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Everolimus rescues multiple cellular defects in laminopathy-patient fibroblasts

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***LMNA* encodes the A-type lamins that are part of the nuclear scaffold. Mutations in *LMNA* can cause a variety of disorders called laminopathies, including Hutchinson-Gilford progeria syndrome (HGPS), atypical Werner syndrome, and Emery-Dreifuss muscular dystrophy. Previous work has shown that treatment of HGPS cells with the mTOR inhibitor rapamycin or with the rapamycin analog everolimus corrects several of the phenotypes seen at the cellular level—at least in part by increasing autophagy and reducing the amount of progerin, the toxic form of lamin A that is overproduced in HGPS patients. Since other laminopathies also result in production of abnormal and potentially toxic lamin proteins, we hypothesized that everolimus would also be beneficial in those disorders. To test this, we applied everolimus to fibroblast cell lines from six laminopathy patients, each with a different mutation in *LMNA*. Everolimus treatment increased proliferative ability and delayed senescence in all cell lines. In several cell lines, we observed that with treatment, there is a significant improvement in nuclear blebbing, which is a cellular hallmark of HGPS and other lamin disorders. These preclinical results suggest that everolimus might have clinical benefit for multiple laminopathy syndromes.**

everolimus | laminopathies | lamin | mTOR | progeria

Laminopathies are a diverse group of disorders caused by a defect in the proteins that make up the nuclear lamina, which is composed of intermediate filament proteins called lamins. In humans, the major lamins are lamin A, lamin B1, lamin B2, and lamin C. All of the A-type lamins, including lamins A and C, are alternatively spliced from the *LMNA* gene (1). There are many laminopathies caused by defects in *LMNA*, including Emery-Dreifuss muscular dystrophy, atypical Werner syndrome, limb-girdle muscular dystrophy type 1B, Charcot-Marie-Tooth disease type 2, dilated cardiomyopathy, Dunnigan-type familial partial lipodystrophy, mandibuloacral dysplasia with type A lipodystrophy, and Hutchinson-Gilford progeria syndrome (HGPS) (2, 3). The relationship between *LMNA* genotype and phenotype is highly complex. Several *LMNA* mutations that cause the same or similar disorders do not cluster in the same functional region of the protein (3). Loss or gain of tissue-specific lamin A/C–protein interactions may explain how *LMNA* mutations cause such different, though at times overlapping, phenotypes.

Two of the disorders that can be caused by *LMNA* mutations, atypical Werner syndrome and HGPS, have some features of premature aging. A majority of classic Werner syndrome cases are caused by mutations in *WRN*, a helicase; however, there are several mutations in *LMNA* that have been observed in patients with atypical Werner syndrome (4). In most cases of HGPS, a de novo G to T mutation in exon 11 of *LMNA* activates a cryptic splice donor. This results in an increase in a form of lamin A, called progerin, with an internal, in-frame deletion of 50 amino acids removing a cleavage site important for protein maturation (5–7). HGPS is a rare premature-aging syndrome that causes an aged appearance, sclerotic skin, small size, alopecia, bone dysplasia, hearing loss, and impaired mobility as a result of joint

contractures (8). Children with HGPS exhibit accelerated atherosclerosis leading to death at a mean age of 14.6 y due to myocardial infarction or stroke (9).

Cells from patients with HGPS exhibit an irregular nuclear shape or “blebbing” of the nucleus (5, 10), altered histone modification patterns, altered transcription (11), impaired DNA damage response (12, 13), early senescence, and decreased replicative ability at later passages (14, 15). These changes at the cellular level are also seen in cells from persons of advanced age (16). Irregular nuclear shape and chromatin disorganization have also been reported in cells from patients with other *LMNA* mutations (17–21).

