Supplemental Material

Methods

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Figure S1. Rapamycin increases autophagic flux in HGPS fibroblasts.

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Methods
Cell culture
In this study we used primary cultures of human dermal fibroblasts obtained from HGPS patients (HGADFN003 and HGADFN127) from Progeria Research Foundation, as an *in vitro* model of HGPS. Although the results observed were similar in both cell lines, for consistency, all results in this report correspond to the results obtained from patient HGADFN003. The results regarding HGADFN127 are shown in Figure S3. We also used primary cultures of human dermal fibroblasts obtained from a skin biopsy of a healthy individual (control fibroblasts). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L D-glucose; Sigma) supplemented with 15 % fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco) and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) and maintained at 37 ºC and 5 % CO_2/air.

Experimental conditions
HGPS and Control fibroblasts were treated with human NPY (100 nM; Phoenix Pharmaceuticals Inc.) every other day for up to 1 week, unless otherwise indicated. Although the results observed were similar in both cell lines, for consistency, all results in this report correspond to the results obtained from patient HGADFN003.

Western Blotting
Cells were lysed on ice in RIPA (radio-immunoprecipitation assay) buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.5% deoxycholate; 0.1 % sodium dodecyl sulphate (SDS); 200 µM phenylmethylsulphonylfluoride (PMSF); 1 mM dithiothreitol (DTT), 1 mM Na3VO4; 10 mM NaF), supplemented with mini protease inhibitor cocktail tablet (Roche). Lysates were incubated for 15 min at 4 ºC, and the insoluble material was pelleted by centrifugation for 10 min at 16,000x g and 4ºC. The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology). The samples were denatured by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10 % SDS, 0.6 M DTT, 0.012 % bromophenol blue) and heating for 5 min at 95 ºC. Samples were stored at -20 ºC until use.

Equal amounts of total protein were loaded per lane and separated by electrophoresis in 4-10% or 4-12% sodium dodecyl sulphate–polyacrylamide gels (SDS-PAGE). Proteins were then transferred electrophoretically in CAPS buffer (0.1 M CAPS, pH 11; 10 % methanol) to polyvinylidenedifluoride membranes (Millipore). The membranes were blocked with 5% low-fat milk in Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) for one hour at room temperature. The membranes were incubated overnight with the primary antibodies at 4 ºC. The primary antibodies used were (all at a dilution of 1:1,000): mouse monoclonal anti-laminA/C (Developmental Studies Hybridoma Bank; MANLAC1 (4A7)) and rabbit anti-LC3B, anti-SQSTM1, anti-MTOR, and anti-phospho-MTOR (Ser2448), from Cell Signaling. After three washes with TBS-T, the membranes were incubated for one hour, at room temperature, with an alkaline phosphatase-linked secondary antibody, specific to mouse or rabbit immunoglobulin G in a 1:20,000 dilution. Protein immunoreactive bands were visualized by chemifluorescence with the ECF substrate (GE Healthcare) in a VersaDoc Imaging System (Bio-Rad). The membranes were reprobed with a monoclonal anti-β-tubulin antibody (1:10,000; Sigma) for equal protein loading control. The optical density of the bands was quantified with the Quantity One Software (Bio-Rad). The results are normalized to β-tubulin and are expressed as the relative amount compared to non-treated cells.

Autophagic flux measurement
LC3B turnover assay
Microtubule-associated protein light chain-3B (LC3B) is now the most widely used marker of autophagy (1). Upon autophagy induction, the soluble cytosolic form of LC3B (LC3B-I) is conjugated to phosphatidylethanolamine to form a lipidated form (LC3B-II). LC3B-II becomes membrane-bound to the phagophores, autophagosomes and autolysosomes. LC3B-II is localized both in the luminal and cytosolic site of the autophagic structures and undergoes degradation within the lysosome. Thus, the steady-state levels of LC3B-II and its turnover within lysosomes can be used to measure autophagic activity in cells. Being autophagy a highly dynamic process, it should be noted, however, that the LCB3-II amount at a given time point does not necessarily estimates the autophagic flux, and therefore, it is important to measure the amount of LC3B-II delivered to lysosomes (1).

