Repression of the Antioxidant NRF2 Pathway in Premature Aging

Graphical Abstract

Highlights

- Impaired activity of the NRF2 antioxidative pathway is a driver mechanism in HGPS
- Suppressed NRF2 activity and oxidative stress recapitulate HGPS aging defects
- Reactivation of NRF2 decreases oxidative stress and reverses cellular HGPS defects

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In Brief

Impairment of the NRF2 pathway is a key contributor to premature aging in Hutchinson-Gilford progeria syndrome (HGPS) via increasing chronic oxidative stress that recapitulates HGPS aging defects.
Repression of the Antioxidant NRF2 Pathway in Premature Aging Phenotype

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SUMMARY

Hutchinson-Gilford progeria syndrome (HGPS) is a rare, invariably fatal premature aging disorder. The disease is caused by constitutive production of progerin, a mutant form of the nuclear architectural protein lamin A, leading, through unknown mechanisms, to diverse morphological, epigenetic, and genomic damage and to mesenchymal stem cell (MSC) attrition in vivo. Using a high-throughput siRNA screen, we identify the NRF2 antioxidant pathway as a driver mechanism in HGPS. Progerin sequesters NRF2 and thereby causes its subnuclear mislocalization, resulting in impaired NRF2 transcriptional activity and consequently increased chronic oxidative stress. Suppressed NRF2 activity or increased oxidative stress is sufficient to recapitulate HGPS aging defects, whereas reactivation of NRF2 activity in HGPS patient cells reverses progerin-associated nuclear aging defects and restores in vivo viability of MSCs in an animal model. These findings identify repression of the NRF2-mediated antioxidative response as a key contributor to the premature aging phenotype.

INTRODUCTION

Aging is a fundamental biological process linked to many common diseases, including cancer and cardiovascular disease. The naturally occurring human premature aging disorder Hutchinson-Gilford progeria syndrome (HGPS) is a powerful tool to study human aging (Gordon et al., 2014). In HGPS a de novo mutation in the LMNA gene, encoding for the nuclear architectural proteins lamin A and C, activates an alternative pre-mRNA splice site, resulting in the expression of a lamin A mutant lacking 50 amino acids (aa), known as progerin, which undergoes incomplete posttranslational processing and consequently retains a farnesylated C-terminal CaaX motif (Gordon et al., 2014). Progerin is also thought to be relevant to the normal aging process, since sporadic usage of the same alternative splice site results in accumulation of progerin during physiological aging (Rodriguez et al., 2009; Scaffidi and Misteli, 2006). Progerin acts in a dominant fashion and causes a variety of cellular defects that compromise the integrity of nuclear architecture, heterochromatin maintenance, DNA repair, and redox homeostasis, and which has been ascribed to reduced levels of key proteins in these pathways (Mateos et al., 2013; Pegoraro et al., 2009; Scaffidi and Misteli, 2006; Viteri et al., 2010). At an organismal level, in vivo attrition of mesenchymal stem cells (MSCs), prone to the detrimental defects of progerin (Pacheco et al., 2014; Rosenstein et al., 2011; Scaffidi and Misteli, 2008), is thought to underlie HGPS tissue defects, in line with observations that HGPS-induced pluripotent stem cells (iPSCs)-derived MSCs have reduced viability in hypoxic niches due to diminished capacity to respond to oxidative stress challenges (Liu et al., 2011a, 2012; Zhang et al., 2011).

Many of the cellular pathways affected in HGPS are highly interdependent, making it difficult to identify and distinguish cellular factors directly affected by progerin and driving HGPS etiology from those secondarily perturbed downstream of progerin. For example, changes in lamin B1 levels observed in HGPS increase reactive oxygen species (ROS) (Malhas et al., 2009), which may compromise the nuclear envelope’s integrity (Pekovic et al., 2011). At the same time, ROS may inflict DNA damage and decrease heterochromatin protein levels (Frost et al., 2014), which, in turn, may activate DNA damage signaling (Pegoraro et al., 2009). The complex interdependencies and the wide range of nuclear abnormalities observed in HGPS and in normal aging (Pegoraro et al., 2009; Zhang et al., 2015) points to the involvement of an upstream effector in the disease. A major goal in understanding HGPS and premature aging is the identification of primary driver mechanisms.

We have developed a cell-based high-throughput, high-content imaging small interfering RNA (siRNA) screening assay to directly assess the involvement of individual factors in bringing about HGPS cellular phenotypes in mammalian cells. Using
this system, we identify the antioxidant NRF2 pathway as a driver mechanism in HGPS.

RESULTS

A Targeted High-Throughput RNAi Screen to Identify Mediators of Progerin-Induced Aging

We set out to identify human genes that drive the formation of progerin-induced aging defects. To this end, we generated human wild-type (WT) skin fibroblasts containing GFP-progerin under control of a doxycycline-inducible (Tet-on) promoter (see the Experimental Procedures). GFP-progerin was nearly undetectable under normal growth conditions, but on exposure to doxycycline was rapidly induced to levels comparable to endogenous lamin A (Figures S1A and S1B), resulting in the formation of nuclear defects typically observed in HGPS patient skin fibroblasts (Kubben et al., 2015; Musich and Zou, 2009; Scaffidi and Misteli, 2006), including nuclear shape distortions, decreased levels of the nuclear architectural proteins lamin B1 and LAP2, reduction of heterochromatin-associated HP1γ and tri-methylated lysine 27 on histone 3 (H3K27me3) (Figures S1A–S1C and S1F), and increased formation of DNA damage foci containing 53BP1 and serine-139 phosphorylated H2AX (γH2AX; Figures S1A, S1C, and S1F).

Using this inducible model, we performed a high-throughput RNAi screen and searched for genes that simultaneously prevent the occurrence of multiple HGPS phenotypes, including loss of lamin B1, increase of γH2AX, and accumulation of GFP-progerin (Figure 1A). Given the widespread involvement of ubiquitin ligases in pathways affected in HGPS and aging (Löw, 2011), as well as the observed selective degradation of a set of nuclear proteins in HGPS (Scaffidi and Misteli, 2006), we used a library containing 320 pools of four siRNAs targeting human ubiquitin E1, E2, and E3 ligases or their direct modulators (Table S3). The screen was conducted in quadruplicate in a 384-well format by reverse siRNA transfection of GFP-progerin fibroblasts, while simultaneously inducing GFP-progerin expression for 96 hr (see the Experimental Procedures; Figure 1A). Lamin B1, γH2AX, and GFP-progerin levels were detected by immunofluorescence (IF) and automated high-throughput imaging of 500–2,000 cells per siRNA pool using custom-built image analysis algorithms (see the Experimental Procedures). As a positive control, siRNA directed against GFP efficiently decreased GFP-progerin levels by 95% and prevented formation of lamin B1 and γH2AX defects, as opposed to a non-targeting siRNA that allowed for full development of the HGPS phenotype and served as negative control (Figures 1A, S1A, and S1C). Z’ scores ranged from 0.15 to 0.74, depending on the cellular readout (see the Experimental Procedures). We identified seven (2.2%) genes that affected multiple HGPS parameters (Figures 1B and S1J; Tables S2 and S3). All candidates partially downregulated progerin, three prevented formation of lamin B1 defects, and four prevented that increase in γH2AX foci formation. Four of the seven candidates (CAND1, WSB1, FBX038, and FLJ25076) significantly downregulated progerin and γH2AX protein levels, with a more moderate effect on lamin B1 defects (see the Experimental Procedures) (Table S2), and were further validated using siRNAs of a different targeting sequence and chemistry to exclude off-target effects. Among the validated targets, RNAi against CAND1 (culin-associated NEDD8-dissociated protein 1) had the strongest effect and lowered progerin levels by 47%, prevented lamin B1 loss by 70%, and γH2AX activation by 89% (all p < 0.05; Figures 1C, 1D, S1F, and S1I; Tables S2 and S3), making CAND1 a promising candidate for further investigation.

