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Fibroblasts from long-lived mutant mice exhibit increased autophagy and lower TOR activity after nutrient deprivation or oxidative stress

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Summary

Previous work has shown that primary skin-derived fibroblasts from long-lived pituitary dwarf mutants resist the lethal effects of many forms of oxidative and nonoxidative stress. We hypothesized that increased autophagy may protect fibroblasts of Pit-1$^{dw/dw}$ (Snell dwarf) mice from multiple forms of stress. We found that dwarf-derived fibroblasts had higher levels of autophagy, using *LC3* and *p62* as markers, in response to amino acid deprivation, hydrogen peroxide, and paraquat. Fibroblasts from dwarf mice also showed diminished phosphorylation of *mTOR*, *S6K*, and *4EBP1*, consistent with the higher levels of autophagy in these cells after stress. Similar results were also observed in fibroblasts from mutant mice lacking growth hormone receptor (GHRKO mice) after amino acid withdrawal. Our results suggested that increased autophagy, regulated by TOR-dependent processes, may contribute to stress resistance in fibroblasts from long-lived mutant mice.

Key words: autophagy; Snell dwarf; GHRKO; aging; oxidative stress; amino acid deprivation.

Introduction

Single-gene mutations that impair the strength of insulin and IGF-1 signals slow aging and extend lifespan in mice, worms, and flies (McCulloch & Gems, 2003; Partridge *et al.*, 2011; Bartke, 2011), and there are at least six mutations that extend mouse lifespan through modulation of levels or effects of growth hormone (GH) and/or its mediator IGF-1 (Brown-Borg *et al.*, 1996; Zhou *et al.*, 1997; Bartke *et al.*, 2001; Holzenberger *et al.*, 2003; Conover, 2010). Working out the cellular mechanisms that connect altered hormonal signals to delay of aging and multiple late-life diseases is a principal challenge for biological gerontology.

Snell dwarf mice (dw/dw) carry a recessive mutation in the gene encoding a transcription factor, Pit-1, critical to embryonic development of the anterior pituitary. Snell dwarf mice have an abnormal hormone profile characterized by primary deficiencies in growth hormone (GH), thyroid-stimulating hormone (TSH), and prolactin, and consequently secondary deficiencies in insulin-like growth factor I (IGF-1) and thyroxine (Snell, 1929; Bartke, 2011; Dozmorov *et al.*, 2002). Snell dwarf mice, like the closely related Prop1 mutant Ames dwarf and mice lacking the GH receptor (GHRKO or Laron dwarf mice), exhibit extended lifespan and show deceleration of multiple aspects of aging (Bartke, 2011).

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Prior investigations from our laboratory involved analysis of dermal fibroblast cell lines derived from young adult Snell dwarf mice (Salmon *et al.*, 2005; Sun *et al.*, 2009; Leiser & Miller, 2010). These cells were found to be resistant, *in vitro*, to many types of cytotoxic stress, including agents that kill cells at least in part via reactive oxygen species (ROS), such as paraquat (PQ), hydrogen peroxide (H_2O_2), cadmium (Cd), and others that cause cell death through other pathways, including UV light, the DNA alkylating agent methyl methanesulfonate (MMS), and heat. Similar resistance profiles were also seen in fibroblast cells derived from two other related long-lived mutants, the Ames dwarf mouse and the GHRKO mice (Salmon *et al.*, 2005). The mechanism of resistance might involve the NF-E2-related factor 2 (Nrf-2)-sensitive antioxidant genes and mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated protein kinases (Erk) pathway (Sun *et al.*, 2009; Leiser & Miller, 2010). A good deal of evidence indicates that autophagy may help to maintain cellular homeostasis and cell survival (Reggiori & Klionsky, 2002; Scott *et al.*, 2011). The three types of long-lived dwarf mice all abrogate the strength of GH signals and diminish plasma levels of IGF-1 and insulin. These alterations in hormones and metabolites may regulate autophagy via the mTOR pathway (Sobolewska *et al.*, 2008). Thus, we considered the idea that dwarf-derived cells might show altered control of autophagy with or without stress exposure *in vitro*.

