INTRODUCTION

Mammalian telomeres are specialized structures that protect eukaryotic chromosome ends from DNA repair reactions, owing to a lariat conformation and the presence of a six-subunit protein complex, called shelterin (1). In humans, telomeres shorten with age, and this interferes with tissue replenishment (2). However, recent studies acknowledged that nongenetic influences can affect telomere attrition and possibly delay aging-related diseases (2, 3). Human telomeres are transcribed from CpG island–containing subtelomeric promoters, located on average 1 kb upstream of TTAGGG repeats, into telomeric repeat–containing RNA (TERRA) molecules (4, 5). TERRA molecules comprise subtelomere-specific sequences at their 5′ end, followed by stretches of UAAGG repeats proportional to telomere length (6), and remains partly associated with telomeres to play crucial protective functions (7). Additional regulatory functions of TERRA will likely emerge in the future. In cycling cells, TERRA levels decline from S phase to G2 before increasing again, ensuring renewal of TERRA pools at each cell division (6, 8, 9). How human telomere transcription is activated is largely unknown, but transcriptional regulators of telomeres include CTCF and ATRX chromatin remodelers, as well as Rad21 cohesin subunit (10–12). Understanding the regulatory mechanisms of human telomere transcription, not only in cycling cells but also in nondividing tissues, is likely to provide new clues for antiaging research.

To gain insight into the regulatory mechanisms of human telomere transcription, we screened the available human subtelomeric sequences for the presence of predicted transcription factor binding sites using an in silico approach. This allowed us to identify nuclear respiratory factor 1 (NRF1) as a key regulator of human telomere transcription and revealed a new link between telomeres and metabolism.

RESULTS

NRF1 binds human subtelomeres

To identify new transcription factors for TERRA, we performed an in silico analysis of human subtelomeric sequences. The "low-confidence" data set (Fig. 1A and table S1) refers to subtelomeric sequences from the study by Stong et al. (13) that were either missing in the GRCh38/hg38 assembly or lacking TTAGGG repeats at their 3′ ends. Sequences of p arms from acrocentric chromosomes are not available. On the basis of a previous study by Nergadze et al. (5), we identified putative transcription sites (TSSs) on 18 of 27 and 4 of 14 subtelomeres from the high- and low-confidence data sets, respectively (Fig. 1A). MatInspector (14) revealed the presence of putative binding sites for NRF1 in 30 of 41 subtelomeric sequences. NRF1 is an important regulator of nucleus-encoded subunits of mitochondrial respiratory complexes, and its disruption leads to early mouse embryonic lethality associated with severe mitochondrial DNA deletion (15). NRF1 targets are coactivated by PGC-1α (peroxisome proliferator–activated receptor γ coactivator 1α), a key regulator of energy metabolism that is activated by endurance exercise or caloric restriction and previously linked to protection against aging (16). This prompted us to further investigate the link between NRF1 and telomere transcription.

We first set up chromatin immunoprecipitation (ChIP) experiments. Knowing that NRF1 binds DNA within CpG-rich sequences and is sensitive to methylation (17), we selected LB37 non–small cell lung carcinoma cell line with hypomethylated subtelomeric promoters and high TERRA expression levels (6) to assess NRF1 binding. Because we could not design primers suitable for quantitative polymerase chain reaction (qPCR) within the repetitive CpG-rich sequences of subtelomeric promoters, qPCRs amplified genomic loci located either directly upstream (21q, 10p–18p) or directly downstream (5p, Xq-Yq, 1q–2q-4q-5p-10q-13q-21q-22q, 15q) of...
CpG islands. In agreement with in silico analysis, NRF1 binding was detected on all subtelomeric sequences with predicted NRF1 binding sites that we tested [from about 3- to 50-fold enrichment over immunoglobulin G (IgG); Fig. 1B]. 7q subtelomere, which lacks predicted TSS and displays only one putative NRF1 binding site, showed enrichment over IgG of only 1.9-fold (see fig. S1A for primer position), in accordance with the extremely low transcriptional activity of that subtelomere (Fig. 1, A to C, and fig. S1, A to D). As expected, NRF1 did not bind to 10p-18p subtelomeric loci located about 1.5 kb upstream of telomeres (Fig. 1B, 10p-18p distal; 1.6-fold over IgG). Furthermore, we could show that the ability of NRF1 to bind 15q subtelomere correlates with 15q TERRA levels in LB37 and Huh-7 hepatocarcinoma cell lines and inversely correlates with the distance from CpG island (Fig. 1, D and E). Together, the above data suggested that NRF1 may play a role in human telomere transcription.

