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Mitochondrial DNA copy number is regulated by cellular proliferation: A role for Ras and p66^{Shc}

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Abstract

The abundance of mitochondria is regulated by biogenesis and division. These processes are controlled by cellular factors, given that, for example, mitochondria have to replicate their DNA prior to cell division. However, the mechanisms that allow a synchronization of cell proliferation with mitochondrial genome replication are still obscure. We report here our investigations on the role of proliferation and the contribution of Ras and p66^{Shc} in the regulation of mitochondrial DNA copy number. Ras proteins mediate a variety of receptor-transduced mitogenic signals and appear to play an essential role in the cellular response to growth factors. P66^{Shc} is a genetic determinant of life span in mammals and has been implicated in the regulation of receptor signaling and various mitochondrial functions. First, we confirmed previous reports showing that mitochondrial DNA is replicated during a specific phase of the cell cycle (the pre-S phase) and provided novel evidences that this process is regulated by mitogenic growth factors. Second, we showed that mitochondrial DNA replication is activated following Ras-induced cellular hyper-proliferation. Finally, we showed that p66^{Shc} expression induces mitochondrial DNA replication, both in vitro and in vivo. We suggest that mitochondria are target of intracellular signaling pathways leading to proliferation, involving Ras and p66^{Shc}, which might function to integrate cellular bio-energetic requirements and the inheritance of mitochondrial DNA in a cell cycle-dependent manner.
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Keywords: DNA; Ras signaling; p66^{Shc} and aging

1. Introduction

1.1. Mitochondrial and cell proliferation

Mitochondria, though mainly considered as specific sites of regulation of the energy metabolism, are certainly multi-functional [1]. Like the nuclear genome, mitochondria have to replicate their DNA prior to cell division. However, the mechanisms that allow the synchronization of cell proliferation with mitochondrial genome replication are still obscure.

Mammalian mitochondrial DNA is a high copy-number, maternally inherited genome that codes for a small number of essential proteins involved in oxidative phosphorylation. Mitochondrial DNA (mtDNA) accumulates somatic mutations during aging, cancer progression and diabetes [2]. In the past decade, it has been demonstrated that a number of protein factors encoded by nuclear genes are involved in the biogenesis of mitochondria and the maintenance of mtDNA copy number [3]. However, it remains unclear how the copy number of mtDNA and the abundance of mitochondria are regulated under different physiological and developmental conditions. Although major advances in our understanding of the molecular bases of replication, transcription and maintenance of mtDNA have been made [4,5], information about how the genetic material is organized and distributed in mammalian mitochondria and how mtDNA molecules segregate before and during mitosis, thus determining the genetic identity of daughter cells, is very limited. Inherited or acquired mtDNA heteroplasmy appears critical not only in the case of mitochondrial diseases, but in an increasing number of age-related diseases, such as diabetes and cancer [1,6]. MtDNA copy number is tightly regulated in a cell- and tissue-specific manner by unknown mechanisms, and it is not clear if and how mtDNA replication is linked to the cell

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cycle [7,8]. Several studies have been reported which showed a correlation between mitochondrial biogenesis and function with specific stages of the cell cycle [9–12]. In particular, it has been demonstrated that, in some cell lines, growth factor stimulation induces nuclear and mitochondrial replication.

1.2. Growth factors signaling and cellular proliferation

Growth factors, both cytokines and hormones, transduce their effect inside the cell through a cascade of events that mainly consist in protein complex association and post-translational modifications. A key step of these signal transduction events is the activation of p21Ras proteins, a family of plasma membrane-associated GTP-binding protein encoded by three mammalian genes: K-, N- and H-Ras. Ras proteins are activated by diverse extra-cellular signals, including integrins and growth factors, that bind to their specific receptors and activate intracellular tyrosine-kinases. The Ras pathway is among the most frequently altered in tumors. Ras genes are themselves the most frequently mutated oncogenes in spontaneously occurring human cancers (up to 30%) [13].

