Fibroblasts from long-lived species of mammals and birds show delayed, but prolonged, phosphorylation of ERK

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Summary

Fibroblasts from long-lived mutant mice show diminished phosphorylation of the stress-activated protein kinases ERK1/2 after exposure to peroxide, cadmium, or paraquat. We have now evaluated the kinetics of ERK phosphorylation in fibroblasts from long-lived and short-lived species of mammals and birds in response to stress by cadmium or hydrogen peroxide. Fibroblasts from the shorter-lived species of rodents and birds showed rapid induction of ERK phosphorylation, with a decline to basal level within 60 min. In contrast, cells from longer-lived species showed slower and more prolonged activation of ERK phosphorylation. These results suggest that fibroblasts from long-lived species may be less susceptible to the early phases of damage from cadmium or peroxide and suggest that altered kinetics of ERK activity may contribute to their stress resistance properties.

Key words: birds; evolution; longevity regulation; life span; mammals; oxidative stress; signalling.

Introduction

The molecular basis for the great diversity in aging rate and maximum longevity among species of mammals and birds is not yet well understood (Austad, 2009). Changes in genotype (Brown-Borg et al., 1996; Flurkey et al., 2001), diet (Orentreich et al., 1993; Weindruch & Sohal, 1997), drug exposure (Harrison et al., 2009), or breeding opportunities (Dammann et al., 2011) can increase maximum lifespan by as much as about 50% within a given species of mammal, but evolutionary processes can lead to differences of nearly 100-fold between the shortest-lived and longest-lived species of mammals, with a range of about tenfold even within some of the mammalian orders. Long-lived species have evolved many times within the mammalian and avian radiations, but it is not yet clear to what extent the mechanisms that contribute to exceptionally long lifespan in one species resemble those with slow aging in distantly related clades.

There is some evidence that cultured cells from long-lived organisms may differ systematically from cells derived from shorter-lived species. Fibroblasts cultured from longer-lived species of mammals are relatively resistant to death induced by a variety of oxidative toxins (Kapahi et al., 1999). Mouse cell lines are more susceptible than human fibroblasts to oxygen-mediated mutation and growth crisis (Busuttil et al., 2003; Parrinello et al., 2007), but do not show telomere-based clonal senescence when cultured at physiologically low oxygen tensions. A study of fibroblasts from 15 rodent species showed (Seluanov et al., 2008) that they could be divided into three classes based on differences in tumor-suppressive mechanisms, with telomere-based growth control characteristic of the larger species. Among the rodent species of small body size, longer lifespan was associated with a slower rate of in vitro cell proliferation. Work in our own laboratory has shown (Harper et al., 2007) that fibroblasts from longer-lived rodent species are resistant, in culture, to death induced by exposure to cadmium and H₂O₂, with similar but nonsignificant trends in cells exposed to heat (P = 0.053) or to the DNA-alkylating agent MMS (methyl methanesulfonate, P = 0.08). Fibroblasts from relatively long-lived species of birds were, similarly, found to be resistant to death induced by cadmium, paraquat, and MMS, with a suggestive trend (P = 0.07) in resistance to H₂O₂. Interestingly, bird fibroblasts were more resistant than rodent fibroblasts to death induced by cadmium, paraquat, H₂O₂, MMS, and ultraviolet (UV) light (Harper et al., 2011), consistent with the relatively longer lifespan of birds compared with rodent species of similar body size (Holmes et al., 2001). These findings parallel the observation that fibroblasts from long-lived mutant mouse stocks, including the Snell dwarf, Ames dwarf, and growth hormone receptor knockout mice, are resistant in culture to death induced by H₂O₂, paraquat, cadmium, heat, UV light, MMS, and arsenite (Murakami et al., 2003; Salmon et al., 2005; Leiser & Miller, 2009), suggesting that at least some of the properties seen in cells from long-lived mutant mice might be related to those through which evolutionary pressures mold life history and aging rates across species.

