higher levels of oxidative macromolecular damage in all tissues, often with no major change in maximum life span (Van Remmen et al. 2003b; Mansouri et al. 2006; Sentman et al. 2006). Although there are data extant showing that overexpression of catalase may increase life span (Schriner et al. 2005; Wu et al. 2007), there are

also many studies showing that overexpression of other antioxidant enzymes involved in scavenging of $O_2^{\bullet -}$ and H_2O_2 in transgenic mice does not result in an extended longevity phenotype (Huang et al. 2000; Mele et al. 2006). Furthermore dietary supplementation with antioxidants often does not retard age-related declines and/or extend life span in laboratory rodents living in a stress-free environment.

However, the aforementioned findings should be interpreted with caution for a number of reasons (Ungvari et al. 2008a). First, most laboratory mice used in biomedical research are derived from fancy mice, which are a product of repeated domestications of mice in both Europe and Asia over a period of several centuries (Potter 1978). During this process, significant subspecies mixing occurred that brought together genes that have evolved away from each other over an extensive period of evolutionary time. These strains have then undergone a century of laboratory evolution and inbreeding and thus have significantly altered endocrine regulation, compromised mitochondrial function, retroviruses integrated into the mouse genome at unexpected chromosomal sites, metabolic defects, and impaired damage repair pathways compared with their wild progenitors (Miller et al. 2002; Harper et al. 2006). Second, laboratory mice are purposely protected from many of the vicissitudes of life, such as infectious diseases, suboptimal diets, and climatic variation. Importantly, in recent years the view has emerged that there is an intimate relationship between longevity and stress resistance phenotypes. Thus, experimental results obtained under these stress-free conditions are likely to differ from those obtained under more realistic and stressful conditions. A more compelling evaluation of the oxidative stress hypothesis of aging should employ a range of species that has not been subjected to inbreeding and laboratory selection.

As Austad (1996) and, subsequently, Miller (Harper et al. 2007) have pointed out, although in mice single-gene mutations that affect cellular redox homeostasis and cellular stress resistance pathways (among other targets) often do not result in a longevity phenotype and can seldom increase life span by more than 40 % (Mele et al. 2006; Ran et al. 2007; Perez et al. 2009b, 2011; Salmon et al. 2010), natural selection has produced significant differences in longevity, among species, of much greater magnitude. Thus, one very promising approach to test the oxidative stress theory of aging is the comparative assessment of cellular ROS homeostasis among wild-caught or wild-derived animals with known and reasonably disparate longevities (Miller et al. 2002; Harper et al. 2006; Ungvari et al. 2008a). In the present review, we provide an (admittedly subjective) overview of recent studies using novel animal models of successful aging, which have provided valuable additional information to support or counter the oxidative stress hypothesis of aging.

Novel Animal Models of Successful Aging: Testing Predictions of the Oxidative Stress Hypothesis of Aging

Mammals have the same cell structure and biochemistry, yet their life span ranges 100-fold. The house mouse (*M. musculus)* is among the fastest aging mammals

(maximal life span potential, ~3.5 years; human MLSP, 122 years) and therefore a popular subject of aging studies. The mouse genome is published and the short life span of mice enables longitudinal studies and experimental manipulations to study mechanisms of aging. Yet, mice are primarily chosen for convenience, rather than for specific features pertinent to human aging (see above).

Long-living species are increasingly recognized as useful models for human aging. In the past decade, a significant number of comparative studies have been published which aimed to explore the large differences in maximum life span and aging patterns to elucidate key mechanisms underlying the aging process. Comparisons of mechanisms related to oxidative stress, oxidative stress resistance, and redox signaling between long-living species and shorter-living ones proved to be particularly relevant.

The oxidative stress theory of aging predicts that long-lived, successfully aging animals utilize one or more of the following three potential strategies to delay/limit oxidative stress-induced cellular damage: (1) lower initial generation of reactive oxygen species (ROS, such as superoxide [$O_2^{\bullet -}$], hydrogen peroxide [H_2O_2]) at young ages, so that it takes longer to reach the critical threshold (beyond which oxidative damage significantly impairs cellular function) even at the same rate of aging; (2) increased tolerance for increases in ROS production (including superior antioxidant defense and/or damage repair mechanisms); and (3) slower rate of age-related increases in ROS generation (increasing the time to reach a critical threshold).

