Increased longevity and refractoriness to Ca$^{2+}$-dependent neurodegeneration in Surf1 knockout mice

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Leigh syndrome associated with cytochrome c oxidase (COX) deficiency is a mitochondrial disorder usually caused by mutations of SURF1, a gene encoding a putative COX assembly factor. We present here a Surf1$^{-/-}$ recombinant mouse obtained by inserting a loxP sequence in the open reading frame of the gene. The frequency of $^{-/-}$, $+/+$ and $+/-$ genotypes in newborn mice followed a mendelian distribution, indicating that the ablation of Surf1 is compatible with postnatal survival. The biochemical and assembly COX defect was present in Surf1$^{loxP-/-}$ mice, but milder than in humans. Surprisingly, not only these animals failed to show spontaneous neurodegeneration at any age, but they also displayed markedly prolonged lifespan, and complete protection from Ca$^{2+}$-dependent neurotoxicity induced by kainic acid. Experiments on primary neuronal cultures showed markedly reduced rise of cytosolic and mitochondrial Ca$^{2+}$ in Surf1$^{loxP-/-}$ neurons, and reduced mortality, compared to controls. The mitochondrial membrane potential was unchanged in KO versus wild-type neurons, suggesting that the effects of the ablation of Surf1 on Ca$^{2+}$ homeostasis, and possibly on longevity, may be independent, at least in part, from those on COX assembly and mitochondrial bioenergetics.

INTRODUCTION

Cytochrome c oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain (MRC), catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen (1). COX is composed of 13 protein subunits, the three largest being encoded by mtDNA genes, and the remaining ten are encoded by nuclear DNA genes (2). A number of accessory factors are necessary for the formation of an active holoenzyme complex (3), including those involved in synthesis of heme a, incorporation of copper atoms and assembly of the protein backbone (4). One of these factors, SURF1, is a 30 kDa hydrophobic protein embedded in the inner membrane of mitochondria. The absence, or malfunctioning, of SURF1p determines the accumulation of COX assembly intermediates, and a drastic reduction in the amount of fully assembled enzyme, in both yeast (5) and humans (6). As a consequence, profound COX deficiency (7) in multiple tissues of Surf1
RESULTS

Generation of Surf1loxP−/− mice

The strategy used to disrupt the mouse Surf1 gene (NM_013677) is shown in Figure 1A. Briefly, a cDNA expressing the Escherichia coli neomycin phosphotransferase (NEO), therefore conferring neomycin-resistance, flanked by two loxP sequences (loxP-NEO-loxP cassette), was inserted into a unique AccI restriction site of Surf1 exon 7. In order to confirm homologous recombination, Southern blot analysis was performed on both extremities of the Surf1loxP-NEOloxP recombinant allele (Fig. 1B and C). Blastocyst injection of two recombinant clones gave rise to eleven Surf1loxP-NEOloxP chimeric mice showing germline transmission. No Surf1loxP−/−, +/+ and +/+− homozygous individuals were obtained by mating Surf1loxP−/− heterozygotes to each other, due to arrest of organogenesis at E8.5−−9.5 (Supplementary Materials, Fig. 1 and Table 1). The NEO cassette and one of the two loxP sequences were then excised by mating Surf1loxP−/−NEOloxP+/− heterozygous animals with animals constitutively expressing the cre recombinase. The resulting recombinant allele (Surf1lox−/−) contains a single loxP insertion (Fig. 1D), which causes the shift of the open reading frame (ORF) from nt 674 downstream of the mouse Surf1 cDNA, and the replacement of the codon encoding N225 into a TAA stop codon (N225X) (Fig. 1A). This mutation predicts the elimination of 81 amino acids on the Surf1p carboxy terminus. No cross-reacting material was detected by western blot immunoassay using polyclonal and monoclonal anti-Surf1 antibodies in several tissues of our Surf1loxP−/− mice (Fig. 1F), indicating that the truncated Surf1p protein is either unstable or fails to be translated, due to mRNA decay (Fig. 1E).

Clinical and biochemical phenotype

The percentages of approximately 800 Surf1loxP−/−, +/+ and +/+− newborn animals followed a mendelian distribution (26, 51 and 23%, respectively; Supplementary Material, Fig. 2A). Newborn Surf1loxP−/−−/− individuals were significantly smaller than their littermates, but this difference progressively disappeared after weaning (Supplementary Material, Fig. 2B). No difference in the clinical phenotype was observed between Surf1loxP−/−−/− individuals and their +/+ or +/+− littermates, including the appearance of neurological symptoms, abnormal reaction to stimuli, aberrant behavior, impaired cognitive abilities and reduced fertility. There was a small but significant reduction at the rotarod test (Supplementary Material, Fig. 2C), indicating mildly decreased motor skills in Surf1loxP−/−−/− versus wt mice.

Biochemically, we observed decreased reaction to COX and increased reaction to succinate dehydrogenase (SDH) in Surf1loxP−/−−/− skeletal muscle (Fig. 2A), similar to, but less severe than, that observed in SURF1 mutant patients. Finally, Surf1loxP−/−−/− brains showed normal cytoarchitecture by thionine and GFAP stainings (data not shown).

Biochemically, there was no difference in the activities of MRC complexes I, II and III, whereas the COX activity in several tissues of Surf1loxP−/−−/− individuals was 30−50% that of control littermates (Fig. 2B). Again, this reduction was much less marked than that observed in LScoxD mice (7). Surf1loxP+/− mice showed no biochemical difference compared to Surf1+/+− littermates, as expected for a recessive trait (data not shown).