Loss of CAND1 Function Prevents and Reverses HGPS Aging Defects

CAND1 is a member of several cullin-containing E3 ubiquitin ligase complexes. CAND1 does not possess ubiquitin ligase activity itself, but functions to modulate the substrate specificity of the E3 ligase complexes (Chua et al., 2011). In addition to the screening phenotypes, CAND1 knockdown also prevented HGPS-associated reduction of LAP2, HP1γ, and H3K27me3 and induction of 53BP1 foci (Figures 1D and S1G), while not affecting nuclear shape (Figure S1H). Importantly, this protective effect was not merely due to a reduction of progerin levels since lowering induction of progerin in shCTRL-treated cells to levels similar to those in shCAND1-treated cells did not prevent the formation of most defects, except for a minor DNA damage protective effect (Figure S1K). CAND1 silencing (~94%; p < 0.05; Figure S1D) was also sufficient to reverse already established aging defects in HGPS fibroblasts, as it decreased endogenous progerin levels by 46%, restored lamin B1, HP1γ, and LAP2 to WT levels, and partially rescued H3K27me3 defects (75%; all p < 0.05; Figures 1E and S1G). γH2AX and 53BP1 levels remained unchanged (Figures 1E and S1G), in line with the reported resistance of DNA damage defects in HGPS cells to reversal (Liu et al., 2006). These data point to a role for CAND1 in a cellular pathway important in establishing and preventing a wide variety of aging defects triggered by progerin.

NRF2-ARE Transcriptional Activity Is Impaired in HGPS

A candidate pathway for the observed CAND1 effects was suggested by the RNAi screening results. Knockdown of the CAND1 interaction partner cullin 3 E3 ligase, but not of other CAND1-interacting cullin proteins tested in the screen, significantly prevented γH2AX foci formation and moderately lowered GFP-progerin levels (~37%, Z = 1.90; Figure S1J; Table S3). In addition, knockdown of RBX1, which interacts with various cullin proteins and is a member of the CAND1/cullin3 complex, prevented formation of lamin B1 defects (Table S3; Z = −2.14), but was slightly toxic. These results were of interest given that one of the major substrates of the CAND1/cullin3/RBX1 complex is the transcription factor NRF2 (nuclear factor [erythroid-derived 2]-like 2), which has been implicated in organismal longevity and stress resistance by transcriptionally activating antioxidant genes upon binding to antioxidant-responsive elements (ARE) motifs in their promoters (Lewis et al., 2010; Lo and Hannink, 2006; Ma, 2013). Indeed, CAND1 knockdown increased NRF2 protein levels (Figure S1E), in line with previous reports linking the cytoprotective effect of CAND1 knockdown to reduced CAND1/cullin3/RBX1-mediated NRF2 protein degradation (Lo and Hannink, 2006).

Given that increased oxidative stress has been implicated in HGPS and normal aging (Viteri et al., 2010), we set out to
determine the status of the NRF2-ARE axis in HGPS. Protein levels of NRF2 and KEAP1, which promote NRF2 degradation via the CAND1/cullin3/RBX1 pathway (Lewis et al., 2010), were unaffected by progerin in HGPS fibroblasts or upon induction of GFP-progerin in WT cells (Figures 2A, 2B, and S2D). Small MAFs (musculoaponeurotic fibrosarcoma) proteins and CBP, two NRF2 transcriptional co-factors, were also unaffected (Figures 2A, 2B, and S2D). Considering that the NRF2 pathway is also regulated at the level of nuclear import, we determined nuclear NRF2 protein levels, which were unchanged in HGPS cells in comparison to WT cells (Figures 2C and 2D), despite a 20% decrease in nuclear import promoting serine 40 phosphorylation.

**Figure 1. High-Throughput RNAi Screen Identifies CAND1 as a Mediator of Progerin-Induced Aging**

(A and B) Schematic (A) and heatmap (B) representation of RNAi screen, indicating identified candidates (Figure S1J; Table S2).

(C) Representative IF images for CAND1 RNAi and controls in progerin-inducible fibroblasts (see the Experimental Procedures). Scale bar, 25 μm.

(D and E) IF quantification in inducible (D) or WT and HGPS (E) fibroblasts. p < 0.05: # ONorHGPS/siCTRL versus OFForWT/siCTRL; * ONorHGPS/siCAND1 versus OFForWT/siCTRL; * ONorHGPS/siCAND1 versus ONorHGPS/siCTRL. n > 300; values represent averages ± SD from at least three experiments. See also Figure S1 and Tables S1, S2, and S3.
NRF2-ARE axis was a global event since 213 of 774 NRF2-Gene expression analysis demonstrated that abrogation of the pathway, we measured its transcriptional activity using ARE-luc assay (see the Experimental Procedures). We conclude that progerin interferes with NRF2 function by impairing its transcriptional activity.

Progerin Causes NRF2 Mislocalization
To investigate potential mechanisms by which progerin interferes with the transcriptional function of NRF2, we probed its subnuclear distribution. Visualization of the NRF2 protein, using a method established to visualize nuclear insoluble fractions (Voncken et al., 2005), revealed an increased accumulation of

(pSer40) of NRF2 (Bloom and Jaiswal, 2003) that could be corrected by a small hairpin RNA (shRNA) targeting progerin (Figure S2C). Furthermore, the NRF2-activating compound sulforaphane equally increased nuclear NRF2 levels in the absence or presence of progerin (Figures S2A and S2B), indicating that NRF2 protein stability and nuclear-cytoplasmic shuttling is unaffected by progerin.

To fully assess the functional status of the NRF2-ARE pathway, we measured its transcriptional activity using ARE-driven luciferase constructs (Wang et al., 2006). ARE-Luc activity was reduced by 51% in HGPS fibroblasts as a consequence of progerin since induction of GFP-progerin in WT cells resulted in a 77% inhibition of ARE-Luc activity (p < 0.05; Figure 2E). Gene expression analysis demonstrated that abrogation of the NRF2-ARE axis was a global event since 213 of 774 NRF2-ARE target genes were significantly repressed in HGPS versus WT cells (∼0.31 Log2 fold change of average expression; p < 0.0001; Figure 2F), while no global change was observed for non-NRF2 or HSF1-regulated control genes. Gene set enrichment analysis confirmed an enrichment of NRF2 target genes in WT versus HGPS cells (Figure S2E; Table S4). We conclude that progerin interferes with NRF2 function by impairing its transcriptional activity.

