Regulatory dynamics of energy metabolism in living cells entails a coordinated response of multiple enzyme networks that operate under non-equilibrium conditions. Here we show that mitochondrial dysfunctions associated with the aging process significantly modify nonlinear dynamical signatures in free radical generation/removal, thereby altering energy metabolism in liver cells. We support our data with a plausible biochemical mechanism for modified bioenergetics that involves uncoupling protein-2 that is up-regulated in aged cells as an adaptive response to mitigate increased oxidative stress. Combining high spatial and temporal resolution imaging and bio-energetic measurements, our work provides experimental support to the hypothesis that mitochondria manifest nonlinear dynamical behavior for efficiently regulating energy metabolism in intact cells, and any partial or complete reduction in this behavior would contribute to organ dysfunctions including the aging process and other disease processes.

Complexity in a dynamic system is characterized by mutually interacting components (nonlinear network dynamics), hypersensitivity to initial conditions (deterministic chaos), and long-term memory processes (power-law scaling) (1, 2). Because of the fact that multiple enzymes, cofactors, and signaling molecules contribute to efficient cell survival amid spatially/temporally varying stimuli, metabolic networks in a living cell are unique biological candidates for studying the role that complexity plays in regulatory enzyme kinetics. Mitochondria serve dual roles in cellular metabolism; that is, as the hub of cellular energetic as well as the centers of programmed cell death (3). The mitochondrial theory of aging postulates that intracellular reactive oxygen species (ROS) produced during normal cellular metabolism cumulatively contributes to the progressive damage of macromolecules and, hence, to organ dysfunction that collectively define aging in organisms (4–6). Earlier studies in isolated mitochondria from young/aged animals were unable to provide a realistic picture since they ignored physiologically relevant regulatory cross-talks between mitochondria and cytosol/nucleus. On the other hand lifespan measurements at the whole animal level may indicate specific conditions (such as calorie restriction) in which lifespan can be increased or decreased, but they do not give any clue on the cellular mechanism involved in the aging process per se. It is, therefore, imperative to develop strategies to probe single-cell responses that can report the mitochondrial dynamics in relation to other relevant metabolic networks in living cells rather than in isolated conditions. Furthermore, it is possible to dissect single cell responses under controlled perturbations, and one can obtain mechanistic insights into the complex dynamics of metabolic networks in living cells. With this motivation we sought to ask how the aging process contributes to modifications in subtle dynamics in metabolic networks and how these modifications determine the overall cellular responses during normal substrate metabolism and under oxidative stress. We used liver cells (primary hepatocytes) isolated from young (5 month) and aged (28 month) mouse models as our experimental model system and employed a unique combination of spatially resolved single cell chemical kinetics, scaling analysis, and biochemical assays. We observed that young liver cells manifest nonlinear dynamics for efficiently regulating ROS generation/removal machinery and that these regulatory correlations in free radical dynamics are diminished in aged cells, suggesting that the aging process modulates chemical dynamics (complexity) in liver cell energy metabolism. As a direct consequence of modulated redox/ROS dynamics, the aged cells up-regulate uncoupling protein homolog possibly for mitigating increased oxidative stress, which in turn contributes to diminished bioenergetics during substrate metabolism. Complex behaviors have been reported recently in various physiological signals such as heart beat intervals, gait dynamics, and respiratory signals, thus challenging the classical paradigm of homeostatic regulation (2, 7–13). Our data demonstrate for the first time that similar physiologically relevant nonlinear dynamical features are operative at the level of single cells for efficient regulation of energy metabolism.