These predictions of the oxidative stress hypothesis of aging have been tested in recent years by a number of laboratories using species with disparate longevity. This approach allows researchers to determine whether oxidative stress features correlate with maximum life span and whether longer-living species exhibit superior resistance against accumulation of oxidative macromolecular damage and cellular injury. Importantly, a recent study by Lambert et al. demonstrated that ROS production by heart mitochondria isolated from diverse mammalian species including muroid rodents, two bat species, naked mole rat, Damara mole rat, guinea pig, baboon, and ox tends to inversely correlate with species life span (Lambert et al. 2007), a finding that is in agreement with the predictions of the oxidative stress theory of aging. Yet, it should be noted that comparative studies on life span and physiology should take into account the possible confounding effects of body mass and phylogeny. There is general correlation between body mass and longevity: short-lived species tend to be relatively small, while the long-lived species have larger body mass. The effects of body mass can be circumvented by comparing animals of similar body mass but differing life spans.

In the present overview, first we will discuss findings from comparative studies aimed to test predictions of the oxidative stress theory of aging using three long-lived but mouse-sized mammalian species, the naked mole rat (*Heterocephalus glaber*), the white-footed mouse (*Peromyscus leucopus*), and the little brown bat (*Myotis lucifugus*). These species have been specifically chosen because relative to that predicted by body size, they are longer living. Next, we discuss evidence in support and against the oxidative stress hypothesis of aging obtained in experiments

using cells derived from multiple rodent species and primates with disparate longevity. We also discuss the results of studies testing predictions of the oxidative stress theory of aging in long-lived birds. Finally, we summarize recent advances from studies using animals from the class *Bivalvia*, which include *Arctica islandica*, the longest-living animal on earth (maximum life span >500 years). Our goal here is not to give an exhaustive review of the literature. We acknowledge that many important studies and exciting model systems are omitted. The studies included in this overview serve to illustrate novel approaches in comparative aging research that predict how the intersecting fields of free radical biology and gerontology may develop in the future.

Studies on Long-Lived Rodents

Naked Mole Rats

The naked mole rats (Rodentia: Bathyergidae; *Heterocephalus glaber*) are mouse-sized African rodents that show exceptional longevity (maximum life span >30 years). The use of the naked mole rat as a model for biogerontological research is a subject of an excellent recent review (Buffenstein 2005). Cells of long-lived species in general tend to produce less ROS than shorter-living ones even at young ages (see below). Although previous studies in these animals could not demonstrate an association between cellular ROS generation and life span in this species (Andziak et al. 2005; Labinskyy et al. 2006), the rate of ROS production in cardiac mitochondria from naked mole rats is less than those from mouse (Lambert et al. 2007). In addition, previous studies demonstrate that in naked mole rats, age-related development of cellular oxidative stress is substantially delayed (Csiszar et al. 2007). Further, NADPH oxidase expression (which shows marked upregulation in aged rats and mice) does not change with age in this extremely long-lived species (Csiszar et al. 2007). The aforementioned findings accord with the prediction based on the oxidative stress hypothesis of aging. Interestingly, naked mole rats in various tissues exhibit similar, or sometimes even greater, levels of accrued oxidative damage to lipids, DNA, and proteins than age-matched mice (Buffenstein and Jarvis 2002; Andziak et al. 2005, 2006; Buffenstein 2005; Andziak and Buffenstein 2006). At present it is unclear what the cause is for the relatively high rate of macromolecular oxidative damage in naked mole rat cells. A recent study reported that naked mole rats exhibit a selective defect in glutathione peroxidase 1 expression in the liver (Kasaikina et al. 2011), which may contribute to the observed cellular redox imbalance in this species. Interestingly, when compared with mouse samples, livers of naked mole rats exhibit significantly higher proteasome activity (Rodriguez et al. 2012), suggesting that proteasomes in this species are primed for the efficient removal of oxidatively damaged and misfolded proteins. Thus, it is possible that naked mole rat cells can handle increased stress and protein damage to maintain their health longer. Further support for this concept comes from studies demonstrating that cells of naked mole rats exhibit a multi-stress resistance phenotype (Labinskyy et al. 2006; Lewis et al. 2012), at least in part, due to the increased

activity of Nrf2-dependent pathways (Lewis et al. 2012). Similar findings were obtained recently in long-lived Damara mole rats (*Cryptomys damarensis*; maximum life span 16 years) and bat species (Ungvari et al. 2008a), in which a negative correlation exists between life span and sensitivity to ROS-induced apoptotic cell death (Labinskyy et al. 2006). It is believed that increased Nrf2 activity is responsible for the cancer resistance phenotype of naked mole rats as well (Lewis et al. 2010, 2012). In that regard it is interesting that Nrf2-dependent pathways were also shown to mediate cancer protection but not prolongevity induced by caloric restriction in laboratory mice (Pearson et al. 2008).