**Cycling endurance exercise up-regulates TERRA levels in human skeletal muscle**

Having shown that NRF1 binds human subtelomeres, we next tested the hypothesis that endurance exercise, a well-established inducer of NRF1 target genes, may affect telomere transcription. Briefly, adenosine 5′-diphosphate (ADP)/adenosine 5′-triphosphate (ATP) ratio increases during exercise, leading to adenosine 5′-monophosphate (AMP)–activated protein kinase (AMPK) activation. In turn, activated AMPK phosphorylates PGC-1α to promote its nuclear accumulation through sirtuin 1–dependent deacetylation. Once in the nucleus, PGC-1α acts as transcriptional coactivator for various transcription factors, including NRF1 (16, 18).

To investigate the impact of exercise on telomere transcription in skeletal muscles, we submitted 10 healthy young volunteers to a cycling endurance exercise for 45 min. To obtain various levels of AMPK activation, subjects were submitted to either low-intensity (50% VO2 peak) or high-intensity (75% VO2 peak) exercise, associated with, respectively, low or high AMPK activation levels. Three muscle biopsies were taken according to a standardized protocol either before (B1), directly after (B2), or 2.5 hours after (B3) exercise (Fig. 2A). Blood lactate was immediately measured at the end of the exercise to evaluate individual responses (Fig. 2B). Fitting with post-exercise blood lactate levels, phosphorylation of acetyl-coenzyme A carboxylase (ACC) in muscle biopsies,
NGF1 and AMPK/PGC-1α axis promote human telomere transcription

The endurance exercise experiment suggested that telomere transcription is regulated by the AMPK pathway. However, although NGF1 is expressed in skeletal muscles (fig. S3), our in vivo experiment did not allow us to test whether the transcription factor is implicated in telomere transcription. To further investigate this, and to gain more insight into AMPK-dependent regulation of TERRA, we used the Huh-7 cell line that responds to phenformin, a biguanide drug that, like metformin, activates AMPK by increasing cellular AMP/ATP ratio (22).

First, we showed that either NGF1 knockdown or overexpression of a dominant negative form that lacks the C-terminal transactivation domain (ΔC NGF1) (23) reduces endogenous TERRA levels by 25 to 45% (Fig. 3A, A and B), suggesting a role for NGF1 in basal transcription of Huh-7 telomeres. Accordingly, overexpression of NGF1 stimulated luciferase activity driven by a portion of 10q promoter (10) containing NGF1 binding sites, whereas ΔC NGF1 had opposite effects (Fig. 3C).

Second, when Huh-7 cells were treated with phenformin, ACC phosphorylation was increased, PGC-1α accumulated in the nucleus, and TERRA levels, measured from various chromosome ends, reached 185 to 400% of the expression detected in untreated cells (Fig. 3D to F). PGC-1α transcriptional induction also occurred, whereas no noticeable change was observed for the three control genes: hTR noncoding telomerase RNA subunit, TRF2 shelterin gene, or β2M which was used to normalize complementary DNA (cDNA) values (Fig. 3F and fig. S4, A and B). Here, too, PGC-1α mRNA up-regulation occurred later than TERRA induction (fig. S4B). In agreement with AMPK activation acting at the level of telomere transcription, phenformin treatment induced 10q-luciferase activity by a factor of 2; induction was further exacerbated by overexpression of wild-type NGF1 (3.3-fold) but mostly lost upon ΔC NGF1 overexpression (Fig. 3G). Mutation of NGF1 binding sites in the 10q promoter-luciferase construct (fig. S5) reduced basal activity of the promoter by 35% (mut3) and 70% (mut4), respectively, and lowered its activation by either overexpressed NGF1 or phenformin treatment (Fig. 3H).