EXPERIMENTAL PROCEDURES

Hepatocyte Isolation and Cell Culture—Primary hepatocytes were isolated from C57BL/6 mice (wild type, young and aged; obtained from NIA, National Institutes of Health) by perfusing the liver as described (14). Mice were anesthetized with ketamine/small animal Rompun mixture, and liver was perfused through the hepatic portal vein with 0.5 mM EGTA in Ca^{2+}-free Earle’s balanced salt solution (EBSS) for 5 min followed sequen-
Nonlinear Dynamics in Liver Cell Metabolism

Initially by wash buffer (0.5% bovine serum albumin and 1.8 mm CaCl₂ in Ca²⁺-free EBSS) and by collagenase buffer (0.06% collagenase, 0.5% bovine serum albumin, and 5 mm CaCl₂ in Ca²⁺-free EBSS) for 10–15 min. The liver was then further perfused with wash buffer for 5 min, and hepatocytes were collected. After washing three times with cold wash buffer, the hepatocytes were cultured in William’s E media supplemented with 5% fetal bovine serum, 4 μg/ml insulin, 1000 unit/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B in collagen-coated chambered cover glasses (Nalge Nunc International, Napervile, IL). Cells were maintained at 37 °C in an atmosphere of 5% of CO₂ and 95% air. To minimize the de-differentiation effects due to cell culture, all the experiments were performed within 9–14 h after isolation, and the results reported in this article correspond closely to in vivo conditions. All animal surgeries were performed according to the institutional animal protocol guidelines.

**Single Cell Kinetics Imaging and Time-series Data Acquisition**—Isolated hepatocytes were cultured in 2-well imaging chambers with a glass coverslip bottom at 37 °C, 5% CO₂ for 8–14 h. Before imaging, medium with serum was replaced by Hank’s balanced salt solution (HBSS) with low glucose. Cultured cells were labeled with two ROS probes: MitoSOX Red (Invitrogen #M36008; 514-nm excitation; 580-nm red emission) and CM-H₂DCFDA (Invitrogen #C6827; 488 nm excitation; 520-nm green emission) in HBSS devoid of any serum. These probes are known to selectively label mitochondrial superoxide and hydrogen peroxide radicals, respectively. All the cells considered in these experiments showed distinct mitochondrial labeling. Hepatocytes are abundant in mitochondria for the energy requirements, and the basal ROS levels in hepatocytes were almost equal in both young and aged hepatocytes. There was no statistical difference observed between the dye uptake of young and aged hepatocytes. These probes were reported earlier to have high response time so that in the time scales of measurement it is ensured that these probes sense the free radicals in real time. Because both these probes yielded identical results, only MitoSox Red data are presented for the sake of simplicity. Zeiss LSM510 confocal microscope (63×, 1.3 NA, oil immersion; 25 °C) was used to measure the time-course kinetics of ROS intensity. For monitoring redox (NAD(P)H) kinetics, the cells were imaged in HBSS medium in a homemade two-photon imaging system with the following imaging conditions: 730 nm two-photon excitation; 440/90 nm emission (15). In a typical kinetics run, a field of view with 6–8 cells were chosen, time-lapse (n = 100 XY scan images) imaging was carried out at regular intervals (~5 s/acquisition for each channel), 5 mM pyruvate/glutamate (PG) mixture (mitochondrial substrate) was added at n = 20, and the rest of the time-lapse was obtained. There was no focus drift due to the addition of PG substrate (10 μl in a 1-ml volume), and the transient rise in ROS levels was solely due to PG substrate metabolism and not due to the buffer. It is known that pyruvate and glutamate can rapidly enter the liver cells through monocarboxylate transporters (16). After the data acquisition, small regions of interest (~20 × 20 pixel size) were selected, each containing nearly 6–8 mitochondria. Average ROS/redox intensity in these regions were calculated using Zeiss 510 software and transferred to an excel spreadsheet for further analysis. Time-course intensity profiles for individual regions were normalized to the intensity at t = 0 for monitoring fractional changes in ROS kinetics. Further similar kinetics profiles were observed in flow cytometric experiments where typically 10⁶ cells were assayed, suggesting that the kinetics profiles reported in the paper are representative of whole cell population (data not shown). ROS time-series in young and aged hepatocytes were acquired as follows; a field of view with ~4–6 cells was chosen before measurements. Instead of XY frame-scan, a line scan was performed so that the chosen line spanned all the cells and nearly 50–80 mitochondria. Zeiss LSM510 data acquisition software allows time-lapse acquisition of the line scan. Because the speed of acquisition was critical, only Mitosox fluorescence was monitored for the fluctuation experiments. n = 6000 time points (Δt = 30–100 ms; pixel dwell time = 30–50 μs) were acquired for the chosen line scan. It was ensured to have minimal laser excitation (~0.5–1% of maximum 20 milliwatt) so that photobleaching was nil or minimum.