Peromyscus Leucopus

The white-footed mouse (*Peromyscus leucopus*) despite its close resemblance to *Mus musculus* has an unusually long life span for its size (the record longevity for *P. leucopus* in captivity is 7.9 years (Burger and Gochfeld 1992; Guo et al. 1993)). Because of these considerations, *P. leucopus* seems to be a useful model of successful aging in small muroid rodents (Ungvari et al. 2008b). Previous studies showed that the rate of mitochondrial ROS production in *P. leucopus* is substantially less than in mice (Sohal et al. 1993; Brunet-Rossinni 2004a, b; Csiszar et al. 2007). Previously we have also demonstrated that in cells of *P. leucopus* there is an attenuated production of ROS from NADPH oxidases (Csiszar et al. 2007). This finding is potentially significant as upregulation of NADPH oxidase was demonstrated to contribute to increased cellular oxidative stress in aged rodents (Hamilton et al. 2001a; Csiszar et al. 2002; Adler et al. 2003). In addition, cells of the *P. leucopus* were shown to exhibit a multi-stress resistance phenotype (Csiszar et al. 2007). Further, previous studies also have shown that tissues of *P. leucopus* have higher activities of catalase and glutathione peroxidase (Sohal et al. 1993; Csiszar et al. 2007) and lower levels of protein oxidative damage as well as lower susceptibility to oxidative damage in response to experimental oxidative stress (Sohal et al. 1993) than those of mice. Taken together, the available data on cellular ROS production, redox homeostasis, and oxidative stress resistance in *P. leucopus* agree with the predictions based on the oxidative stress hypothesis of aging.

Comparative Studies on Cells of Long-Lived Rodent Species

The laboratory of Richard A. Miller has pioneered a novel and innovative approach to comparative aging studies: investigation of mechanisms of cellular stress resistance in fibroblast cell lines developed from skin biopsies of long-lived rodent species (Harper et al. 2007), including the white-footed mouse, deer mouse (*Peromyscus maniculatus*; maximum life span 8.3 years), Norway rat (*Rattus norvegicus*; maximum life span 5 years), red squirrel (*Tamiasciurus hudsonicus*; maximum life span 7 years), fox squirrel (*Sciurus niger*; maximum life span 18 years), North American porcupine (*Erethizon dorsatum*; maximum life span 18 years), and the North American beaver (*Castor canadensis*; maximum life span 24 years). Studies on these long-lived rodent species are particularly useful for aging research given their relationship to the most commonly used laboratory aging models: the house mouse and the Norway rat. The findings that species

longevity in this group of animals was associated with resistance to cell death induced by H_2O_2 and other stressors are consistent with the idea that evolution of long-lived rodents may require development of a multi-stress resistance cellular phenotype, including superior cellular resistance to oxidative stress (Harper et al. 2007).

Bats

On average, the life span of bats (order Chiroptera) is approximately three times greater than a nonflying placental mammal of similar size (Brunet-Rossinni and Austad 2004; Podlutsky et al. 2005). Thirteen species in the genus Myotis, ranging in size from 7 to 25 g, have been documented to live at least 20 years in the wild (Podlutsky et al. 2005). The exceptional longevity of bats, which is unusual for mammals of such a small size and a high metabolic rate, renders them an interesting animal model of slow aging. Previous studies testing predictions of the oxidative stress hypothesis of aging in bat species yielded similar results than studies in *P. leucopus* and other long-lived rodents. The available data show that ROS generation in cardiac mitochondria of *M. lucifugus* is significantly attenuated (Brunet-Rossinni 2004a, b; Lambert et al. 2007). Similar conclusions were reached by studies measuring ROS production in intact cells of *M. lucifugus* (Ungvari et al. 2008a). Although SOD activity may not differ between bats and shorter-living species (Brunet-Rossinni 2004a, b), the cellular content of low molecular weight antioxidant compounds in various organs of bats was reported to be severalfold higher than those usually found in rat and mouse tissues (Wilhelm Filho et al. 2007). Importantly *M. lucifugus* cells are also significantly more resistant to the effects of H_2O_2 than mouse cells (Harper et al. 2007; Ungvari et al. 2008a). Bat cells also repair DNA damage faster than mouse fibroblasts (Podlutsky et al. 2005). Comparison of mice with two bat species, the Brazilian or Mexican free-tailed bat (Tadarida brasiliensis; maximum life span ~12 years) and the cave myotis (Myotis velifer; maximum life span ~12 years), showed that bat cells exhibit lower protein oxidation (Salmon et al. 2009) and more resistant to acute oxidative stress. Interestingly, proteins derived from bats are resistant to urea-induced protein unfolding relative to the level of unfolding detected in protein fractions from mice (Salmon et al. 2009). These bat species also show low levels of protein ubiquitination in total protein lysates along with reduced proteasome activity, suggesting diminished protein damage and removal in bats (Salmon et al. 2009). Taken together, the aforementioned results are consistent with the idea that evolution of long-lived bats required development of cellular resistance to oxidative stress, as predicted by the oxidative stress theory of aging.