Blood lactate was higher in Surf1loxP−/−−/− mice than in wt littermates, indicating partial block in the aerobic utilization of pyruvate (Supplementary Material, Fig. 2D).

As exemplified in Fig. 2C, fully assembled COX was variably reduced in different tissues of Surf1loxP−/−−/− mice, to an extent compatible with the levels of residual COX activity. Early assembly COX intermediates were also present in isolated mitochondria of Surf1loxP−/−−/− mice, similar to, but much lesser than, what is found in SURF1 mutant patients.
Taken together, these data are remarkably similar to those previously reported for the surviving Surf1\textsuperscript{NEO}/2 individuals (11).

Increased longevity of Surf1\textsuperscript{loxP}/2 mice

In order to evaluate whether the lack of Surf1p could determine a late-onset phenotype, Surf1\textsuperscript{loxP}/2 mice and control littermates were maintained under continuous observation in the same standard breeding conditions. No neurological or other clinical symptoms were ever seen in any individual. However, the lifespan was markedly different between the two groups. At the level of 25/43 Surf1\textsuperscript{loxP}/2 mice died during the observation period, against 30/48 deaths recorded in the control group. The median survival was 793 days for the Surf1\textsuperscript{loxP}/2 group and 654 days for the control group, the latter being the standard median survival reported for laboratory mice (12). As shown in Figure 3A, the difference in the Kaplan–Meier survival

Figure 1. Generation of Surf1\textsuperscript{−−} mice (see Materials and Methods). (A) Schematic representation of the Surf1 locus, Surf1\textsuperscript{loxP}-NEO-loxP and Surf1\textsuperscript{loxP} alleles. Surf1\textsuperscript{cDNA} and Surf1\textsuperscript{protein} proteins. P1 and P2 refer to the probes used for Southern-blot analysis shown in panels B and C. Numbers in the scheme of the Surf1 gene and Surf1 cDNA refer to exons ‘E’. In the nucleotide sequence of the Surf1\textsuperscript{loxP} allele, exon 7 is in gray, the loxP sequence is in black and italicized, the TAA stop codon is underlined. In the scheme of the Surf1 protein (bottom part of panel A), the putative mitochondrial targeting peptide is in gray. (B and C) Southern-blot analysis of the recombinant Surf1\textsuperscript{loxP} allele. (D) PCR-based genotyping of the Surf\textsuperscript{loxP} versus Surf\textsuperscript{wt} alleles. (E) RT-PCR analysis of the Surf\textsuperscript{loxP} versus Surf\textsuperscript{wt} alleles. The GAPDH cDNA fragment serves as a control. (F) Western blot analysis. Surf1p cross-reacting material is absent in mitochondrial membranes isolated from different Surf\textsuperscript{loxP}/2 organs probed with a monoclonal anti-Surf1p antibody (upper panel) and with a polyclonal antibody against the Surf1p mid portion (middle panel). The 30 kDa SDH-B subunit was used as a loading control (bottom panel). Lane 1, control fibroblasts; lanes 2 and 4, brain; lanes 3 and 5, liver.
probability, calculated by the logrank test, was highly significant between the two groups ($P = 0.0002$), irrespective of the gender (Fig. 3B and C).

**Surf1loxP$^{-/-}$ mice are protected from Ca$^{2+}$-related excitotoxic brain damage**

In order to determine whether our Surf1loxP$^{-/-}$ mice were more susceptible to stress-induced neuronal damage, we used kainic acid, an epileptogenic glutamate agonist that has extensively been used to test neuronal response and survival to Ca$^{2+}$-mediated excitotoxicity. We injected *intra peritoneum* (i.p.) a total of 145 three-month old mice (73 controls and 72 Surf1loxP$^{-/-}$) with 30 mg/kg of kainic acid. The mortality rate, as well as the frequency, time lapse, severity and duration of the kainate-induced seizures were similar between the KO and control groups (Table 1), suggesting that the pharmacokinetics of the drug did not differ in the two groups. Following a standard protocol (13), only animals surviving the most severe level-5 seizure were further investigated (Table 1). Whole-brain pathological examination was carried out using the neurodegeneration-sensitive FluoroJadeB (FJB) fluorochrome (14) and thionine stainings. Strong FJB-positive neurons were detected in virtually all the glutamergic areas of the control brains, including the Cornu Ammonis (CA) areas 1−4 of the hippocampus, the cerebral cortex, the amygdala and the thalamic and olfactory nuclei. The most severely
Glutamate-induced cytosolic Ca\(^{2+}\) signals and consequent neuronal cell death are reduced in Surf1\(^{loxP}/-\) neurons