Autophagy is a catabolic lysosome-dependent process for the degradation of misfolded proteins and damaged organelles such as mitochondria (Reggiori & Klionsky, 2002). Autophagy is maintained at a basal level in most tissues benefiting the balance of cellular homeostasis (Scott *et al.*, 2011). During aging, production of reactive oxygen species (ROS) increases, resulting in elevated levels of oxidative stress, with subsequent cellular stress responses (Finkel & Holbrook, 2000). Increased ROS or oxidative stress response has been shown to induce autophagy (Scherz-Shouval *et al.*, 2007). The primary functions of autophagy are housekeeping and quality control of proteins and organelles. Autophagy can promote cell survival by recycling amino acids and fatty acids for energy utilization during nutrient withdrawal and by removal of oxidized proteins or damaged organelles during stress. Alternatively, autophagy can lead to cell death if cells are severely damaged. Multiple lines of evidence have demonstrated that the efficiency of autophagic degradation declines with aging and contributes to accumulation of intracellular waste (Brunk & Terman, 2002; Cuervo *et al.*, 2005). The accumulation of impaired macromolecules and organelles, a consequence of impaired autophagy, is a frequent characteristic of age-related change in many cell types. Some findings have suggested that modulation of autophagy might contribute to preserved cellular function in several models of delayed aging and extended longevity. In mice, damaged proteins and organelles accumulate in cytoplasm as a consequence of manipulations that knock down function of several autophagy-related genes (Komatsu *et al.*, 2006). In *Caenorhabditis elegans*, abrogation of autophagy abolished lifespan extension of *daf-2* mutants (Meléndez *et al.*, 2003). In Drosophila, adult mutants lacking autophagy-related gene 7 (Atg7$^{-/-}$) are short-lived and hypersensitive to nutrient and oxidative stress (Juhász *et al.*, 2007). SIRT1, mTOR, Foxo3, NF-kB, and P53, each of which is thought to have a role in longevity determination, all modulate

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autophagy, although the connections between autophagy and aging are still not well-defined in mammals (Salminen & Kaarniranta, 2009). Rapamycin, which activates autophagy by down-regulating the mTOR pathway, enhances mouse lifespan (Harrison *et al.*, 2009).

These findings in *C. elegans*, Drosophila, and mammals suggest an important role for autophagy as a regulator of the aging process and cellular stress responses, but little is known about autophagic function in mouse mutants in which longevity is increased by reduction of pathways controlled by GH and/or IGF-1. In our study, we tested the hypothesis that fibroblasts grown from Snell dwarf or GHRKO mice might have enhanced autophagy when subjected to oxidative stress or deprivation of amino acids. We also evaluated mTOR/S6k pathways, which regulate autophagy, in fibroblast cells from mutant and control mice.

Results

Increased autophagy in fibroblasts derived from Snell dwarf mice after amino acid deprivation

We evaluated autophagy in fibroblasts grown from Snell dwarf and control mice by measuring isoforms of LC3II, with and without bafilomycin A1 as an inhibitor of lysosomal degradation of LC3II. One hour of incubation in medium without amino acids induced autophagy in fibroblasts of both Snell dwarf and littermate control mice, with higher levels of LC3II accumulation in the cells from the Snell dwarf mice (Fig. 1A,C). Bafilomycin A1 increased levels of LC3II in both cell types, again with higher levels in dwarf-derived compared with control cells (Fig. 1A,C). An evaluation using immunofluorescence to quantitate LC3 punctae showed a similar pattern of results (Fig. S1). To confirm these findings, we used an assay based on levels of the ubiquitin binding protein p62, which binds to autophagosomal membrane protein LC3/Atg8. Lysosomal degradation of autophagosomes leads to a decrease in p62 levels during autophagy (Bjørkøy *et al.*, 2005). Consistent with the LC3 results, cells from Snell dwarf mice showed increased degradation of p62 after amino acid withdrawal (Fig. 1B,D).

Increased autophagy in fibroblasts derived from Snell dwarf mice after exposure to oxidative stress inducers H_2O_2 and paraquat

Cells were washed once with PBS and cultured in serum-free medium [Dulbecco's modified Eagle's medium (DMEM) with 2% BSA] for 14–16 h prior to initiating oxidative stress treatments. H_2O_2, PQ, or Cd was then added, with or without bafilomycin A1, in fresh serum-free DMEM for 2 h prior to the measurement of LC3II or p62 levels (Fig. 2). Peroxide induced higher levels of LC3II in dwarf cells, compared with control cells, at each tested dose and regardless of bafilomycin treatment; the results were statistically significant except at the higher peroxide dose without bafilomycin. The level of LC3II was higher in bafilomycin-exposed dwarf cells than in control cells even prior to peroxide treatment (Fig. 2A,B). Similarly results were observed using PQ as a source of oxidative stress (Fig. 2E,F), with significant differences noted at either

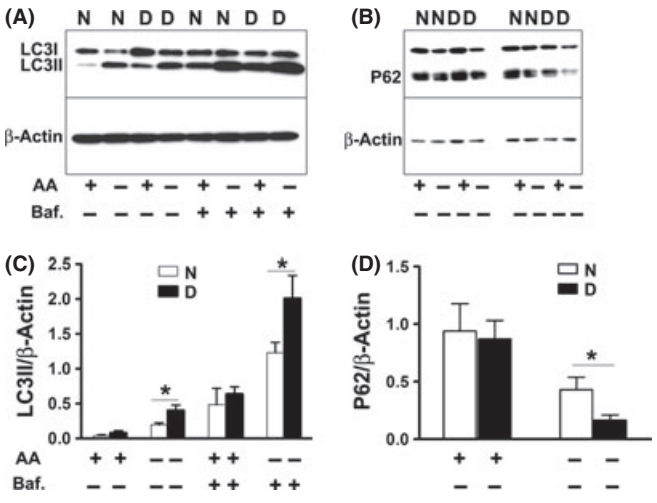

Fig. 1 LC3II and p62 protein levels after amino acid deprivation in fibroblasts from Snell dwarf and control mice. Cells were incubated with minimum essential medium (MEM) supplemented with 10% dialyzed fetal bovine serum (FBS). Amino acid deprivation was initiated by replacing the medium with Earle's balanced salt solution (EBSS) including 10% dialyzed FBS and vitamin mix for 1 h. Autophagic flux was studied by measuring LC3II with and without the presence of bafilomycin A1 at 10 nM. Abbreviations: N, normal cells; D, Snell dwarf cells; AA+, amino acids present; AA–, amino acids absent; Baf., bafilomycin. $N = 7$, Mean ± SEM, $^*P < 0.05$.