Fine regulators of Ras activation are the Shc proteins. The Shc proteins were initially recognized as 'adaptor' proteins that specifically bind to phosphorylated tyrosine residues on the cytoplasmic motif of growth factor receptors. There are three Shc proteins in mammals, the 46 kDa Shc ($p46^{Shc}$), the 52 kDa Shc ($p52^{Shc}$), and the 66 kDa Shc ($p52^{Shc}$). Upon growth factor stimulation, $p52^{Shc}$/$p46^{Shc}$ proteins are rapidly and efficiently tyrosine-phosphorylated by all tyrosine kinases (TKs) tested and recruit the Grb2–Sos complex on the plasma membrane [14]. The hypothesis that Shc proteins are involved in the regulation of Ras is supported by the findings that over-expression of $p52^{Shc}$/$p46^{Shc}$ increases Ras signaling and proliferation upon stimulation with EGF, GM-CSF and PDGF [15]. Indeed, evidences for divergent regulation of $p66^{Shc}$ and $p52^{Shc}$/$p46^{Shc}$ emerged from studies demonstrating that $p66^{Shc}$, like $p52^{Shc}$/$p46^{Shc}$, is a target of TK receptors (EGFR, INSR, PDGFR) [15,16] and binds the Grb2/Sos complex, but it exerts a negative effect on the Ras-MAPK-Fos pathway upon EGF [17] or cytokines stimulations of fibroblasts or lymphocytes, respectively [18]. Recently, $p66^{Shc}$ has been shown to exert an inhibitory effect on the Erk pathway [19].

$P66^{Shc}$ localizes partially within the inter-membrane space of mitochondria where it oxidizes cytochrome *c*, thus producing hydrogen peroxide [20]. Electrochemical experiments demonstrated that the amino terminal portion of p66Shc contains a redox center that mediates electron transfer from reduced cytochrome *c* to molecular oxygen, producing H_2O_2. We have demonstrated that the H_2O_2 formed by $p66^{Shc}$ may favor the swelling of mitochondria, which then triggers the opening of the mitochondrial permeability transition pore and increases intracellular oxidative stress [20]. Notably, the deletion of $p66^{Shc}$ in mice reduces apoptosis and oxidative stress and prolongs life span of about one third, suggesting that $p66^{Shc}$ is a genetic determinant of aging in mammals [20–26].

2. Materials and methods

2.1. Mice, cells and reagents

In this study, we used 129SvEv WT and $p66^{Shc}$−/− mice at different ages. Whole kidneys, liver and skin were dissected and immediately frozen for DNA preparation.

Mouse NIH3T3 fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Mouse embryonic fibroblasts (MEFs) and mouse adult skin fibroblasts (MAFs) were prepared according to standard procedures and grown in DMEM 5% (v/v) FBS. Media were supplemented with 1% penicillin-streptomycin. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. Brown adipocytes (ADPC) were generated from newborn WT and $p66^{Shc}$−/− mice according to standard procedures and differentiation of these cells was induced with 20 nM insulin.

2.2. Cellular treatments

Centrifugal elutriation was performed in a Beckman JE 5.0 centrifuge and a JE-5S rotor equipped with the standard separation chamber. Logarithmically growing NIH/3T3 cells were introduced into the separation chamber. Cells were elutriated at 4 °C in PBS. Elutriation was executed at a constant rotor speed of 2800 rpm. The fractionation of cells into various cell cycle subpopulations was accomplished by increasing the pump speed stepwise. To test the cell cycle distribution of cells within each fraction, an aliquot of cells was fixed with ethanol, stained with propidium iodide and analyzed by a Becton-Dickinson FACScan flow cytometer.

Mutated Ras, $p66^{Shc}$ WT and $p66^{Shc}$ mutant proteins were expressed in MEFs using the pBABE-puro retroviral vector. Retroviral vector DNAs were transfected into the Phoenix packaging cell lines and after 48 h supernatants were used to infect target cells, which were then selected with 1 μg/ml puromycin. Protein expression was analyzed by PAGE-SDS followed by Western Blotting using anti-Ras or anti-Shc (Transduction Lab.) antibodies.

2.3. DNA preparation

Total genomic DNA was isolated with the QIAamp kit (Qiagen). MtDNA content was determined in cells and tissues utilizing a quantitative real-time PCR (QPCR) by the TaqMan method (ABI PRISM7700 Sequence Detection System); specific primers were used to amplify a fragment of the mitochondrial D-loop region:(MDF) 5′-AAT CTA CCA TCC TCC GTG AAA CC and (MDR) 5′-TCA GTT TAG CTA CCC CCA AGT TTA A, probe 5′-FAM-CGCCCACCAATGCCCCTCTTC-TAMRA or a fragment of nuclear genomic telomerase gene region TERTF, 5′-CTA GCT CAT GTG TCA AGA CCC TCT T and TERTR 5′-GCC AGC ACG TTT CTC TCG TT, probe 5′VIC-TCC TTA CCA GGT GTC ATC CCT GAA AGA GC-TAMRA. PCR reactions were performed according to standard conditions for TaqMan (Applied Biosystems): 50 °C for 2′; 95 °C for 10′; 40 cycles at 95 °C 15″, 60 °C 1′. PCR assays were performed in triplicate for each DNA sample. The expression of mtDNA copy number relative to nuclear DNA was determined using the $2^{-\Delta CT}$ method.