Kainic acid treatment mimics glutamate-induced excitotoxicity, which is characterized by delayed Ca\(^{2+}\) deregulation (DCD) and loss of mitochondrial potential (ΔΨ\(_{m}\)) (15,16). Glutamate stimulation leads to the activation of metabotropic (mGlu) and ionotropic (AMPA, kainate and NMDA-type) plasma-membrane receptors in neurons. To further investigate the impact of Surf1 ablation on these phenomena, we used 7-day-old cortical/hippocampal primary neuronal cell cultures obtained from Surf1\(^{loxP}/-\) mice and controls. Activation of the metabotropic receptor leads to inositol-triphosphate (IP\(_3\))-induced Ca\(^{2+}\) release from the endoplasmic reticulum (ER). Metabotropic Ca\(^{2+}\) responses were not detected in cultured neuronal cells, as confirmed by the use of the specific mGlu agonist APB (data not shown). Activation of ionotropic receptors causes Na\(^{+}\)-mediated cell depolarization and subsequent Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels, as well as direct Ca\(^{2+}\) influx through activation of the NMDA channel. To measure cytosolic Ca\(^{2+}\) signals, we loaded neurons with the low-affinity Ca\(^{2+}\) dye fura-FF. Neurons were then challenged with 10 or 100 μM glutamate for 30 min, and cytosolic [Ca\(^{2+}\)] \(_{c}\) ([Ca\(^{2+}\)]\(_{c}\)) changes were measured in single cells. As shown in Figure 6A and B, the increase in [Ca\(^{2+}\)]\(_{c}\) induced by 10 μM glutamate was not significantly different in Surf1\(^{loxP}/-\) versus Surf1\(+/-\) neurons (ΔF/F 0.10 ± 0.01 in Surf1\(^{loxP}/-\) versus 0.12 ± 0.01 in Surf1\(+/-\), \(n > 100\) for each group, \(P = 0.31\), but it was much lower after exposure to 100 μM glutamate (ΔF/F = 0.26 ± 0.02 in Surf1\(^{loxP}/-\) versus 0.45 ± 0.03 in Surf1\(+/-\), \(n > 140\) for each group, \(P < 10^{-5}\)).

Sustained stimulation with high doses of glutamate induces deregulation of neuronal Ca\(^{2+}\) homeostasis, which manifests as a secondary, delayed and irreversible [Ca\(^{2+}\)]\(_{c}\) increase in the supramicromolar range, eventually leading to cell death (17). We used fura-FF to detect glutamate toxicity by calculating the number of cultured neurons showing the characteristic secondary Ca\(^{2+}\) increase during sustained (up to 30 min) glutamate stimulation. The percentage of neuronal death after stimulation with 10 μM glutamate was reduced in Surf1\(^{loxP}/-\) neurons as compared to Surf1\(+/-\) neurons, and it remained significantly lower at 100 μM glutamate stimulation (% cell death at 10μM glutamate: Surf1\(+/-\) +17.1 ± 5.0% versus Surf1\(^{loxP}/-\) -6.7 ± 2.9%; at 100 μM glutamate: Surf1\(+/-\) +32.5 ± 5.0% versus Surf1\(^{loxP}/-\) -16.9 ± 2.4% \(P < 10^{-5}\); Fig. 6C).

Reduced mitochondrial Ca\(^{2+}\) uptake is responsible for reduced Ca\(^{2+}\) influx in Surf1\(^{loxP}/-\) neurons

The extent of mitochondrial Ca\(^{2+}\) uptake, strategically located at plasma membrane Ca\(^{2+}\) entry sites, has been shown to regulate Ca\(^{2+}\) influx through different plasma membrane channels, such as capacitative or ligand-induced Ca\(^{2+}\) influx channels (17). Ca\(^{2+}\) buffering in the sub-plasma membrane space was shown to reduce Ca\(^{2+}\) feedback inhibition of capacitative Ca\(^{2+}\) influx channels as well as of diverse subunits of NMDA channels (18,19). In order to verify that the reduction of [Ca\(^{2+}\)]\(_{m}\), following glutamate stimulation in Surf1\(^{loxP}/-\) neurons, it was correlated to a modification of mitochondrial Ca\(^{2+}\) homeostasis, determining reduced Ca\(^{2+}\) influx, we measured mitochondrial [Ca\(^{2+}\)] \(_{m}\) ([Ca\(^{2+}\)]\(_{m}\)) in intact and permeabilized neurons, using a mitochondrially targeted low affinity aequorin probe (mitAEQmut). Neurons were transfected with the mitAEQmut probe and [Ca\(^{2+}\)]\(_{m}\) was measured in Surf1\(+/-\) and Surf1\(^{loxP}/-\) cell populations after stimulation with glutamate at low (10 μM) and high (100 μM) concentrations. Stimulation with 100 μM glutamate induced very high increase of [Ca\(^{2+}\)]\(_{m}\), leading to immediate consumption of the probe. However, mitochondrial Ca\(^{2+}\) transients could be measured at a lower dose of glutamate (10 μM). In these conditions (Fig. 7A), the mitochondrial Ca\(^{2+}\) uptake was drastically reduced in Surf1\(^{loxP}/-\) versus Surf1\(+/-\) cells. Maximum [Ca\(^{2+}\)]\(_{m}\) was 28.95 ± 2.50 mM in Surf1\(^{loxP}/-\) neurons (n = 24) versus 50.95 ± 3.36 mM in Surf1\(+/-\) neurons (n = 21), \(P < 10^{-5}\).