Rapamycin and its analogs are of interest as potential new treatments for disorders associated with deposition of insoluble protein aggregates (22). Rapamycin inhibits TOR (target of rapamycin), a serine/threonine kinase that responds to environmental conditions and growth factors by regulating cell growth and metabolism (23). In mammals, the single *TOR* gene encodes mTOR (mammalian target of rapamycin), which functions as a component of two complexes, mTORC1 and mTORC2. Rapamycin inhibits mTORC1 but can also inhibit mTORC2 with prolonged treatment (24, 25). When cellular environmental conditions are favorable, mTORC1 signaling promotes ribosome biogenesis, transcription, and protein synthesis. If conditions are unfavorable (hypoxia,

Significance

The lamin proteins are an important component of the nuclear scaffold. Abnormal lamins can cause a variety of disorders called laminopathies, one of which is the premature aging disorder Hutchinson-Gilford progeria syndrome (HGPS). Cells from HGPS patients show improvement when treated with rapamycin and everolimus. These drugs stimulate the cellular system that disposes of abnormal proteins. Several other laminopathies also produce abnormal lamin proteins, so we hypothesized that everolimus would also be beneficial. We treated cells from six different laminopathy patients with everolimus and saw improved nuclear shape in most cells. Treatment also increased the cells' ability to multiply and delayed senescence. These results suggest that everolimus might have clinical benefit for multiple laminopathy syndromes.

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Conflict of interest statement: F.S.C. and T.W.G. are coauthors on a 2017 research article. They did not collaborate directly on this work.

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patient (GM23780); and one line from an HGPS patient with the most common causative mutation (HGADFN167). We also included the normal cell line HGFDFN168 (father of HGADFN167). For each experiment, cells were treated with either 0.1 μ M everolimus (dissolved in ethanol) or the same volume of ethanol (vehicle control).

To assess biological effect of the drug treatment, we measured reduction in the level of phosphorylated ribosomal protein S6 (pRPS6), which is commonly used as a biomarker to reflect mTOR inhibition. We observed a reduction in pRPS6 levels in all cell lines after 2 wk of everolimus treatment (Fig. 2).

Lobulations, or blebs, of the nuclear membrane have been observed at a much higher rate in HGPS cells compared to normal cells, especially after multiple passages in culture. Blebs have also been observed in cells with other *LMNA* mutations. We explored whether everolimus could improve the nuclear morphology of cells from patients with different laminopathies. Given the limitation of subjective assessments, we developed a machine vision approach to identify nuclei in images of immunofluorescence-labeled cells, define the borders of the nuclei, and measure aspects of the nuclei. The metric mean negative curvature (MNC) was used to assess the severity of the blebbing (Fig. 3). Cells were treated three times per week for 2 wk with everolimus or vehicle control, and then nuclei were immunofluorescently labeled with a lamin A/C antibody and imaged. First, we assessed whether nuclear blebbing is a feature of the six laminopathy cell lines in this study. Two of the vehicle-treated lines, AG04110 and PSADFN425, did not demonstrate a difference in blebbing compared with the vehicle-treated normal control ($P = 0.58$ and $P = 0.25$, respectively, Wilcoxon rank sum test, two sided), so increased nuclear blebbing does not appear to be associated with these mutations. In most of the cell lines, the median MNC of the everolimus-treated cells was less than the median MNC of the control-treated cells, including the normal control (Fig. 4). The one exception was GM23780, which shows a higher median MNC with everolimus treatment. GM23780 nuclei have an unusually elongated shape, which may make MNC a less informative metric for measuring improvement in this line.

The proliferative ability of HGPS cells in culture is reduced compared with normal cells, and it has been previously shown that rapamycin treatment can improve this defect. We observed the proliferation rates of the laminopathy fibroblasts with everolimus or control treatment and found that with everolimus treatment, several of the lines were able to proliferate better than their control-treated counterparts (Fig. 5). The everolimus-treated cells did show a decrease in proliferation initially, which has been noted elsewhere (27, 28), but later surpassed the control-treated cells in most of the lines. Several of the everolimus-treated cell lines (AG04110, PSADFN414, and PSADFN425) continued growing for 30 d or more past the time when control-treated cells of the same line stopped dividing. Normal fibroblasts (cell line HGFDFN168) showed increased proliferation with everolimus treatment as well, which has been observed previously with rapamycin treatment (27).