3. Results

To investigate mtDNA biogenesis and nuclear DNA replication we took advantage of synchronized cell populations. In a first set of experiments we analyzed mtDNA copy number in cell fractions of NIH3T3 fibroblasts obtained by elutriation. The cell cycle profile of each fraction is shown in Fig. 1A. Cell cycle distribution of the various cell fractions revealed a good separation between G0/G1, S and G2/M (Fig. 1B). The mtDNA content of various cell fractions was measured by QPCR, using oligonucleotides primers specific for a fragment of the mitochondrial D-loop region, and normalized with the content of nuclear DNA. Results showed that mtDNA copy number was

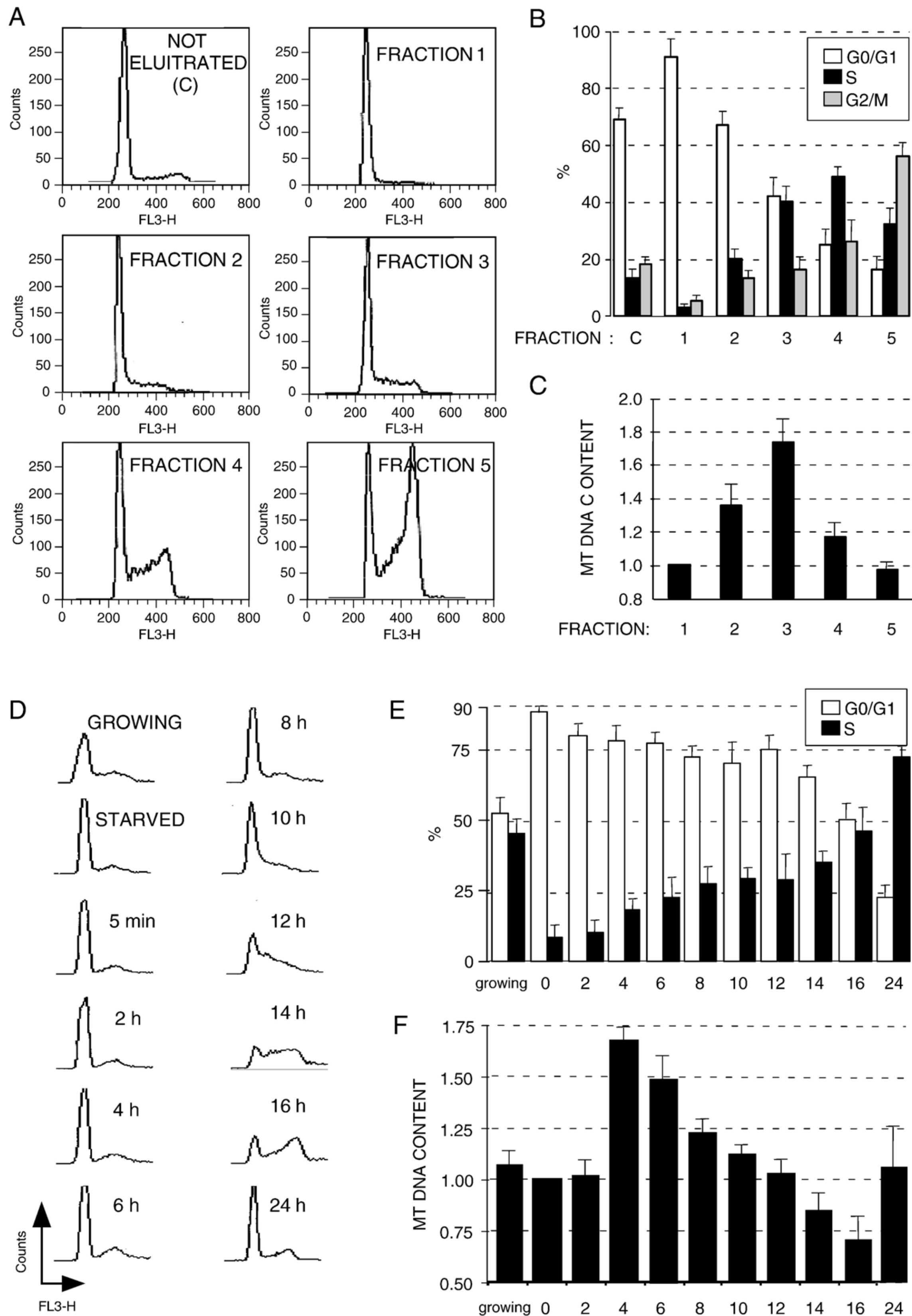

Fig. 1. (A–C) MtDNA copy number in various cell cycle fractions of NIH3T3 fibroblasts. Cell fractions, enriched at specific phases of the cell cycle, were obtained by elutriation. The FACS profile of each fraction is shown in panel A, their cell cycle distribution is shown in panel B. MtDNA content was measured by real-time PCR and normalized with nuclear DNA. The results are expressed as a ratio relative to the value of fraction 1 (C). (D–F) Mt DNA replication after growth factor stimulation of NIH-3T3 fibroblasts NIH3T3 cells were G0–G1 synchronized by serum starvation and then induced to re-enter the cell cycle by the addition of serum. FACS profiles and cell cycle distribution at each time point are shown in panels D and E respectively. MtDNA copy number, normalized with nuclear DNA, is shown in panel F (the results are expressed as a ratio relative to the serum-starved fraction).

higher in the S phase enriched population (Fig. 1C), suggesting that it is regulated during the cell cycle. To obtain an independent confirmation of this hypothesis, we then analyzed mtDNA copy number during the re-entry into the S-phase of G0/G1-synchronized cells. To this end, NIH3T3 cells were serum-starved, to induce their exit of the cell cycle, and then treated with serum, to allow their synchronous re-enter (see Fig. 1D for representative FACS profiles and Fig. 1E for their cell cycle distribution). Notably, analysis of the mtDNA copy number revealed a marked increase during late G0/G1 and early S phase, just before the beginning of nuclear DNA replication (Pre-S phase) (compare Fig. 1E and 1F). It appears, therefore, that mtDNA is replicated during a specific phase of the cell cycle (the pre-S phase) and that this process is regulated by mitogenic growth factors.