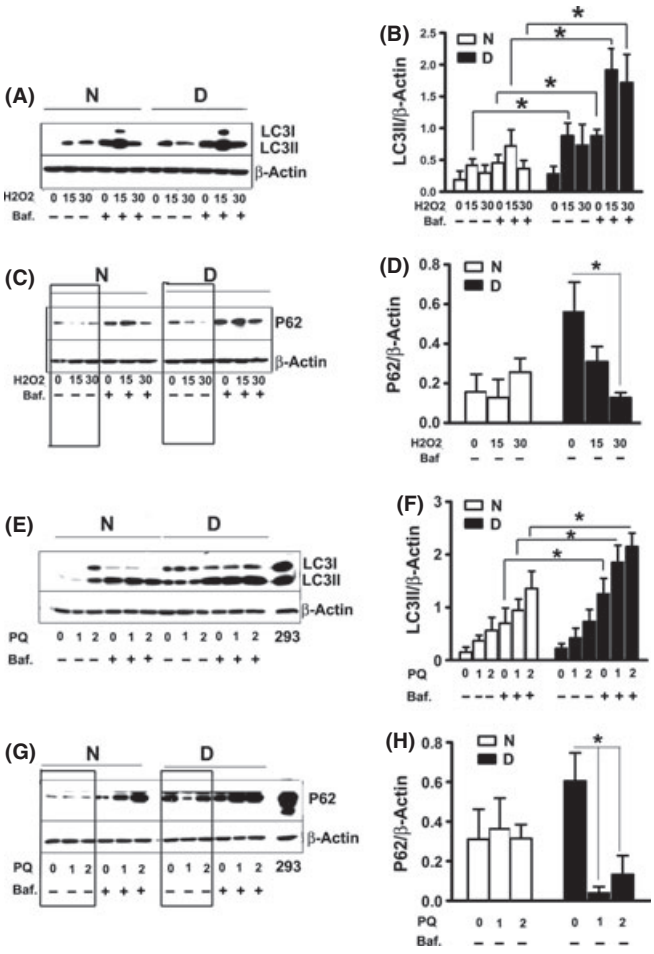

Fig. 2 LC3II and p62 protein levels under oxidative stress conditions in fibroblasts from Snell dwarf and control mice. Cells were grown in medium without serum [Dulbecco's modified Eagle's medium (DMEM) with 2% BSA] for 14–16 h prior to oxidative stress. Then, cells were treated by H_2O_2 (15 μM or 30 μM) or paraquat (PQ, at 1 mM or 2 mM) for 2 h. LC3II and p62 were measured by immunoblotting. Bafilomycin A1 at10 nM was added to some cultures as indicated. Human embryonic kidney 293 cell lysate was used as a positive control in panels G and H. $N = 8$, mean ± SEM, $^*P < 0.05$.

PQ dose in the presence of bafilomycin. A similar pattern was also seen using LC3 immunofluorescence (Fig. S2). P62 was also evaluated in parallel for further confirmation. Prior to stress exposure, dwarf fibroblasts had higher p62 levels, but exposure to H_2O_2 or PQ, in the absence of bafilomycin, led to a decline in p62 levels, an index of autophagy, mainly in the dwarf cells (Fig. 2C,D,G,H). Neither cells from Snell dwarf mice nor cells from control mice showed any accumulation of LC3II after exposure to cadmium over a range of 1–20 μM (data not shown).

Diminished phosphorylation of mTOR, S6K, and 4EBP1 in Snell dwarf fibroblasts after amino acid deprivation

The mTOR signaling pathway is known to regulate autophagy, and inhibition of mTOR results in the activation of autophagy (Ravikumar *et al.*, 2004). We therefore evaluated the phosphorylation of mTOR and two of its substrates, S6K and 4EBP1, to see whether these might contribute to the increased tendency of dwarf cells to initiate autophagy after amino acid reduction. In control cells, removal of amino acids led to little if any change in mTOR phosphorylation and a modest decline in the phosphorylation of S6K and 4EBP1 (Fig. 3). Cells from dwarf mice, in contrast, showed a significant decline in mTOR phosphorylation and a more dramatic decline in the phosphorylation of S6K and 4EBP1 as compared to control cells. Dwarf and control cells showed no difference in the total amount of mTOR protein. Interestingly, addition of bafilomycin during amino acid deprivation further decreased the levels of phosphorylation of mTOR, S6K, and 4EBP1 in both control and dwarf fibroblasts.