Figure 2. NRF2 Transcriptional Activity Is Impaired in HGPS
(A) Western blot (WB) analysis of total progerin-inducible fibroblasts (P1 cells; see the Experimental Procedures) extracts (two biological replicates). Dotted line indicates separate WBs.
(B) WB of total cell extract of WT and HGPS fibroblast (two biological replicates for four cell lines; see the Experimental Procedures).
(C) WB of nuclear P1 cell extracts (three biological replicates of P1 cells). β-actin serves as the cytoplasmic control.
(D) NRF2 IF staining (Abcam 62352) in formaldehyde-fixed WT and HGPS fibroblasts. n > 300; scale bar, 10 μm.
(E) ARE-luc assay (see the Experimental Procedures). *p < 0.05.
(F) Line graph of Log2 mRNA expression changes between WT and HGPS fibroblasts for indicated transcriptional targets. A left shift of the NRF2 targets plot indicates an increased frequency of NRF2-regulated target genes downregulated in HGPS patients (p < 0.0001).

See also Figure S2 and Table S4.
NRF2 at the nuclear periphery and intranuclear aggregates mirror the distribution of progerin, both in progerin- and HGPS cells, but not in uninduced and WT controls (Figures S3A–3C, S3A, and S3B). To test whether NRF2 mislocalization may be caused by a differential interaction of NRF2 with progerin compared to WT lamin A, we tested progerin-NRF2 interactions using an orthogonal One-STrEP (OST) pull-down assay in cellular extracts (Kubben et al., 2010). The interaction of nuclear OST-tagged NRF2 with progerin was strongly enhanced compared to WT lamin A (248%; p < 0.05; Figure 3D). Conversely, OST-progerin pulled down significantly more NRF2 than OST-lamin A (142%; p < 0.05; Figure 3E). For further validation, we directly probed progerin-NRF2 interactions in vitro by testing the ability of recombinant progerin-NRF2 to bind to streptactin-purified, immobilized OST-lamin A, and OST-progerin. In line with its co-localization and in vivo interaction, NRF2 preferentially bound to OST-progerin (275%; p < 0.05; Figure 3F). These data suggest that progerin impairs the transcriptional activation of NRF2-ARE-regulated antioxidants through sequestration of NRF2 away from its transcriptional targets.

**NRF2 Impairment Causes Oxidative Stress and Recapitulates the Progeroid Phenotype**

To determine whether abrogation of NRF2 activity contributes to the progeroid phenotype, we investigated the effect of NRF2 knockdown in WT fibroblasts. NRF2 knockdown (−45%; Figures S4A and S4B) was sufficient to recapitulate all tested HGPS defects in WT cells. As expected, NRF2 RNAi increased progerin levels in WT-inducible cells (Figure S4A), but further exacerbated nuclear defects in HGPS cells in which NRF2 pathway activity was already suppressed without affecting progerin protein levels (Figures 4A, 4B, and S4B–S4D), demonstrating that NRF2 affects the establishment of HGPS disease phenotypes by mechanisms other than modulating progerin levels.

In line with NRF2 impairment driving HGPS etiology, NRF2 knockdown in WT fibroblasts reduced expression of antioxidant genes, similarly to the effect of progerin (n = 10, Figures S4E, S4F, and S5E), thereby increasing ROS levels (146%, p < 0.05) compared to HGPS cells (+79%; p < 0.05; Figures 4C and 4D). Furthermore, elevated levels of oxidative stress itself, induced by H2O2 pro-oxidant treatment, was sufficient for WT fibroblasts to phenotypically mimic HGPS cells for all tested defects, with the anticipated exception of lamin B1 (Barascu et al., 2012) (Figures 4E and S4G–S4I). In line with the NRF2 RNAi experiments, H2O2 treatment of HGPS cells further worsened LAP2, HP1γ, H3K27me3, γH2AX, and 53BP1 defects, as well as increased both lamin A (+55%) and progerin (+46%) levels, while not affecting lamin C levels (Figures 4E, 4F, and S4G). Importantly, the observed increase in progerin levels did not account for lamin B1, LAP2, H3K27me3, and 53BP1 defects since individual cells expressing high levels of progerin had a nearly similar extent of HGPS-associated defects compared to the total population of HGPS fibroblasts (Figure S4J). These results demonstrate that chronic oxidative stress due to impaired NRF2 activity, as found in HGPS cells, is sufficient to recapitulate cellular HGPS aging defects.

**Reactivation of the NRF2 Pathway Reverses Progerin-Induced Aging Defects**

To test whether NRF2 pathway reactivation ameliorates HGPS defects, we introduced constitutively activated NRF2 (caNRF2; see the Supplemental Experimental Procedures) in WT and HGPS cells (Figure S5A). In line with CAND1 RNAi results, caNRF2 expression prevented the formation of all tested nuclear defects, as well as lowered GFP-progerin levels (−60%, p < 0.05; Figure S5B) in WT fibroblasts, and fully restored lamin B1, LAP2, HP1γ, and H3K27me3 (Figures 5A, 5C, and S5F). NRF2 pathway reactivation in HGPS cells decreased progerin protein levels by 59% (p < 0.05; Figure 5B) and reduced lamin A (−25%, p < 0.05) without altering lamin C levels. In addition, we also tested the effect of four small-molecule NRF2-activating agents on HGPS fibroblasts. Oltipraz (Magesh et al., 2012) ameliorated lamin B1 and HP1γ levels by 73% and 53% (100% = difference between WT and HGPS cells), respectively (p < 0.05; Figure S5C), and showed some effect in reducing LAP2 and H3K27me3 defects (52%–79%, p < 0.05), as did the compound AI-1 (Magesh et al., 2012). CPDT, however, increased γH2AX levels (+35% absolute change; p < 0.05), which we did not observe for other tested compounds (Figure S5C). TAT-14, a cell-penetrating NRF2-stabilizing short peptide (Steel et al., 2012) restored HP1γ levels and ameliorated H3K27me3 defects (62%; p < 0.05; Figure S5C). All beneficial effects of NRF2-activating compounds occurred without affecting progerin levels (Figure S5C).

caNRF2 expression also improved the expression of progerin-repressed NRF2-regulated antioxidant genes (n = 10; Figure S5E) and lowered ROS levels in HGPS cells to only 39% of WT levels (p < 0.05; Figure 5D). To test whether reducing oxidative stress itself is sufficient to reverse established HGPS aging defects, we treated HGPS patient cells with the broad-acting antioxidant N-acetyl cysteine (NAC). NAC treatment partially improved γH2AX (18% of WT/HGPS difference, p < 0.05), restored LAP2 and HP1γ levels, and ameliorated lamin B1 and H3K27me3 defects (38%–54% of WT/HGPS difference; p < 0.05; Figure 5E), without affecting endogenous progerin or lamin A or C levels (Figure 5D). The observation that increased NRF2 activity by caNRF2 expression or CAND1 knockdown in WT cells does not alter lamin B1, LAP2, HP1γ, H3K27me3, γH2AX, and 53BP1 protein levels (Figures S5F and S5H), but as expected lowers ROS levels (Figure S5I), further indicates that NRF2 pathway reactivation specifically corrects HGPS patient cellular defects, while not affecting the physiological levels of key proteins in these pathways. Overall, these data demonstrate that restoration of the impaired NRF2 pathway in HGPS patient cells ameliorates aging defects in vitro by lowering oxidative stress.