Since Ras is critical for the cell-cycle control mediated by growth factors and for growth arrest in non-transformed, check point competent cells [27] we then investigated the role of Ras in mitochondrial biogenesis. To this end, we expressed an oncogenic mutant of Ras (rasV12 [28,29]) into NIH3T3 fibroblasts and primary mouse embryonic fibroblasts (MEFs). Measurement of mtDNA copy number revealed that expression of activated Ras is able to increase mtDNA content only in NIH3T3 fibroblasts, while it has no effect, or a slight inhibitory effect, in primary fibroblasts (Fig. 2A–B). The biological consequences of mutated Ras expression in immortalized (NIH3T3) and primary (MEFs) fibroblasts are profoundly different, since it induces hyper-proliferation and transformation of NIH3T3 cells and replicative senescence of MEFs, (Fig. 2C) the latter being the consequence of the activation of a p53-dependent checkpoint [27]. It appears, therefore, that Ras is not sufficient, per se, to trigger mtDNA replication; instead, mtDNA replication appears to be the consequence of the Ras-induced activation of a signaling pathway which then leads to nuclear DNA replication.

A growing body of evidence suggests that reactive oxygen species (ROS) regulate a number of diverse intracellular pathways including those triggered by Ras [28]. Considering that $p66^{Shc}$ is implicated in the regulation of Ras and mitochondrial production of ROS, we investigated the role of $p66^{Shc}$ in mtDNA replication. We first analyzed the content of mtDNA in MEFs, MAFs (Mouse Adult Fibroblasts) and ADPC (Adipocytes) derived from WT or $p66^{Shc}$-null ($p66^{Shc}-/-$) mice. Notably, in all cases, a marked decrease of mtDNA content was observed in the absence of $p66^{Shc}$ expression (Fig. 3A). Consistently, the over-expression of $p66^{Shc}$ into WT or p66Shc−/− MEFs induced a marked increase (of about 2-folds) of mtDNA content (Fig. 3B). Together, these findings indicate that levels of $p66^{Shc}$ expression regulate mtDNA replication.