Diminished phosphorylation of mTOR/S6K/4EBP1 in fibroblasts derived from Snell dwarf mice exposed to H_2O_2

The phosphorylation of mTOR and its two substrates S6K and 4EBP1 was evaluated after exposure to 30 μM H_2O_2 for 30 or 60 min (Fig. 4). Prior to H_2O_2 exposure, levels of phosphorylated mTOR were higher in dwarf fibroblasts than in controls, presumably due to overnight incubation in serum-free medium prior to peroxide exposure. Consistent with this observation, levels of p-4EBP1 were higher in dwarf than in control cells prior to H_2O_2 exposure, and p-S6K showed a similar, though nonsignificant, trend. Peroxide exposure for 60 min increased mTOR phosphorylation in the control cells, but did not change mTOR phosphorylation in the dwarf cells, so that levels of phospho-mTOR were higher in control than in dwarf cells after 60-min exposure. We saw no difference in total levels of mTOR between cells derived from dwarf as compared to control cells. Exposure to 30 μM H_2O_2 for 30 min enhanced phosphorylation of S6K in cells from both Snell dwarf and control mice, but the response in control cells was more robust (Fig. 4C,D). Control cells, but not dwarf cells, also showed higher levels of 4EBP1 phosphorylation after 60 min of exposure to 30 μM H_2O_2 (Fig. 4E,F).

Increased autophagy in fibroblasts derived from GHRKO mice after amino acid deprivation

To see whether similar effects were produced in cells from a different long-lived, stress resistant mouse stock, we evaluated autophagy in cells from GHRKO mice after withdrawal of amino acids. In contrast to cells from Snell dwarf mice (Fig. 1), 1-h amino acid withdrawal without

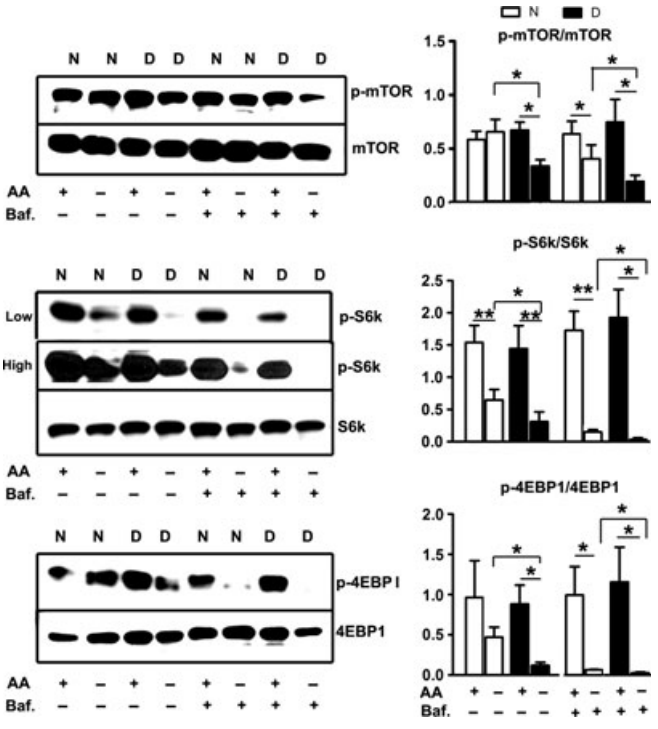

Fig. 3 Phosphorylation of mTOR, S6K, and 4EBP1 after amino acid deprivation in fibroblasts from Snell dwarf and control mice. Cells were deprived of amino acids for 1 h. Phosphorylation and total protein levels of mTOR, S6K, and 4EBP1 were evaluated by Western blot. $N = 6$, mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$.