**NRF2 Pathway Activation Restores In Vivo Viability of HGPS Mesenchymal Stem Cells**

A prominent in vivo defect in HGPS is the reduced ability of MSCs to respond to oxidative stress, limiting their viability in vivo (Zhang et al., 2011) and contributing to reduced stem cell pools in HGPS (Pacheco et al., 2014; Rosengardten et al., 2011). To test the effect of NRF2 pathway modulation on HGPS MSCs, we generated iPSCs from HGPS patient fibroblasts (henceforth...
Figure 3. Progerin Mislocalizes NRF2
(A) NRF2 IF staining (Sc-722) in methanol-fixed progerin-inducible fibroblasts (P1 cells; see the Experimental Procedures). Arrows indicate overlapping IF signals. Scale bar, 10 µm.
(B) IF stain for NRF2 (Sc-722) and lamin B1 (Sc-6217) in methanol-fixed WT and HGPS fibroblasts. Arrows indicate overlapping IF signals. Scale bar, 10 µm.
(C) Line graphs indicating IF signal intensity across the dotted lines in (A) and (B).

(legend continued on next page)
HGPs-iPSCs; see the Experimental Procedures), and their isogenic controls in which the LMNA C1824T mutation was genetically corrected (GC-iPSCs; Figure S6A) (Liu et al., 2011b). As expected, since LMNA is inactive in iPSCs (Constantinescu et al., 2006; Liu et al., 2011a, 2011b), expression of NRF2-regulated antioxidants was not downregulated in HGPs-iPSCs compared to GC-iPSCs (Figure 6A). However, in line with progerin impairment of NRF2 activity, reactivation of the LMNA gene upon differentiation of iPSCs into MSCs reduced NRF2 target gene expression in HGPs-iPSC-MSCs compared to GC-iPSC-MSCs (Figures 6A, S6B, and S6C). This effect was a direct consequence of progerin expression since induced GFP-progerin expression in GC-iPSC-MSCs similarly reduced NRF2 target gene expression (Figures S6D and S6E). Reduced NRF2 activity was sufficient to mimic HGPs defects since NRF2 knockdown in GC-iPSC-MSCs decreased expression of NRF2 antioxidant genes (Figure 6B), increased oxidative stress (Figure 6C), reduced levels of lamin B1, LAP2, and HP1γ proteins (p < 0.05; Figures 6D and S6G), increased frequencies of senescence-associated β-galactosidase (SA-β-gal) positive cells (Figure 6E), and, ultimately, reduced cell survival (Figure 6F) comparable to HGPs-iPSC-MSCs (Figures S6F–S6H). Conversely, as expected, expression of caNRF2 in GC-iPSC-MSCs increased expression of NRF2 antioxidant genes and thereby lowered ROS levels (Figures 6H and S6I). To test the effect of the progerin-NRF2 pathway in vivo, we used an established animal model assay system (Zhang et al., 2011) in which MSCs are implanted into the tibialis anterior muscle and engraftment and survival is measured (Pan et al., 2016; Zhang et al., 2015) (see the Experimental Procedures). As expected by our in vitro observations, the presence of progerin in HGPs-iPSC-MSCs or knockdown of NRF2 in GC-iPSC-MSCs resulted in increased stem cell attrition in the in vivo niche (p < 0.05; Figure 6L). In contrast, overexpression of caNRF2 (EB2G; see the Experimental Procedures) or Oltipraz-mediated NRF2 activation in HGPs-iPSC-MSCs increased expression of NRF2-regulated antioxidants (Figure 6G), decreased ROS levels (Figure 6H), rescued HGPs nuclear defects (Figure 6I), and reduced the number of apoptotic (Figure 6J) and SA-β-gal-positive cells (Figure 6K). Similarly, knockdown of CAND1 in HGPs-iPSC-MSCs reactivated all tested NRF2 target genes (Figures S6J and S6K). Importantly, NRF2 pathway reactivation by expression of caNRF2 restored the in vivo viability of HGPs-MSCs (Figure 6L). A similar effect was observed by pre-treatment of cells with the mild NRF2 inducer Oltipraz (Figure 6L), indicating that the NRF2-mediated rescue effect is not due to massive hyperactivation of the pathway. These data from an HGPs-relevant animal model support an in vivo role of NRF2 as a driver in HGPS pathology.

**DISCUSSION**

The lamin A mutant isoform progerin causes extensive architectural, epigenetic, redox, and DNA damage defects in the premature aging syndrome HGPS (Gordon et al., 2014; Scaffidi and Misteli, 2006; Viteri et al., 2010). It is unclear how progerin triggers these defects and discovery of HGPS driver mechanisms is crucial to a molecular understanding and the identification of therapeutic targets of premature and normal aging. Using a high-throughput, high-content cell-based assay, we identify impairment of the longevity-promoting transcription factor NRF2 by progerin as a driver mechanism in HGPS etiology.

NRF2 is a major stress responder that transcriptionally activates antioxidant and cytoprotective genes through binding to ARE motifs (Lewis et al., 2010). The sum of our results delineates a mechanism by which progerin sequesters NRF2, and in this way reduces its availability for transcriptional activation of antioxidant genes, resulting in elevated oxidative damage and consequentially an array of HGPS defects. Given that progerin is a highly expressed long-lived protein (Boisvert et al., 2012), whereas NRF2 is low in abundance and short-lived (McMahon et al., 2004), progerin/NRF2 sequestration is expected to drastically reduce the availability of NRF2 for the formation of functional transcription factor complexes at ARE motifs. In agreement, we find that experimental elevation of NRF2 levels in HGPS cells restored NRF2 pathway activity, further suggesting that the availability of NRF2, and not of other NRF2/ARE complex co-factors, is limited in HGPS.

A likely scenario, based on this study and others, for how increased ROS levels contribute to HGPS etiology suggests that aberrant NRF2/progerin interaction reduces expression of antioxidants, increases ROS levels, and thereby causes loss of heterochromatin and increased levels of DNA damage (Frost et al., 2014; Pegoraro et al., 2012; Richards et al., 2011). We hypothesize that the elevated oxidative damage in HGPS cells is the result of increased ROS formation, due to defective mitochondrial oxidative phosphorylation (Rivera-Torres et al., 2013), a pathway that is maintained by the progerin-impaired NRF2 target NQO1 (Kwon et al., 2012), as well as a diminished ROS-counteracting antioxidative capacity due to impaired NRF2 activity. Subsequently, increased ROS levels can cause a severe loss of heterochromatin (Frost et al., 2014), which precedes and by itself is sufficient to activate DNA damage signaling as observed in progerin-inducible fibroblasts (Pegoraro et al., 2009). That NRF2 activation fully prevents γH2AX and 53BP1 foci formation upon progerin induction in a WT background, but does not reduce these defects in HGPS cells, suggests that progerin renders DNA damage foci partially irreparable through NRF2 pathway-independent mechanisms, in line with previously observed minimal effects on DNA damage in HGPS cells by farnesyl-transferase inhibitors (Liu et al., 2006) and NAC (Richards et al., 2011). Similarly, nuclear shape alterations, the formation of which was prevented by NRF2 activation in progerin-inducible WT cells, but could not be reversed once established in HGPS patient cells, may be due to insufficient reduction of progerin levels by caNRF2.

