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The p66^{Shc} knockout mice are short lived under natural condition

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Summary

Deletion of the p66^{Shc} gene results in lean and healthy mice, retards aging, and protects from aging-associated diseases, raising the question of why p66^{Shc} has been selected, and what is its physiological role. We have investigated survival and reproduction of p66^{Shc}–/– mice in a population living in a large outdoor enclosure for a year, subjected to food competition and exposed to winter temperatures. Under these conditions, deletion of p66^{Shc} was strongly counterselected. Laboratory studies revealed that p66^{Shc}–/– mice have defects in fat accumulation, thermoregulation, and reproduction, suggesting that p66^{Shc} has been evolutionarily selected because of its role in energy metabolism. These findings imply that the health impact of targeting aging genes might depend on the specific energetic niche and caution should be exercised against premature conclusions regarding gene functions that have only been observed in protected laboratory conditions.

Key words: aging genes; environment; fat; fertility; fitness; survival.

Introduction

P66^{Shc} is a vertebrate protein (Luzi *et al.*, 2000) whose deletion in mice (p66^{Shc}–/–) induces resistance to obesity (Berniakovich *et al.*, 2008; Ranieri *et al.*, 2010), atherosclerosis (Napoli *et al.*, 2003; Graiani *et al.*, 2005), ischemic injury (Zaccagnini *et al.*, 2004; Carpi *et al.*, 2009), and diabetes (Menini *et al.*, 2006; Fadini *et al.*, 2010; Ranieri *et al.*, 2010). P66^{Shc} regulates the intracellular redox balance (Nemoto & Finkel, 2002) and related processes, including apoptosis and cellular growth (Giorgio *et al.*, 2005). It increases intracellular levels of reactive oxygen species (ROS) by enhancing ROS production by mitochondria (Giorgio *et al.*, 2005; Pinton *et al.*, 2007) and plasma membrane oxidases (Khanday *et al.*, 2006), and inhibiting expression of ROS-scavenging enzymes (Nemoto & Finkel, 2002). Notably, oxidative stress is reduced in p66^{Shc}–/– mice (Napoli *et al.*, 2003; Menini *et al.*, 2006; Carpi *et al.*, 2009) and they showed extended lifespan (Migliaccio *et al.*, 1999).

The multiple benefits of p66^{Shc} deletion raise the question of how this gene has been selected during evolution and what is its physiological function. Testing subtle impairments of biological fitness under laboratory conditions, however, is a difficult task, particularly when the relevance of the gene for survival under natural conditions is unknown. Walker *et al.* (2000) have demonstrated that during starvation-cycles mimicking field condition, a mutation that greatly increased the lifespan of the nematode *Caenorhabditis elegans* resulted in a fitness cost. Yet, scarce information is available on the early-life fitness effects of mutations that delay aging. To address these questions, we have analyzed the effects of p66^{Shc} deletion on early-life fitness by exposing mice to natural selection under outdoor conditions in a harsh environment.

Results

P66^{Shc} deletion is counterselected in wild-mimicking condition

As only hybrid populations of different laboratory mouse strains survive and expand in natural conditions, probably owing to segregation of adverse genetic factors in inbred mouse strains (Lewejohann *et al.*, 2004), we produced a hybrid population of F1 mice by crossing heterozygous mutant mice maintained on either a C57BL/6 or a 129 background. Mice were housed in a specific pathogen-free animal facility at the European Institute of Oncology (Milan, Italy) until they reached the age of 3–4 months. At that time, mice were genotyped, transferred to Russia, and released in a large outdoor pen at the biological station of 'Chisty Les' in the western Tver province (Abbott, 2007) (more details are provided in the Experimental Procedures section). They lived and reproduced in a 20 × 20 m enclosure containing a protected shelter (2 × 2 m, 1 m deep) filled with hay, planks, and branches and had the opportunity to roam the entire enclosure. Mice were provided with standard laboratory mouse-food, delivered twice a week through a food hopper in the shelter, and drinking water from bottles replenished every second day. Environmental temperature varied from +30°C in summer to −20 °C in winter. Except for the regular supply visits, mice were left unattended and were exposed to avian predators (terrestrial predators were barred from the pen by an electrical cattle fence mounted on the pen walls).

A total of 61 3- to 4-month-old mice were released in the pen in August 2008, including 22 p66^{Shc}+/+ (14 females and eight males), 20 p66^{Shc}+/– (12 females and eight males), and 19 p66^{Shc}–/– mice (11 females and eight males). Eight months later (April 2009), after snow melt, 62 mice (eight of which founders) were captured, subjected to a tail biopsies, and released again. Analysis of genotypes revealed that 35 (including four founders) of the 62 mice were p66^{Shc}+/+, 17 (three

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founders) p66^{Shc}+/–, and 10 (only one founder) p66^{Shc}–/–, (Fig. 1A), of either sex. These data indicate that our laboratory-derived population could survive a winter with several weeks of temperatures below zero and suggest the existence of a selection effect against the mutated allele (binomial test: $P < 0.01$).