To preliminarily investigate the molecular mechanisms underlying the effect of $p66^{Shc}$ expression on mtDNA replication, we analyzed the ability of relevant $p66^{Shc}$ mutants to increase the mtDNA content into $p66^{Shc}-/-$ MEFs. To this end, we used phosphorylation-defective $p66^{Shc}$ Ser36Ala (p66*a*) and redox defective p66Shc Glu132Gln,Glu133Gln (p66*qq*) $p66^{Shc}$ mutants. Both mutants are unable to execute the

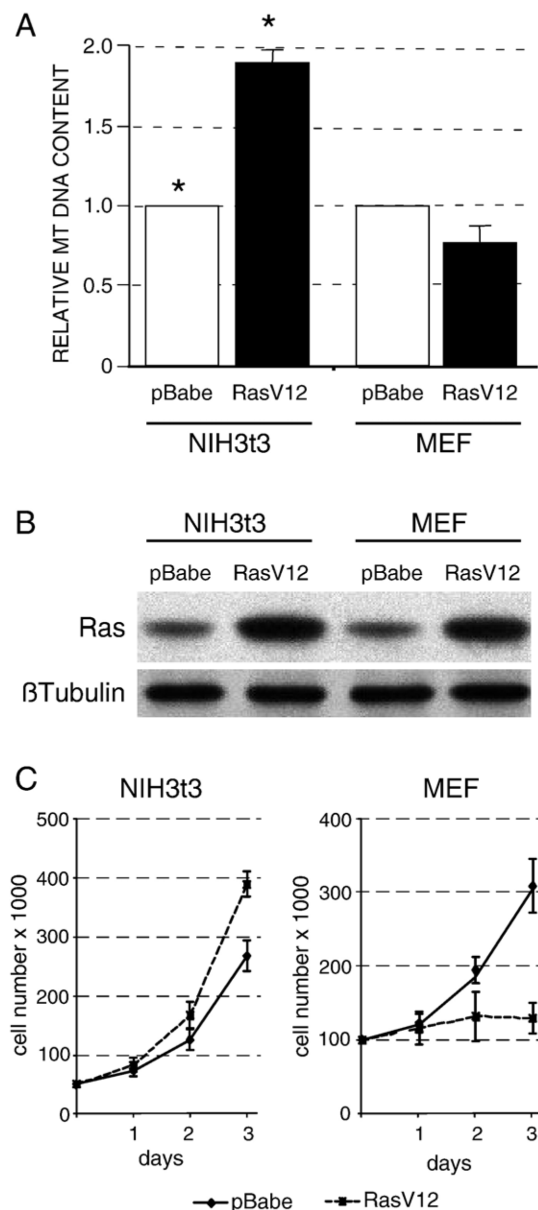

Fig. 2. Ras controls of mtDNA copy number. Activated Ras (RasV12) was expressed by retroviral infection in NIH3T3 cells and in MEFs, as indicated, and the mtDNA content was measured by QPCR and normalized with nuclear DNA. Results are expressed as a ratio relative to the value of the pBabe retroviral control infection (A). Results from WB analysis of the expression levels of Ras (B) and of growth properties (C) in the infected cells are reported.

pro-apoptotic programme of $p66^{Shc}$, by interfering with different properties of p66Shc: the ability to undergo oxidative stress-induced phosphorylation at the serine 36 residue, and the ability to generate mitochondrial ROS, respectively [20,21]. Strikingly, both mutants were able to increase mtDNA content, upon retroviral-mediated gene transfer into $p66^{Shc}-/-$ MEFs (Fig. 3C), thus indicating that this property of $p66^{Shc}$ relies on novel molecular mechanisms, regardless from their ability to induce ROS production (Fig. 3 D–E).