**Rate Constant Analysis**—A biphasic pharmacokinetics model was adopted for analyzing the single cell kinetics data. The rationale for this choice is that liver cells displayed a rapid, instantaneous absorption and an immediate fast decay followed by a slower decay, indicating that there are two distinct phases, the first denoting instantaneous response (α phase) and the other denoting steady-state response (β phase). It is known that hepatocytes express monocarboxylate transporters in their plasma membrane which facilitate rapid pyruvate/glutamate transport into the cytosol thereby justifying the assumptions of well mixed container and rapid mixing while analyzing the substrate-induced redox/ROS kinetics (16). The reversibility of the fluorogenic probe Mitosox Red was tested in a separate experiment where repeated substrate-stimuli yielded identical response times. ROS (elimination) decay was assumed to follow pseudo-first-order kinetics, K_{el} = −d[I]/dt = K_{el}. From this differential equation, it is clear that the rate (d[I]/dt) changes as the concentration changes; however, for a linear model, the rate constant K_{el} is constant. For statistical comparison of young/aged groups, a one-way analysis of variance test was adopted, and statistical significance was set for values p < 0.05.

**Scaling Analysis; Detrended Fluctuation Analysis**—The major premise of scaling analysis can be summarized as follows. Any cellular process is a manifestation of interactions among many interdependent chemical reactions. If the cellular process is regulated by specific metabolic components (for example, oxidative phosphorylation enzymes and tricarboxylic acid cycle enzymes together regulate substrate metabolism in mitochondria), then monitoring the process-specific fluorescence signals can shed light on the underlying regulatory dynamics. Scaling analysis is an elegant way to probe real-time regulatory dynamics by analyzing subtle signal fluctuations (fluorescence fluctuations in the present case) that attempts to look for time-correlations between the signal (or process) at any instant and the same signal (or process) at some other instant. If there are no apparent correlations between the signal in various time-windows, then the underlying process can be assumed to be a “random” process. Brownian diffusion is a classical example of a
random process (brownian noise) with no apparent long-range time correlations. On the other hand, a non-random scaling behavior may indicate positive regulatory correlations in time-scales that are relevant to the cellular process that is being probed. In the recent past, new analytical strategies have been developed to look for scaling behaviors in physiological signals such as heart beat interval, gait dynamics, etc. In the present work we attempt to undertake a novel application of similar strategies to ROS dynamics at the level of single cells as described below.