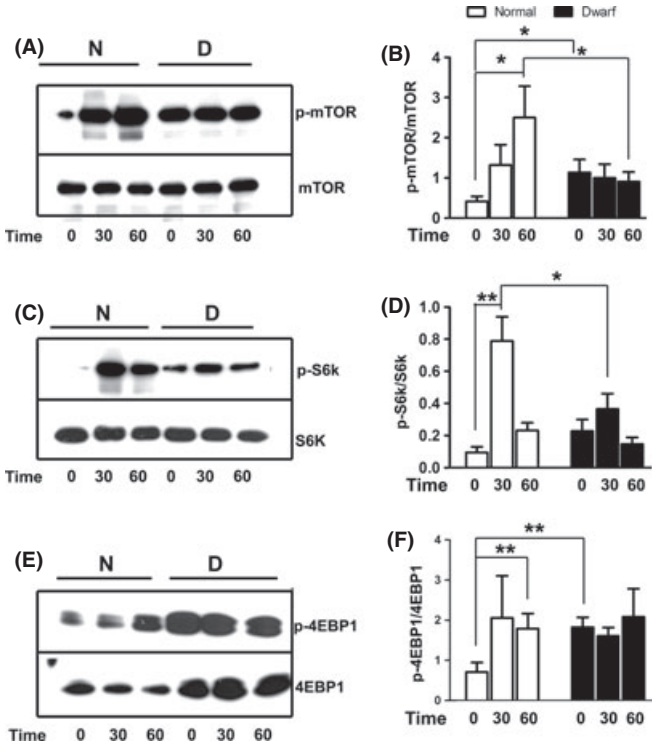

Fig. 4 Phosphorylation of mTOR, S6K, and 4EBP1 after exposure to H_2O_2 in fibroblasts from Snell dwarf and control mice. Cells were cultured overnight in serum-free medium and then challenged by 30 μM H_2O_2 for 0, 30, or 60 min. Phosphorylation and total protein levels of mTOR, S6K, and 4EBP1 were examined. $N = 6$, mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$.

bafilomycin did not lead to a pronounced increase in LC3II accumulation either in control or in GHRKO cells, perhaps as a result of more robust lysosomal degradation of LC3II in this system. When autophagic flux was evaluated after the addition of bafilomycin, LC3II accumulation was enhanced both in control and in GHRKO cells and was higher in the GHRKO cells (Fig. 5), consistent with the results seen in Snell dwarf cells (Fig. 1). A similar pattern of results was seen in the immunofluorescence system (Fig. S3). Analysis of P62 levels (Fig. 5) supported the conclusion that amino acid withdrawal leads to stronger induction of autophagy in fibroblasts from GHRKO mice than in control cells.

Diminished phosphorylation of S6K and 4EBP1 in GHRKO fibroblasts after amino acid deprivation

In parallel, we also evaluated mTOR signaling pathways in cells from GHRKO mice (Fig. 6). mTOR phosphorylation was significantly lower in GHRKO cells even prior to amino acid withdrawal. Amino acid starvation for 1 h decreased mTOR phosphorylation in control and KO cells, but no significant difference was seen between control and KO cells after amino acid withdrawal. As expected, incubation without amino acids led to lower levels of phosphorylation of both S6K and 4EBP1, and this decline was significantly more dramatic in GHRKO KO, consistent with the observations on cells from Snell dwarf mice (Fig. 3).

Discussion

We show here that fibroblasts from Snell dwarf mice differ from controls in several ways: (i) dwarf cells show more vigorous induction of autophagy in response to amino acid deprivation and to oxidant stress; (ii) amino acid withdrawal induces a more dramatic decline in the phosphorylation of mTOR and its substrates in dwarf-derived cells; and (iii) dwarf cells are more resistant than controls to mTOR activation induced by exposure to hydrogen peroxide. Consistent results were seen using three methods: LC3II accumulation, p62 reduction, and immunofluorescent assessment of LC3 punctae. Similar results were seen using fibroblasts from another long-lived mouse with altered GH and IGF-1 signals, the GHRKO mutant. These observations are consistent with a model in which *in vivo* exposure to the altered hormonal milieu of the Snell dwarf and GHRKO mice leads to long-lasting epigenetic changes, retained in fibroblast cells *in vitro*, that increase their resistance to multiple forms of lethal stress (Salmon *et al.*, 2005), augment plasma membrane redox pumps, and other Nrf-2-sensitive defences (Leiser & Miller, 2010) and enhance expression of some ERK-dependent immediate early genes despite the lower induction of ERK1/2 phosphorylation (Sun *et al.*, 2009).

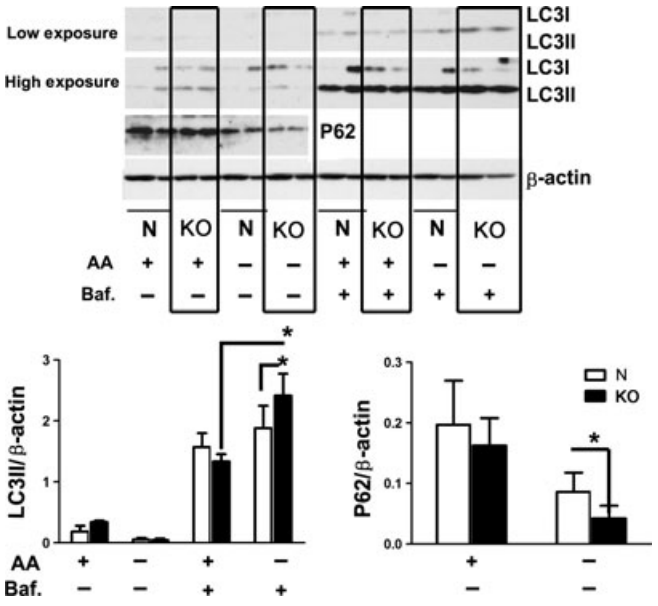

Fig. 5 LC3II and p62 protein levels after amino acid deprivation in fibroblasts from GHRKO and control mice. Amino acid deprivation was performed by incubating the cells with EBSS including 10% dialyzed FBS and vitamin mix for 1 h. Autophagic flux was studied by measuring LC3II with and without the presence of bafilomycin A1 at 10 nM. Abbreviations: N, normal cells; KO, growth hormone receptor knockout cells; AA+, amino acids present; AA–, amino acids absent; Baf., bafilomycin; EBSS, Earle's balanced salt solution; FBS, fetal bovine serum. $N = 4$, mean ± SEM, $^{*}P < 0.05$.