(D and E) WB analysis of the interaction between immunoprecipitated OST-NLS-mCherry-NRF2 with HA-lamin A or HA-progerin (D) or (E) immunoprecipitated OST-lamin A and OST-progerin with FLAG-NLS-NRF2 in HEK293FT cells (three biological replicates; see the Experimental Procedures). *p < 0.05.

(F) WB analysis to probe the interaction between WT immunoprecipitated OST-lamin A or OST-progerin with recombinant HIS6x-NRF2. *p < 0.05.

See also Figure S3.
Figure 4. NRF2 Impairment Causes Oxidative Stress and Recapitulates the Progeroid Phenotype

(A) IF analysis in WT and HGPS fibroblasts. p < 0.05; * WT/shCTRL versus HGPS/shCTRL; # WT/shCTRL versus WT/shNRF2; * HGPS/shCTRL versus HGPS/shNRF2.

(B) WB analysis of HGPS fibroblasts (two biological replicates).

(C and D) DCFDA-based ROS quantification in WT fibroblasts expressing indicated shRNAs (C) or in WT and HGPS fibroblasts (D) (N > 300; * p < 0.05). Scale bar, 10 μm.

(E) IF analysis in WT and HGPS fibroblasts treated with H$_2$O$_2$ (250 μM; 4 days). p < 0.05; * WT/vehicle versus HGPS/vehicle; ** WT/vehicle versus WT/H$_2$O$_2$; * HGPS/vehicle versus HGPS/H$_2$O$_2$.

(F) WB analysis of HGPS fibroblasts treated with H$_2$O$_2$ (250 μM; 4 days) (two biological replicates). * p < 0.05. For all IF-based panels: n > 300; values represent averages ± SD from at least three experiments.

See also Figure S4 and Table S1.
Several lines of evidence support the conclusion that the protective function of NRF2 in HGPS is largely independent of an effect on progerin levels. First, lowering ROS levels in HGPS fibroblasts by NAC antioxidant treatment ameliorates HGPS defects without lowering progerin levels. Second, further impairment of NRF2 activity in HGPS fibroblasts by...
Figure 6. NRF2 Activation Alleviates HGPS Mesenchymal Stem Cell Viability Defects

(A) Heatmap of NRF2 target mRNA expression levels. Values represent the averages from at least three experiments.

(B) NRF2 target mRNA expression. *p < 0.05, GC/shCTRL versus GC/shNRF2.

(C) H2DCFDA-based ROS quantification.

(D) IF analysis of lamin B1 (manual count of low lamin B1-expressing cells), LAP2, and HP1γ (see the Experimental Procedures). Scale bar, 10 μm. n > 300.

(E and F) Frequency of SA-β-gal-positive cells (E) and relative cell survival rate (F) (see the Experimental Procedures). p < 0.05: *GC/shCTRL versus GC/shNRF2; *GC/shCTRL versus HGPS/shCTRL.

(G) NRF2 target mRNA expression levels. *p < 0.05, HGPS/CTRL versus HGPS/caNRF2.

(H) H2DCFDA-based ROS quantification for the indicated cell types.

(I) IF quantification (see D) in vehicle or Oltipraz-treated (20 μM; 3 weeks) HGPS-iPSC-MSCs. n > 100. *p < 0.05.

(legend continued on next page)
NRF2 knockdown worsens all tested defects without altering progerin protein levels. Third, using single-cell-level analysis, we observed that even a 50% above-average increase in progerin levels in HGPS patient cells did not account for the increased defects observed in response to H$_2$O$_2$ pro-oxidant treatment. Fourth, increased oxidative stress levels through NRF2 knockdown or H$_2$O$_2$ treatment in WT cells was sufficient to mimic HGPS defects in the absence of progerin. Fifth, when comparing two cell populations with similar progerin levels, but one with activated NRF2 signaling due to CAND1 knockdown, the reduction of progerin alone was insufficient to prevent the formation of other nuclear defects. A possible mechanism for how NRF2 may decrease progerin comes from previous observations that the NRF2-ARE pathway also transcriptionally activates 20S proteasome subunits to aid in the clearance of ROS damaged proteins (Pickering et al., 2013). A plausible scenario is that reactivation of NRF2 activity in HGPS cells increases the capacity of the proteasome system, which is slightly attenuated in HGPS cells (Viteri et al., 2010) and thereby aids in clearing ROS-damaged proteins, as well as progerin aggregates.

Our results are in line with observations at the organismal level that have demonstrated a contribution of the NRF2 pathway to multiple aspects of premature aging. Genetic disruption of the Nrf2 gene decreases the lifespan of female mice (Yoh et al., 2001), and hypomorphic mutants of SKN-1, the Caenorhabditis elegans NRF2 homolog, shorten C. elegans lifespan nearly 2-fold (An and Blackwell, 2003). Furthermore, Nrf2 knockout mice show decreased adipogenic differentiation and high-density lipoprotein (HDL) levels (Tanaka et al., 2008), intriguingly similar to the global loss of adipose tissue and altered blood lipid profiles in HGPS patients (Gordon et al., 2003), and in addition suffer from aging-associated skeletal muscle wasting, retinopathies, and dermal photo-aging (Hirotta et al., 2011; Miller et al., 2012; Zhao et al., 2011). As expected, reported phenotypes in NRF2-targeted mice are milder than in HGPS mice models (Chan et al., 1996), most likely due to redundancy and biological differences between complete genetic gene disruption and a hypomorphic effect on NRF2 in HGPS.

Although our primary goal was to identify HGPS driver mechanisms, our observations suggest that restoration of NRF2 activity may offer a novel therapeutic strategy for HGPS. Clinical activation of NRF2 in HGPS patients may increase bone mineral density, which is reduced in HGPS patients (Rankin and Ellard, 2006) and has been observed in Nrf2 knockout mice (Ibanez et al., 2014). NRF2 activation may also decrease the prevalence of atherosclerotic lesions and stroke (Li et al., 2009), the main causes of death in HGPS patients (Gordon et al., 2014). Cardiovascular protective effects may further occur due to NRF2 inhibiting NFkB inflammatory responses (Li et al., 2008), which are detrimental to HGPS (Osorio et al., 2012). Given that increased NRF2 activity restored in vivo HGPS-iPSC-MSC viability, we expect NRF2 activation to counteract the premature exhaustion of adult stem cell populations that occurs in HGPS patients (Halaschek-Wiener and Brooks-Wilson, 2007), due to their diminished capacity to respond to oxidative challenges in their hypoxic niches (Liu et al., 2012, 2011a; Zhang et al., 2011). Further beneficial effects may originate from the fact that adult stem cells are highly dependent on NRF2 activity to maintain self-renewal ability and lineage commitment (Murakami et al., 2014; Pan et al., 2016) and show altered differentiation in response to reduction of NRF2-regulated MnSOD antioxidant (Michaeloudes et al., 2011) and elevated ROS levels (Mateos et al., 2013).