In order to verify that the reduction of mitochondrial Ca\(^{2+}\) uptake in Surf1\(^{loxP}/-\) cells is due to lack of Surf1p, rather than to reduced cytosolic Ca\(^{2+}\) response consequent to reduced Ca\(^{2+}\) influx through the plasma membrane, neurons expressing the mitAEQmut probe were treated with low-dose digitonin, which selectively permeabilizes the plasma membrane, and endogenous cytosolic Ca\(^{2+}\) was washed out by perfusion with Ca\(^{2+}\)-free intracellular buffer. Mitochondrial Ca\(^{2+}\) uptake was then triggered by the addition of 1 μM

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Table 1. Kainic acid treatment

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\(^{a}\)L5, level-5 seizures (13).
Ca$^{2+}$ to the buffer. Again, the velocity of mitochondrial Ca$^{2+}$ uptake was drastically reduced in Surf1$^{\text{loxP}+/\text{loxP}^{-}}$ cells ($\text{Surf1}^{\text{loxP}^{-}/\text{loxP}^{-}} 0.34 \pm 0.07 \mu\text{M/s} \ n = 23$ versus Surf1$^{+/+}$ $1.10 \pm 0.12 \mu\text{M/s} \ n = 26$, $P < 10^{-7}$) (Fig. 7B). This result indicates that the reduction of mitochondrial Ca$^{2+}$ uptake is intrinsic to Surf1$^{\text{loxP}^{-}/\text{loxP}^{-}}$ mitochondria. Since the inhibition of mitochondrial Ca$^{2+}$ uptake has been shown to augment the feedback inhibition of plasma-membrane Ca$^{2+}$ channels in neurons, the impairment of mitochondrial Ca$^{2+}$ buffering in Surf1$^{\text{loxP}^{-}/\text{loxP}^{-}}$ neurons might ultimately prevent them from Ca$^{2+}$ overload, which could explain their refractoriness to DCD and cell death.
Surf1 deficiency impairs mitochondrial Ca^{2+} uptake without changing the mitochondrial structure and membrane potential

The mitochondrial membrane potential (ΔΨ_m) drives the mitochondrial Ca^{2+} uptake by sustaining the activity of the mitochondrial Ca^{2+} uniporter (20,21). Therefore, reduction of ΔΨ_m can decrease the driving force of Ca^{2+} entry and the activity of the Ca^{2+} uniporter machinery in the inner mitochondrial membrane, thus determining reduced Ca^{2+} uptake into the organelle. Since Surf1p plays a role in the formation of COX, and the latter is in turn involved in the maintenance of mitochondrial transmembrane H^{+} gradient and ΔΨ_m, the reduction of Ca^{2+} uptake observed in Surf1^{loxP−/−} neurons could depend on a reduction of ΔΨ_m. We then measured the ΔΨ_m in Surf1^{+/+} and Surf1^{loxP−/−} neuronal populations.
by steady-state loading of neurons with the ΔΨₐₐₐₐ-sensitive dye teramethyl-rhodamine-methylester (TMRM), followed by fluorimetric measurement of the intensity of the dye in mitochondria. Our results showed no difference in the steady-state distribution of TMRM in Surf1+/+ versus Surf1loxP²/² neurons (31.19 ± 0.23 in Surf1+/+ versus 32.12 ± 0.29 in Surf1loxP²/², fluorescence intensity in arbitrary units, Fig. 8A), demonstrating that the mild reduction observed in COX activity in various tissues of Surf1loxP²/² animals failed to result in significant changes of ΔΨₐₐₐₐ.

We previously showed that mitochondrial fragmentation in epithelial cells leads to an average reduction of mitochondrial Ca²⁺ load upon IP₃-induced Ca²⁺ release (22). Therefore, we next investigated whether Surf1 deficiency can change the shape of the mitochondrial network. Neurons were transfected with mitochondrially targeted DsRed probe (mtDsRed) and the structure of mitochondria in 300 Surf1loxP²/² and 300 Surf1+/+ individual cells was imaged by digital microscopy (22). As exemplified in Fig. 8B, the overall arrangement of the mitochondrial network did not appear to be disturbed in Surf1loxP²/² neurons with respect to controls.

On the basis of these results, we concluded that Surf1p deficiency in mice does not lead to reduced ΔΨₐₐₐₐ or altered mitochondrial structure, suggesting that the effect of Surf1p on mitochondrial Ca²⁺ uptake could be independent from its role on COX assembly and maintenance of ΔΨₐₐₐₐ.

To further confirm this hypothesis, we analyzed the effects of SURF1 overexpression on global cellular and mitochondrial Ca²⁺ signals in HeLa cells. HeLa cells were co-transfected with a vector expressing human SURF1 (hSURF1) (6) and with mitAEQmut or its non-targeted cytosolic variant (cytAEQ). Cells were then challenged with histamine (100 μM), which induces IP₃-dependent Ca²⁺ release from the ER. The resulting rise in [Ca²⁺]c stimulates the mitochondrial Ca²⁺ uptake at sites located in proximity of the ER Ca²⁺ release sites. As shown on Figure 8C, the cytosolic Ca²⁺ responses remained unaltered in cells overexpressing SURF1 (peak [Ca²⁺]c 3.06 ± 0.08 mM in controls versus 2.86 ± 0.09 mM in hSURF1 overexpressing cells); however, mitochondrial Ca²⁺ uptake was significantly increased by SURF1 overexpression (peak [Ca²⁺]c 78.07 ± 5.32 mM in controls versus 94.51 ± 6.04 mM in overexpressing cells, P < 0.05, Fig. 8D). This increase was not due to variations of the ΔΨₐₐₐₐ, as measured by TMRM uptake (data not shown).