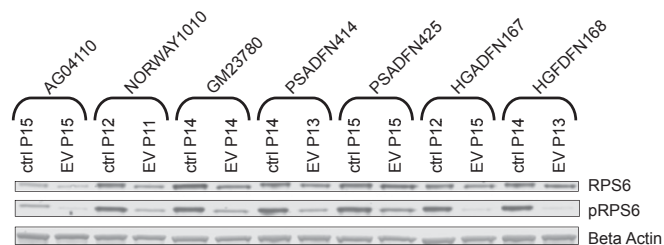


Fig. 2. Reduction in pRPS6 levels with everolimus treatment. Western blots of RPS6 and pRPS6 are shown for everolimus-treated and vehicle control-treated cell lines. All cell lines were treated three times per week for 2 wk with 0.1 μ M everolimus (EV) or vehicle control (ctrl). Passage (P) number at collection is indicated.

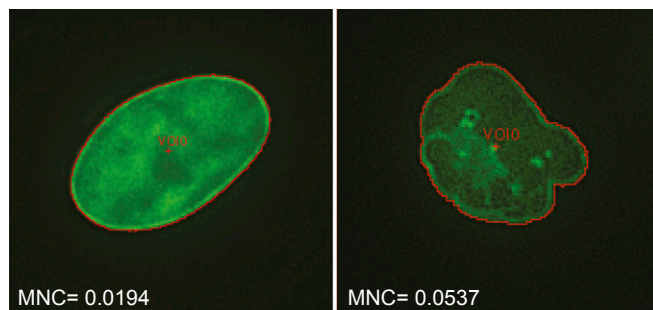


Fig. 3. MNC is an objective measurement of nuclear blebbing that can be determined by machine vision. Shown are two example nuclei stained with a lamin A/C antibody, one unblebbed and one blebbed. The perimeter of each nucleus, which is used to determine MNC, is shown in red.

Another defect observed in HGPS cells is premature senescence. To assess senescence, everolimus- and control-treated cells from each line were stained for β -galactosidase activity. In all of the lines, we observed β -galactosidase-positive cells in the control-treated cells, but none in the everolimus-treated cells (Fig. 6 and Fig. S1).

Lastly, we assessed whether everolimus treatment results in a quantitative reduction in lamin proteins. We did not observe an overall reduction in lamin A or C in the fibroblast lines after everolimus treatment for 2 wk (Fig. S2).

Discussion

Fibroblast cells from laminopathy patients with six different *LMNA* mutations showed improvement in at least two key phenotypic measures of cellular health upon everolimus treatment. First, all everolimus-treated laminopathy cultures showed an increase in proliferative ability, with three of five lines continuing to proliferate after control cultures had ceased to grow (AG04110, PSADFN414, and PSADFN425). Second, all treated cell lines displayed a reduction in senescence by β -galactosidase staining. In addition, of the four laminopathy cell lines that exhibited nuclear blebbing, three demonstrated significant improvement in nuclear morphology (a reduction in nuclear MNC) after everolimus treatment.

A previous study showed that the benefit of rapamycin treatment of HGPS fibroblasts is based, at least in part, on increased autophagy of the mutant progerin protein (27). Although most of the laminopathy fibroblast lines studied here do not produce progerin, they each produce some form of abnormal lamin A proteins that might well accumulate as insoluble complexes in the nucleus and cytoplasm after multiple mitoses. We therefore hypothesized that mTOR inhibition could provide benefit for these cell lines through a similar mechanism, by increasing autophagy and eliminating aggregated abnormal lamin protein. Unfortunately, our experiments with autophagy inhibitors in concert with everolimus were unsuccessful due to the extreme cell toxicity of the autophagy inhibitors. The autophagy inhibitors were not tolerated for the length of time needed for everolimus treatment.