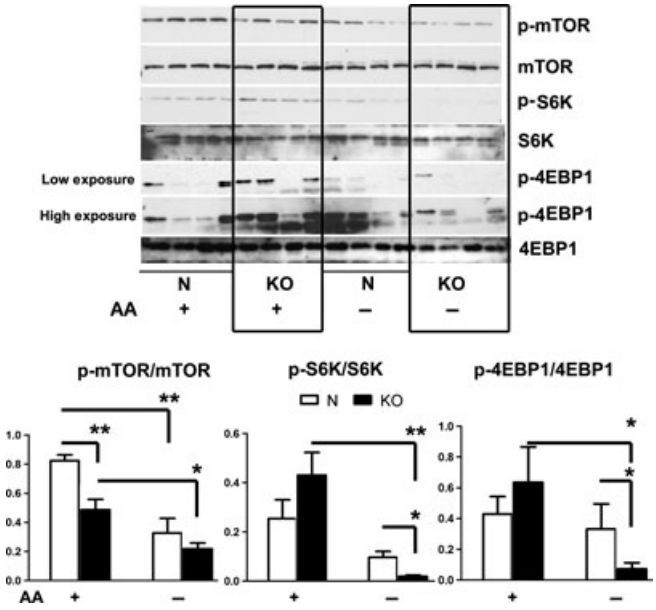

Fig. 6 Phosphorylation of mTOR, S6K, and 4EBP1 after amino acid deprivation in fibroblasts from GHRKO and control mice. Cells were deprived of amino acids for 1 h. Phosphorylation and total protein levels of mTOR, S6K, and 4EBP1 were evaluated by Western blot. $N = 4$, mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

Autophagy serves housekeeping functions by self-digestion of long-lived proteins and organelles and is essential for maintaining healthy cells. Once autophagy is initiated, cytoplasmic materials become enclosed in a double-membrane structure, which subsequently fuses with a lysosome leading to the degradation of damaged or unwanted components. The formation of autophagosomes requires a process that is called light chain 3 (LC3) modification. During this process, LC3II is formed by lipidation of LC3I, an ortholog of yeast Atg8, and becomes incorporated into the autophagosomal membrane. Thus, conversion from LC3I to LC3II has been used as an index of autophagy (Kabeya *et al.*, 2004). However, LC3II itself is degraded by autophagy, and therefore, it is often helpful to measure autophagic flux by comparing the amount of LC3II in the presence and absence of lysosome protease inhibitors such as bafilomycin A1, a specific inhibitor of vacuolar-type (H^{+}) ATPase (Mizushima & Yoshimori, 2007). P62, also named sequestosome 1 (SQSTM1), binds to LC3 and is involved in the navigation of polyubiquitinated protein aggregates to the autophagic machinery (Bjørkøy *et al.*, 2005). The level of p62 can also be used as an index of autophagy, because autophagic degradation of p62 in autophagosomes leads to decline in p62 levels, and conversely, autophagy inhibitors stabilize p62 levels. Accumulation of LC3 into lysosomes can be

evaluated by immunofluorescence microscopy, providing a third index of induced autophagy (Klionsky *et al.*, 2008). Although each of these autophagy indicators is potentially subject to different forms of technical artifacts, the agreement among all of these assay systems increases our confidence that fibroblasts from dwarf mice are indeed more prone to autophagy than control cells in response to both amino acid withdrawal and oxidative stress.

Insufficient autophagy is thought to be at least partly responsible for a range of age-related diseases (Brunk & Terman, 2002; Cuervo *et al.*, 2005). Levels of expression of the autophagy-related gene Atg7 and of LC3 protein exhibit a significant decline, as levels of p62 and polyubiquitin accumulate concomitant with decreased autophagy in aging rat kidney (Cui *et al.*, 2012). Similar phenomena have been documented in liver and thymus of aging mice (Uddin *et al.*, 2012). Abolishment of several autophagy-related genes results in increased aggregates of damaged proteins and organelles in mice (Komatsu *et al.*, 2006), abrogates lifespan extension in *daf-2* mutants of *C. elegans* (Meléndez *et al.*, 2003), and shortens the lifespan in Drosophila adults of stocks hypersensitive to nutrient and oxidative stress (Juhász *et al.*, 2007). Many of the pathways implicated in the control of lifespan in invertebrates, mice, and humans, including SIRT1, mTOR, Foxo3, NF-kB, and P53, are known to modulate autophagy (Salminen & Kaarniranta, 2009). Similarly rapamycin, an activator of autophagy, extends lifespan from *C. elegans* to mammals (Harrison *et al.*, 2009; Bjedov *et al.*, 2010), suggesting that manipulation of autophagy may be able to provide insights into the molecular mechanism of aging processes.