Detrended fluctuation analysis (DFA) is a modified root mean square fluctuation analysis of random walk originally developed by C.-K. Peng et al. (17) to quantify statistical correlations in time-series signal. The original time-series (of length $N$) is first integrated and then divided into boxes of equal size ($n$). In each box the integrated profile is fit to a polynomial that gives a local trend in that box. Next, this local trend is subtracted from the integrated profile in each box which is termed “detrending.” Finally, root mean square fluctuation $F(n)$ is calculated from the integrated and detrended signals in each box. These steps are repeated for different values of box size ($n$) to generate $F(n)$ for broad range of scale sizes $n$. Intuitively $F(n)$ will increase as $n$ increases, and for scale-invariant signals with power-law correlations, there is a scaling relationship $F(n) = n^\alpha$. In a nutshell, DFA dissects the original time series data into many windows with progressively increasing scale size ($n$) and calculates the root mean square fluctuations $F(n)$ for every scale size ($n$) to yield a scaling function $F(n) = n^\alpha$. The scaling exponent $\alpha$ is a quantitative measure of extent of correlations in the signal. $\alpha$ can characterize randomness ($\alpha = 0.5$ for white noise; $\alpha = 1.5$ for Brownian noise) or correlations ($\alpha < 0.5$ for anti-correlations, and $0.5 < \alpha < 1.5$ for persistent power law-like correlations) regardless of the nature or source of the fluctuations. Similar crossover in exponents has been noted in earlier studies of physiological signals. An important advantage of the DFA algorithm over other time-series analysis methods is that it can be reliably used for non-stationary signals also since local detrending eliminates the errors associated with being non-stationary. Although this algorithm has been successfully used in quantifying correlations in physiological signals such as heartbeat dynamics and human gait dynamics, to the best of our knowledge there is no systematic application of time-series analysis in single cell dynamics as presented in this paper. We further verified that the DFA plots for simulated noise functions, microscope photomultiplier tube noise, and a fluorescent solution specimen (rhodamine 6G in water) yielded $\alpha = 0.5$, indicating that the observed correlated dynamics in intact cells is mitochondrial regulation-specific and not due to instrument artifacts. More details about this algorithm can be found in Peng et al. (17), and source code can be obtained from the webpage of National Research source. Further information on application of this algorithm to mitochondrial signals was recently reported (18).

Scaling Analysis; Fast Fourier Transform—Fast Fourier transform (FFT) is another well established power spectrum analysis method to quantify correlations in a time-series signal. The basic idea is to convert the signal in time-domain (s) to frequency domain (Hz) and compute the different frequency components in the signal. A periodic wave (sine wave for example) oscillating with a period $T$ in time-domain will have a single frequency component $f = 1/T$ in FFT amplitude output. Usually time-series analysis output is represented as power spectral density, which is the square of the FFT amplitude. For an uncorrelated noise input, the power spectral density will be flat for the entire frequency scale implying that all frequencies have almost equal amplitudes. This flat power spectral density output is the source of the name “white noise” for uncorrelated randomness. To quantify correlations in an unknown signal, the FFT power spectral density is plotted as a function of frequency (log-log scale). Power spectral density follows a scaling relationship, $P(f) = f^{-\beta}$, where the exponent $\beta$ characterizes randomness ($\beta = 0$ for white noise, and $\beta = 2$ for Brown noise). FFT plots were obtained by using the FFT tool in Origin 5.0 (OriginLab Corp., Northampton, MA). These plots clearly indicate long-range correlations in redox/ROS fluctuations and further substantiate the results obtained independently from detrended fluctuation analysis.

Scaling Analysis; Poincare’ Phase-Plane Plots—Poincare’ plot analysis is a quantitative visual technique whereby the shape of the plot is categorized into functional classes (19). The Poincare’ plot is a scatterplot of the current ROS fluctuation (instantaneous value) plotted against the preceding instantaneous ROS fluctuation. The plot provides summary information as well as detailed instant-to-instant information on the behavior of the ROS dynamics. Points above the line of identity indicate ROS fluctuations that are longer than the preceding ROS fluctuation, and points below the line of identity indicate a shorter ROS fluctuation than the previous one. Accordingly, the dispersion of points perpendicular to the line of identity (the “width”) reflects the level of short-term variability. This dispersion can be quantified by the S.D. of the distances the points lie from the line of identity. This measure is equivalent to the S.D. of the successive differences of the ROS fluctuations.