DISCUSSION

High embryonic lethality was a major feature of a mouse knockout model for Surf1p, an accessory assembly factor of COX, based on the replacement of a region of several kb in
the midportion of the Surf1 gene with a NEO cassette (Surf1\textsuperscript{NEO}). The new mouse model reported here carries a recombinant null allele, consisting in the insertion of the 35 bp \textit{loxP} sequence within exon 7 of the murine Surf1 gene (Surf1\textsuperscript{loxP}). The restoration of mendelian distribution of the genotypes in newborn recombinant Surf1\textsuperscript{loxP} mice indicates that the embryonic lethality observed in the previous Surf1\textsuperscript{NEO} model was not a consequence of the ablation of the Surf1 gene itself, but was rather due to the presence of the NEO cassette or to the elimination of regulatory elements contained in the deleted region of the Surf1 gene. This conclusion is further supported by the observation that the Surf1\textsuperscript{loxP-NEO-\textit{loxP}} allele, from which the Surf1\textsuperscript{loxP} allele is derived, was again associated with 100\% embryonic lethality, when present in homozygosity. Several recent reports (reviewed in 23) have shown that the maintenance of a NEO cassette in recombinant alleles can be associated with a number of unpredictable effects, including the creation of hypomorphic alleles, altered gene expression and embryonic lethality, mostly due to the promotion of illegitimate splicing of either the targeted gene or neighboring genes. The latter phenomenon was likely to occur in the \textit{surfeit} genomic region, which is packed with six housekeeping genes, some of which share common regulatory elements (24).

Although SURF1p is a ubiquitously expressed mitochondrial protein, and its ablation leads to an early onset, invariably fatal encephalopathy in humans, no clinical disease phenotype could be observed in our Surf1\textsuperscript{loxP-}mice at any age. This situation is similar to that reported for the Surf1\textsuperscript{NEO-}animals that survived embryonic selection (11). The absence of neurological and extra-neurological abnormalities was associated with a biochemical phenotype that showed a specific and generalized defect of COX activity, however less severe than that observed in Surf1 mutant patients (7). The COX defect was likely too mild to cause brain failure or impairment, but was possibly sufficient to determine the modest functional and morphological alterations found in skeletal muscle of adult Surf1\textsuperscript{loxP-}mice.

The mild biochemical and the virtually absent clinical phenotypes of Surf1\textsuperscript{loxP-}mice suggest that, in spite of the ubiquitous expression and high evolutionary conservation of Surf1p, the function of this protein in COX assembly is either ancillary or redundant, that is, it can partly be overtaken by other unknown factors. To some extent, this may well be true also in Surf1-less mutant patients, in whom fully assembled COX is diminished, but not absent (6). The different severity of the phenotype associated with the absence of Surf1p may then depend on the efficiency and efficacy of compensatory genetic or epigenetic mechanisms acting in different organisms, notably fungi, mice and men.

In an attempt to determine whether the reduced COX activity found in Surf1\textsuperscript{loxP-}animals could make their brain more sensitive to energy stress, we used an excitotoxic glutamate agonist, kainic acid, which triggers epileptic seizures in experimental animals. Kainic acid acts on the AMPA-kainate glutamate receptors present on the neuronal cell membrane (25). When activated, the Na\textsuperscript{+}-channel component of these receptors opens up, thus determining Na\textsuperscript{+} influx and membrane depolarization. As a consequence, massive influx of Ca\textsuperscript{2+} occurs through the NMDA receptors and the VOCC channels of the plasma membrane (26). In addition to determining the epileptic discharge, the marked rise of [Ca\textsuperscript{2+}]\textsubscript{c} can promote a cascade of secondary effects that may ultimately lead to cell death (27).

These experiments were aimed at challenging the OXPHOS reserve of neuronal cells provoked by epileptic discharge. The same approach has been used in the recent past to precipitate catastrophic neurodegeneration in the Mitochondrial Late-Onset Neurodegeneration (MILON) mouse, a conditional TFAM knockout model, characterized by loss of mtDNA in neurons of the frontal cortex (28). To our surprise, however, the elicitation of level-5 seizures failed to cause any neuronal degeneration and neuronal loss in Surf1\textsuperscript{loxP-}mice, while these lesions were consistently observed in glutamatergic areas of control brains. Since kainate-associated neurodegeneration is largely dependent on perturbation of Ca\textsuperscript{2+} homeostasis, we investigated the cytosolic and mitochondrial Ca\textsuperscript{2+} fluxes in primary neuronal cell cultures from Surf1\textsuperscript{loxP-}and control mice. The results of these experiments can be summarized as follows.

First, the ablation of Surf1 drastically reduces the glutamate-induced increase of [Ca\textsuperscript{2+}]\textsubscript{c} in both cytosolic and mitochondrial compartments.

Figure 7. Mitochondrial Ca\textsuperscript{2+} uptake of primary cultured Surf1+/+ and Surf1\textsuperscript{loxP-} neurons measured by the recombinant low affinity mitAEQmut probe targeted to mitochondria. (A) Representative traces of luminescent values converted to [Ca\textsuperscript{2+}]\textsubscript{c} are shown on the left panel; mean ± SEM of [Ca\textsuperscript{2+}]\textsubscript{c} peaks are shown on the right panel. (B) The same experiment as in A was carried out on cells treated with 25 \textmu M digitonin for 1 min. Representative traces of [Ca\textsuperscript{2+}]\textsubscript{c} values are shown on the left panel; mean ± SEM of Ca\textsuperscript{2+} uptake velocity (micromol/s) are shown on the right panel.
Second, the reduction of mitochondrial Ca\textsuperscript{2+} uptake is directly consequent to the absence of Surf1p.