At the beginning of September 2009, 13 months after the release of the founders, all mice in the pen (101 animals) were recaptured, carefully inspected, and genotyped. They appeared healthy and without external parasites. Comparison of the numbers of mice released in July 2008 and recaptured in September 2009 showed that the colony of p66^{Shc}+/+ mice had expanded approximately 3-fold (from 22 to 74 members, including three founders, 34 adults, and 37 sub-adults), the p66^{Shc}–/– decreased of approximately 4 fold (from 19 to 5 members: all adults and not including founders), while the number of p66^{Shc}+/– mice remained near constant (from 20 to 22 members: one founder, eight adults, and 13 sub-adults). No significant difference in sex ratio was observed between founders and recaptured mice in any of the three genotypes (Fig. 1B). In contrast, we observed a similar percentage of survivors of the three genotypes (p66^{Shc}+/+: 45% males and 40% females; p66^{Shc}+/–: 42% and 41%; p66^{Shc}–/–: 47% and 46%) in an aged-matched colony of 105 F1 C57/129 hybrids (p66^{Shc}+/+: 17 males and 18 females; p66^{Shc}+/–: 18 and 17; p66^{Shc}–/–: 18 and 17) maintained in our animal facility from summer 2008 up to now.

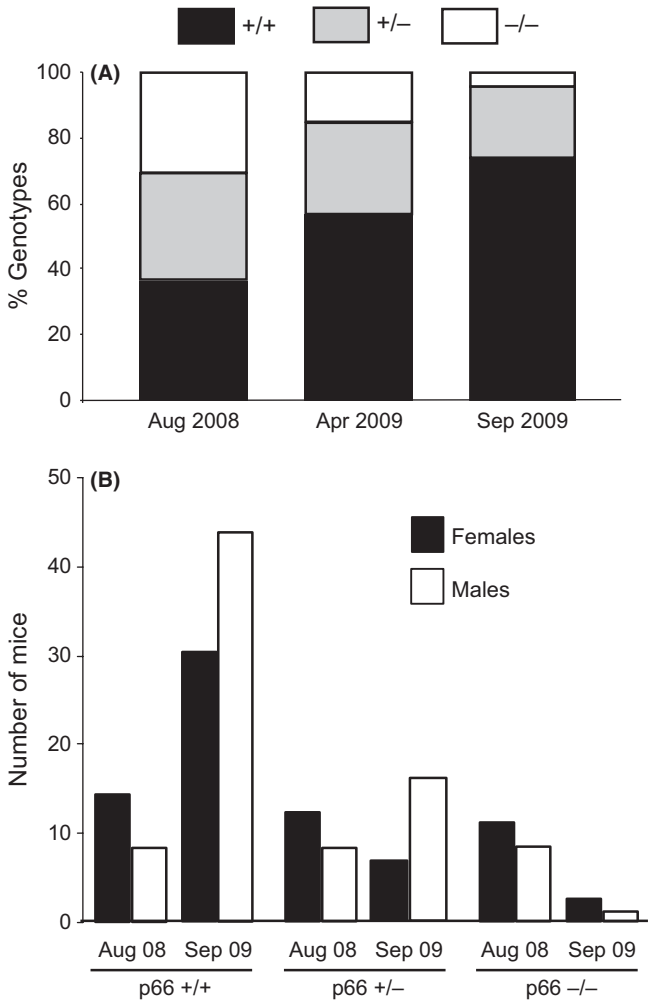

Fig. 1 Natural selection of p66^{Shc}–/– mice. P66^{Shc} allelic frequencies (A) and population sizes (B) at the release of mice (August 2008), after 8 months (April 2009) and after 1 year (September 2009) in wild.

These data demonstrate that the outdoor conditions of the 'Chisty Les' pen exerted a highly significant selection effect against the p66^{Shc} null allele ($P < 0.00001$, Fisher's exact probability test). Under the general assumption that fitness values for the three genotypes behave as: $f(-/-) < f(+/-) \leq f(+/+)$ or $f(-/-) \leq f(+/-) < f(+/+)$ (Hamilton, 2009) as suggested by the experimental data, the mathematical modeling of the trajectory of the allelic frequencies over time clearly shows that after a sufficient number of generations the frequency of the null allele will approach zero, suggesting that the p66^{Shc} mutated allele might disappear from the population over time.

P66^{Shc}–/– mice are sensitive to starvation and cold

Death in a natural setting could be owing to behavioral abnormalities (careless exploratory behavior, cognitive problems, sensorimotor deficits, increased intraspecific competition), vulnerability to infections, or metabolic impairment [including starvation- and cold-sensitivity (Berry & Bronson, 1992)].