mTOR inhibitors have shown benefit in mouse models with a *Lmna* deletion or mutation. Rapamycin treatment increases survival in *Lmna*-knockout homozygous mice, a model that develops dilated cardiomyopathy and skeletal muscle dystrophy (32). Ramos et al. demonstrated that mTORC1 signaling is increased in tissues linked to the pathology and that rapamycin treatment reduces this signaling and, thus, increases autophagy (32). Unlike the laminopathy cell lines studied here that have an aberrant form of lamin A and/or C, these mice have a lack of lamin A and C altogether. Clearly, rapamycin is not improving the phenotype of these mice through clearing lamin A and/or C, but perhaps clearance of other deleterious proteins is causing the improvement. In *Lmna*^{H222P/H222P} mice, a model of Emery-Dreifuss muscular dystrophy, treatment with the rapamycin analog tesirolimus has been shown to improve heart function (33,

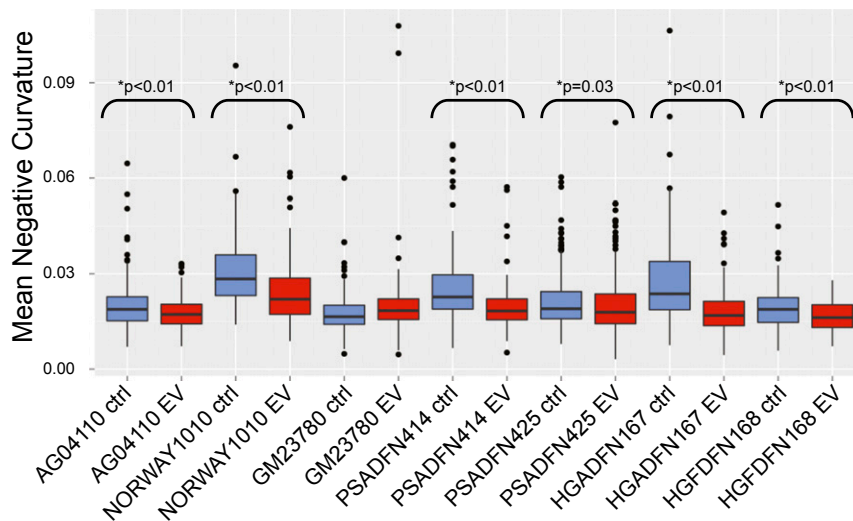


Fig. 4. MNC values for everolimus-treated and control-treated cell lines. All cell lines were treated three times per week for 2 wk with 0.1 μ M everolimus or vehicle control. Cells were fixed and labeled with lamin A/C antibody, and nuclei were photographed with an immunofluorescent microscope. All *P* values were calculated using a one-sided Wilcoxon rank sum test.

34). Again, an increase in mTORC1 signal was present in disease-relevant tissue, and treatment increased autophagy, although lamin A/C aggregates or a reduction in lamin A/C were not observed. It is possible that improvement in overall autophagy is responsible for the amelioration of the phenotype observed in both of these models and in the laminopathy cell lines treated in this study with everolimus as well.

Previous treatment strategies for HGPS have focused on farnesyltransferase inhibitors, since the toxic protein in this disorder is permanently farnesylated as a result of loss of a cleavage site used in the final posttranslational step (7). Inhibiting farnesylation

(and thus quantitatively reducing the amount of progerin) has shown benefit in HGPS cell models (35), mouse models (36), and patients in a clinical trial (37). The molecular defect in some of the other laminopathies is not the overproduction of a permanently farnesylated prelamin, but rather the production of a mature lamin containing a missense mutation in the protein. Although farnesyltransferase inhibitors have proved useful in treating HGPS, their use in other laminopathies does not have the same rationale. Alternative therapeutic approaches are needed.