Next, to probe more directly for PGC-1α involvement in TERRA transcription, we transduced Huh-7 cells with adenoviral particles containing either mouse PGC-1α (mPGC-1α) coding sequence or GFP cDNA as control (fig. S6A). Luciferase activity driven by 10q promoter was up-regulated by a factor of 4.7 upon mPGC-1α overexpression and of 13.8 when cells were simultaneously overexpressing wild-type, but not ΔC, NGF1 (Fig. 3I). Accordingly, mPGC-1α overexpression up-regulated endogenous TERRA levels by factors of 1.4 to 1.6 (Fig. 3J). Although modest, the induction was significant and only twofold less than the induction of hCYC1 gene, a well-established AMPK activator, increased with blood lactate concentration revealed a significant correlation (P < 0.005) (Fig. 2H). Compared to matching B1, TERRA levels in samples and in 80 to 100% of B3 samples, depending on the chromosomal test (Fig. 2H).

Interestingly, quantitative reverse transcription PCR (qRT-PCR) against distinct TERRA 5′ ends revealed up-regulation in 50 to 90% of B2 samples and in 80 to 100% of B3 samples, depending on the chromosome end tested (Fig. 2I). Compared to matching B1, TERRA levels in B3 reached an average of 186 and 131% in the high- and low-intensity exercise group, respectively (Fig. 2I). The different induction timing observed for TERRA (already in B2) and PGC-1α (in B3) transcription may result from distinct mechanisms of PGC-1α coactivation. As a transcriptional activator, PGC-1α interacts with multiple and various DNA binding factors, the nature of which depends on the target gene. For PGC-1α transcription, NGF1 is not involved (20). Our data fit with the observation that, in response to exercise, NGF1-dependent mitochondrial biogenesis occurs before the up-regulation of PGC-1α levels in rat muscles (21). Plotting TERRA induction in B3 against post-exercise blood lactate concentration revealed a significant correlation (P < 0.05) (Fig. 2J). Because blood lactate concentrations correlated with AMPK activity in muscle tissues (P < 0.005) (Fig. 2K), these data suggest that the kinase regulates telomere transcription. Together with our demonstration that most telomeres from muscle cells are probably covered with TERRA (Fig. 2K and fig. S2), this finding suggests that exercise provides a means to renew TERRA pools and protect telomeres in muscle.
**Fig. 2.** Endurance exercise up-regulates TERRA in human muscle. (A) Design of the in vivo experiment. (B) Blood lactate concentrations (mM) before (gray) and at the end (black) of exercise in subjects (S) (50% VO2 peak, low-intensity exercise; 75% VO2 peak, high-intensity exercise). (C) Representative Western blots of ACC phosphorylation (P-ACC) in B1, B2, and B3 biopsies from S5 (high lactate), S6 (medium lactate), and S12 (low lactate). Total ACC was used to evaluate the P-ACC/ACC ratio, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (D) P-ACC/ACC ratios in B2 and B3 normalized to B1 for all subjects. (E) P-ACC/ACC ratios plotted against blood lactate concentration after exercise. (F) PGC-1α abundance in nuclear fractions from S12, S6, and S5 biopsies normalized to Ku80 and to matching B1. (G) Representative qRT-PCR analysis of TERRA (15q, 16p, and 1q-2q-4q-10q-13q-22q) in B2 and B3, normalized to βM cDNA and to matching B1. (H) Average induction of TERRA (all three qRT-PCRs pooled) in B3 compared to matching B1 for 50% VO2 peak group (n = 5) and 75% VO2 peak group (n = 5). Error bars indicate SD. (J) TERRA fold induction in B3 plotted against blood lactate concentration after exercise. (K) TERRA-FISH (red) and TRF2 detection (green) in muscle biopsies. Blue, 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 5 μm.
Fig. 3. NRF1 and AMPK/PGC-1α axis promote human telomere transcription. (A) NRF1 protein in siLuci- and siNRF1-treated Huh-7 cells; TERRA cDNA normalized to β2M cDNA and siLuci (n = 3). (B) NRF1/ΔNRF1 overexpression in Huh-7 cells; TERRA cDNA normalized to β2M cDNA and to NRF1-overexpressing cells (n = 3). (C) Transcriptional activity of 10q promoter in Huh-7 cells overexpressing increasing amounts of NRF1/ΔNRF1 (n = 3). (D) Phenformin treatment in Huh-7: ACC phosphorylation and PGC-1α nuclear translocation. Scale bar, 100 μm. (F) qRT-PCR analyses of TERRA, PGC-1α, and hTR upon phenformin treatment normalized to β2M cDNA and to untreated cells (n = 3). (G) Transcriptional activity of 10q promoter in phenformin-treated Huh-7 cells overexpressing NRF1 or ΔNRF1 normalized to pcDNA3-transfected control cells (n = 5). (H) Impact of NRF1 binding site mutation on 10q transcriptional activity. Data were normalized to untreated cells transfected with wild-type (WT) 10q promoter and pcDNA3 (n = 4). Empty: No promoter. (I) 10q transcriptional activity upon mPGC-1α overexpression, in combination with NRF1/ΔNRF1. Data were normalized to pcDNA3-transfected/adenovirus–green fluorescent protein (Ad-GFP)–transduced cells (n = 6). (J) TERRA cDNA in mPGC-1α-overexpressing Huh-7 normalized to β2M cDNA and Ad-GFP control cells (n = 5). (A to J) Error bars indicate SD. (K) TIF formation upon NRF1 knockdown in Huh-7 cells. Telomeres were detected by FISH (red) and DNA damage with 53BP1 antibody (green). Data are representative of three independent transfections. Scale bar, 5 μm.