Due to these health-promoting effects of NRF2, identification of NRF2-activating small-molecular compounds is a high-value target in drug discovery (Magesh et al., 2012). Our observation that the FDA-approved drug Oltipraz, as well as other experimental NRF2-activating compounds, improved multiple aging defects in HGPS cells further indicates the potential of NRF2-activating drugs as a novel treatment type in HGPS. In this light, it is also noteworthy that both sulforaphane and resveratrol, both reported to alleviate HGPS symptoms (Gabriel et al., 2015; Liu et al., 2012), are dependent on NRF2 for their general health-promoting effects in non-HGPS animal models (Ungvari et al., 2010; Zakkar et al., 2009). Finally, and maybe most intriguingly, the recent finding that lamin A mutants that cause muscular dystrophy cause cytoplasmic KEAP1 aggregation, increased NRF2 activity, and reductive stress (Dialynas et al., 2015) raises the possibility that the defective NRF2 pathway activity may not just be a driver of HGPS, but a common denominator among laminopathies.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Treatments**

hTert immortalized doxycycline GFP-progerin inducible human skin fibroblasts (P1 cells) were generated and induced (96 hr) as described (Kubben et al., 2015). Human HGPS-iPSCs (C1824T LMNA mutation) and their gene-corrected isogenic counterparts (GC-iPSC; no LMNA mutation) were generated as described (Liu et al., 2011a). iPSC MSC differentiation was performed using established protocols (Duan et al., 2015; Zhang et al., 2015; Pan et al., 2016). Primary HGPS patient skin fibroblasts (Coriell Cell Repository (CCR), AG01972 and AG06297), and age-matched control cell lines (American Type Culture Collection, CRL-1474; CCR, GM00038) were used under conditions as previously described (Pegoraro et al., 2009). Cell treatments included A1-1 (125 μM), CPDT (60 μM), H$_2$O$_2$ (100, 125 and 250 μM), NAC (10 mM), oltipraz (20, 100 μM), and sulforaphane (5 μM), TAT-14 (100 μM). All compounds were purchased from Sigma, except TAT-14 (Millipore). H$_2$O$_2$, NAC and TAT-14 were dissolved in PBS, all others in DMSO. Media with compounds were replaced every 48 hr, except for H$_2$O$_2$ which was left unreplaced for 96 hr (Figure 4), or was replaced with vehicle containing medium after 1 hr of stimulation (Figure S4G).

(J and K) Relative amounts of apoptotic cells (J) and SA-β-galactosidase-positive cells in GC- and HGPS-iPSC-MSC-expressing calNRF2 or treated with Oltipraz (K) (20 μM; 3 weeks), n > 300. *p < 0.05.

(L) In vivo MSC implantation assay for the indicated conditions (Oltipraz: 20 μM; 3 weeks) (n = 3–5). *p < 0.05 for indicated comparisons: HGPS versus GC; GC/shNRF2 versus GC/shCTRL; HGPS/canNRF2 versus HGPS/CTRL; HGPS/Oltipraz versus HGPS/vehicle. For all bar graphs, values represent averages ± SD from at least three experiments.

See also Figure S6 and Table S1.
siRNA Transfection in a 384-Well Format
Reverse siRNA transfections were carried out in quadruplicate in a 384-well format (Perkin Elmer Cell carrier plates) in the presence of doxycycline (1 μg/ml) as described (Pegoraro et al., 2009) with pooled siRNA oligos (50nM; 4 siRNAs/target) from the Dharmacon siGENOMESMARTpool siRNA Human Ubiquitin Conjugation subset 1 and 2 libraries. Positive and negative controls consisted of GFP-targeting and non-targeting siRNA (50nM; Ambion, #AM4662, #AM4611G), respectively. Transfected cells were incubated over-night, after which 60 μl of antibiotic and doxycycline (1 μg/ml) containing medium was added and cells were incubated for another 3 days (37°C, 5% CO2).

High-Throughput High-Content Imaging Analysis
IF staining was performed (see the Supplemental Experimental Procedures), after which cells were imaged in a single optimal focal plane (24 fields/well, 20x water immersion objective) on an Opera high-throughput spinning-disk confocal microscopy system (Perkin Elmer), using sequential acquisitions with a 405/640, 488 and a 568 nm excitation laser. Immunofluorescence (IF) stainings were analyzed at the singular cell level with a customized algorithm (Kubben et al., 2013) using DAPI-based nuclear segmentation to quantify the total nuclear intensity for all indicated targets, and a contrast-based algorithm to detect 53BP1 foci numbers and total integrated intensity for γH2AX foci (Kubben et al., 2015). 500-2000 cells were analyzed per condition, and quantifications were depicted as an average of the total cell population. iPSC IF experiments were captured with a Leica SP5 confocal microscope, and total nuclear intensity was quantified with ImageJ software. Normalized Z-score RNAi screen values were calculated using CellHITS2 (Pegoraro et al., 2012). Z’-scores for the assays’ parameters were 0.15 (lamin B1), 0.38 (γH2AX) and 0.74 (progerin), siRNA pools with cell numbers above 50% of the negative control, and with a Z-score < -1.65 for lamin B1 or > 1.65 for γH2AX, together with a Z-score > 2.0 for progerin, were selected initial candidates, out of which the strongest candidates (Z-score < -2.0 for combined laminB1 and γH2AX effects; see Table S2) were pursued for secondary validation using On-Target Plus pooled siRNAs (Dharmacon, 4/target).

Statistical Analysis
All RNAi screening follow-up experiments are depicted as averages of three biological replicates, each consisting of at least three technical replicates, unless stated otherwise, and analyzed by Student’s t test in Graphpad Prism.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.05.017.

AUTHOR CONTRIBUTIONS
N.K. and T.M. designed the study; G.-H.L., W.Z., and J.Q. designed iPSC experiments; N.K. performed fibroblasts and HEK293FT-based experiments (Figure 1, 2, 3, 4, and 5); W.Z. and J.Q. performed iPSC-based experiments (Figure 6); L.W. aided in iPSC culture and differentiation; and with a Z-score < -1.65 for lamin B1 or > 1.65 for γH2AX, together with a Z-score > 2.0 for progerin, were selected initial candidates, out of which the strongest candidates (Z-score < -2.0 for combined laminB1 and γH2AX effects; see Table S2) were pursued for secondary validation using On-Target Plus pooled siRNAs (Dharmacon, 4/target).

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**Figure S1. Validation of the GFP-Progerin-Inducible Assay and RNAi Screening Candidates, Related to Figure 1**

(A) Representative IF image of non-targeting or GFP-targeting siRNA transfected GFP-progerin inducible fibroblasts (96 hr). Scale bar, 10μm.