In laboratory tests, the p66^{Shc}–/– mice did not show cognitive deficits or signs of intraspecific aggressiveness (but for maternal cannibalism; see below), while they showed retarded aging-associated decay of physical strength and spatial orientation, as compared to age-matched wild type (WT) mice (Berry *et al.*, 2007, 2008). In addition, p66^{Shc}–/– mice showed more robust immune response to vaccination and infection challenges (Finetti *et al.*, 2008; Berry *et al.*, 2010). Together, these data argue against the possibility that behavioral abnormalities or infection vulnerability were responsible for the negative-selection effect of the natural environment against the p66^{Shc} null allele. The p66^{Shc}–/– mice, instead, have altered glucose homeostasis, reduced adipogenesis, and cold susceptibility (in laboratory conditions) (Berniakovich *et al.*, 2008), suggesting a metabolic origin of their frailty in natural conditions.

Thus, we investigated the sensitivity of the p66^{Shc}–/– mice to cold stress and starvation in our laboratory mouse-husbandry. Simultaneous exposure to cold (+12 °C) and starvation led to 50% mortality of the p66^{Shc}–/– mice within 3 days, as compared to 0% of WT mice (six mice per group). Neither of the two conditions alone affected the survival of WT or mutant mice, under the same experimental conditions, thus suggesting that starvation and cold might have acted synergistically in the outdoor enclosure to counterselect the p66^{Shc} deletion.

Mammals modify their metabolic rate, social and reproductive behavior utilizing fat depots when temperature drops and/or food is scarce. In these circumstances, adequate fat accumulation and utilization become essential. Indeed, genetic factors that regulate fat-tissue functions and lipid storage itself have evolved to adapt species to their energetic niches (Pond, 1998). A main consequence is that survival and fecundity increase as a function of fat depots, resulting in improved fitness for that species (Kennedy & Mitra, 1963). Based on previous findings that p66^{Shc} is essential for adipogenesis (Berniakovich *et al.*, 2008; Ranieri *et al.*, 2010), we investigated whether alterations in fat-tissue functions in p66^{Shc}–/– mice are responsible for their survival deficit when energetic resources are scarce and temperature is low. The C57/129F1-hybrid p66^{Shc}–/– mice showed reduction of all body fat depots investigated (approximately 30% and approximately 40% in female and male mice, respectively; $P < 0.001$), as previously observed in the C57BL/6 or 129 inbred strains (Berniakovich *et al.*, 2008) (Fig. 2A), as well as decreased production and reduced circulating levels of adipokines, including leptin, adiponectin, TNF-alpha, and Pai-1 (Fig. S1). The finding in p66^{Shc}–/– mice of reduced