Responses of cells to stress are modulated, in part, by enzymes in the mitogen-activated protein kinase (MAPK) signaling pathway. This group, which includes sets of ERK, JNK, and p38 kinases (Roux & Blenis, 2004), consists of ubiquitously expressed signaling proteins that are involved in many facets of cellular regulation, including proliferation, differentiation, adaptive responses to hormones, nutrient availability, and injurious stresses. Many of these kinases have been reported to play a role in various diseases and have been implicated in cell injury (Chang & Karin, 2001; Narasimhan et al., 2009). The extracellular-signal-regulated protein kinase ERK typically exists in two isoforms, ERK1 and ERK2, also called p42 and p44 kinases, and serves as one of the major transducers of various diseases and have been implicated in cell injury (Chang & Karin, 2001; Narasimhan et al., 2009). The extracellular-signal-regulated protein kinase ERK typically exists in two isoforms, ERK1 and ERK2, also called p42 and p44 kinases, and serves as one of the major transducers of extracellular signals connecting the membrane-bound receptors to changes in cellular function (Costa et al., 2006). In mammals, ERK1 and ERK2 have 83% amino acid identity and are expressed in varying amounts and proportions in all tissues (Chen et al., 2001; Roux & Blenis, 2004). In quiescent cells, ERK1/2 is typically dispersed throughout the cell, but a significant population of ERK1/2 accumulates in the nucleus after cell stimulation (Chen et al., 1992).

ERK1 and ERK2 are both activated through a cascade of kinases downstream of various receptor tyrosine kinases (RTKs) and cytokine receptors (Chang & Karin, 2001). In the MAPK pathway, ligand binding induces the activation of an RTK that initiates a signal transduction cascade, resulting in the activation of the GTPase Ras and the Ser/Thr kinase Raf. Activated Raf then binds to and phosphorylates MEK.
(MAPKK), resulting in its activation. MEK, a Ser/Thr and Tyr kinase, activates ERK by phosphorylating Thr202/Tyr204 and Thr185/Tyr187 of ERK1 and ERK2, respectively (Chang et al., 2003).

Activated ERK1 and ERK2 phosphorylate various substrates in multiple cellular compartments, including membrane proteins (CD120a, Syk, and calnexin), nuclear substrates (SRC-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, c-Myc, and STAT3), cytoskeletal proteins (neurofilaments and paxillin), and several MAPK-activated protein kinases (Chen et al., 2001).

Stress resistance in fibroblasts from Snell dwarf mice is accompanied by delays in kinetics of phosphorylation of ERK in the first hour after exposure to cadmium, paraquat, and H₂O₂, but not in cells exposed to UV light (Sun et al., 2009). The current work was undertaken to determine whether alterations in kinetics of ERK phosphorylation also distinguish fibroblasts of long-lived from short-lived species of mammals and birds.

Results

To see whether interspecies differences in lifespan in the MEK/ERK system, we measured pERK levels in fibroblasts of 21 species of small mammals, including 18 species of rodents, with maximum lifespan (MLS) ranging from 2.2 to 23.4 years (Table S1, Supporting information). Cells were exposed to Cd or to H₂O₂ for 5–60 min, and the ratio of pERK/ERK measured as an index of MEK function. Figure 1 shows two representative images. For the red squirrel (left panel, maximum lifespan 9.8 years), ERK phosphorylation increases dramatically in the first 5 min after exposure to either stress and subsides to near-baseline (unstressed) levels by 30 min. For the chinchilla (right panel, maximum lifespan 17 years), ERK phosphorylation begins more gradually and continues to increase for 20–30 min before declining to baseline levels. Figure 2 shows the time course for the pERK/ERK ratio for six species in responses to cadmium (panels A and B) or to H₂O₂ (panels C and D). In cells from the relatively short-lived species (panels A and C), ERK phosphorylation increases quickly and then quickly returns to baseline. In cells from the longer-lived species (panels B and D), peak levels of pERK are not seen until the 30-min time point.