ATP Measurements—Isolated hepatocytes were transferred to William’s E medium and incubated for 30 min at 37 °C, 5% CO2. For different treatments mentioned in the text (complex inhibition, glycolytic inhibition, and OXPhos uncoupling), separate microcentrifuges each containing ~1 million cells/ml were centrifuged at 3000 $g$ for 5 min at room temperature. The serum-containing medium was removed, and the cell pellet was washed twice in HBSS before resuspending in 1 ml of HBSS. The cellular ATP extraction was carried out as described earlier (23). Briefly, the control and inhibitor-treated cells were centrifuged at 10,000 $g$ for 10 min, 4 °C to obtain a solid cell pellet. The cellular ATP was then extracted by adding 1 ml of boiling water to the pellet. After vortexing and centrifugation (12,000 $g$; 5 min; 4 °C), the supernatant was carefully removed and immediately transferred to a fresh microcentrifuge maintained in ice. Bioluminescent detection of ATP was carried out by using Enliten ATP assay system (#FF2000, Promega Corp., Madison, WI) which detects ATP by using the reaction ATP + d-luciferin + O2 → oxyluciferin + AMP + pyrophosphate + CO2 + light (560 nm). When ATP is the limiting component in the luciferase reaction, the intensity of the emitted light is proportional to the concentration of ATP. Luminescence measurements were carried out by using a luminometer (TD20/20,
Turner Biosystems, Sunnyvale, CA). ATP standards were prepared and measured in identical conditions to yield a standard curve. Results presented are representative of three different experiments performed in identical conditions, each in duplicate.

**Western Blots**—Proteins in whole cell lysates were resolved by electrophoresis on SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blotted for molecules of interest with anti-UCP2 (1:500; Calbiochem), anti-complex II (1:10,000, MitoSciences), anti-cytochrome c (1:1000, MitoSciences), anti-SOD2 (1:250, SantaCruz Biotechnologies) overnight. The bound primary antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies followed by detection with a SuperSignal enhanced chemiluminescence kit (Pierce). Student’s t test was employed where necessary to obtain statistical significance (p < 0.05) in densitometry.

**RESULTS**

Young and Aged Cells Show Significant Differences in Single Cell ROS/Redox Kinetics—To establish and validate single cell chemical kinetics approach, we first monitored redox (NADH/NAD+)) and ROS kinetics in intact hepatocytes. Fig. 1 shows representative fluorescence intensity images of young and aged hepatocytes showing superoxide-selective Mitosox Red labeling (confocal) and two-photon excited autofluorescence (NAD(P)H) emission signals. Average kinetics profiles (redox and ROS) in young and aged hepatocytes from 6–8 independent experiments are shown, and as can be seen, young and aged cells show distinct kinetics profiles upon mitochondrial substrate stimulus. In addition to the transient increase and a subsequent decay in redox signals, aged cells show an additional broad peak that was absent in young cells. Similar to redox kinetics, there is a corresponding increase in steady-state ROS levels in both young and aged cells. However, aged cells clearly show the difference in removal rates of steady-state ROS levels indicating differences in antioxidant responses to transient perturbations in young and aged hepatocytes. A biphasic pharmacokinetics model (α, initial substrate oxidation phase; β, steady-state substrate oxidation phase) was adopted to analyze the ROS removal kinetics, and these parameters are summarized in Fig. 1, d and e. We observed that aged cells have a slower ROS removal rate than young cells. To investigate the effects of respiratory chain inhibition on ROS kinetics parameters, we selectively preconditioned the young and aged cells with respective inhibitors. Fig. 1 summarizes the pharmacokinetics parameters obtained after respiratory chain inhibition at complex I site (10 μm rotenone) or complex III site (10 μm antimycin A) before carrying out the substrate-induced ROS kinetics. This quanti-
Nonlinear Dynamics in Liver Cell Metabolism

tatively verifies that young and aged cells differ in their ability to respond to transient perturbations to steady-state ROS levels. It is interesting to note that transient perturbations in fact enable the cells to explore new steady states when challenged with a stimulus of mitochondrial substrates.

Quantitative analysis of basal NAD(P)H oxidation kinetics (Fig. 2a) revealed that young cells have a significantly higher oxidation rate than aged cells, which may indicate an impaired complex I function in aged cells. Fig. 2b shows the cellular response to multiple PG stimuli, demonstrating rapid substrate mixing in cells and, thus, justifying the assumed biphasic pharmacokinetics model.

In contrast to the case where the cells were stimulated with complex I substrates (pyruvate/glutamate), aged cells show a higher redox signal when stimulated by complex II substrate (Fig. 2d) succinate, in contrast to the case where the cells were stimulated with complex I substrates (pyruvate/glutamate), and this feature will be discussed in a later section of this article.