Autophagic flux is often inferred on the basis of LC3-II turnover, measured by Western blotting, in the presence and absence of lysosomal degradation. The relevant parameter in LC3B turnover assays is the difference in the amount of LC3B-II in the presence and absence of saturating levels of inhibitors, which can be used to examine the transit of LC3B-II through the autophagic pathway and its accumulation within lysosomes. This assay will reflect the net amount of LC3B-II delivered to lysosomes, which in turn will be the measure of autophagy flux. If flux is occurring, the amount of LC3B-II will be higher in the presence of the inhibitor. Lysosomal degradation

can be prevented through the use of compounds that neutralize the lysosomal pH such as chloroquine or NH_4Cl inhibiting lysosomal degradation (1).

In our study, we determined autophagic flux by measuring the amount of LC3B-II delivered to the lysosomes by comparing the LC3B-II amount in the presence and absence of the lysosomal inhibitor chloroquine (Chq; 100 μM) by Western blotting. Chloroquine was added to cell culture medium 30 min before the addition of NPY (6 h treatment). For each experimental condition, untreated HGPS cells (used as control) and NPY-treated HGPS cells, autophagic flux, expressed as "LC3B-II net flux", was determined by subtracting the densitometric value of the LC3B-II band of chloroquine-untreated sample (Chq - LC3B-II) from the densitometric value of the LC3B-II band of the corresponding chloroquine-treated sample (Chq + LC3B-II). For each independent experiment, the values obtained upon subtraction for each condition were normalized to the control condition (untreated HGPS cells). The results are represented as mean values for each experimental condition.

The LC3B-net flux results are shown in parallel with the results regarding the quantification of the steady-state levels of LC3B-II (densitometric values of LC3B-II are normalized to the loading control β-tubulin) in the presence or absence of chloroquine in untreated (HGPS cells) and NPY-treated cells to assess the effect of NPY on LC3B-II levels as well as demonstrate the accumulation of LC3B-II levels upon chloroquine treatment.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were lysed, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers and eluted with 30 μL of RNase-free water by centrifugation. Total RNA amount was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific), and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm. RNA samples were treated with RNase-free DNAse (Qiagen) to eliminate any contamination with genomic DNA. RNA samples were kept at -80 ºC until use.

Reverse transcription into cDNA was carried out using the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Briefly, 1 μg of total RNA from each sample was reverse transcribed into cDNA in a 30 μL reaction containing 1x iScript reaction buffer, and 1 μL of iScript reverse transcriptase. Reverse transcription reactions were performed in a thermocycler at 25 °C for 5 minutes, 46 °C for 30 minutes, 95 °C for 5 minutes, and 4 °C for 5 minutes. cDNA samples were then stored at -20 °C until use.

HGPS cells mRNA expression was measured by qRT-PCR performed in the StepOnePLus™ Real-Time PCR System (Applied Biosystems) using 96-well optical plates (Thermo) and the SsoAdvanced™ Universal SYBR® Green Supermix (BioRad). For amplifying human progerin mRNA, the sequence of the forward PCR primer was 5′-CTCAGGAGCCCAGAGCC-3′ and for the reverse primer was 5′-GGCATGAGGTGAGGAGGAC-3′. GAPDH was used as housekeeping gene to normalize progerin expression; the sequence of the forward PCR primer was 5′-TGTTCGACAGTCAGCCGCATCTTC-3′ and for the reverse primer was 5′-CAGAGTTAAAAGCAGCCCTGGTGAC-3′.

A master mix was prepared for each primer set, containing the appropriate volume of 2× SsoAdvanced™ Universal SYBR® Green Supermix and 500 nM of each specific gene primer.