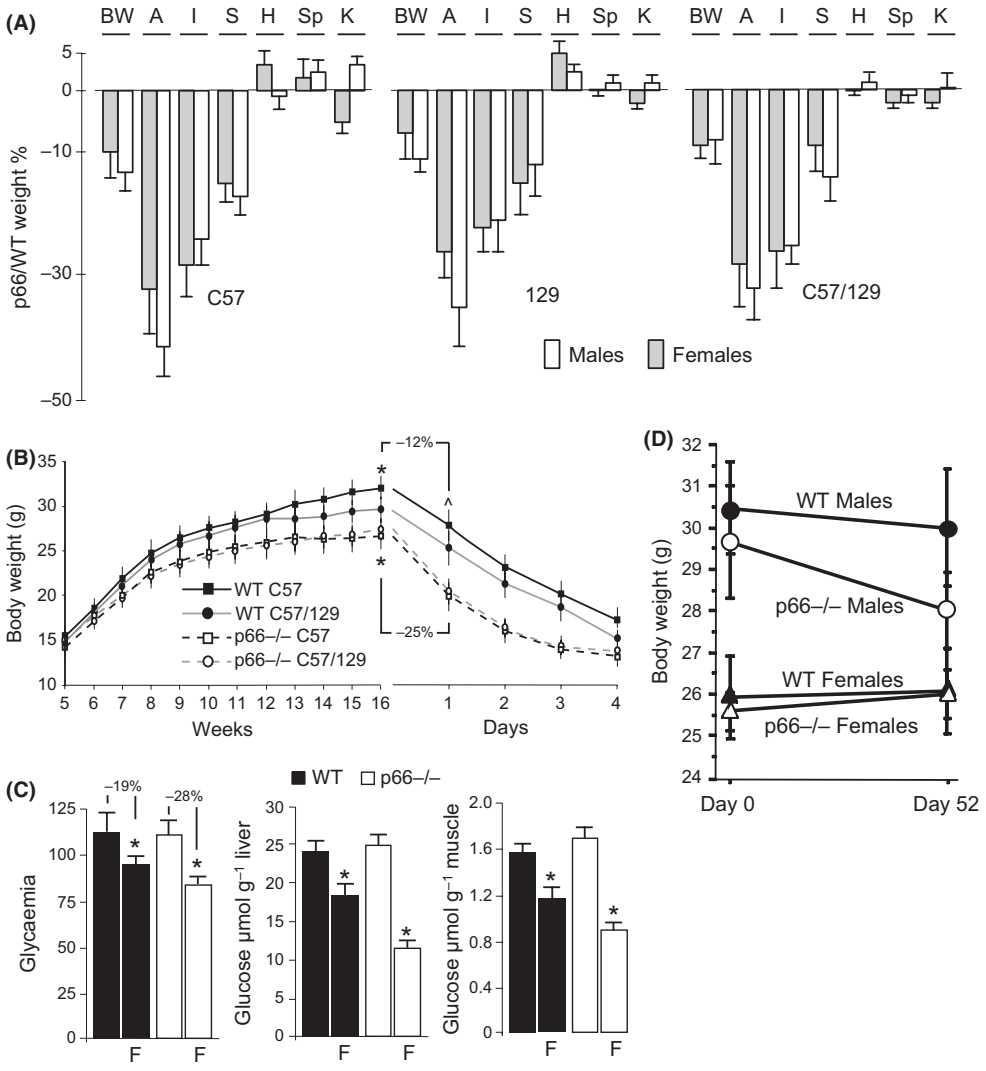

Fig. 2 Decreased fat in p66^{Shc}–/– mice. (A) Percent differences of 4-month-old p66^{Shc}–/– vs. WT mice [males or females; 129, C57Bl/6 (C57), or C57/129F1 (C57/129) background strains, as indicated] in weights of total body (BW), fat pad (A: abdominal; I: inguinal; S: interscapular), heart (H), spleen (Sp), and kidney (K) (n = 20 per group, P < 0.01 for A, I, and S). (B) Body weight gains (from 5 weeks to 4 months of age) of C57 or C57/129 p66^{Shc}–/– and WT male mice and weight drops after 4 days of fasting (n = 10, * WT vs. p66^{Shc}–/– P < 0.01; ^ day 1 fasted vs. day 0 fasted P < 0.01 in both WT and p66^{Shc}–/– groups). (C) Glucose content in WT and p66^{Shc}–/– C57 male mice in normal and fasted (F) conditions (n = 8; difference among *: P < 0.01). (D) Body weights of WT and p66^{Shc}–/– C57 males and females exposed to cold for 3 h per day.

adiponectin levels, which, usually, inversely correlate with fat depots, suggests that adipogenic dysfunctions prevail on the effect of this hormone on lipid metabolism.

To investigate the sensitivity of p66^{Shc}–/– mice to weight loss upon starvation, 16-week-old male mice were starved for 4 days and weighed at day intervals. The drop of body weight, at 24 h after starvation, was markedly enhanced in the p66^{Shc}–/– mice, regardless of their genetic background (approximately 25% and approximately 10% in the p66^{Shc}–/– and WT mice, respectively; P < 0.001) (Fig. 2B). Starvation induces mobilization of glucose, to sustain energetic metabolism. Notably, the decrease in glycaemia and tissue glucose (liver and skeletal muscles) after 16 h of starvation was approximately 50% greater in the p66^{Shc}–/– animals (P < 0.001), indicating rapid exhaustion of carbon sources in the starved p66^{Shc}–/– mice (Fig. 2C). Liver-glycogen levels after starvation, instead, did not differ in the p66^{Shc}–/– and WT mice, implying that hepatic glycogen mobilization was not sufficient to maintain tissue glucose levels in the starved p66^{Shc}–/– mice. Finally, we investigated the sensitivity of p66^{Shc}–/– mice to cold. We reported previously that the drop in body temperature after brief exposures to cold (+4 °C) is faster in the p66^{Shc}–/– mice (Berniakovich *et al.*, 2008). Strikingly, chronic cold-exposure (3 h *per* day at +4 °C) provoked a significant drop in body weight in the p66^{Shc}–/–, but not in the WT male mice (Fig. 2D).

In conclusion, p66^{Shc}–/– mice are leaner and more sensitive to cold and starvation, suggesting that alterations in energy storage and utilization are responsible for their diminished survival when energetic resources are scarce and temperature is low. Thus, p66^{Shc} might function to maximize fat accumulation in response to variations of the energetic environment (food intake and temperature). In this respect, we noticed that the levels of tissue p66^{Shc}-expression among individuals are highly variable in the fat, even among C57/BL6 inbred mice, while generally constant in all the others tissue (Fig. 3A), suggesting that p66^{Shc}-expression in the fat is tightly regulated. Notably, fat levels of p66^{Shc} are up-regulated in mice fed high-fat diet or exposed to cold, while they are down-regulated upon starvation (Fig. 3).