Aged Cells Show Characteristic Reduction in Nonlinear Scaling Behavior in ROS Dynamics—ROS-sensitive signal fluctuations labeling in cultured hepatocytes was carried out as described under “Experimental Procedures.”

Fig. 3a shows representative time-series data indicating ROS-sensitive signal fluctuations. Self-similarity of fluctuations can be seen in different time-windows as indicated. Fig. 3b shows a summary of scaling behavior obtained from DFA of the time-series data in intact cells (representative of n = 6 experiments). Short-term (0 < log n < 1.5) scaling behavior resembles white noise limit (scaling exponent α = 0.5). However, there is a distinct crossover at larger time scales (1.5 < log n < 4) where both young and aged cells display scaling exponent α ∼ 1.1 that is much lower than the brown noise limit of 1.5, indicating that both young and aged hepatocytes display non-random, positive correlations during resting metabolic status. Also shown in the figure is the summary of scaling behaviors in young and aged cells when metabolizing mitochondrial substrate pyruvate/glutamate. Under these conditions of transient metabolic perturbations, the young cells maintain their scaling integrity (α = 1.16), whereas the aged cells display a reduction in scaling behavior as manifested in the long-term scaling exponents (α = 1.38). DFA scaling plot of the randomized time-series ROS data in both young and aged hepatocytes yielded a value of α = 0.5 as shown in the figure. This indicates that the observed scaling exponents in hepatocytes are genuinely related to intrinsic dynamics and not an analytical artifact. Because white noise (α = 0.5) and brown noise (α = 1.5) limits can be interpreted as measures of uncorrelated randomness, any deviation from these values will indicate the extent to which a system exhibits nonlinear dynamical scaling behavior.

Fig. 3c shows plots of deviation from randomness (Δα) in young and aged hepatocytes as calculated from Fig. 3b. This quantitatively confirms the fact that aged cells display a characteristic reduction in scaling behavior during substrate metabolism. Also shown in the figure is Δα for non-viable, dead cells that further confirms the classical notion that living cells are open systems far away from thermodynamic equilibrium. The above analysis was independently confirmed by plotting Poincare’ phase-plane plots (Fig. 3d) and by fast-Fourier transform analysis (Fig. 3e) of the time-series data. Young and aged hepatocytes clearly indicate different dynamics in unperturbed (control) and metabolic perturbed (pyruvate/glutamate substrate) states. The minor and major axes of the ellipse correspond respectively to short- and long-term regulatory correlations and the complexity of the system is measured by the degree of dispersion of the data points in the delayed map.
As can be seen clearly, young cells display larger dispersion than aged cells even in their resting metabolic status, and this becomes more pronounced during substrate metabolism. On the other hand, substrate stimulus induces only a small change in dispersion along the major axis in aged cells, further confirming that aged cells have reduced regulatory capacity during mitochondrial substrate metabolism.

**Mitochondrial Dysfunction in Aged Cells Leads to (OxPhos-Glycolysis) Metabolic Switching**—Having confirmed the existence of nonlinear dynamic features in mitochondrial regulatory enzyme network, we wanted to further test the robustness of this complexity by selective perturbations to both glycolytic and mitochondrial metabolic pathways by monitoring the total ATP under these perturbations (Fig. 4). ATP measurements were carried out in liver cells as described (21) under different metabolic perturbations. Fig. 4a shows that under glycolytic inhibition conditions, young cells have much higher ATP during substrate metabolism, indicating that mitochondrial functioning is intact in these cells. However, aged cells are unable to increase ATP even under glycolytic inhibition, suggesting that mitochondrial bioenergetics in aged cells is severely compromised plausibly due to respiratory inhibition and/or uncoupling of substrate oxidation from ADP phosphorylation as confirmed by preconditioning with complex inhibitors (rotenone and antimycin A) and synthetic uncoupler (2,4-dinitrophenol), respectively. Glycolytic inhibition is expected to amplify mitochondrial metabolism as an adaptive response, and as can be seen, mitochondrial metabolism is more efficient in young cells as compared with aged cells. Furthermore, mitochondrial complex inhibition is expected to decrease ATP in both normal and glycolytic-inhibited conditions. These data show how cellular energetics is tightly regulated by mutually interacting nonlinear feedback circuits (glycolysis/electron transport chain/ATP synthesis).