For a more systematic analysis of the relation of lifespan to the kinetics of ERK phosphorylation, we calculated two indices for each species for each stress agent. We calculated S₁₀/₅ as the ratio of pERK/ERK at 10 min, divided by the pERK/ERK ratio at 5 min. High values of S₁₀/₅ indicate rapid induction of ERK phosphorylation. We calculated S₃₀/₁₀ as the ratio of pERK/ERK at 30 min divided by the ratio at 10 min. High values of S₃₀/₁₀ indicate species in which ERK phosphorylation remains high 30 min after stress exposure. We then used least squares regression to test the idea that S₁₀/₅ or S₃₀/₁₀ was associated with differences in MLS among the mammalian species. Figure 3 shows scatterplots of these associations, with the corresponding regression lines; significance levels for the regressions are provided in Table 1. Both for cadmium and for H₂O₂, longer lifespan is associated with low values of S₁₀/₅ (i.e. slower onset of ERK phosphorylation) and higher values of S₃₀/₁₀ (i.e. relatively prolonged maintenance of ERK phosphorylation). The associations are fairly strong, with R² values ranging from 0.36 to 0.90, each significant at P < 0.002. When the analysis was limited to the 18 rodent species (Table S1, Supporting information), the association remained strong, with P < 0.03 for S₁₀/₅ in cadmium responses and P < 0.006 for the other three associations. When regression analyses were performed in which the species body mass and pERK kinetic indices were both included as predictor values, the association for the pERK indices remained significant, both for the mammalian data set as a whole and for the rodent species per se. Thus, we infer that skin-derived fibroblasts from longer-lived mammalian species, including rodent species, tend to activate MEK/ERK signals more slowly than cells from short-lived species and to retain elevated ERK activity for somewhat longer.

To see whether a similar relationship applied across a set of avian species, we also measured pERK levels in fibroblasts of 27 species of birds, with maximum lifespan (MLS) ranging from 7 to 50 years (Table S2, Supporting information). Similar to the assay used for mammals, avian cells were exposed to Cd or to H₂O₂ for 5–60 min, and the ratio of pERK/ERK was quantified as an index of MEK function. Figure 4 shows two representative images. For the Carolina Wren (left panel, maximum lifespan 9 years), ERK phosphorylation increases considerably in the first 5 min after exposure to either Cd or H₂O₂ and continues to increase through the 10-min time point, but then declines to near-baseline levels by 30 min. For the Canada goose (right panel, maximum lifespan 42 years), ERK phosphorylation increases more slowly, reaching a peak 30 min after exposure to either stress agent, before declining to baseline levels by 60 min.

Figure 5 shows quantitative estimates of the pERK/ERK ratio for six species in responses to cadmium (panels A and B) or to H₂O₂ (panels C and D). Panels A and C show short-lived species, and in these cases, pERK phosphorylation increases quickly in the early stages (time points 5 and 10 min) and then quickly returns to baseline (time points 20, 30, and 60 min). Panels B and D represent long-lived birds, and in these cases, peak levels of pERK do not appear until 20–30 min after stress exposure. Figure 6 shows the scatterplots and regression lines for all 27 avian species. Significance levels for the regressions are provided in Table 2. The associations are strongest for the S₃₀/₁₀ parameter, with R² values ranging from 0.36 to 0.76, each significant at P < 0.0001, in good agreement with the mammalian data set. For birds, there was less consistency in the early activation rate measured by the S₁₀/₅ parameter. The association was significant for cadmium (R² = 0.16, P = 0.02) but not for H₂O₂ (R² = 0.02, P = 0.25). A regression analysis in which both log (mass) and the pERK/ERK indices were used as predictors of maximum lifespan, also shown in Table 2, reduced the strength of the association, and only S₃₀/₁₀, for H₂O₂, remained at P < 0.05 in these regressions.