Reduced fertility of p66^{Shc}–/– mice

A reduction of the fat function in energetically stressful conditions might have further implications for fitness. Caloric restriction and cold not only affect early survival of the species, but also fecundity. Reproduction, in fact, is a high energy-cost process (involving, at least in mammals, gonadal function, pregnancy, and lactation) that relies substantially on energetic and endocrine fat functions (Hausman & Barb, 2010), suggesting that the counterselection of the p66^{Shc} null allele in the 'wild' might also

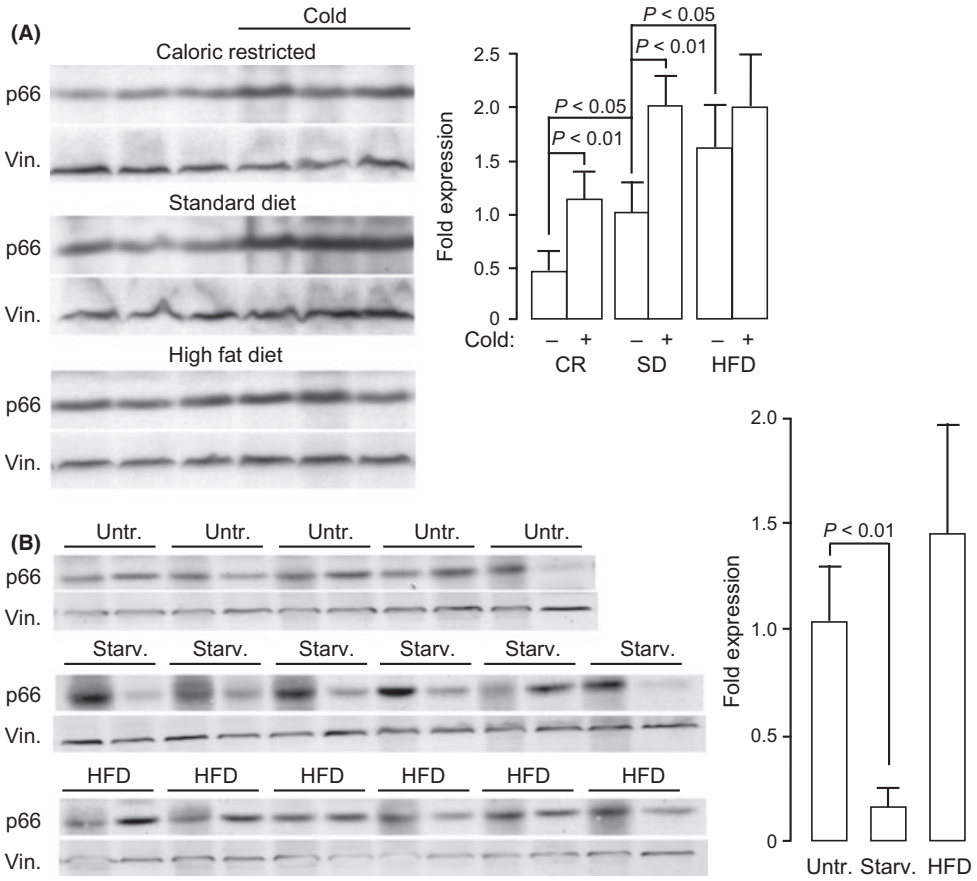

Fig. 3 P66^{Shc} expression regulation by diet and cold. (A) Western blot of p66^{Shc} and vinculin (vin) expression in abdominal white-fat from six representative C57-WT 4-month-old males that were calorie restricted (CR), or fed either standard diet (SD) or high-fat diet (HFD). The graph on the right summarizes the densitometric analysis of a similar experiment where 24 mice in each group (CR, SD, HFD) were exposed (n = 12), or not (n = 12), to cold (10 °C for 24 h before killing). P66^{Shc} expression levels were normalized with vinculin and expressed as fold variations with respect to SD. (B) Effect of different diet regimens on p66^{Shc} protein-expression levels in the same individuals (C57-WT 3-month-old female mice). Western blots were performed on lysates of the fat pad surrounding ovaries, surgically removed from the same individuals before and after diet treatments (no treatment: Untr.; 1 day starvation: Starv.; 1 week of HFD: HFD) (see Data S1 in Supporting information for details).

be owing to reduced fertility. Thus, we investigated fecundity of the p66^{Shc}–/– mice under laboratory conditions. Mean numbers of pups *per* litter were comparable in the p66^{Shc}–/– vs. wild-type mice (4.54 and 4.26, respectively; Table 1), despite p66^{Shc}–/– females were leaner [genotype main effect: $F(1,32) = 5.770$; $P = 0.0223$; Fig. 4A]. However, when evaluated over repeated breeding cycles of the same pair, fecundity decreased more rapidly in the p66^{Shc}–/– mice (a significant reduction was observed from the fourth breeding cycle; Fig. 4B). Notably, the number of full-term pregnancies was significantly reduced also in the p66^{Shc}–/– virgin females that had been exposed to an inverted dark-light cycle, an established procedure to induce stress in rodents (Table 1). Together, these results suggest that stressful conditions cause reduced reproductive capacity in the p66^{Shc}–/– mice.