Third, Surf1\textsubscript{loxP}\textsuperscript{-/-} cultured neurons are much more resistant to glutamate toxicity than control neurons. How could this effect be linked to the observed reduction of mitochondrial Ca\textsuperscript{2+} uptake? One possibility is that reduced buffering capacity by Surf1\textsubscript{loxP}\textsuperscript{-/-} mitochondria can determine the saturation of the Ca\textsuperscript{2+} microdomains in the contact sites between mitochondria and the plasma membrane or the ER. This effect could in turn promote the feedback closure of the Ca\textsuperscript{2+} channels in the above structures, thus inhibiting the [Ca\textsuperscript{2+}]c transient rise (18). Although speculative, this hypothesis can offer a mechanistic explanation for the neuro-protection observed in vivo. A second possibility is that the ablation of Surf1 may alter the expression of nuclear genes encoding proteins engaged in Ca\textsuperscript{2+} homeostasis. As a preliminary result, quantitative PCR analysis failed to show different expression of the Ca\textsuperscript{2+}–Na\textsuperscript{+} plasma membrane exchanger (NCX1) in Surf1\textsubscript{loxP}\textsuperscript{-/-} versus wt brains (data not shown). More work is needed to expand this analysis to other Ca\textsuperscript{2+}-related genes.

Fourth and last, the reduction of the mitochondrial [Ca\textsuperscript{2+}] uptake seems not to be dependent from a decrease of the $\Delta$Ψ\textsubscript{m}, as a consequence of partial defect of COX activity.

Figure 8. (A) Mitochondrial membrane potential is unchanged between Surf1\textsubscript{loxP}\textsuperscript{-/-} and +/- neuronal cells. A.U., arbitrary units. (B) Mitochondrial structure remains unchanged in Surf1\textsubscript{loxP}\textsuperscript{-/-} neurons. Representative images of whole neurons and somata (zoomed insets) are shown. (C and D) Analysis of cytosolic (C) and mitochondrial (D) Ca\textsuperscript{2+} homeostasis in HeLa cells overexpressing hSURF1 protein. Representative traces are shown on the left panels. Right panels show the mean ± SEM peak values after histamine stimulation from >10 experiments.
and mitochondrial respiration. This conclusion, which was also supported by the results of hSURF1 overexpression in HeLa cells, suggests that Surf1p could play a direct role on mitochondrial Ca\(^{2+}\) handling, partially or completely independent from its function as a COX assembly factor. More work is necessary to test this hypothesis, but it is interesting to observe that an increasing number of mitochondrial proteins have been established to carry out multiple functions. A well known example is cytochrome c, which acts as both a redox electron shuttle of the MRC, and as an apoptogenic messenger (29). Likewise, the apoptosis-inducing factor (AIF), a flavoprotein closely associated with the mitochondrial inner membrane, has been implicated as both a cell death-promoting molecule and a regulator of activity and protein expression of MRC complex I (30). Lastly, p66\(^{shc}\) is an electron-transfer redox enzyme of the mitochondrial intermembrane space, which controls the production of reactive oxygen species (ROS), and regulates Ca\(^{2+}\) transport by acting on plasmamembrane Ca\(^{2+}\)-ATPases. These two independent activities can ultimately converge and synergize in mediating p66\(^{shc}\)-dependent apoptosis (31). Targeted disruption of p66\(^{shc}\) is associated with prolonged lifespan in mice, possibly related to its role on the control of ROS production.

Similar to the p66\(^{shc}\)\(-\)/\(-\), our Surf1\(^{loxP}\)\(-\)/\(-\) mouse model displays significantly increased longevity. Of note, increased longevity was also observed in a CNS-restricted conditional Surf1 knockdown (KD) model in Drosophila melanogaster, whereas the corresponding constitutive model was embryonic lethal (32). We do not have an explanation for this observation, which is in striking contrast with the early onset, invariably fatal phenotype associated with the loss of SURF1p function in humans. It is possible that the COX defect in Surf1\(^{loxP}\)\(-\)/\(-\) mice is not severe enough to confer selective disadvantage in a ‘protected’ environment such as an animal care facility. These data are nevertheless surprising, considering that COX deficiency associated with Surf1p disruption should in principle determine an increase of ROS (33), which are proposed to play a major role in the aging process (34). An attractive possibility is that the effect on longevity is due to the role of Surf1p on mitochondrial Ca\(^{2+}\) uptake and cellular Ca\(^{2+}\) homeostasis. This effect is likely to be hidden in organisms, such as S. cerevisiae and Homo sapiens, in which lack of Surf1p leads to severe impairment of COX assembly and faulty OXPHOS, whereas it would be unmasked in other organisms, such as Mus musculus and the conditional D. melanogaster KD model, characterized by less severe impairment of COX assembly and OXPHOS phenotype.

**MATERIALS AND METHODS**

**Creation of Surf1\(^{loxP\_NEO\_loxP}\)\(+\)/\(-\) and Surf1\(^{loxP}\)\(-\)/\(-\) recombinant mice**

The list of all primers is provided in the Supplementary Material, Table 2.

For the construction of the Surf1 recombinant alleles, we used a 10 kb HindIII-EcoRI fragment containing the entire Surf1 and Surf2 genes, and part of Surf4 and Surf3 genes, cloned in BlueScript SK. A fragment of approximately 1.2 kb, composed of the gene encoding the neomycin phosphotransferase (NEO cassette) flanked by two 35-mer identical loxP sites (loxP\_NEO\_loxP), was inserted in the unique AccIII site contained in exon 7 of the Surf1 murine gene corresponding to nt 670 of the Surf1 cDNA. The DNA vector was verified on both strands by automated sequence analysis using the big-dye terminator kit and protocol (Applied Biosystems), on a 3100 ABI apparatus.