(A) Time

| Time | 0 | 5 | 10 | 20 | 30 | 60 |
|------|---|---|----|----|----|----|
| p-ERK | Cd | p-ERK | H₂O₂ | ERK | β-actin |

(B) Time

| Time | 0 | 5 | 10 | 20 | 30 | 60 |
|------|---|---|----|----|----|----|
| p-ERK | Cd | p-ERK | H₂O₂ | ERK | β-actin |

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Standard regression methods make the assumption that the data for each species, both lifespan and ERK kinetic data, are independent, but this assumption is not correct, because closely related species reflect fewer evolutionary changes than species further from their most recent shared ancestor (Felsenstein, 1985). We therefore evaluated our data using the standardized independent contrast method (Garland & Adolph, 1994), which weights associations between lifespan and ERK based on the phylogenetic distances among groups of species. For mammals, we found no significant relationship for the S10/5 (early response) parameter, either for H2O2 ($r^2 = 0.13$, $P = 0.12$) or for

![Fig. 2](A) Phosphorylation of ERK1/2 in skin-derived primary fibroblasts from mammals represented as the ratio of phosphorylated ERK to total ERK at the indicated times after exposure to 80 μM cadmium (top panels) or 100 μM H2O2 (bottom panels). Panels A and C show three short-lived species, and panels B and D show three long-lived species.

![Fig. 3](B) Scatterplots representing association of species lifespan (MLS in years) as a function of kinetic parameters (early slope S10/5 and later slope S30/10) for phosphorylation of ERK1/2 in skin-derived primary fibroblasts from 21 species of mammals. Upper panels show responses to cadmium, and lower panels show responses to H2O2. Rodent species indicated by circles and nonrodent species by triangles. The regression coefficients and p-values shown reflect calculations without adjustment for body mass.

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cadmium ($r^2 = 0.09$, $P = 0.2$), but the relationship for the S30/10 parameter remained strong and statistically significant for both sources of stress (Fig. 7, top panels). Similarly, S10/5 was not significantly related to species lifespan among bird species ($r^2 = 0.0004$, $P = 0.93$ for H$_2$O$_2$ and $r^2 = 0.1$, $P = 0.13$ for cadmium), but S30/10 showed a significant relationship for both sources of stress (Fig. 7, bottom panels). Because passerine species make up nearly half of the bird species in our collection, we repeated the independent contrast analysis using an approach that collapsed all of the passerine species to a single point. Despite the fact that this analysis reduced our functional sample size by nearly half, there was still a suggestion of association for S10/5 for the cadmium data set ($r^2 = 0.27$, $P = 0.055$) but not for the H$_2$O$_2$ stress ($r^2 = 0.01$, $P = 0.7$), and the association with S30/10 levels remained statistically significant (cadmium: $r^2 = 0.35$, $P = 0.03$; H$_2$O$_2$: $r^2 = 0.31$, $P = 0.04$, data not shown). We conclude that the relationship between sustained ERK activation (S30/10) and lifespan in mammals and birds is

| Endpoint   | Dose ($\mu$M) | $P$-value | $r^2$ | $P$-value (log mass adjusted) | Rodents only $P$-value | Rodents only $P$-value (log mass adjusted) |
|------------|---------------|-----------|-------|-------------------------------|-----------------------|-------------------------------------------|
| S10/5, H$_2$O$_2$ | 300           | 0.001     | 0.45  | 0.003                         | 0.006                 | 0.02                                       |
| S30/10, H$_2$O$_2$ | 300           | 0.000     | 0.90  | 0.000                         | 0.000                 | 0.000                                      |
| S10/5, cadmium  | 140           | 0.002     | 0.36  | 0.001                         | 0.03                  | 0.02                                       |
| S30/10, cadmium | 140           | 0.000     | 0.62  | 0.000                         | 0.000                 | 0.001                                      |

Where $P$-value is given as 0.000, this represents $P < 0.001$. Other $P$-values are given rounded to the nearest significant figure.

Table 1: Statistics from regression analyses for mammalian species

Fig. 4 Representative autoradiographs of western blots for phosphorylated and total forms of ERK1/2 in skin-derived primary fibroblasts from rodents, in responses to cadmium (top row) or H$_2$O$_2$ (second row). Rows 3 and 4 show, respectively, total ERK and $\beta$-actin. Panel A: Carolina Wren. Panel B: Canada goose.