Table 1 Fertility of p66^{Shc}–/– mice

Percentages of	WT	P66–/–
Standard breeding conditions		
Dams found pregnant	58.0	47.7
Dams found not pregnant	42.0	52.3
Total *n*.	119	128
Stressful breeding conditions		
Dams found pregnant	36.1	11.4*
Dams found not pregnant	63.9	88.6
Total *n*.	36	35
Nests with no dead pups	86.9	91.8
Nests with dead pups	13.1	8.2
Total *n*.	69	61
Dams exhibiting proper care	98.5	82.0
Dams exhibiting cannibalism	1.5	18.0**
Total *n*.	69	61
Mean number of pups per dam	4.54	4.26

Percentages of pregnant dams in standard and stressful (inversion of light cycle) breeding conditions; nests with death newborns; dams exhibiting proper care or cannibalism and number of pups per dam, in WT and p66^{Shc}–/– mice (*P < 0.05; **P < 0.01).

We then investigated the ability of p66^{Shc}–/– dams to care for their newborn pups and observed higher frequency of cannibalistic behavior (defined as visible physical damage to the pups, owing to repeated bites, leading to death) in p66^{Shc}–/– litters, as compared to WT controls (11/61 vs. 1/69 dams exhibiting cannibalism; $P = 0.0014$; Table 1). Reduced energy stores are frequently associated with the redirection of maternal activities, which might lead to increased cannibalistic behavior. Analysis of maternal behavior during the first week after delivery revealed decreased frequency of proper-care activities (Fig. 4C), and increased foraging behavior (Fig. 4D). Consistently, time course analysis showed that reduced pup care occurred when foraging behavior increased (see Fig. 4C,D, postnatal days 5 and 8). In addition, p66^{Shc}–/– dams moved and explored more frequently than WT controls [$F(1,14) = 9.999$, $P = 0.0069$ and $F(1,14) = 8.299$, $P = 0.0121$, respectively for moving-exploring and rearing behaviors], in agreement with previous reports showing higher and more prompt exploratory activity of p66^{Shc}–/– mice in tests of spontaneous behavior (Berry *et al.*, 2007, 2008). In summary, p66^{Shc}–/– dams showed altered behaviors, characterized by increased locomotor activities and foraging activities and cannibalism. As metabolic needs (hunger) are thought to increase locomotor activity and adaptive