MtDNA content has been shown to increase with age and in several age-associated diseases [7,30]. Thus, we analyzed mtDNA content in different tissues from WT and $p66^{Shc}-/-$

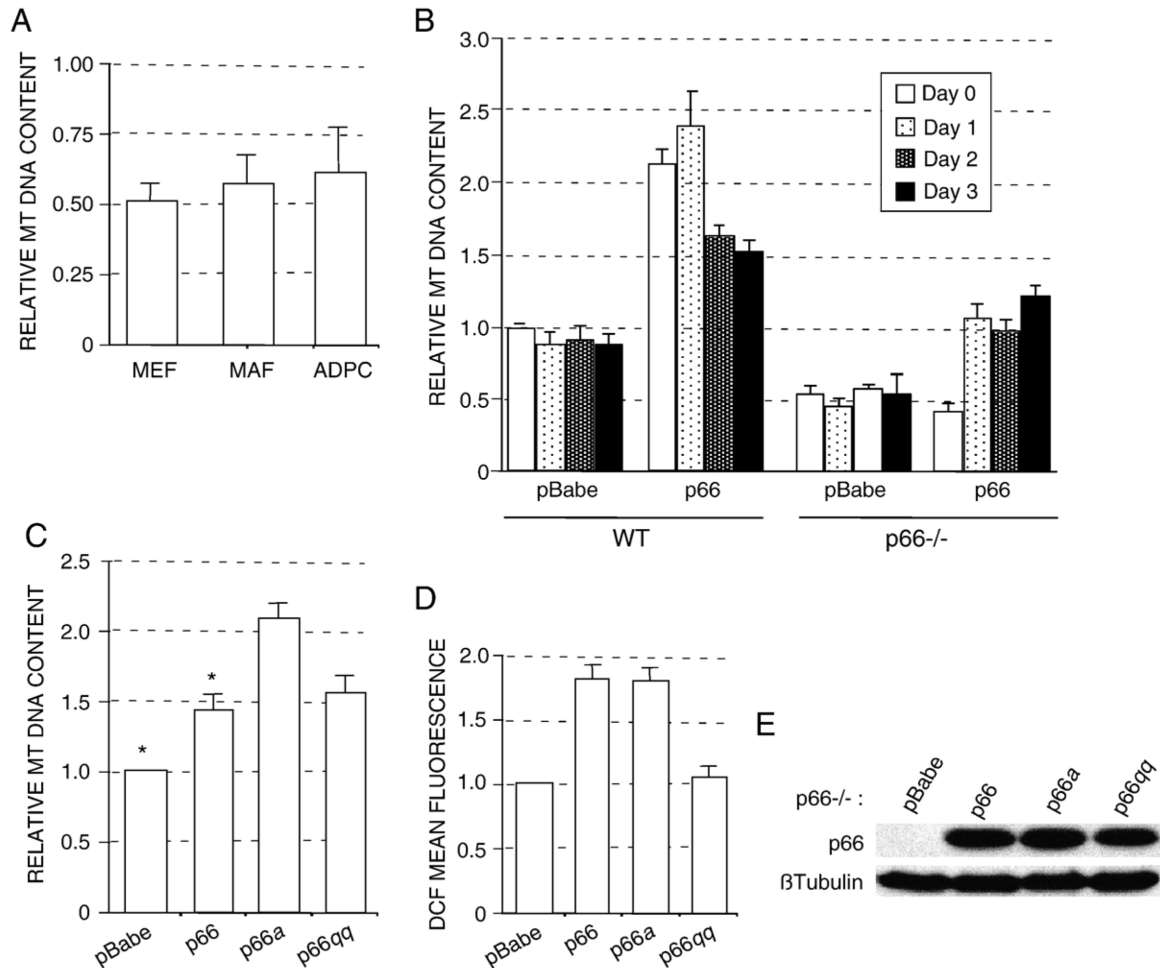

Fig. 3. P66[Shc] increases mtDNA copy number. MtDNA copy number was measured by QPCR and normalized with nuclear DNA in MEFs, MAFs and ADPC from WT and p66[Shc]−/− mice. Results are expressed as a ratio of p66[Shc]−/− versus WT samples (A). p66[Shc] was reintroduced in WT and p66[Shc]−/− MEFs by retroviral infection and mtDNA content was measured by QPCR at different days from the end of puromycin selection (day 0 corresponds to the first day after selection). Results are expressed as a ratio relative to the pBabe control infection of WT cells (B). P66[Shc] WT protein, p66*a* and p66*qq* mutants were expressed by retroviral infection into p66[Shc]−/− MEFs, as indicated, and mtDNA content was measured by QPCR. Results are expressed as a ratio relative to the value of the pBabe control (C); p66 [Shc]−/− MEFs expressing different p66 [Shc] mutants were stained with DCFDA and analyzed by FACS (D); levels of p66 [Shc] expression were analyzed by WB using anti-Shc and anti-βtubulin antibodies (E).