For each reaction, 7 μL of master mix were added to 3 μL of template cDNA (1:50). All reactions were performed in duplicates (two cDNA reactions per RNA sample). No template control (NTC) and no reverse transcriptase control (NRT) were used as negative controls. The reactions were performed according to the manufacturer's recommendations: 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 sec and 59ºC for 15 sec. The melting curve protocol started immediately after amplification. The amplification efficiency for each gene and the threshold values for threshold cycle determination (Ct) were determined automatically by the StepOnePLus Software (Applied Biosystems). Relative mRNA quantification was performed using the ΔCt method for genes with the same amplification efficiency. The results are expressed as the relative amount compared to non-treated cells.

Immunocytochemistry

After the treatments, cells were washed twice with pre-warmed PBS (pH 7.4) at 37ºC and then fixed with ice-cold 4% paraformaldehyde for 15 minutes. Next, cells were rinsed three times with ice-cold PBS and permeabilized with 0.1 % (v/v) TX-100/PBS for 10 minutes at room temperature. Cells were washed twice with PBS and blocked with 3% BSA/10% goat serum/ PBS for 1h at room temperature. Then, cells were incubated with primary antibody overnight at 4ºC. The primary antibodies used were mouse monoclonal anti-progerin (1:500; Sigma), mouse monoclonal anti-Lamin A/C (1:500; Millipore) mouse monoclonal anti-H2AX (1:500; Millipore) and mouse monoclonal anti-Ki67 (1:400; Dako). After incubation, cells were washed three times with PBS and incubated with the respective secondary antibody for 1h at room temperature. The secondary antibodies used were Alexa-Fluor 488- or Alexa Fluor 594-conjugated goat anti-mouse IgG. The nuclei were stained with Hoechst 33342 (2 μg.mL-1; Sigma-Aldrich) during secondary antibody incubation. Lastly, cells were rinsed

three times with PBS and the coverslips were mounted on glass slides with Aqua-Polymount (Polysciences, Inc.) mounting medium. Cells were analyzed in an Axio Observer Z1 fluorescence microscope or LSM 710 confocal microscope (Carl Zeiss).

Quantification of misshapen/blebbing nuclei and Ki-67-positive cells was done by direct counting in 40 randomly chosen, non-overlapping fields (x400 magnification), normalized to the total number of nuclei stained with Hoechst for each coverslip of each experimental condition. Results represent the mean ± SEM of four independent experiments (at least 400 cells analyzed for each experimental condition) and are expressed as percentage of non-treated cells. The analysis of nuclei morphology parameters was performed using FIJI (Fiji is Just ImageJ) software, through the use of a home-made macro. Briefly, images were thresholded and nuclei were automatically detected based on their size (100-infinite) and circularity (0.3-1). Then, all images were manually reanalyzed to confirmed the automatic selection. Finally, the following parameters were measured for each selected nucleus: area, perimeter and circularity ($4\pi*area/perimeter^2$).

For H2AX foci quantification 20 randomly choosen, non-overlapping z-stacking images were acquired on a Carl Zeiss LSM 710, using a Plan ApoChromat (63x/1,4 oil-objective, and laser49 lines DPSS 561-10 for H2AX staining and Diode 405 for Hoechst staining. First, the nuclei in all images using the nuclear morphology parameters macro above described. Then, the FindFoci plugin was applied (Herbert et al. 2014) in order to identify the peak corresponding to each foci in each nuclei, and setting the minimum peak size above saddle on 5. The number of foci/nuclei, the number of foci/nuclei area and the percentage of area were calculated for each nuclei and condition.

Senescence associated β galactosidase assay (SA- β-Gal)

Senescence, a hallmark of aged fibroblasts, is characterized by a loss of proliferation and an increase in senescence-associated β-galactosidase (SA-β-Gal) activity (2). Medium from HGPS treated cells was removed and cells were washed twice with PBS at room temperature. Next, cells were fixed with ice-cold 4% paraformaldehyde for 3 minutes. Cells were then rinsed twice with PBS at room temperature. Afterwards, cells were exposed to fresh SA- β-Gal staining solution (2 mL) composed of 1x citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl2, and 1 mg/mL X-Gal in water and were incubated overnight in a non-CO_2 incubator, at 37ºC. Staining solution was washed away, cells were rinsed twice with PBS and, lastly, the coverslips were mounted on glass slides with Aqua-Polymount mounting medium). Cells were analyzed by brightfield microscopy on a Zeiss Axio Imager Z2 microscope (Carl Zeiss, Oberkochen, Germany). Quantification of SA-β-Gal-positive cells was performed by direct counting of positive cells in 30 randomly chosen, non-overlapping fields (x200 magnification), for each coverslip of each experimental condition.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post test, or Student's unpaired t test with two-tailed p value, as indicated in figure legends. A value of $p<0.05$ was considered significant. Prism 5.0 (GraphPad Software) was used for all statistical analysis.