Everolimus is approved for the management of transplant rejection and for cancer treatment and has been administered to

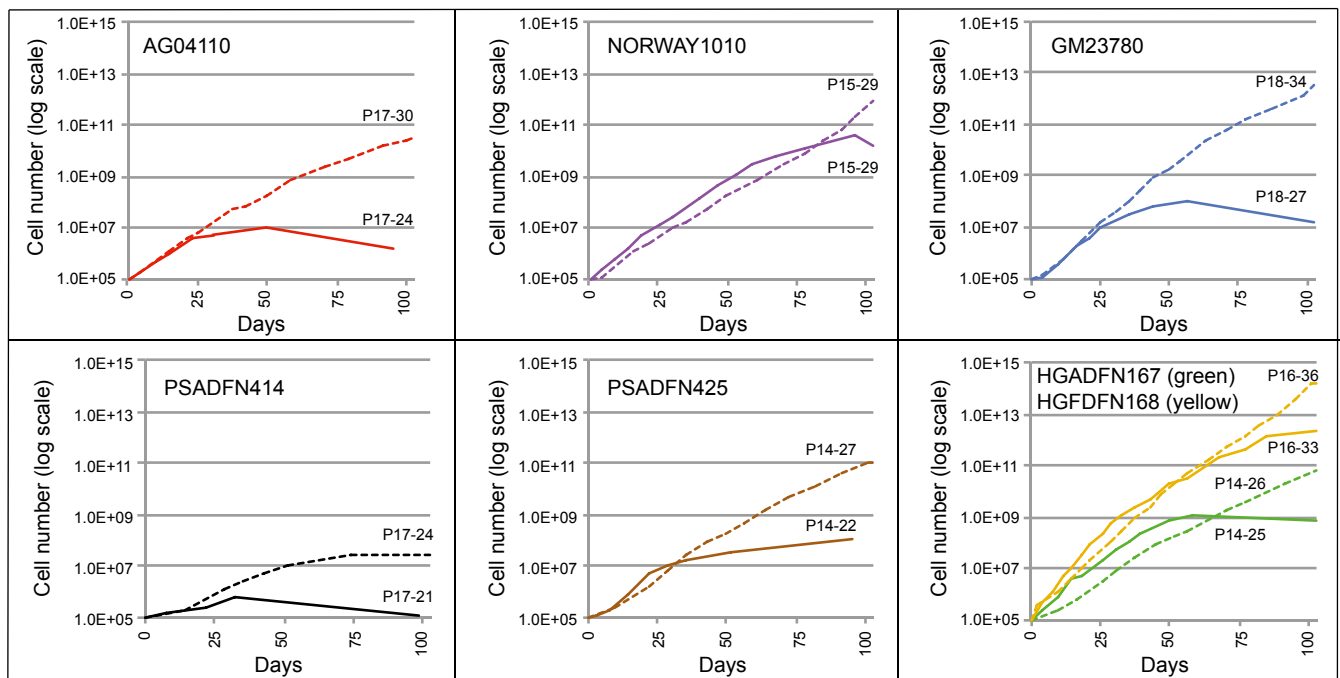


Fig. 5. Proliferation is improved with everolimus treatment. Cell numbers over time of everolimus-treated (dashed lines) and vehicle control-treated (solid lines) cell lines are shown. All cell lines were treated three times per week with 0.1 μ M everolimus or vehicle control. The cells were treated for 102 d, or until they ceased reaching confluence for at least 30 d. At each division, the cells were counted. Passage (P) number at the start and end of treatment for everolimus-treated and vehicle control-treated cells is indicated.

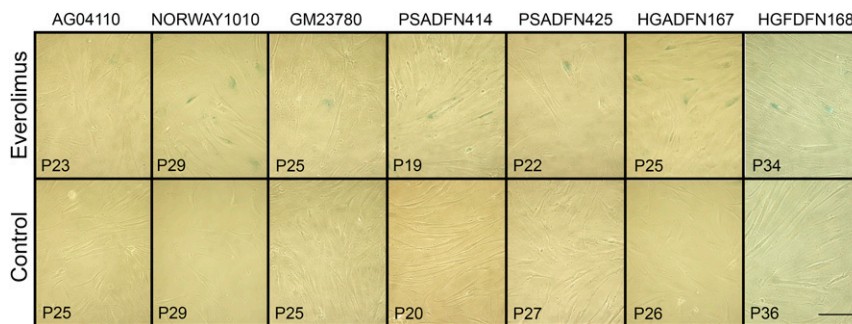


Fig. 6. Senescence-associated β -galactosidase staining of everolimus-treated and control-treated cells. Cells were treated three times per week with 0.1 μ M everolimus or vehicle control. Passage (P) number of each cell line is indicated in the lower left corner. (Scale bar: 100 μ m). All control lines have β -galactosidase-positive cells (indicated by blue staining), but everolimus-treated lines are negative.