Previous studies have also demonstrated a link between autophagy and oxidative stress. Cellular oxidative stress and increased generation of reactive oxygen species (ROS) have been identified as positive regulators of autophagy, which may lead the cell either to survival or to death (Scherz-Shouval *et al.*, 2007). Induced autophagy by oxidative stress contributes to the removal of damaged oxidized proteins and supports cell survival (Xiong *et al.*, 2007). Conversely, autophagic cell death occurs when oxidative stress is so severe that repair cannot compensate for cellular damage (Kirkland *et al.*, 2002). Our results with peroxide and paraquat suggest that higher levels of autophagy may act as a rescue mechanism that cells from Snell dwarf mice can use to escape from cell death induced by oxidative stressors.

Amino acid withdrawal is a well-known inducer of autophagy. Through autophagy, amino acids are recycled or re-utilized, promoting cell survival (Mortimore & Schworer, 1977). In our experiment, as expected, amino acid withdrawal stimulated autophagy in cells from both Snell dwarf and control mice. Strikingly, more robust autophagy activities were observed in Snell dwarf-derived cells when amino acids were removed, suggesting that these cells may be prone to autophagy whether induced by nutrient or oxidative stress. Previous work from this laboratory has shown that fibroblasts from Snell dwarf mice are resistant to metabolic changes induced by incubation in medium with very low glucose levels (Leiser *et al.*, 2006), probably reflecting differences in Nrf2-dependent regulation of the plasma membrane redox system (Leiser & Miller, 2010). Studies from other groups also demonstrated the involvement of autophagy in the modulation of cell death after oxidative stress, including studies of Atg7 mutation in Drosophila exposed to starvation or oxidative stress. The mutant flies are shorter lived and show progressive decline of neuronal function, accumulated ubiquitin-positive particles in the brain, and have increased autophagic cell death (Juhász *et al.*, 2007).

Target of rapamycin (TOR), an evolutionarily conserved serine/threonine kinase, is a negative regulator of autophagy (Ravikumar *et al.*, 2004). Reduction of TOR signaling has been implicated in lifespan extension in the context of lower protein biosynthesis (Hansen *et al.*, 2007). mTORC1 affects cell growth and translation via at least two important substrates: the ribosomal p70S6 protein kinase (S6K) and the eukaryotic initiation factor 4E-binding protein 1 (4EBP1) (Sonenberg & Hinnebusch, 2009). As an index of TOR pathway activation, we evaluated the phosphorylation of mTOR on Ser2448 after amino acid deprivation and found dephosphorylation of mTOR only in Snell dwarf cells. Bafilomycin-mediated inhibition of lysosomal activity increased the sensitivity of TOR Ser2448 to amino acid withdrawal, but did not abrogate the difference between dwarf and control cells. Dwarf fibroblasts also showed a more pronounced decline in the phosphorylation of both S6K and 4EBP1 after withdrawal of amino acids, consistent with the data on TOR phosphorylation. Similar results were noted after amino acid withdrawal using cells from GHRKO mice. Diminution of mTOR signaling pathways in response to nutrient deficits is considered to be an adjustment to physiological states that require cell maintenance and repair (Foster & Fingar, 2010), and so it seems likely that the more pronounced decline of mTOR signals seen in dwarf-derived cells may contribute to the enhanced autophagy seen in these cells after amino acid withdrawal. Inhibition of autophagy by bafilomycin rendered both dwarf and control cells more sensitive to the effects of amino acid withdrawal on the phosphorylation of mTOR, S6K, and 4EBP1. This observation implies that interplay between mTOR signaling and autophagy includes a positive feedback loop that amplifies autophagy activity.

Phosphorylation of mTOR, S6K, and 4EBP1 was also evaluated in cells treated with H_2O_2. mTOR/S6K/4EBP1 signaling activities were intensified in cells from control mice by H_2O_2, but these responses to oxidant stress were blunted in Snell dwarf cells. Lower levels of TOR function after peroxide stress may also contribute to higher autophagy (Fig. 2) and diminished cell death (Salmon *et al.*, 2005) in Snell dwarf fibroblasts confronted with oxidant stress.

Our data thus suggest that the stimulation of autophagy-mediated clearance of damaged cellular components may protect Snell cells from various lethal and nonlethal sources of stress, perhaps through changes in baseline and/or stimulated levels of TOR signals. The stress resistance of primary fibroblast cultures grown from long-lived Snell dwarf mice presumably reflects long-lasting epigenetic changes induced, *in vivo*, by maturation of fibroblast precursor cells in a postnatal environment lacking GH and IGF-1. Differences in stress resistance between Snell dwarf and control-derived cells are not apparent when cultures are established from mice that are only 7 days old (Salmon *et al.*, 2005) and can be prevented or reversed by injecting Ames dwarf mice with GH initiated at 2 weeks of age (Panici *et al.*, 2010). It remains to be seen to what extent the differences seen in cultured fibroblasts also affect cells of other lineages in living mice, although there is evidence (Sun *et al.*, 2011) that some of the differences in stress-kinase function seen in cultured cells also affect liver cells in mice before and after exposure to diquat. Further studies of autophagy and its links to TOR function in intact mice may shed light on the cellular basis of disease resistance and longevity in pituitary dwarf mice.