(B) Western blot of GFP-progerin inducible fibroblast (no versus 96 hr induction).

(C) IF quantification of (A). N > 300; *p < 0.05, OFF/siCTRL versus ON/siCTRL.

(D) Quantification of nuclear invaginations in non-targeting or CAND1 targeting siRNA expressing GFP-progerin inducible fibroblasts that were uninduced or induced for 96 hr. *p < 0.05, OFF/siCTRL versus ON/siCTRL.

(E) Venn diagram of the identified hits in the RNAi screen. Individual target genes and are listed by their effect on lamin B1, GFP-progerin and H2AX nuclear defects (see Table S2).

(F) Quantification of nuclear invaginations in non-targeting or CAND1 targeting siRNA expressing GFP-progerin inducible fibroblasts that were uninduced or induced for 96 hr. *p < 0.05, OFF/siCTRL versus ON/siCTRL.

(G) Representative IF image of non-targeting or GFP-targeting siRNA transfected GFP-progerin inducible fibroblasts (96 hr). Scale bar, 10μm.

(H) Quantification of nuclear invaginations in non-targeting or CAND1 targeting siRNA expressing GFP-progerin inducible fibroblasts that were uninduced or induced for 96 hr. *p < 0.05, OFF/siCTRL versus ON/siCTRL.

(I) Quantification of GFP-mRNA levels upon CAND1 knockdown in GFP-progerin inducible fibroblasts induced for 96 hr.

(J) Venn diagram of the identified hits in the RNAi screen. Individual target genes and are listed by their effect on lamin B1, GFP-progerin and γH2AX or combination thereof. Out of the 7 candidates affecting multiple protein defects, CAND1, WSB1, FLJ23076 and FBXO38 also had a positive effect on the combined lamin B1 and γH2AX nuclear defects (see Table S2).

(K) Quantification of indicated proteins by IF in GFP-progerin inducible fibroblasts with control or CAND1-targeting siRNA, and in which GFP-progerin was either uninduced, or induced by doxycycline amounts at levels comparable to the RNAi screen (full induction), or at lower doxycycline concentrations resulting in GFP-progerin levels half those of the RNAi screen (half induction). Significant changes (p < 0.05) are indicated with the following symbols: *control siRNA transfected cells in which GFP-progerin was induced at full strength versus half strength. #control versus CAND1 siRNA transfected fibroblasts in which GFP-progerin was fully induced. *control siRNA transfected cells in which GFP-progerin was induced at half strength versus fully induced CAND1 siRNA transfected cells.
Figure S2. NRF2 Intracellular Localization and Transcriptional Activity in HGPS, Related to Figure 2

(A) IF staining for NRF2 in formaldehyde fixed GFP-progerin inducible fibroblasts (induction for 96 hr) treated with 5 μM sulforaphane (SF) for 8 hr. Values represent averages ± SD from at least 3 experiments. N > 300; *p < 0.05. Scale bar, 10 μM.

(B) IF staining for NRF2 (Abcam, 62352) in formaldehyde fixed WT and HGPS fibroblasts treated with 5 μM sulforaphane (SF) for 8 hr. Values represent averages ± SD from at least 3 experiments. N > 300; *p < 0.05. Scale bar, 10 μM.

(C) IF staining for serine 40 phosphorylated NRF2 (Abcam, Ab76026) in formaldehyde fixed WT and HGPS fibroblasts infected with non-targeting and progerin targeting shRNA. Values represent averages ± SD from at least 3 experiments. N > 300; *p < 0.05. Scale bar, 10 μM.

(D) IF staining of KEAP1, small MAFs and CBP in formaldehyde fixed GFP-progerin inducible fibroblasts (induction for 96 hr). Scale bar, 10 μM.

(E) Geneset enrichment analysis (GSEA) (see the Experimental Procedures) of mRNA datasets for WT and HGPS human dermal fibroblasts for gene signatures of NRF2 and HSF1 regulated genes. Normalized Enrichment Score (NES), and false discovery rates (FDR) q-values are indicated. NRF2 gene signatures are significantly enriched in WT cells (FDR q-value < 0.01; see also Table S4). HSF1 gene signatures were not enriched for in either wild-type or HGPS patient cells (FDR q-value > 0.01; see also Table S4).
Figure S3. Lamin B1 and Progerin Localization in HGPS Fibroblasts, Related to Figure 3
(A) IF staining of lamin B1 (SantaCruz, Sc-6217) and progerin (Alexis Biochemicals, ALX-804-662) in methanol fixed HGPS patient dermal fibroblasts. Scale bar, 10μM.
(B) Line graphs of IF signal intensity for progerin and lamin B1 across the indicated dotted line in (A).
Figure S4. Effects of Decreased NRF2 Pathway Activity on HGPS Nuclear Defects, Related to Figure 4

(A) IF image and quantification in progerin inducible cells expressing non-targeting and NRF2 targeting shRNA. Values represent averages ± SD from at least 3 experiments. Scale bar, 10 μM. *p < 0.05.

(B) IF staining in WT fibroblasts expressing non-targeting or NRF2-targeting shRNA. Scale bar, 10 μM.

(C) Quantification of NRF2 knockdown in WT fibroblasts expressing NRF2-targeting shRNA. Values represent averages ± SD from at least 3 experiments. *p < 0.05.

(D) Quantification of nuclear invaginations in WT and HGPS patient fibroblasts expressing non-targeting or NRF2 targeting shRNA. Values represent averages ± SD from at least 3 experiments. N > 300. p < 0.05: *WT/shCTRL versus WT/shNRF2; #HGPS/shCTRL versus HGPS/shNRF2.

(E) mRNA levels of NRF2 transcriptional targets as well as the non-NRF2 regulated target TBP in WT cells upon knockdown of NRF2. Values represent averages ± SD from at least 2 experiments. *p < 0.05.

(F) mRNA levels of NRF2 regulated transcriptional targets and the non-NRF2 regulated target TBP in GFP-progerin inducible fibroblasts (uninduced versus induced for 96 hr). Values represent averages ± SD from at least 3 experiments. *p < 0.05.

(G) Quantification of indicated proteins for WT and HGPS patient fibroblasts treated with hydrogen peroxide (100 μM) for 1 hr, and allowed to recover for 30 hr in normal growth medium. p < 0.05: *WT/vehicle versus WT/H2O2; #HGPS/vehicle versus HGPS/H2O2; *WT/vehicle versus WT/H2O2. Values represent averages ± SD from at least 3 experiments.

(H) Representative IF images of vehicle or H2O2 treated (250 μM; 96 hr) WT and HGPS patient fibroblasts. Values represent averages ± SD from at least 3 experiments. Scale bar, 10 μM.