Gene targeting by electroporation of the HindIII-linearized vector into AB1 ES cells, derived from 129/SvEvBr [\(\neq\) / Hprt-bm2 mouse substrain (a kind gift from Alan Bradley), and generation of chimeras, were performed as described (35). Three hundred colonies that survived selection with the neomycin analogue drug G-418 (200 \(\mu\)g/mL) were screened for homologous recombination by PCR and Southern blot analyses.

For diagnostic PCR analysis, a 2 kb DNA fragment was amplified using a forward primer corresponding to a region inside the NEO cassette and a reverse primer corresponding to a sequence within the Surf3 gene located outside the recombinant region. For diagnostic Southern blot analysis (Fig. 1B) on the 5’ end of the recombinant region, 10 \(\mu\)g of ES genomic DNA was digested with EcoRI, run through a 0.8% agarose gel in 1X TE buffer, blotted on a nitrocellulose filter and hybridized with a 0.7 kb PCR fragment (probe P1 in Fig. 1) radiolabeled with \([\alpha^32P]\)-dCTP (NEN, Boston, MA, USA) using the ‘Ready-to-go’ random priming kit (Amersham, Piscataway, NJ, USA). P1 corresponds to a region of the Surf3 gene outside but contiguous to the recombinant region. The wt Surf1 allele corresponds to a 19 kb hybridization band, whereas the Surf1\(^{loxP\_NEO\_loxP}\)\(-\) recombinant allele corresponds to 13 and 7 kb bands due to the presence of an EcoRI site within the NEO cassette. For diagnostic Southern blot analysis on the 3’ end, DNA was digested with EagI and HindIII, separated by electrophoresis and blotted as above. Hybridization was then carried using a 0.8 fragment corresponding to a sequence on the Surf4 gene outside but contiguous to the recombinant region (probe P2 in Fig. 1). The wt allele corresponds to a hybridization band of 9.2 kb, whereas the Surf1\(^{loxP\_NEO\_loxP}\)\(-\) recombinant allele corresponds to a band of 7.5 kb, again due to the presence of an extra EagI site within the NEO cassette.

Two of the five ES clones that showed homologous recombination were injected in B6D2F1[\(\neq\) C57/B6J] blastocysts (36). Chimeric pups were identified by the presence of agouti hair and, on maturity, mated with B6D2F1 (C57/Bl6J, DBA2) females to check for the contribution of the ES cells to the germline.

For genotyping, 250 ng genomic DNA extracted from tail tips was PCR amplified in 50 \(\mu\)l of 1 \(\times\) MgCl\(_2\)-PCR buffer (Applied Biosystems), 200 \(\mu\)M dNTPs, 0.6 \(\mu\)M each dNTPs and 0.03 \(\mu\)M Taq Polymerase (Applied Biosystems), 5% DMSO. After an initial denaturation at 94°C for 2 min, each of the 35 PCR cycles was as follows: 94°C for 30 s, 58°C for 60 s, 72°C for 90 s. Final extension was at 72°C for 5 min. For the Surf1\(^{loxP\_NEO\_loxP}\) allele, we used a single...
forward primer (LNL-FW) and two distinct reverse primers, one internal to the NEO cassette (LNL-RV1) specific to the recombinant allele, and another corresponding to a Surf1 region (LNL-RV2) specific to the wt allele. The recombinant allele generates a PCR fragment of 583 bp, whereas the wt allele generates a PCR fragment of 458 bp. For the Surf1<sup>loxP</sup> allele, we used the forward primer LP-FW, corresponding to a region of Surf1 exon 6; and the reverse primer LP-RV, corresponding to a region of Surf1 exon 7. The PCR produces a 305 bp wt DNA fragment and a 340 bp recombinant fragment. The cre transgene was detected by using the forward primers CRE-FW and the reverse primer CRE-RV (PCR fragment 590 bp).

Animal studies were approved by the animal welfare Ethics Committee of the National Neurological Institute in accordance with the Institutional Animal Care and Use Committee guidelines. Standard food and water were given ad libitum.

**RT-PCR analysis**

To evaluate the presence of recombinant Surf1<sup>loxP</sup> transcript, total RNA was extracted from brain, liver, muscle and heart of four Surf1<sup>loxP</sup><sup>/−</sup> and four Surf1<sup>+/+</sup> adult animals, using the RNeasy lipid tissue kit (QIAGEN Sciences, MD, USA) following the manufacturer’s protocol. Total RNA was used as a template for reverse transcription, using the ‘cDNA cycle’ kit and protocol (Invitrogen, Carlsbad, CA, USA). Total cDNA was purified and resuspended in a final volume of 20 µl and used for PCR amplification of individual cDNA fragments corresponding to GAPDH and Surf1 genes; 5 µl were then used in each 50 µl PCR reaction containing 1 × MgCl<sub>2</sub>-PCR buffer, 200 μM dNTPs, 5% DMSO, 0.6 µM of each primer and 0.03 U/µl of Taq-Gold polymerase (Invitrogen, Carlsbad, CA, USA). GAPDH was detected using primers GAPDH-FW and GAPDH-RV. The wt and Surf1<sup>loxP</sup> transcripts were PCR-diagnosed using exonic primers LP-FW and LP-RV (Fig. 1E).