Studies on Primate Cells

It is generally accepted that exceptional longevity evolved independently many times in various mammalian orders, but it is still debated whether mechanisms of

successful aging are conserved among these various groups (Austad 2009). Primates are among the longest-lived mammals (they live on average more than twice as long as a standard mammal for their body size (Austad and Fischer 1992; Austad 2009)). Cells of nonhuman primates, similar to human cells, were shown to exhibit increased oxidative stress with aging (Ungvari et al. 2011a; Csiszar et al. 2012b). To assess whether decreased cellular ROS levels and/or resistance to oxidative stress-induced cellular injury might be causally involved in primate longevity, we have recently assessed ROS production and oxidative stress resistance in cultured fibroblasts from 13 primate species ranging in body size from 0.25 to 120 kg and in longevity from 20 to 90 years, including five great apes (human, chimpanzee, bonobo, gorilla, and orangutan), four Old World monkeys (baboon, rhesus and crested black macaques, and patas monkey), three New World monkeys (common marmoset, red-bellied tamarin, and woolly monkey), and one lemur (ring-tailed lemur) (Csiszar et al. 2012a). We found an inverse correlation between longevity and steady state or metabolic stress-induced mitochondrial $O_2^{\cdot -}$ production, but this correlation was lost when the effects of body mass were removed and the data were analyzed using phylogenetically independent contrasts (Csiszar et al. 2012a). On the basis of the aforementioned findings, it can be concluded that increased longevity in this sample of primates is not causally associated with low cellular ROS generation. Importantly, cells from longer-lived primate species also exhibited superior resistance to oxidative stress-induced cellular injury than cells from shorter-living primates (Csiszar et al. 2012a). This finding accords with the prediction based on the oxidative stress hypothesis of aging and warrants further studies to better understand the molecular mechanisms underlying increased cellular resistance to oxidative stressors in long-lived primates.

Studies on Long-Lived Birds

Bird species live about three times as long as an average mammal of similar size, despite the fact that they exhibit elevated body temperature, a rapid metabolic rate, and high blood glucose levels (Austad 2011). There are well-conceived extant studies that aim to understand the role of antioxidant protection in bird longevity and to circumvent the effect of body mass on life span by comparing birds such as pigeons (maximal life span ~35 years) with rats that have similar body masses (Ku and Sohal 1993; Barja 1998; Barja and Herrero 1998; Herrero and Barja 1998; Ogburn et al. 1998; Perez-Campo et al. 1998; Lambert et al. 2010). In general, the results from these studies accord with the predictions of the oxidative stress theory of aging. The available evidence suggests that the capacity to show a low rate of cellular and mitochondrial ROS production and to exhibit increased cellular resistance to oxidative stressors is a general characteristic of birds (Ku and Sohal 1993; Barja 1998; Barja and Herrero 1998; Herrero and Barja 1998; Ogburn et al. 1998; Perez-Campo et al. 1998; Lambert et al. 2010). However, in these cases there are issues associated with phylogeny as it is likely

that certain traits (such as macromolecular damage, metabolism) are different in all birds for reasons unrelated to their longevity. Another interesting approach to test predictions of the oxidative stress hypothesis of aging is to compare birds with disparate longevity.