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context of healthy nondividing muscle cells. To this end, we differentiated human deltoid-derived myoblasts into myotubes (fig. S7). To our knowledge, the presence of TERRA at telomeres of these cells has not been checked before. Combined RNA/DNA–fluorescence in situ hybridization (FISH) experiments in myotubes revealed an average of 36 telomeric signals and 38 TERRA foci per nucleus (Fig. 4, A and B, and fig. S8). Telomeric signal abundance agrees with a previous report that telomeres are clustered in human cells (28). An average of 35 TERRA foci colocalized with telomeres, suggesting that, although not all telomeres are transcribed, about 97% of them may be covered by TERRA if RNA molecules were provided in trans. Conversely, and for still unknown reasons, two to three TERRA foci per nucleus were not associated with telomeres.

AMPK pathway activation in primary myotubes treated with either phenformin, metformin, or AICAR (5-aminoimidazole-4-carboxamide ribonucleotide), another well-known AMPK activator (18, 22), led to ACC phosphorylation (Fig. 4C) and was associated with consistent and significant up-regulation of TERRA levels by factors of 1.6 to 2.2 (P < 0.001; Fig. 4D). To assess NRF1 involvement in AMPK-induced TERRA levels, we transfected myoblasts on day 1 of differentiation with siNRF1, before treatment with phenformin at day 4 and harvesting at day 5. The increase in TERRA expression upon phenformin treatment was lost when cells were transfected with siNRF1 but not with nonrelevant siRNA (siLuci) (Fig. 4, E and F). The absence of siNRF1 impact on basal TERRA levels may be due to the fact that myotubes are noncycling cells and TERRA turnover may thus be different from cells in which degradation is linked to cell cycle progression (8). In this view, it is expected that longer incubation times with siNRF1 may be required to detect any impact on basal TERRA levels. However, this extended treatment would have a negative impact on cell integrity. Together, these results show that AMPK activation induces NRF1-dependent increase in TERRA levels in noncycling human myotubes.