Experimental procedures

Animals

Snell dwarf (dw/dw) mice and heterozygote (dw/+) controls were bred as the progeny of (DW/J × C3H/HeJ)F1 dw/+ males and (DW/J × C3H/HeJ)F1 dw/+ females. GHRKO mice and normal littermate controls were produced by mating heterozygous (+/–) carriers of the disrupted GHR/GHBP gene or by mating homozygous knockout (–/–) males with (+/–) females. Both male and female tail skin biopsies were taken from 4- to 6-month-old mice as in our previous work. Mutant and control mice were gender matched within each experiment.

Primary and secondary cell cultures

Tail skin biopsies (1.5–2 cm) were obtained, and primary fibroblast cells were cultured as previously described (Salmon *et al.*, 2005). Briefly, skin samples were rinsed, minced, and digested overnight in collagenase type II (400 U mL^{-1}; Gibco-Invitrogen, Carlsbad, CA, USA) dissolved in Dulbecco's modified Eagle's medium (DMEM) at 37 °C with 5% CO_2 in air. Then, cells were dislodged, centrifuged, resuspended in DMEM with serum, and seeded at approximately 2.5×10^5 cells in 5 mL of medium into tissue culture flasks of 25-cm^2 surface area. These initial cultures were considered 'passage 0'. Cells were fed at day 3 with the replacement of two-thirds of the medium and then at day 7 were removed by trypsinization and transferred to 75-cm^2 flasks at 7.5×10^5 cells/flask in 12 mL complete medium ('passage 1'). This process was repeated, and cells at the third passage were plated at a confluent density of 10^5 cells/cm^2 for the assessment of responses to oxidative and nutritional stresses.

Antibodies

The following antibodies were used: LC3B antibody with catalog #2775, p62/SQSTM1 antibody with catalog #p0067, p-mTOR (S2448) with catalog #2971, mTOR with catalog #2972, p-p70 S6 kinase (T389) with catalog #9205, and p70 S6 kinase with catalog #9202 are from Cell Signaling Technology, Inc. (Danvers, MA, USA); p-4ebp1 (Ser 65/Thr 70) with catalog #Sc-12884-R, 4ebp1 with catalog #Sc-6936, goat anti-rabbit IgG-HRP with catalog #Sc-2301, and β-actin conjugated with horseradish peroxidase (HRP) with catalog # Sc-47778 HRP are from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell treatments

For the amino acid deprivation assay, the growth medium was replaced by fresh minimum essential medium (MEM), with 10% dialyzed fetal bovine serum (FBS) 24 h before assay. Amino acid deprivation was achieved by replacing the MEM with Earle's balanced salt solution (EBSS) supplemented with vitamin mix and dialyzed FBS for the indicated times (Kaushik *et al.*, 2008). For H_2O_2, paraquat (PQ), and cadmium (Cd) treatment, cells were washed once with PBS and cultured in medium without serum (DMEM with 2% BSA) for 14–16 h. The cells were then exposed to fresh serum-free DMEM containing H_2O_2, PQ, or Cd for the indicated times and doses (Hariharan *et al.*, 2011). Bafilomycin A1 (10 nM), a lysosomal inhibitor, was added to the medium where indicated to compare steady-state autophagic activity with measures of cumulative LC3-II concentrations (Mizushima & Yoshimori, 2007).

Immunoblots

Lysates for Western blots were prepared in radioimmuno-precipitation assay (RIPA) buffer (Leiser & Miller, 2010). Lysates were collected and stored at −80 °C prior to analysis. LC3 levels were evaluated using SDS–PAGE gels containing 12% acrylamide, and p-mTOR, mTOR, p-S6K, and S6K blots were run on SDS–PAGE gels containing 6% acrylamide (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to an Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA). Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% (w/v) nonfat dry milk (Bio-Rad) for 1 h. After blocking, membranes were probed overnight with primary antibodies with shaking at 4 °C, followed by incubation with secondary antibody for 1 h, with shaking, at room temperature. Antibody detection was accomplished with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Quantification was performed using IMAGEQUANT software (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

Statistical analyses were performed using paired *t*-tests (two-tailed) for one-factor comparisons such as comparisons of treated cells with control cells. Analysis of variance (ANOVA) was used to compare effects and interactions of two factors, for example genotype and treatment. Graphs show the mean of combined experiments, and error bars represent the standard error of the mean.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1 Immunofluorescence assay.

Fig. S1 Fluorescent analysis of LC3 punctae of fibroblasts from Snell dwarf and control mice under amino acid deprivation.

Fig. S2 Fluorescent analysis of LC3 punctae of fibroblasts from Snell dwarf and control mice under oxidative stress conditions.

Fig. S3 Fluorescent analysis of LC3 punctae of fibroblasts from GHRKO and control mice under amino acid deprivation.

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