Among birds, parrots are exceptionally long lived with some species living more than 70 years (Montgomery et al. 2012), whereas quails are among the shortest-living bird species (maximum life span potential ~5.5 years). To test predictions of the oxidative stress theory of aging, a recent study measured total antioxidant capacity, reduced glutathione levels, activities of superoxide dismutase, glutathione peroxidase and catalase, and markers of oxidative macromolecular damage in three species of long-living parrots and two species of short-living quails (Montgomery et al. 2012). The data from the aforementioned study suggest that although there appears to be higher protection against some aspects of oxidative stress in long-lived parrots than shorter-living quails, the accumulation of oxidative damage is not a primary determinant of maximum life span potential in these bird species (Montgomery et al. 2012). In contrast, primary fibroblasts derived from long-lived budgerigar (*Melopsittacus undulatus*; maximum life span ~20 years) resistant to oxidative damage was reported to be greater than in cells derived from short-lived Japanese quail (*Coturnix coturnix japonica*; maximum life span ~5 years) (Ogburn et al. 2001). Similarly, an important recent study showed that in primary fibroblast, cultures from 35 species of free-living birds resistant to cell death caused by cadmium, paraquat, H_2O_2, and methyl methanesulfonate correlate with species longevity (Harper et al. 2011), extending previous findings from the same group of investigators (Harper et al. 2007). Importantly, in the aforementioned study, the correlation between cellular multi-stress resistance and longevity was discernible even after accounting for the influence of body mass and phylogeny (Harper et al. 2011). In accordance with the results of previous studies, avian fibroblasts in general were significantly more resistant than fibroblasts derived from rodent species to each of the tested stressors (Harper et al. 2011). Further, recent studies demonstrate that dermal fibroblasts from tropical birds resist chemical agents that induce oxidative stress better than do cells from temperate species, consistent with the hypothesis that birds that live longer exhibit superior cellular oxidative stress resistance (Williams et al. 2010). It should be noted, however, that these studies have only measured a handful of oxidative stress parameters and far-reaching conclusions on the validity of the oxidative stress theory of aging in birds cannot yet be drawn. Future comparative studies on bird models of successful aging using more advanced methodologies may reveal novel cellular and molecular mechanisms of resistance to age-related oxidative stress and senescence. Dr. Steven N. Austad, the doyen of comparative aging research, has recently compiled a list of bird species that deserve special attention for development as models of successful aging, including budgerigars, canaries, zebra finches, the European starling, and the house sparrow (Austad 2011). Future comparative aging studies will be able to take advantage of the availability of the whole-genome sequence of multiple bird species and use more sophisticated analyses of redox homeostasis in exceptionally long-lived birds.

Studies on Bivalve Models of Exceptional Longevity

Within the class *Bivalvia*, maximum life span differs more than 200-fold, more than any other non-colonial animal group (from an excess of 500 years to less than 2 years). This natural variation of life span makes them ideal model organisms to test predictions of major hypotheses of aging (Ridgway et al. 2011; Ungvari and Philipp 2011; Ungvari et al. 2011, 2012). Using the burrowing clam *Arctica islandica* (ocean quahog), which is the longest lived of all non-colonial animal species on earth (maximum species life span >500 years), we recently demonstrated that in bivalve models of extreme longevity, there is an association between maximum species life span potential and resistance to oxidative stress-induced mortality and also a marked resistance to oxidative stress-induced apoptotic cell death (Ungvari et al. 2011). Extreme longevity in *A. islandica* is also associated with an attenuated cellular ROS production and reduced protein carbonyl content (Ungvari et al. 2011). These findings are consistent with the oxidative stress hypothesis of aging and provide justification for detailed evaluation of pathways involving repair of oxidative stress-induced macromolecular damage and regulation of apoptosis in the world's longest-living non-colonial animal.

Perspectives

Collectively, studies examining cellular and mitochondrial free radical generation and oxidative stress resistance in a broad array of successfully aging species seem to concur in general with predictions based upon the oxidative stress theory of aging. Future studies should determine whether long-lived animals are more resistant to age-related diseases, whose development is facilitated by oxidative stress. The cellular and molecular mechanisms underlying superior oxidative stress resistance in long-lived species are likely multifaceted. Future studies should investigate mechanisms related to ROS homeostasis, protein recycling, and DNA repair that might be informative about successful aging of these species. One promising area of research is targeting pathways that appear to be involved in the longevity phenotype in multiple species. We hope that this chapter will stimulate interest among researchers to consider long-lived species as study organisms in a comparative approach to free radical biology and aging research.

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