Fig. 5 Phosphorylation of ERK1/2, in skin-derived primary fibroblasts from birds represented as the ratio of phosphorylated ERK to total ERK at the indicated times after exposure to 140 $\mu$M cadmium (top panels) or 300 $\mu$M H$_2$O$_2$ (bottom panels). Panels A and C show three short-lived species, and panels B and D show three long-lived species.
Discussion

Primary skin fibroblast cell lines isolated from adults of 27 avian species (seven orders, with maximum lifespan ranging from 7 to 50 years) and 21 mammalian species (mostly rodents, maximum lifespan range from 2 to 23 years) were used to examine the relationship between species lifespan and the pace of response to cadmium and H₂O₂ as measured by changes in phosphorylation of the stress-activated protein kinases ERK1/2. Previous work from our laboratory has shown that primary fibroblast cultures established from the skin of long-lived mutant Snell dwarf, Ames dwarf, and growth hormone receptor knockout (GHRKO) mice are resistant to lethal injury induced by cadmium and H₂O₂ (Murakami et al., 2003; Salmon et al., 2005). Studies of the Snell and GHRKO fibroblasts showed slower rates of phosphorylation of ERK1/2 after exposure to peroxide, cadmium, or paraquat (Sun et al., 2009). Our current results suggest that differences in the kinetics of stress-induced activation of ERKs are also seen in cross-species contrasts. For both birds and mammals, species with longer lifespan show slower onset of ERK phosphorylation, in that the ratio of pERK at 30 min to pERK at 10 min is higher in long-lived species, both for cadmium and for H₂O₂ exposure. For the mammals, the same pattern can be seen in the first 10 min after stress exposure, with high ratios of pERK at 10 min to pERK at 5 min, characteristic of shorter-lived species for both sources of cellular injury. The regression coefficients for rodents, which made up 18 of the 21 tested mammals, are similar to those seen in the larger mammalian data set. In qualitative terms, cells from shorter-lived species tend to turn on ERK activity sooner, and turn it off sooner, than cells from the longer-lived species.

Interpretation of regression analyses after adjustment for species differences in body mass is complicated by several considerations. Because larger species also tend to live longer than smaller ones, traits that are closely related to mass, such as metabolic rate, would show a correlation with longevity even if there were no causal links between the trait and the rate of aging. For this reason, some analysts (Speakman, 2005) place little value on associations of longevity with physiological measurements across species unless these associations survive correction for body mass. Other workers (Hulbert, 2005; Hulbert et al., 2006) note that adjustment for mass is likely to obscure associations that do indeed reflect causal associations and that diluting statistical trends by adjustment for a common covariate (mass) will increase type II errors. We have therefore reported the strength of association for our ERK parameters both with and without adjustment for species mass (Tables 1 and 2), and note that the significant associations seen for both kinetic parameters, for both stresses, survive adjustment for species mass in the rodents, but are weakened by this adjustment (P = 0.02 for H₂O₂ and P = 0.12 for cadmium) among the bird species.

Both our mammal and bird data are heavily weighted toward certain phylogenetic groups. Eighteen of our 21 mammal species are rodents, and 13 of our 27 bird species are passerines. However, even when we analyzed the data adjusting for phylogeny, the pattern for prolonged phosphorylation of ERK in our longer-lived species in response to both H₂O₂ and cadmium persisted.