children over long periods of time with a good safety record. It is being tested for treatment of HGPS in an ongoing clinical trial (<https://clinicaltrials.gov/>). Cellular data presented here, including improvements in the growth, senescence, and nuclear morphology of cell lines from several different laminopathies, indicate that everolimus might also be a viable treatment option for other disorders in this category of rare diseases.

Materials and Methods

Laminopathy Fibroblast Cell Lines and Patient Phenotypes. Cells and information (genotype and patient phenotype) for the HGDFN168, HGADFN167, PSADFN414, and PSADFN425 cell lines were obtained from the Cell and Tissue Bank and the Medical and Research Database, respectively, of the Progeria Research Foundation (www.progeriaresearch.org). PSADFN414 and PSADFN425 have been described previously (identified as APS 400.5 and APS 500.3, respectively) (38).

PSADFN425 is a compound heterozygote for the mutation listed in Table 1 and for *LMNA* intron 6, 1158-44 C>T. The mutation found in intron 6 is present in the 1000 Genomes Project database (39) and in one of the patient's unaffected parents and is unlikely to be causative. AG04110 and GM23780 were purchased from the Coriell Institute for Medical Research (Coriell Cell Repositories, <https://ccr.coriell.org/>) and genotype and patient phenotype information was obtained from the online listing for those lines. NORWAY1010 was a gift from Junko Oshima, University of Washington, Seattle, and genotype and patient phenotype information was previously published (18).

Control and Experimental Treatments. Cells were treated with media containing 0.1 μ M everolimus (S1120; Selleck Chemicals) in ethanol or with media containing vehicle control (the same volume of ethanol) three times per week (Monday, Wednesday, and Friday). The media used was Minimum Essential Media (10370021; Life Technologies) supplemented with 13% heat-inactivated FBS (10438018; Life Technologies), 1 \times L-Glutamine (25030081; Life Technologies), and 1 \times Antibiotic-Antimycotic (15240096; Life Technologies).

Proliferation Studies. Each cell line was trypsinized and counted, and two 25-cm² culture-treated vented flasks were seeded with 1 \times 10⁵ cells each. The media on the cells was changed as described above, and split 1:3 when approaching 90% confluency. One-third of the cells were seeded into a new 25-cm² flask. Cells were counted on a Z2 COULTER COUNTER Cell and Particle Counter (Beckman Coulter), and the media/cell volume was recorded. The total number of cells was calculated and then multiplied by 3 for each split the line had undergone between the start of the assay and the count to adjust for cells that were removed at each split. If the cells were less than 90% confluent 30 d after the last split, the cells were counted and not cultured further. The data are available in Table S1.

Senescence-Associated β -Galactosidase Assay. Cells from 0.1 μ M everolimus-treated and control-treated flasks that had been growing for the same amount of time (not necessarily the same passage due to differences between the proliferation rates of the drug-treated and vehicle control-treated cells) were trypsinized and seeded onto a six-well plate. HeLa cells transfected with a β -galactosidase expression plasmid were used as a positive control, and nontransfected HeLa cells were used as a negative control. Cells were stained using a senescence-associated β -galactosidase cell staining kit according to the manufacturer's protocol (9860; Cell Signaling Technologies). Photographs were taken on a light microscope at 20 \times magnification.

Western Blots. For RPS6/pRPS6 and lamin A/C detection in laminopathy and control fibroblasts (Fig. 2 and Fig. S2), cells were treated with 0.1 μ M everolimus or vehicle control three times per week for 2 wk, and the cells were last treated ~4 to 5 h before collection. Cells were suspended in 1 \times LI-COR protein loading buffer diluted in ddH₂O (928-40004; LI-COR). Cells were boiled for 3 min and cooled at room temperature for 5 min. Ten micrograms of protein was used per sample, and NuPAGE Sample Reducing Agent was added before loading (NP0004; ThermoFisher Scientific). Protein was loaded into each well of a 10% Bis-Tris gel and electrophoresed in 1 \times 3-(*N*-morpholino) propanesulfonic acid buffer.