References

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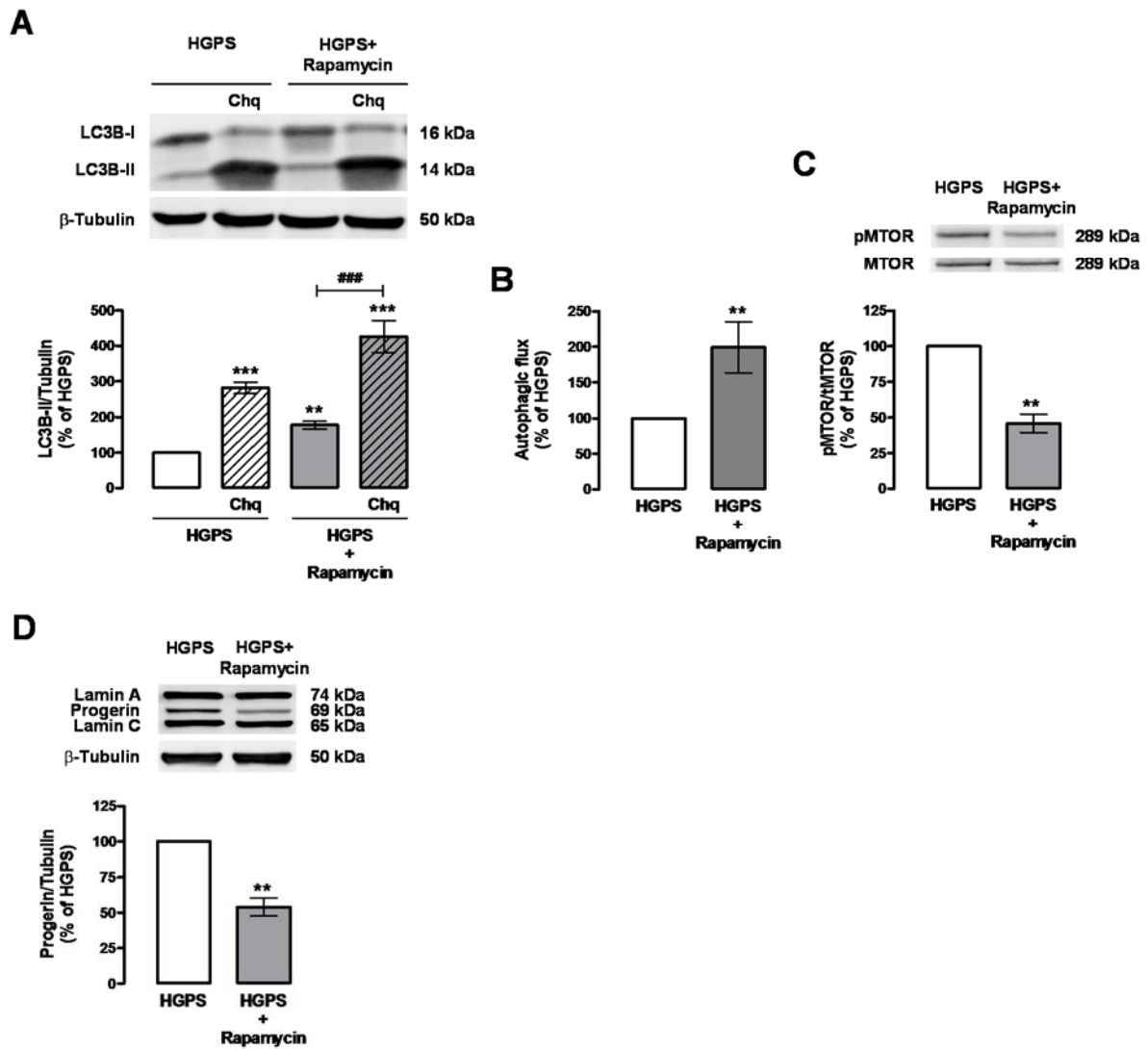
Figure S1