Fig. 4. AMPK activation in human myotubes induces NRF1-dependent increase in TERRA levels. (A) TERRA-FISH (green) combined with telomeric DNA FISH (red) in myotubes. Blue, DAPI. Scale bar, 5 μm. (B) Quantification of (A) on 25 nuclei. (C) ACC phosphorylation in myotubes treated with either AICAR, metformin, or phenformin. (D) qRT-PCR analysis of TERRA levels in treated myotubes normalized to β2M cDNA and to untreated cells. Error bars indicate SD (n = 4). (E) Western blot analysis of NRF1 knockdown in myotubes. (F) qRT-PCR analysis of TERRA in siNRF1-treated myotubes and upon phenformin treatment. Values were normalized to β2M cDNA and to siLuci-treated cells without phenformin. Error bars indicate SD (n = 3). (G) Unified theory of aging (34) revisited with data from this study (green). See text for details.
DISCUSSION

The association between physical activity and telomere length is still an open question. Although endurance training may provide protective effects on telomere length and attenuate biological aging (29), acute exposure to ultradistance endurance trail races was reported to shorten telomeres (30) as a possible consequence of excessive reactive oxygen species (ROS) production (31). Because we found that telomere transcription is activated by NRF1 antioxidant factor, it is tempting to speculate that TERRA up-regulation may be part of the antioxidant response that skeletal muscles set up to counteract exercise-induced ROS (32). In this view, we hypothesize that, because of their high content in guanine residues, $UUAGGG$ repeats may be highly prone to oxidation and telomere-bound TERRA may possibly shield $TTAGGG$ telomeric repeats from ROS. TERRA oxidation may also occur as a daily consequence of cellular respiration, and exercise would thus help in renewing TERRA pools by eliminating oxidized molecules, thereby ensuring maintenance of TERRA-protective functions. It would be interesting to test that hypothesis in the future. Alternatively, the telomere transcription process per se may positively affect telomeres, possibly through chromatin remodeling or telomeric loop formation. TERRA also recently emerged as a telomerase regulator, but it is still unclear whether telomeric RNA species act as positive or negative regulators of telomerase (7). The demonstration that short telomeres of budding yeast cells display higher transcriptional activity to promote telomerase recruitment (26), together with similar data recently obtained from fission yeast (27), favors the positive regulator hypothesis. Although our data show that AMPK activation up-regulates TERRA regardless of telomerase expression status (HuH-7 cells express telomerase, whereas myotubes do not), we propose that exercise may also positively affect telomeres by promoting telomerase recruitment at telomeres of somatic stem cells that express the enzyme.

Our study also provided a new link between telomeres and PGC-1α. First connections between telomeres, mitochondria, and PGC-1α were established by Sahin and colleagues (33), who reported that telomere dysfunction compromises mitochondrial and metabolic functions in mice through transcriptional repression of $PGC-1\alpha/\beta$. Hence, in the context of the unified theory of aging involving PGC-1 as a key regulator (34), we propose that AMPK-activated transcription of telomeres may be part of a compensatory mechanism that aims to prevent telomere dysfunction–induced decline of postmitotic tissues and stem cells (Fig. 4G). These observations agree with the report that exercise-induced up-regulation of PGC-1α reverts sarcopenia and, more generally, rescues progeroid aging in mitochondrial DNA mutant mice (35). Finally, our study agrees with the recent classification of dietary restriction, yet another AMPK/PGC-1 activation mechanism, with exercise, metformin, and sirtuin activators as possible interventions with translational potential that may delay aging (2, 3).

MATERIALS AND METHODS

In silico analysis of human subtelomeric promoters

Subtelomeric sequences obtained from the University of California, Santa Cruz genome browser using the December 2013 human reference sequence assembly (GRCh38/hg38) were assigned to the “high-confidence” data set. Missing subtelomeric sequences or sequences that did not have $TTAGGG$ telomeric regions at the $3'$ ends in GRCh38/hg38 assembly were obtained from the study by Stong et al. (13) and were classified as low confidence. Sequences of p arms from acrocentric 13, 14, 15, 21, and 22 chromosomes are missing. For promoter identification, 1.6 kb of upstream regions from final $TTAGGG$ was analyzed using the Genomatix software with PromoterInspector for the identification of putative RNA polymerase II promoter regions and MatInspector for the identification of putative transcription factor binding sites. Predicted TSSs were based on the study by Nergadze et al. (5).