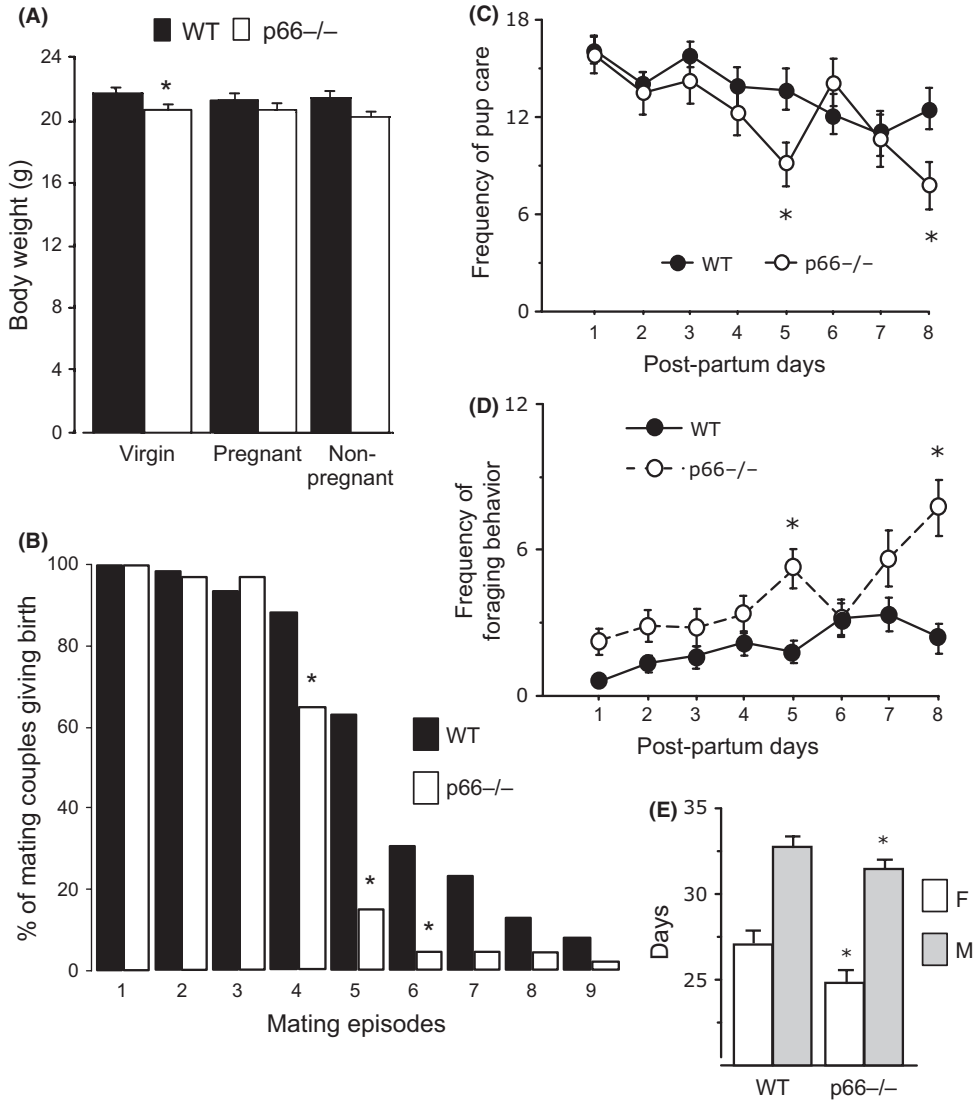

Fig. 4 Reproductive performance of p66^{Shc}–/– mice. (A) Body weight of WT and p66^{Shc}–/– virgin females (n = 18 for both WT and P66^{Shc}–/– breeding pairs; *: $P < 0.05$). Mutant mice showed reduced body weight (main effect of genotype) and normal ability to become pregnant (interaction between genotype and pregnancy condition). (B) Reproductive success is reduced in reproductive p66^{Shc}–/– pairs on the 5th and 6th subsequent breeding cycles. Data are expressed as mean + SEM (n = 40 for WT and P66^{Shc}–/–). (C,D) Time course (postpartum days) of pup care (C) and foraging (D) behavior in WT and p66^{Shc}–/– dams. P66^{Shc}–/– dams showed decreased parental care and higher frequency of foraging especially on PNDs 5 and 8. Data are expressed as mean ± SEM (n = 9 for WT and 7 for P66^{Shc}–/–). (E) Days to reach the onset of puberty in females and males, WT and p66^{Shc}–/– mice, (mean + SEM; n = 19 WT and 32 P66^{Shc}–/– males; 10 WT and 19 P66^{Shc}–/– females). *Post hoc* comparisons: * $P < 0.05$.

foraging behavior (Overton & Williams, 2004), we propose that the behavioral changes of the p66^{Shc}–/– mice are motivated by enhanced food searching that, under natural conditions, leads to cannibalism and lower survival of the offspring, ultimately contributing to reduced fitness.

Finally, we investigated the timing of puberty in p66^{Shc}–/– and control mice. Sexual maturation strictly depends on energy metabolism and has a great impact on fitness. Surprisingly, p66^{Shc}–/– pups experienced early puberty [p66^{Shc}–/–, 29.059 ± 0.565 vs. WT, 30.96 6 ± 0.745; $F(1,76)$ = 14.250, P = 0.0003; main effect of sex: females, 25.690 ± 0.566 vs. males 32.059 ± 0.355; $F(1,76)$ = 91.315, $P < 0.0001$, see Fig. 4E]. It is unlikely, however, that such anticipation can represent an evolutionary-significant compensatory mechanism for the survival disadvantage of p66^{Shc}–/– mice; this suggests that the retarded aging of the p66^{Shc}–/– mice under laboratory conditions is not owing to a delay in the lifespan cycle *per se*, rather to a favorable interaction with the artificial environment.

In conclusion, these data suggest that reduced fertility might contribute to the decreasing density of the p66^{Shc}–/– population in natural conditions. Thus, multiple factors might have acted synergistically against the expansion of the p66Shc–/– genotype, including the reduced survival of adult individuals, the reduced fecundity of females, and the increased frailty of their offspring, owing to decreased maternal behaviors and increased sensitivity to cold temperatures.

Discussion

Taken together, and within the experimental limits of an investigation performed in a natural environment, our data indicate a function of the p66^{Shc} protein in adapting the organism to changes in the energetic niche, e.g., food access and environmental temperature, and suggest that, mechanistically, p66^{Shc} exerts this function by regulating the fat tissue. These metabolic effects of p66^{Shc} increase fitness when food is scarce and energetic resources are to be stored, suggesting that the degree of fat accumulation in mammals is evolutionarily set early in life for optimal reproduction and survival in the wild. When food is constantly available and individuals are protected from low temperatures, as it occurs in mammals in captivity and humans with westernized lifestyles, fat accumulation becomes instead detrimental, by predisposing to diseases such as diabetes, cardiovascular disease, and cancer, eventually leading to accelerated aging (Neel, 1962). The negative effect of the fat

tissue on aging-associated diseases is not programmed, manifest late in-life and in protected environments. In conclusion, p66^{Shc} functions may adapt populations to the changeability of resources, thus increasing the distribution of species, and become disadvantageous if food availability becomes excessive, as it has happened with western diets and habits.