Figure S1. Rapamycin increases autophagic flux in HGPS fibroblasts.
HGPS fibroblasts were exposed to Rapamycin (100 nM) for 6 h (HGPS + Rapamycin) in the presence or absence of chloroquine (Chq, 100 μM), a lysosomal degradation inhibitor. Untreated cells were used as control (HGPS). Whole cell extracts were assayed for LC3B (A), pMTOR/MTOR (C), LaminA/Progerin/Lamin C (D) and β-tubulin (loading control) immunoreactivity through Western blotting analysis. Representative Western blots for each protein are presented above each respective graph. Autophagic flux analysis in HGPS cells (B) is shown. Autophagic flux was determined in the presence of the lysosomal inhibitor chloroquine, and expressed as "Autophagic flux" calculated by subtracting the densitometric value of LC3B-II – Chq from those corresponding LC3B-II + Chq values. The results represent the mean ± SEM of, at least, four independents experiments, and are expressed as percentage of HGPS. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different compared to HGPS; ###$p<0.001$, significantly different compared to NPY, as determined by ANOVA, followed Bonferroni's post test, or Student's t test.
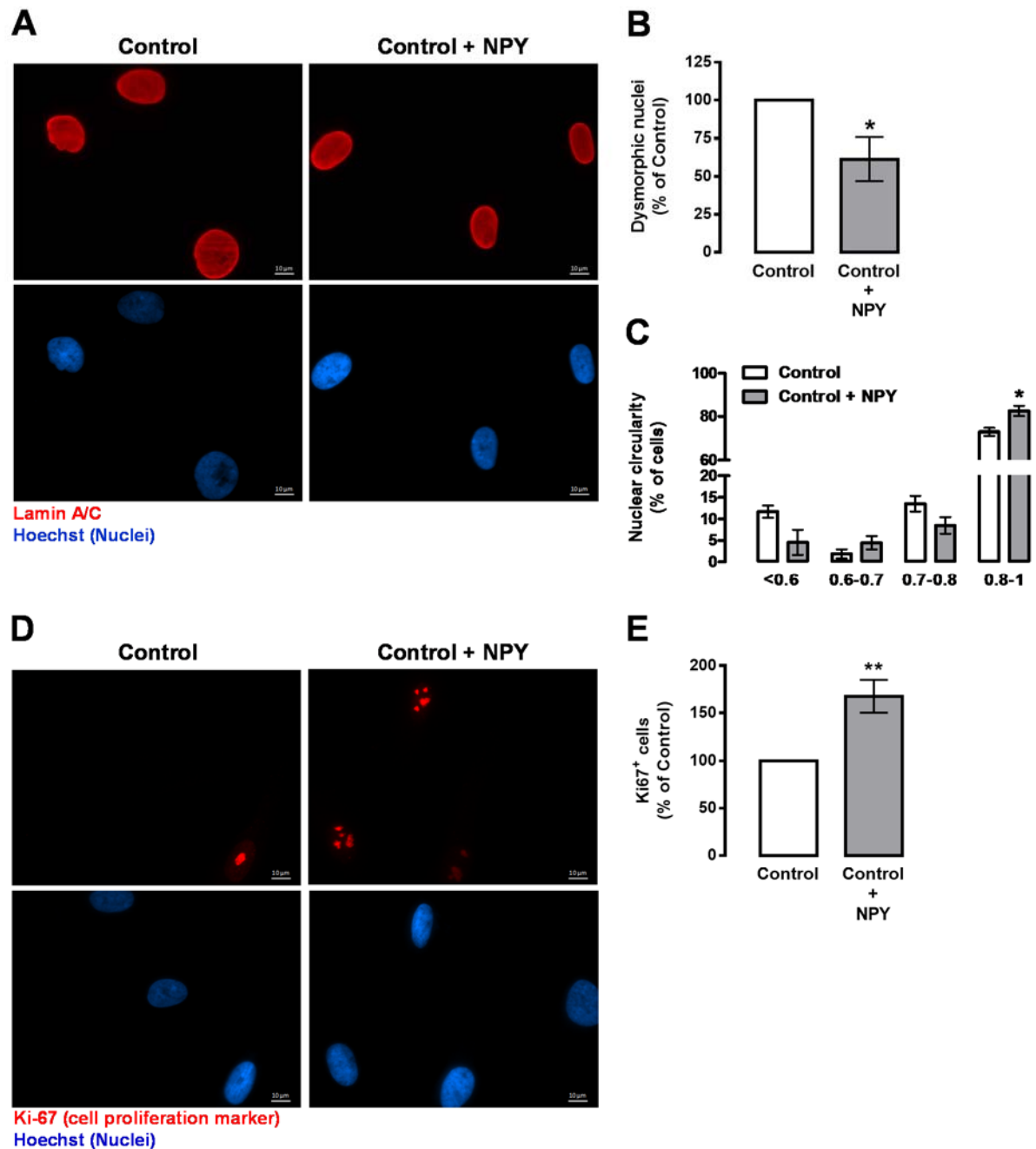
Figure S2