Table 2 Statistics from regression analyses for avian species

| Endpoint   | Dose (µM) | P-value | R²  | P-value (log mass adjusted) |
|------------|-----------|---------|-----|-----------------------------|
| S10/5, H₂O₂ | 300       | 0.24    | 0.05| 0.6                         |
| S30/10, H₂O₂| 300       | 0.000   | 0.76| 0.02                        |
| S10/5, cadmium| 140     | 0.02    | 0.19| 0.9                         |
| S30/10, cadmium| 140     | 0.000   | 0.66| 0.12                        |

Fig. 6 Scatterplots representing association of species lifespan (MLS in years) as a function of kinetic parameters (early slope S10/5 and later slope S30/10) for phosphorylation of ERK1/2 in skin-derived primary fibroblasts from 27 species of birds. Upper panels show responses to cadmium, and lower panels show responses to H₂O₂. Passerine species are indicated by circles, and nonpasserine species by triangles. The regression coefficients and p-values shown reflect calculations without adjustment for body mass.

not an artifact of shared phylogenetic descent, but we cannot rule out this idea for the early stages of ERK activation (S10/5) using our current data set.
There is a good deal of evidence supporting the idea that differences in response to cellular stresses may contribute to variation in the rate of aging and in lifespan (Johnson et al., 1996). Enhanced stress resistance is associated with extended longevity in worms, flies, and yeast, with a significantly positive relationship between individual ability to respond to stress and its longevity (Johnson et al., 1996). Among isogenic worms, those individuals with a high capacity to react to thermal stress are relatively long-lived. Studies of fibroblasts have also shown relationships between species lifespan and resistance to lethal stress in mammals (Kapahi et al., 1999), birds (Harper et al., 2011), and rodents (Harper et al., 2007), as well as differences in clonal growth patterns and responses to contact inhibition (Seluanov et al., 2008). Our data extend these earlier reports by suggesting altered kinetics of ERK activation as indices of upstream responses to stress and potentially as mediators of cellular responses to injury.

Even though it is known that ERK1/2 is activated by oxidative injury, the exact mechanism of activation of MEK and ERK by agents such as cadmium and hydrogen peroxide is not yet well defined. The pathways by which these agents induce MEK and ERK activity may well differ. For instance, H$_2$O$_2$ has been shown to directly stimulate ERK-1 and ERK-2 phosphorylation in human fibroblasts after 5 min of exposure to H$_2$O$_2$, with the activity peaking after 20 min (Ceolotto et al., 2004), via a sequential activation of PKC, Raf-1, and MEK1 (Abe et al., 1998). Cadmium also causes oxidative injury as a secondary effect of its inhibition of a multitude of enzymatic reactions, but does not seem to interfere directly with cellular oxygen metabolism (Jonak et al., 2004).

We do not know the molecular basis for the interspecies differences in the pace of ERK activation in our cell lines. The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptor tyrosine kinases (RTKs) to transcription factors, which in turn regulate gene expression (Kolch, 2005). In some cell systems, activation of ERK1/2 promotes cell survival, while activation of JNK or p38 induces apoptosis (Marshall, 1995). In other cell systems, activation of ERKs can generate pro-apoptotic signals as well (Chandra et al., 2002; Recchia et al., 2004).

ERK activation by 17β-estradiol differs between osteoblasts and osteoclasts; in the former cell type, the effect of the hormone was brief, with return to baseline within 30 min, and protected against apoptosis, while the response of the latter cell type was sustained for at least 24 h and promoted cell death, with corresponding differences in the timing of nuclear accumulation of ERK (Chen et al., 2005). The timing and duration of nuclear accumulation of ERKs are modulated by nuclear phosphatases responsible for ERK dephosphorylation including dual-specificity phosphatases (DUSPs) and MAPK-selective phosphatases (MKP) (Khokhlatchev et al., 1998; Woods & Johnson, 2006). More generally, transient modulation of MAPK (and specifically ERK) activity is thought to reflect complex interactions involving effects of spatially restricted phosphatases and scaffolding proteins, including negative feedback to limit continued stimulation through upstream kinases (Khodolenko & Birtwistle, 2009).

Our data, limited to the first 60 min after stress exposure, do not reveal whether additional oscillations in ERK function might be detected over a longer time course and do not provide insights into species-specific variation in ERK-binding scaffolds or spatial localization of key kinases and phosphatases. Computational models (Bhalla et al., 2002) of growth factor action suggest that variations in a MAPK-specific phosphatase, MKP, can control whether extracellular signals lead to graded ERK responses or alternately to an all-or-none switch between two stable states. Studies of 3T3 cells are consistent with the idea that arachidonic acid derivatives can also provide negative feedback signals on ERK activity by the control of protein kinase C function (Bhalla et al., 2002). Clarification of the potential role of feedback signals in species-specific modulation of ERK kinase, and cellular resistance to lethal stress, will provide attractive topics for additional investigation.