**Nonlinear Dynamics of Metabolic Networks Manifests in Compensatory Protein Expression**—Stability of a dynamical network can be evaluated by monitoring its ability to compensate for structural-functional loss in any part of the network (27). In the context of mitochondrial enzyme network, any perturbation to respiratory chain may evoke compensatory responses in overall metabolism. We observed that young cells displayed compensatory up-regulation of complex II subunit protein levels (Fig. 5, a and b) when challenged with acute (2 days) complex I/III inhibition or chronic complex I inhibition (up to 6 days). The disproportionate responses to 100 nM and 1 μM rotenone treatment in young cells further validate nonlinear dynamical paradigm in liver cells even at the protein expression levels. On the other hand, aged cells displayed constitutively higher basal levels of complex II (probably due to age-related chronic complex I/III dysfunction), and these levels do not change appreciably in response to complex I/III inhibition, suggesting that aged cells are not as robust as young cells in eliciting compensatory responses. Succinate oxidation (complex II activity) was constitutively higher in aged cells in accordance with the higher protein level expression (Fig. 2d).

**DISCUSSION**

Our observations of single cell kinetic responses during substrate metabolism together with scaling analysis as described in this article point to an interesting strategy for analyzing subtle metabolic modifications with high spatial and temporal resolution. A common theme in redox/ROS kinetics is that despite the fact that initial transient flux look similar, there are significant differences between young and aged hepatocytes in the signal decay profiles. We speculate that these differences arise from nonlinear network interactions among glycolysis, gluconeogenesis, and mitochondrial electron transport chain (Fig. 6b). Pyruvate dehydrogenase is the key enzyme that converts glycolytic product pyruvate to acetyl-CoA as an input to mitochondrial pathway, and it is known that pyruvate dehydrogenase is inhibited by excess NADH/acetyl-CoA (product inhibition). If the mitochondrial pathway is defective in aged cells,
unmetabolized pyruvate will be converted back to glucose by gluconeogenesis, which then will initiate the glycolytic pathway. Because NADH generation/oxidation rates are different in glycolytic and mitochondrial pathways, the second redox peak in aged cells (Fig. 1b) points out to a defective mitochondrial electron transport chain. It has been reported earlier that aging is associated with ROS-induced chronic dysfunction of mitochondrial respiratory chain either at site I or III and that mitochondria isolated from aged animals show reduced sensitivity to complex I inhibitor (28, 29). Similar trends were observed in intact hepatocytes (Figs. 1d). These data point out that aging in liver is accompanied by a dysfunctional mitochondrial network that can be due to structural or functional respiratory defects or both. The present observation of positive, long-range correlations at the level of single cells confirms the general notion that living cells operate far from equilibrium in a complex-phase space and display nonlinear dynamical behavior to efficiently regulate energy metabolism. The fact that aged cells displayed diminished regulatory correlations further confirms that the aging process systematically modulates energy metabolism, driven by structural and/or functional defects in metabolic networks (Fig. 6a). Intriguingly, these differences in regulatory correlations seem to have direct influence on cellular bioenergetics.