Figure S2. NPY rescues nuclear morphology and increases cell proliferation in control fibroblasts.

Control fibroblasts were exposed to NPY (100 nM; Control + NPY) for 1 week. Untreated cells were used as control (Control). (A) Control fibroblasts were immunolabeled for Lamin A/C (red) and nuclei were stained with Hoechst (blue). Representative images of four independent experiments are shown. Scale bar, 10 µm. Quantification of the number of abnormal nuclei (B) and nuclear circularity (C) upon NPY treatment. For each condition, an equal number of nuclei (>400) were randomly analyzed. Circularity (defined as: $4*\pi*area/perimeter^2$) was measured using ImageJ. A circularity value equal to 1 corresponds to perfectly circular nuclei. (D NPY increases cell proliferation, as determined by Ki-67 immunoreactivity. Cells were immunolabeled for Ki-67 (red) and nuclei were stained with Hoechst (blue). Representative images of five independent experiments are shown. Scale bar, 10 µm. (E) Quantification of the number of Ki-67-positive cells in untreated and NPY-treated Control cells. The results represent the mean ± SEM of five independents experiments, and are expressed as percentage of Control. *p<0.05, ***p<0.001, significantly different from Control, as determined by Student's t test.
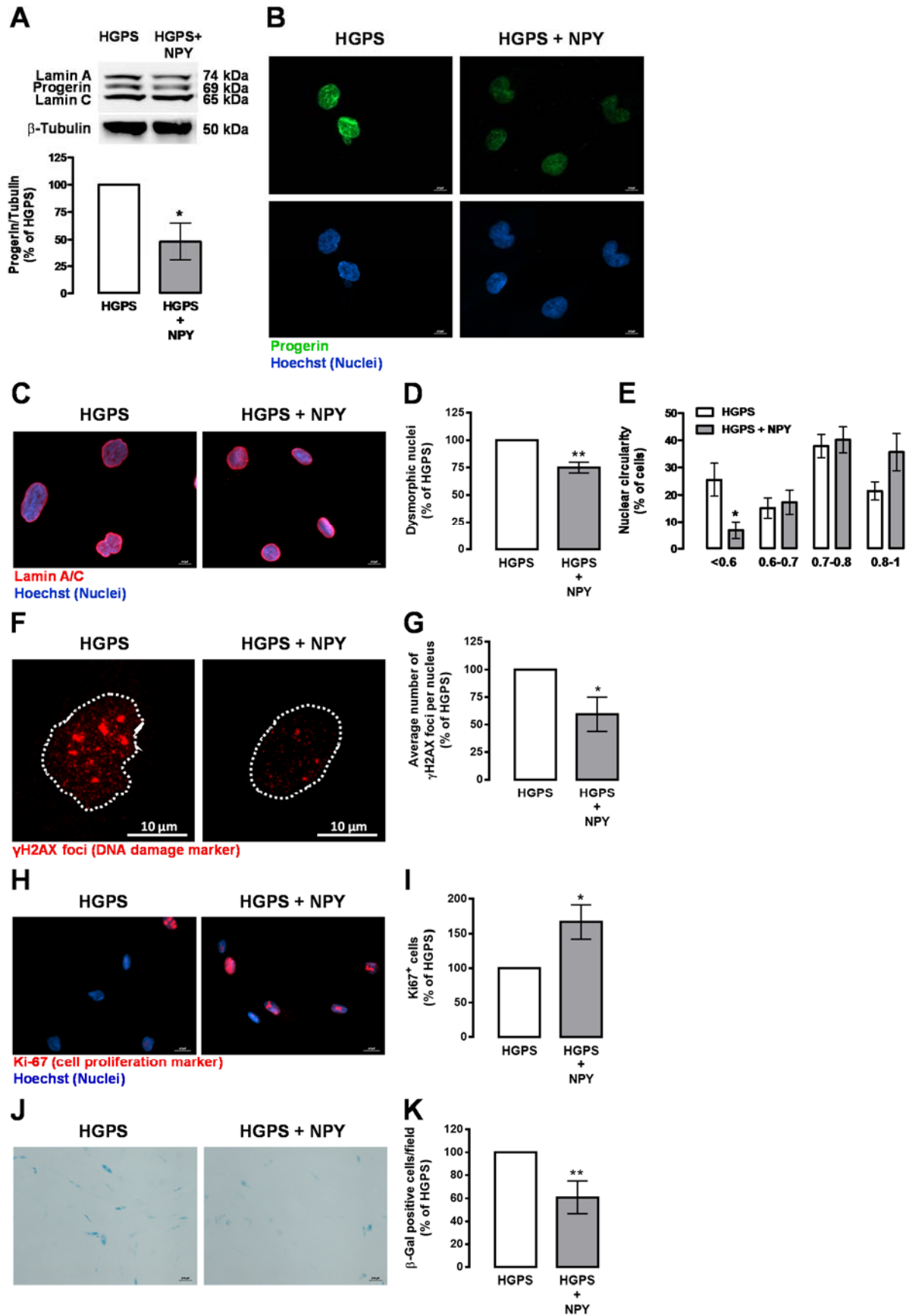
Figure S3