Cell culture, transfections, and treatments

Huh-7 hepatocarcinoma cell line (American Type Culture Collection) was grown in Dulbecco’s modified Eagle’s medium (DMEM) (Lonza). LB37 non–small cell lung carcinoma cell line (6) was grown in Iscove’s modified Dulbecco’s medium (Lonza). Culture media were supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% glutamine (Gibco), and 1% penicillin/streptomycin (Lonza). Transfections with siRNAs were performed as previously described (6) using Lipofectamine 2000 (Invitrogen) and either siNRF1 (table S2) or siLuci (6) (Eurogentec). For plasmid transfections, we used polyethyleneimine (PEI) reagent (Polysciences, catalog no. 23966-2). Human myoblasts, provided by G. Carnac (INSERM U1046, University of Montpellier, France), were isolated from deltoid muscle biopsies and purified as previously described (36). Myoblasts were plated at a density of $1 \times 10^6$ cells/cm$^2$ in DMEM supplemented with 20% FBS and 0.5% Ultrasphere G (Pall BioSepra). When cultures reached 80% of confluence, myogenic differentiation was induced by changing the medium to DMEM/2% FBS. Myotubes were obtained after 5 days. Cell treatments were performed as follows: 1 mM phenformin (Sigma-Aldrich) for 16 hours, 1 mM metformin (Sigma-Aldrich) for 8 hours, or 1 mM AICAR (Sigma-Aldrich) for 4 hours. For NRF1 knockdown in myotubes, myotubes were transfected with siRNAs during the first day of differentiation using Lipofectamine RNAiMAX (Invitrogen), and cells were treated with phenformin on the fourth day of differentiation before harvesting 16 hours later. Ad-PGC-1α and Ad-GFP constructs were provided by B. Viollet and M. Foretz (Institut Cochin, Paris), and adenoviral particles were prepared using the human embryonic kidney 293 (HEK293) cell line according to standard procedures (37).

Human study

Ten healthy and moderately active young men [ age, 20.0 ± 0.6 years; body mass index, 21.7 ± 0.7 kg m$^{-2}$; peak oxygen consumption (VO$_2$ peak), 47.2 ± 2.9 ml min$^{-1}$ kg$^{-1}$; maximal power output ($W_{\text{max}}$), 226.9 ± 5.5 W ] volunteered to participate in this study, which was approved by the local ethics committee (Université catholique de Louvain) and was in conformity with the Helsinki Declaration (www.wma.net/en/30publications/10policies/b3/index.html). Subjects were asked to refrain from vigorous physical activity for 2 days and to abstain from alcohol consumption the day before the experiments. A medical checkup was done to detect any contraindications for maximal exercise testing, and written consent was obtained from all subjects after all potential risks of the study were explained.