The outcome of c-Src inhibition of osteoclasts is also regulated by the timing of pERK activation (Recchia et al., 2004). In this system, c-Src...
inhibition led to rapid loss of pERK within 15 min, followed by the increase in pERK, 2 h later, to levels above those seen in untreated control cells. Pharmacologic inhibition of this secondary increase in pERK reduced apoptosis. In a third system, activation of c-Fos was shown to require only transient pERK activity in CCL9 hamster fibroblasts, whereas sustained pERK was needed for the activation of JunB and Fra-1 and for cell cycle entry (Balmanno & Cook, 1999).

Similarly, sustained phosphorylation and nuclear localization of ERK regulate S phase entry in Swiss 3T3 fibroblasts cells. Both PDGF and EGF lead, within 10–15 min, to phosphorylated active ERK and translocation of pERK to the nucleus. ERK phosphorylation is transient after EGF exposure, returning to baseline within 30–45 min, while PDGF-induced pERK is sustained for at least 240 min, leading to S phase entry (Murphy et al., 2002).

Each of these experimental systems suggests that modulation of the kinetics of ERK activity and deactivation can have functional consequences, but further work will be needed to evaluate the causes and consequences of differential kinetics of ERK activation in fibroblasts from long-lived and short-lived species. One possibility is that c-Fos may function as a molecular sensor regulating consequences downstream of ERK phosphorylation and nuclear localization (Murphy et al., 2002). Newly synthesized c-Fos protein has a half-life of about 30–45 min (Curran et al., 1984), which can be extended to 2 h or more after ERK-mediated phosphorylation (Chen et al., 1996). Therefore, in cells where ERK is rapidly inactivated, c-Fos is present in the nucleus, but is not phosphorylated, and is therefore unstable and degraded. In contrast, delayed inactivation of ERK can result in an efficient phosphorylation of c-Fos and its stabilization for several hours (Marshall, 1995; New et al., 2001; Murphy et al., 2002). Alteration in the timing of ERK-dependent signals may contribute to species-specific variation in cellular properties in vitro and give clues to cellular properties by which evolution controls aging rate and disease risk across species.

**Experimental procedures**

**Cell culture and conditions**

Fibroblast cultures from 27 bird species and 21 mammalian species were obtained from cells grown from cryopreserved aliquots (P1) stored in liquid N2 at 10⁶ cells mL⁻¹ in DMEM supplemented with 40% fetal bovine serum and dimethyl sulfoxide (DMSO) at a final concentration of 20%. The establishment of the cell lines used in this research has been described previously (Harper et al., 2007, 2011). Cells were grown at 37°C in a humidified incubator with 5% CO₂ in 3% O₂, as there is evidence that exposure to 21% O₂ can lead to early growth crisis relative to cells grown at lower oxygen tension (Busuttil et al., 2007, 2011). Each species was represented by a single donor in this study.

Cells thawed from cryopreserved aliquots were cultured for 7–14 days in Dulbecco’s modified Eagle’s medium (DMEM) [high glucose variant (4.5 mg mL⁻¹), with sodium pyruvate (110 mg mL⁻¹) containing 10 mM HEPES], supplemented with 10% heat-inactivated fetal bovine serum, 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin, and 2% heat-inactivated chicken serum was added for bird cell lines. These cells were then harvested and used for the assessment of ERK activation kinetics.