Protein was transferred from the gel to a nitrocellulose membrane (wet transfer) using an iBlot gel transfer device (Invitrogen), and the membrane was blocked with shaking overnight at 4 $^{\circ}$ C in 1:1 1 \times PBS:Odyssey Blocking Solution (927-40000; LI-COR). The membrane was incubated with two primary antibodies (one for the protein of interest and one for the control protein) and two secondary antibodies (each with shaking at room temperature for 3 and 1 h, respectively, in 1:1 1 \times PBS:Odyssey Blocking Solution with 0.1% Tween 20 and antibody).

The following primary antibodies were used: S6 Ribosomal Protein (5G10) Rabbit mAb (2217; Cell Signaling Technology), Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP Rabbit mAb (4858; Cell Signaling Technology), Anti-beta Actin antibody (mouse, ab8226; Abcam; used for Fig. 2 and Fig. S2), and Lamin A/C (N-18) Antibody (goat, sc-6215; Santa Cruz Biotechnology, Inc.; used for Fig. S2). The following secondary antibodies were used as appropriate: IRDye 680RD Donkey (polyclonal) Anti-Mouse IgG (926-68072; LI-COR), IRDye 680RD Donkey (polyclonal) Anti-Rabbit IgG (926-68073; LI-COR), IRDye 800CW Donkey Anti-Mouse IgG (926-32212; LI-COR), IRDye 800CW Donkey Anti-Rabbit IgG (926-32213; LI-COR), and IRDye 800CW Donkey Anti-goat IgG (925-32214; LI-COR). Membranes were scanned using a LI-COR Odyssey CLx.

Immunofluorescence. After 2 wk of 0.1 μ M everolimus or vehicle control treatment, cells were trypsinized, counted, and seeded onto eight-chamber treated culture slides (354108; Corning) at 25,000 cells per well. The cells were allowed to attach overnight. The next day, the cells were fixed with 4% paraformaldehyde (15710; Electron Microscopy Science) in 1 \times PBS and permeabilized with 0.5% Triton X-100 in 1 \times PBS. Slides were blocked overnight with 4% BSA in 1 \times TBS at 4 $^{\circ}$ C. Primary antibody to lamin A/C (MAB3211; Millipore) was diluted 1:50 in 4% BSA in 1 \times TBS and applied for 5 h at room temperature, and then washed. Secondary antibody, Alexa Fluor 594 Donkey Anti-Mouse IgG (H+L) (A21203; Invitrogen) was diluted 1:1,000 in 4% BSA in 1 \times TBS and applied for overnight at 4 $^{\circ}$ C, and then washed. VECTASHIELD Antifade Mounting Medium with DAPI (H1200; Vector Laboratories, Inc.) was mixed with VECTASHIELD Antifade Mounting Medium (H1000; Vector Laboratories, Inc.) at a 1:4 ratio and applied to the wells, and the coverslip was affixed.

Nuclear Imaging and Analysis. Images were taken using a DeltaVision Elite (GE Healthcare Life Sciences) fluorescent microscope with a 63 \times oil objective. Images were run through the MIPAV (Medical Image Processing, Analysis, and Visualization) application with the Nuclei Segmentation plugin. Segmented nuclei were viewed, and any with improper segmentation were removed. Remaining segmented nuclei were analyzed with the Nuclei Statistics plugin to determine the MNC. The Nuclei Statistic plugin was used to measure the features of the nucleus, including MNC. To determine the MNC, the perimeter of the nucleus is divided into 100 segments, and all segments with a negative change in slope (concave) are averaged.

The data are available in Table S2. The cell lines used in this work can be requested from their respective sources. MIPAV and relevant plugins are available at <https://mipav.cit.nih.gov/>.

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