Experimental procedures

Animals

Experiments were carried out on Sv/129, C57Bl/6, and Hybrid F1 C57Bl/6Sv/129 strains generated at the European Institute of Oncology and maintained in a temperature-controlled room with a 12-h light/12-h dark cycle. HF (D12492 with 60% kcal from fat equivalent to 5.2 kcal g^{-1}) and standard (2018S, 3.3 kcal g^{-1}) diets were purchased from Research Diets Inc., New Brunswick, NJ, USA and Harlan Teklad, Bresso, Italy, respectively. Body weight and food consumption were controlled biweekly. Semi-natural conditions were reproduced in the field station 'Chisty Les' (Clear Forest), Bubonizi (Pozhnia, Tvier Region, Western Russia, 56°44′7.99″N; 31°31′34.44″E) where the annual temperature range was –13/20 °C and the average rainfall was around 600 mm per year. A photograph of the area is in Fig. S1.

The experimental pen measured 20 × 20 m and was surrounded by a smooth wall topped with a band of zinced iron, 1 m high and sunk 50 cm into the soil, totally preventing animal escaping. Inside the pen the ground was grass with *Artemisia* species and several young pine trees all around. The pen was organized to allow inter-individual competition for food, water, shelter, and mating, and exposure to natural weather conditions and aerial predation. Terrestrial predators were kept away by an electrically charged wire on top of walls, as used for cattle fences. The pen contained a closed shelter of 2 × 4 m and 70 cm depth filled with hay, straw, and branches. Mice were initially released in these shelters. Commercial standard ('Complete One' from Mest firm, Moscow, Russia) mouse-food pellets were delivered in two feeders consisting of a tray, which allowed the feeding of several animals at a time, filled *ad libitum* with standard lab chow under a wooden roof.

Two feeders were used to reduce inter-male competition. The food and two 1L water bottles near the feeders were checked and replenished every 2–3 days.

Reproductive behavior

Reproductive success was evaluated by counting, over eight breeding cycles of virgin females, numbers of pregnant subjects, delivered pups, litters with dead pups, and litters in which cannibalistic episodes took place.

Maternal behavior

Maternal behavior was monitored each day from PND 1 to PND 8 by an observer blind to the genotype, taking 20 instantaneous samplings per hour (inter-observational interval of 3 min) four times a day. Maternal behaviors were scored as '1' or '0' depending on whether each behavioral component occurred or not.

Onset of puberty in the offspring

The onset of puberty was assessed in male (19 WT and 32 KO) and female (10 WT and 19 KO) pups by examining balano-preputial separation and vaginal opening (VO) every day, starting from the day of weaning (PND 21).

Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA) with genotype as between-subject factor (maternal behavior, onset of puberty, mean number of delivered pups per litter) and time blocks as within-subject repeated measures (maternal behaviors). *Post hoc* comparisons were performed using the Tukey's test.

P66^{Shc} expression

Analysis of p66^{Shc} expression after diet treatment was performed both in groups of mice (treated or not treated) and in the same individuals. In the latter case, mice were anesthetized with IP avertin before diet treatment and subjected to extraction of fat depot. To this end, each mouse was placed on its left side and treated surgically to create a small hole in the skin and peritoneum. Correct positioning of fine forceps allowed hooking and dragging-out of the right part of perigonadal deposit, from where one piece of fat was surgically removed, snap frozen in liquid nitrogen, and used for subsequent analyses of p66^{Shc} expression. The surgical hole in the mouse skin was closed with clips and the mouse left until awaked on a 37 °C pad. The mouse was then left to recover for 2 weeks without disturbance. Mice survived this procedure without visible complications. After the recovery period, mice were treated with high-fat or normal diet for 10 days, or with normal diet for 8 days followed by 48 h starvation, or left on normal diet. Mice were then anesthetized and another symmetrical part of perigonadal depot was extracted following the same procedure described above. Tissue lysis was performed at 4 °C in a buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X100, 1.5 mM orthovanadate, supplemented with a protease inhibitors cocktail from Calbiochem-Merck KGaA, Darmstadt, Germany. Protein concentration was determined using the Bradford reagent on collected supernatants. A calibration curve was obtained by plottering BSA solution in the same lysis buffer. Western blots were performed as described[5], using the BD Transduction Laboratories (Lexington, KY, USA) anti-p66^{Shc} antibody. All expression data are expressed as mean ± SD and analyzed by Student's *t*-test. Differences between means were assessed by one-way analysis of variance.

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Supporting Information

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

Fig. S1 Blood leptin, adiponectin and TNF-a levels in WT and p66^{Shc}−/− C57 4-months old male mice fed standard diet (SD) or high-fat diet (HFD) (n = 12; * vs. *, # vs. #, ° vs. ° and ^ vs. ^: $P < 0.01$).

Fig. S2 A spring view of the pens at the field station 'Chisti Les', Bubonizi (Pozhnia, TvierRegion, Western Russia).

Data S1 Supplementary methods.

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