At least 1 week before the experimental session, a maximal incremental exercise test was performed on a cycle ergometer (Cyclos2, RBM electronics) to determine VO$_2$ peak and $W_{\text{max}}$. The starting load was 70 W, incremented by 40 W every 3 min, until exhaustion. Heart rate (Polar) and respiratory exchanges (Medisoft) were continuously monitored. Participants were randomly divided into two groups: a low-intensity exercise group (50% VO$_2$ peak) and a high-intensity exercise group (75% VO$_2$ peak). On the day of the experimental session, subjects arrived after an overnight fast and received a standardized
breakfast (715 kcal; 74% carbohydrate, 17% lipid, and 9% protein). One hour after breakfast, a first biopsy sample, with the needle pointing proximally, was taken from the midportion of the vastus lateralis muscle from the right leg under local anesthesia (1 ml of Xylocaine 2%; AstraZeneca). Samples were immediately frozen in liquid nitrogen and stored at −80°C before further analysis, except for ~15 mg samples, which were embedded in the Tissue-Tek O.C.T. Compound (Sakura) for immunofluorescence (IF) staining and RNA-FISH experiments. A capillary blood sample (5 μl) was collected from the earlobe to determine the blood lactate concentration (Lactate Pro) before cycling exercise. IF was performed as previously described ([36]–[38]). Revelation was performed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, catalog no. 37076) according to the manufacturer's instructions using rabbit primary antibodies (1 : 500 dilution) and a goat anti-rabbit secondary antibody (1 : 5000 dilution) (Asgaard, catalog no. 4021) with a signal to noise ratio of 1 : 5. Western blotting was performed according to standard procedures using antibodies described in table S2. The supernatant was kept as cytosolic fraction. The pellet was then resuspended into 100 μl of suspension buffer containing 20% glycerol, 1 mM Na3VO4, 10 mM p-nitrophenyl phosphate, 10 mM β-glycerophosphate, and 5 mM NaF (Sigma-Aldrich) before 1:1 dilution with the same buffer containing 0.8 M NaCl. Samples were rotated for 30 min at 4°C before centrifugation at 10,000 g for 10 min at 4°C, and supernatant was kept as nuclear fraction.

**Cellular fractionation from human muscle biopsies**

About 20 mg of frozen muscle biopsies was homogenized with a Polytron mixer in 200 μl of ice-cold suspension buffer (20 mM Heps, 5 mM NaF, 1 mM Na2MoO4, and 0.1 mM EDTA; protease inhibitor cocktail) (Sigma-Aldrich) supplemented with 0.5% NP-40 (Fluka). The homogenate was incubated on ice for 5 min and centrifuged at 10,000 g for 30 s at 4°C. The supernatant was kept as cytosolic fraction. The pellet was then resuspended into 100 μl of suspension buffer containing 20% glycerol, 1 mM Na3VO4, 10 mM p-nitrophenyl phosphate, 10 mM β-glycerophosphate, and 5 mM NaF (Sigma-Aldrich) before 1:1 dilution with the same buffer containing 0.8 M NaCl. Samples were rotated for 30 min at 4°C before centrifugation at 10,000 g for 10 min at 4°C, and supernatant was kept as nuclear fraction.

**Western blotting**

Total cell extracts were prepared with radioimmunoprecipitation assay buffer [150 mM NaCl, 50 mM tris-HCl (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA]. Western blotting was performed according to standard procedures using antibodies described in table S3. Revelation was performed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, catalog no. 34080). Signal quantification was done using ImageJ software (National Institutes of Health).