Figure S3. NPY decreases progerin levels and delays cellular senescence in HGPS cells from HGADFN127 patient.

Primary HGPS fibroblasts were exposed to NPY (100 nM; HGPS + NPY) for 1 week. Untreated cells were used as control (HGPS). (A) NPY decreases progerin protein levels. Whole cell extracts were assayed for Lamin A/Progerin/Lamin C and β-tubulin (loading control) immunoreactivity through Western blotting analysis. (B) NPY decreased progerin immunoreactivity. Cells were immunolabeled for progerin (green) and nuclei were stained with Hoechst (blue). Scale bar, 10 μm. (C-E) NPY decreases the number of abnormal nuclei and improves nuclear circularity HGPS fibroblasts were immunolabeled for Lamin A/C (red) and nuclei were stained with Hoechst (blue). Scale bar, 10 μm. (F-G) NPY decrease the number of γH2AX foci. Cells were immunolabeled for γH2AX (red). Scale bar, 20 μm. (G) (H-I) NPY increases cell proliferation, as determined by an increase of Ki-67-positive cells. Cells were immunolabeled for Ki-67 (red) and nuclei were stained with Hoechst (blue). Scale bar, 10 μm (J-K) NPY delays cellular senescence, as determined by a decrease in the number of SA-β-Gal-positive cells Scale bar, 100 μm. The results represent the mean ± SEM of, at least, three independents experiments, and are expressed as percentage of HGPS. *p<005, **p<0.01, significantly different compared to HGPS, as determined by Student's t test.