A major contribution of cellular ATP comes from mitochondria in the presence of oxygen, whereas glycolytic process (glucose metabolism) in the cytosol generates ATP continuously but much reduced in content as compared with the mitochondrial ATP. Transient stimulus with glucose or other mitochondrial substrates leads to a corresponding increase in ATP. Because of an intricate nonlinear circuit of regulatory and inhibitory enzymes, it is not always possible to develop quantitative models of ATP generation, as this depends on multiple enzyme fluxes and metabolic status of the cell. For efficient cell survival with varying rates of ATP demand and utilization, it is expected that mitochondrial ATP synthesis is in synchrony with cytosolic glycolysis. Under normal conditions, the cellular demand of ATP is fulfilled by both glycolytic and mitochondrial contributions; however, the net ATP levels can
fall significantly lower than the functional threshold when mitochondrial dysfunctions are dominant. Unlike previous studies in isolated mitochondria, our design allowed us to investigate the role of mitochondrial perturbations in the context of the whole cell as well as to probe any possible cross-talk between mitochondria and the cytosol. Recently it has been suggested that “mild uncoupling” is an adaptive response to mitigate high steady-state ROS levels by reducing the mitochondrial membrane potential, and superoxide (through its lipid-peroxidation products) can act as a possible activator of UCPs as a part of a negative feedback mechanism (22–26). We believe that the uncoupling that is observed in aged cells is a similar adaptive mechanism that has become constitutively active because of high steady-state ROS levels (Fig. 1c) during the aging process. This also explains the severely compromised mitochondrial contribution to total ATP under glycolytic-inhibition conditions in aged cells.

The primary step in the complex, cellular decision-making process is the “substrate processing” that is akin to information processing in a computational network. Multiple interdependent signaling pathways and metabolic landscapes coordinate the cellular responses under various perturbation conditions, and this makes cellular dynamics amenable to nonlinear dynamical perspectives. The observation of long-range dynamical correlations and scaling behavior together with the biochemical data support the view that the above paradigm is operative in liver cells (Fig. 6). Liver is the major organ for detoxification of oxygen compounds along with its vital role in synthesis of plasma proteins and carbohydrate/lipid metabolism (30). Mitochondrial ROS are generated during normal cellular metabolism and are removed via numerous anti-oxidant defense systems, preventing deleterious effects on cells and their survival (31). This is carried out by orchestrating an ensemble of structural and functional hierarchy of metabolic networks and by regulating homeostasis as in free radical regulation. Oxidative stress-induced functional modifications in liver cells during the aging process are, therefore, expected to induce imbalance in the functions of other organs through hemodynamic changes in hepatic blood. Our data directly demonstrate that the aging process chronically modulates nonlinear dynamical behavior in free radical regulation in liver cells, which in turn influences energy metabolism significantly, thus offering a plausible cellular basis for the free radical hypothesis of aging. The physiological significance of nonlinear dynamics is to enhance performance of the cellular system that is operating under the control of multiple stimuli and over various spatial and time scales (32). We envisage that the present work may lay a foundation for analysis of similar scaling relationships in other vital processes such as the role of noise in gene expression, protein signaling pathway, and hormonal regulation of metabolic networks. Our data support the free radical theory of aging and attempt to explain how modifications in mitochondrial energy metabolism may contribute to normal aging process. Although the mitochondrial theory of aging has been steadily accumulating experimental evidence for the past five
Nonlinear Dynamics in Liver Cell Metabolism

decades, there have been notable exceptions to this premise. For instance, lifespan studies in mouse models heterozygous for mitochondrial antioxidant enzyme, Mn-SOD, do not show significant differences from the wild type, although complete knock-out of Mn-SOD enzyme makes them embryonically lethal (33). On the other hand, evolutionary theories of aging such as mutation accumulation theory and the antagonistic pleiotropy theory are gaining attention in the limelight of accumulating evidence for evolutionary perspectives on aging (34, 35)

The general idea behind these evolutionary concepts is that aging is an inevitable result of declining force of natural selection with age. Over successive generations, late-acting deleterious mutations will accumulate, leading to an increase in mortality rates late in life. Evolutionary gerontologists tend to think of aging as a fundamental consequence of evolutionary forces rather than due to biochemical degeneration of cells and tissues from free radical damage etc. Regardless of the differences in basic tenets of various theories, it is possible that multiple mechanisms (both genetic and biochemical perspectives) may operate synergistically to contribute to normal aging process.

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