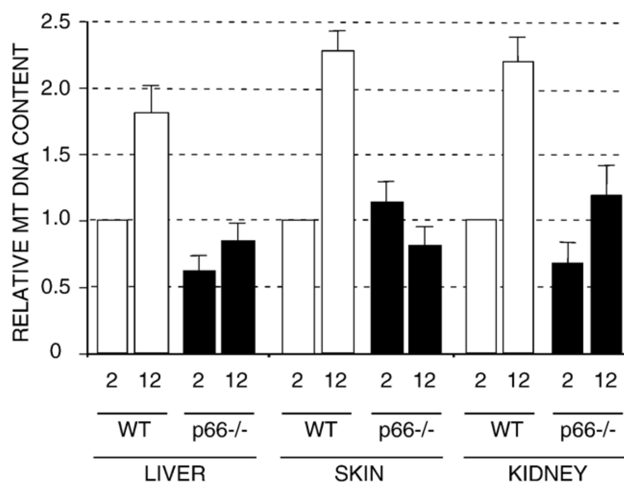

Fig. 4. P66[Shc] controls age-dependent accumulation of mtDNA. MtDNA content was measured by QPCR in the liver, skin and kidney from 2 to 12 months old p66[Shc]−/− mice and compared with matched controls. Results are normalized with nuclear DNA and expressed as a ratio relative to the value of WT samples at 2 months.

mice of different age (Fig. 4). As expected, mtDNA content was significantly higher in the liver, skin and kidney of WT old mice (as observed comparing 12 versus 2 months old animals). In old p66[Shc]−/− mice, instead, mtDNA content did not increase (skin) or increased modestly (liver and kidney) (Fig. 4). It appears, therefore, that the age-dependent increase of tissue mtDNA is lost or significantly reduced in p66[Shc]−/− mice.

4. Discussion

Though recent progress has been made in mitochondrial genetics, several fundamental questions remain unanswered. Mitochondrial replication must be tightly regulated when cells divide, when they differentiate, increase in size or respond to changes in their environment. However, we still do not know how the ratio of mitochondrial to nuclear DNA is maintained, how the number of mitochondria is adjusted to meet various cellular needs and how mtDNA contributes to aging and various age-associated diseases, including cancer.

Cellular demands for increased energy supply are often met by an increase in respiratory activity, usually accompanied by an increase in mitochondrial mass. An increase in mitochondrial proliferation presents a unique challenge to cells, because mitochondrial biogenesis requires protein products that are encoded by both nuclear and mitochondrial genomes, suggesting that a variety of intracellular and extra-cellular signals have to be integrated. We measured mtDNA copy number during cell cycle progression and, consistent with previous reports [9–11], we found that mtDNA is mostly replicated in the pre-S phase, suggesting a tight correlation between mitochondrial biogenesis and specific stages of the cell cycle. We then demonstrated that serum-induced cell cycle re-entry correlates with increased mtDNA copy number, suggesting that growth factors are part of the molecular mechanisms which couple mitochondrial proliferation to environmental signals. Ras proteins function as a point of convergence for different signaling pathways and have been implicated in both aging and cancer development. Notably, we observed that activated Ras (RasV12) is able to regulate mtDNA replication. This effect, however, only occurs in cells where Ras induce a full hyper-proliferative response, suggesting that Ras-induced mtDNA replication is tightly linked to the ability of Ras to also induce nuclear DNA replication. It appears, therefore, that Ras functions as a molecular switch in cell cycle regulation by coupling cell surface receptors to the nucleus, cytoskeleton and also mitochondria. The molecular determinants of this novel function of Ras are unknown and may involve the fraction of Ras proteins that localizes within mitochondria and its ability to stimulate intracellular ROS production [31,32].

Our data suggest that $p66^{Shc}$ is also part of the signaling pathway that regulates mtDNA replication, though its mechanistic relation with Ras remains to be determined. We found that $p66^{Shc}$ expression stimulates mtDNA copy number both in vitro and in vivo. Notably, this effect of $p66^{Shc}$ likely reflects its involvement in other signaling pathways, with respect to those already known. In fact, $p66^{Shc}$ mutants that are defective for the ability of the WT protein to induce ROS production and apoptosis are still able to stimulate mtDNA replication.

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