**Western blot analysis**

Western blot analysis was performed on electroblotted denaturing sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and two-dimension blue native electrophoresis (2D-BNE), as described previously (6). Approximately 100 µg non-collagenous protein was used for each sample in SDS-PAGE and 20 µg of isolated mitochondria in 2D-BNE. Chemiluminescence-based immunostaining (ECL kit, Amersham) was performed using the following antibodies: polyclonal antibody AS182–196 (6) raised against amino acid sequence 82–96 of mouse Surf1p, which is at the N-terminal of the truncated protein predicted by the Surf1<sup>loxP</sup> allele; monoclonal antibodies against subunits COX I and COX IV (Molecular Probes, Eugene, OR, USA) and Surf1p (Mitosciences LLC, Eugene, OR, USA).

**Morphological analysis**

For light microscopy, samples from different organs were frozen in liquid-nitrogen-cooled isopentane. Standard histological and histochemical techniques for the detection of mitochondrial alterations and muscle fiber distribution were performed on serial cryostat cross sections as previously described (37).

**Biochemical analysis**

Biochemical assays of individual respiratory complexes were carried out on tissue homogenates (38). Specific activities of each complex were normalized to that of CS, an indicator of the number of mitochondria. Blood lactate was measured using the ‘Lactate reagent’ kit and protocol (Sigma, St Louis, MO, USA).

**Rotarod test**

Motor performance tests were given to 13 wt mice and to 13 Surf1<sup>loxP</sup><sup>/−</sup> mice. All animals were 3 months old. The rotating rod test was performed on a Rotarod apparatus for mice (Ugo Basile) (39).

**Kainate-induced seizures and brain analysis**

Kainic acid was dissolved in isotonic saline (pH 7) with a drop of 1 m NaOH and administered i.p. Mice were monitored continuously for at least 3 h after injection to determine the onset and level of seizures according to Spork et al., (13). For histological analysis of the brain, animals were treated with a lethal injection of 4% chloral hydrate before intracardiac perfusion with 4% paraformaldehyde in PBS. Brains were rapidly dissected and post-fixed overnight in the same solution. Serial rostro-caudal, 50 µm thick coronal brain sections were obtained using a Vibratome (Leica) and collected in 0.1 M PB at pH 7.2 with 0.01% NaN<sub>3</sub>. One every 12 sections was labeled with FJB (Histo-Chem, Jefferson, AR) according to manufacturer’s instructions. Adjacent sections were stained with 0.1% thionine. Next adjacent sections were labeled by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) (Apoptag <i>in situ</i> Apoptosis Detection kit; Intergen, Purchase, NY) according to the manufacturer’s instructions. Fluorescent images were acquired on a confocal microscope (Radiance 2100 confocal microscope, Bio-Rad, Hercules, CA, USA) using an FITC filter and optical photographs were acquired using a Nikon Eclipse E400 microscope and a Nikon DS-U1 digital camera.

**Cortical/hippocampal primary neuronal cell cultures**

Cortical/hippocampal neurons were prepared from 1- to 3-day-old newborn mice, according to Pasti et al. (40), and neurons were resuspended in NeurobasalA Medium (Gibco) with supplement B-27 (Gibco), GlutaMax (Gibco) and penicillin/streptomycine (Gibco) rigorously at 37°C and plated onto glass coverslips, coated with poly-D-lysine (Sigma).

**Dynamic in vivo [Ca<sup>2+</sup>] measurements with targeted aequorin probes**

The construction and use of luminescent Ca<sup>2+</sup> sensitive aequorin probes were previously described (41). All
aequorin measurements were carried out in KRB containing 1 mM CaCl$_2$ (KRB/Ca$^{2+}$), Krebs-Ringer modified Buffer: 135 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 0.4 mM K$_2$HPO$_4$, 1 mM CaCl$_2$, 15 mM glucose, 20 mM HEPES, pH 7.4). For HeLa cells KRB contained 5 mM glucose. Experiments in permeabilized neurons were performed as previously described for HeLa cells (42), except that 25 µM digitonin was used, in order to preserve mitochondrial integrity.

**Imaging procedures**

Cortical cultures were loaded for 20 min with 3 µM fura-2FF/AM (or fura-2) (Teflabs, Austin, TX, USA) at 37°C in the cell culture medium (K$_2$ of fura-2FF or fura-2 for Ca$^{2+}$ is 5 and 55 µM, respectively; 43). Images were acquired on an epifluorescence inverted microscope Axiosvert 200 (Zeiss, Germany) equipped with a 40× fluoro objective. [Ca$^{2+}$]$_i$ was monitored in single cells using two excitation light wavelengths, at 340 nm and 380 nm (Sutter Instrument Co., CA, USA). Emitted fluorescence light was selected by a 505–530 nm filter. Images were acquired by CCD camera (Roper Scientific, USA). All imaging data were collected and analyzed using the Metafluor 6.1 software. For the visualization of the mitochondrial network, Z-series of images of neurons were 3D deconvolved and reconstructed using a custom-made software (44).

**Statistical analysis**

Two-tailed, unpaired, unequal variance Student's t-test was used for statistical analysis. Survival probability was calculated using the Kaplan–Meier and log-rank tests.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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**Conflict of Interest statement.** None declared.

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