**IF, DNA-FISH, and RNA-FISH**

IF was performed as previously described ([39]) using antibodies described in table S3. For TIF detection, IF was first performed using anti-53BP1 antibody (table S3). Then, cells were fixed again with 3.7% paraformaldehyde (PFA) (VWR)/phosphate-buffered saline (PBS) for 2 min at room temperature, washed with PBS, and treated with ribonuclease (RNase) A (100 μg/ml; Sigma-Aldrich) for 1 hour at 37°C before another incubation with permeabilization buffer for 10 min at room temperature. Cells were briefly washed with PBS, refixed with 3.7% PFA/PBS, and washed with PBS before successive baths in 70, 80, 90, and 100% ethanol for 2 min each. Hybridization with 50 nM TeloG Exiqon LNA red probe (TAMRA)GGGTtAGGGtAgGTTAGGGtAGGGtAGGGtTA (TAMRA) (small letters indicate LNA-modified bases) was next performed in 50% deionized formamide (Millipore)/2× SSC in 1× blocking reagent (Roche) for 2 hours at room temperature after denaturation for 3 min at 85°C. Samples were dried out and mounted with DAPI (Sigma-Aldrich) after two washes with 50% formamide/2× SSC, 20 mM tris (pH 7.4) for 15 min at room temperature, three washes with 50 mM tris (pH 7.4), 150 mM NaCl, 0.05% Tween 20 (Sigma-Aldrich) for 5 min at room temperature and dehydration with ethanol bath series. RNA/DNA-FISH on primary myotubes differentiated onto coverslips was adapted from Basu et al. (40). Briefly, following TERRA-FISH performed as previously described (6) using TeloC green Exiqon LNA probe (FAM)CCCTAaaCCCTaACCTaaACCTaACCTaA(FAM) (small letters indicate LNA-modified bases), cells were fixed with 4% PFA (VWR) for 15 min at room temperature and rinsed once with PBS. Samples were next denatured at 80°C for 10 min in 70% deionized formamide (Millipore)/2× SSC before successive baths in 70, 85, and 100% ethanol. Denatured TeloG Exiqon LNA red probe (400 nM) was then added into the hybridization buffer [50% deionized formamide, 10% dextran sulfate (Pharmacia Biotech), 2× SSC, 0.2% bovine serum albumin (Sigma-Aldrich), 20 mM ribonucleoside- vanadyl complex (New England Biolabs)], and coverslips were incubated overnight at 37°C in a dark and humid environment. After three washes with 0.1× SSC at 37°C and one wash with 2× SSC, dehydration with ethanol bath series was performed again, and coverslips were dried out and mounted with DAPI (Sigma-Aldrich). TERRA-FISH combined with IF in muscle biopsies is described in Supplementary Methods. Images were acquired with a confocal microscope (Cell Observer Spinning Disc, Zeiss) with 100× objective and analyzed using the ImageJ software (National Institutes of Health).

**Luciferase activity measurements**

pGL3-10q construct containing the 10q subtelomeric promoter cloned upstream of Firefly luciferase gene (10) was provided by P. Lieberman (The Wistar Institute, Philadelphia). Luciferase activity normalization was obtained with pGL4.75(hRluc/CMV) vector containing the Renilla luciferase gene under the constitutive cytomegalovirus (CMV) promoter (Promega, catalog no. E6931). Mutation of NRF1 binding sites was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, catalog no. 200515) according to the manufacturer’s protocol, using primers described in table S2. Mutated DNA sequences are provided in fig. S5. For luciferase activity measurements, 8 × 104 Huh-7 cells per well were seeded into 12-well culture plates for 24 hours before transfection with PEI reagent (Polysciences, catalog no. 23966-2). Cells were transfected with a total of 0.5 μg DNA comprising 0.3 μg pGL3-10q, 0.005 μg pGL4.75, and 0.2 μg of either pcDNA3.1 empty plasmid, pcDNA3.1-Flag-NRF1, or pcDNA3.1-Flag-ΔC-NRF1 constructs (23) provided by K. Kohno (University of Occupational and Environmental Health Japan, Fukuoka). Cells were collected 24 hours after transfection, and relative luciferase activity was measured using the
Acknowledgments: We thank P. Lieberman, B. Viollet, M. Foretz, K. Kohno, and G. Carnac for reagents; R. Van Thienen for muscle biopsies; all subjects for their dedication to the experiment; and N. Arnoult for critical comments on the article. Funding: A.D. has a fellowship from Télévie/ Fonds National de la Recherche Scientifique (FNRS). J.B., H.E., L.B., and A.D.C. are supported by the FNRS. F.P. has a grant from Fonds pour la formation à la Recherche dans l’Industrie et dans l’Agriculture. J.R. is supported by ProCell sprl. M.F. is supported by the Walloon Region. Author contributions: A.D. and J.B. performed most of the experiments and carried out data analysis, with assistance from F.P., J.R., M.P., and H.E. L.B. provided assistance with adenovirus production and guidance for AMPK activation. M.F. and L.D. designed and supervised the human study. A.D., L.D., and A.D.C. wrote the paper. A.D.C. conceived the project, directed the research, and assisted in data analysis. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Correspondence and request for materials should be addressed to A.D.C. (anabelle.decottignies@uclouvain.be).
Nuclear respiratory factor 1 and endurance exercise promote human telomere transcription

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Sci Adv 2 (7), e1600031.
DOI: 10.1126/sciadv.1600031