**Reagents and antibodies**

Cadmium chloride hemi (pentahydrate) (CdCl₂·2H₂O) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). CdCl₂ was dissolved in H₂O, with the pH of 5 µM CdCl₂ at 7.1. FBS was from Invitrogen/GIBCO (Grand Island, NY, USA). Antibodies against phospho-p44/42 ERK (Thr202/Tyr204) and p44/42 ERK were from Cell Signaling Technology (Beverly, MA, USA). β-actin was from Sigma-Aldrich Corp. (St. Louis, MO, USA). Goat anti-rabbit and goat anti-mouse antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Cd and H₂O₂ dosage relative to LD₅₀**

Because bird fibroblasts are more resistant to lethal stress than rodent fibroblasts (Harper et al., 2011), we used different concentrations of cadmium and H₂O₂ for the induction of ERK phosphorylation, depending on the clade. Mammalian cells were exposed to 80 µM cadmium and 100 µM peroxide, in each case corresponding roughly to twice the mean LD₅₀ for rodent species in our previous publication (Harper et al., 2011). For bird cells, we used 140 µM cadmium and 300 µM peroxide, again based on previous estimates of LD₅₀.

**Cellular stress assay**

Cells were placed at 5 × 10⁵ in each well of a six-well plate and incubated overnight in 2 mL of complete DMEM in 3% O₂. On the next day, the medium was replaced with DMEM containing 2% BSA but without serum. The following day, the cells were washed in PBS and placed on ice. Cadmium or peroxide dissolved in DMEM was added, and the culture dish placed at 37°C for intervals of 5–60 min, after which cells were washed once in PBS and harvested for immunoblot analysis.

**Western blot analysis**

Cells were washed with ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 6 mM Na₂HPO₄, and 1 mM KH₂PO₄, pH 7.4) and then harvested in 100 µL of lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton 100, with protease inhibitor cocktail and phosphatase inhibitor cocktails (Sigma-Aldrich Corp.)]. Lysates were centrifuged at 15300 g for 30 min at 4°C, and supernatants collected. Protein concentration was measured using the bicinchoninic acid assay (Pierce Corp., Rockford, IL, USA). The protein samples were mixed with 2× loading buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and heated at 100°C for 5 min. Ten micrograms of total protein was then subjected to SDS polyacrylamide gel electrophoresis and separated according to size using Criterion XT Precast Gel (Bio-Rad Laboratories) for 60 min at 150 V. The resolved proteins were then wet-transferred to nitrocellulose membranes for 1 h at 100 V and blocked with 5% BSA in Tris buffer saline (TBS, pH 7.6) containing 0.05% Tween 20. Proteins were then wet-transferred for 1 h at 100 V at room temperature. The membranes were then washed with TBST and incubated with the primary antibody diluted in the appropriate blocking solution at 4°C overnight with shaking. After incubation, the protein blots were washed five times (15 min each) with TBST and incubated with the appropriate secondary antibody. The protein blots were visualized by the incubation with enhanced chemiluminescence reagent from Pierce (Thermo Scientific, Rockford, IL, USA); the luminescent signals were analyzed using an ImageQuant LAS 4000 scanner from GE Healthcare (Piscataway, NJ, USA). Quantification of immunoblot signals was performed using the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA, USA).

**Statistical analysis**

Statistical analysis was carried out using Logger Pro, using linear regression between the reported maximum lifespan and the level of
phosphorylation of ERK for each of the 27 species for birds and the 21 mammal species. Information on adult body mass and maximum recorded lifespan was obtained from the Age Database of Animal Ageing and Longevity, at http://genomics.senescence.info/species/. Phylogenetic analyses were performed using the method of standardized independent contrasts (Felsenstein, 1985; Garland & Adolph, 1994). Mammalian phylogenies were constructed from the studies of Mercer & Roth (2003), Fabre et al. (2012), and O’Leary et al. (2013). Avian phylogenies were from the study of Jetz et al. (2012).

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Conflict of interest

None of the authors has a conflict of interest to declare.

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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** Phylogenetic relationships among mammalian species.

**Fig. S2** Phylogenetic relationships among avian species.

**Table S1** Taxonomy of birds used in this study, with maximum life span and mass.

**Table S2** Taxonomy of mammals used in this study, with maximum life span and mass.