(I) Quantification of nuclear invaginations in WT and HGPS patient fibroblasts treated with vehicle or hydrogen peroxide (250 μM; 4 days). Values represent averages ± SD from at least 3 experiments. N > 300. *p < 0.05, WT/vehicle versus WT/H2O2; #vehicle versus H2O2 treated total cell populations. *vehicle treated total cell population versus vehicle treated high progerin expressing population, #H2O2 treated total cell population versus vehicle treated high-progerin expressing subpopulation. Values represent averages ± SD from at least 3 experiments.
Figure S5. Effect of NRF2 Pathway Reactivation on HGPS Aging Defects, Related to Figure 5
(A) Representative IF staining of HGPS fibroblasts infected with HA-tagged caNRF2. Scale bar, 10 μM.
(B) Quantitative analysis of IF staining for indicated proteins in WT fibroblasts containing control or constitutively GFP-progerin and inducible caNRF2 expression constructs (96 hr). Values represent averages ± SD from at least 3 experiments. N > 300. *control versus GFP-progerin expressing WT fibroblasts in which caNRF2 remained uninduced; #caNRF2 uninduced versus caNRF2 induced WT cells that constitutively express GFP-progerin. **WT cells that constitutively express control vector and in which caNRF2 expression remained uninduced versus WT cells that constitutively express GFP-progerin and in which caNRF2 was induced. Values represent averages ± SD from at least 3 experiments. N > 300.
(C) Quantitative analysis of IF staining in WT and HGPS patient fibroblasts treated with NRF2 activating compounds (10 days; Oltipraz 125μM; see the Experimental Procedures for used concentrations of other compounds). N > 300; values represent averages ± SD from at least 3 experiments. *p < 0.05 (Student’s t test): HGPS/compound versus HGPS/vehicle.
(D) Western blot of HGPS patient fibroblast that were vehicle or N-Acetyl cysteine (10 mM; 96 hr) treated.
(E) mRNA levels of NRF2 transcriptional targets and the non-NRF2 target TBP in control and caNRF2 infected WT and HGPS patient fibroblast. N > 300; values represent averages ± SD from at least 3 experiments. p < 0.05: #WT versus HGPS. *HGPS versus HGPS/caNRF2. **WT versus HGPS/caNRF2.
(F) Quantification of nuclear invaginations of HGPS patient fibroblasts expressing control or caNRF2. Values represent averages ± SD from at least 3 experiments. N > 300.
(G) IF quantification of indicated proteins in WT fibroblast expressing caNRF2. Values represent averages ± SD from at least 3 experiments. N > 300.
(H) IF quantification of indicated proteins in WT fibroblast expressing CTRL or CAND1 targeting shRNA.
(I) DCFDA-based ROS quantification in WT fibroblasts expressing caNRF2. *p < 0.05. Values represent averages ± SD from at least 3 experiments. N > 300.
Figure S6. Status of the NRF2 Pathway in Mesenchymal Stem Cells Derived from Induced Pluripotent Stem Cells, Related to Figure 6
(A) Genomic sequencing of MSCs derived from HGPS-iPSCs as well as from C1824T LMNA gene corrected HGPS-iPSCs (GC-iPSC-MSC; see the Experimental Procedures), and corresponding validation of progerin mRNA levels in both cell types. Values represent averages ± SD from at least 3 experiments. *p < 0.05.
(B) FACS analysis of CD73, CD90 and CD105 MSC markers in indicated cell types.
(C) Western blot analysis of indicated proteins in GC- and HGPS-iPSC-MSCs. N = 3, *p < 0.05.
(D) mRNA levels of NRF2 transcriptional targets in GC-iPSC-MSC in which GFP-progerin expression is uninduced or induced for 2 weeks.
(E) H2DCFDA-based ROS measurement by FACS in HGPS-iPSC-MSC and GC-iPSC-MSC containing the inducible GFP-progerin construct (uninduced or induced for two weeks).
(F) Representative IF images and quantification of indicated proteins (lamin B1: manual count of cells with strong decrease of lamin B1 IF signal; LAP2, H3K27me3, and HP1γ: IF nuclear intensity) in GC-iPSC-MSC and HGPS-iPSC-MSC. Values represent averages ± SD from at least 3 experiments. N > 300. *p < 0.05.
(G) Representative IF images and quantification of indicated proteins (lamin B1: manual count of cells with strong decrease of lamin B1 IF signal; LAP2, H3K27me3, and HP1γ: IF nuclear intensity) in GC-iPSC-MSC and HGPS-iPSC-MSC. Values represent averages ± SD from at least 3 experiments. N > 300. *p < 0.05.
(H) Quantification of apoptotic cells (see the Experimental Procedures) in GC- and HGPS-iPSC-MSCs treated with vehicle or hydrogen peroxide (125 μM, 72 hr). Values represent averages ± SD from at least 3 experiments. *p < 0.05, GC-iPSC-MSC/vehicle versus HGPS-iPSC-MSC/vehicle; *p < 0.05: GC-iPSC-MSC/H2O2 versus HGPS-iPSC-MSC/H2O2.
(I) mRNA levels of NRF2 transcriptional targets in GC-iPSC-MSC expressing control or caNRF2.*p < 0.05.
(J) Western blot and quantification of indicated proteins in HGPS-iPSC-MSC expressing non-targeting or CAND1-targeing shRNA. Values represent averages ± SD from at least 3 experiments. *p < 0.05.
(K) Heatmap of mRNA levels of NRF2 transcriptional targets in HGPS-iPSC-MSC expressing non-targeting or CAND1 targeting shRNA. Values represent averages ± SD from at least 3 experiments.
Supplemental Information

Repression of the Antioxidant NRF2 Pathway in Premature Aging

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Supplemental Experimental Procedures

Plasmid construction

shRNA directed against human CAND1 or NRF2 were, respectively, directed against 5'-CGTGCACAATGTACACTA-3', 5'-GCACAGGGACATTGAGC-3' recognition sequences and cloned into the pSiH1puro plasmid (System Biosciences). pSiH1puro-shLuc (System Biosciences) was used as a non-targeting control in experiments. NRF2 knockdown experiments as described in figure 6 were performed with shRNA directed against the 5'-GTAAGAAGCCAGATGTAA-3' recognition sequence and cloned into the pLVTHM/GFP plasmid (Addgene, #12247), further using pLVTHM-shGL2 (Zhang et al., 2015) as a non-targeting control. The pRetrosuperblast plasmid expressing progerin shRNA was kindly provided by P. Fernandez (NCI, NIH, Bethesda). Constitutively active human NRF2 (caNRF2) was generated by sticky PCR using 5'-TCACCGGTTACTCGAGCAATTCTCCTCAATTCAGCAGCCAGCACATCC-3' and 5'-GAAGAATGTACTGTTGAAAGCCTTTCGAAAGTG-3' as well as 5'-CACCTTTCGAACGCTTTACACACCAGTAGATTCTTC-3' and 5'-GCGGCGCGGATATCCTGTTTTTCTTAACATCTGG-3'.

For immunoprecipitation experiments NRF2, lamin A and progerin constructs were cloned into pCDNA3.1 (Life Technologies). pcDNA3.1 OST-Neh2 and OST-Neh6 destabilizing domains were removed (Kubben et al., 2012) from previously published plasmids (Addgene, #22661& #22662) into the pcDNA3.1 OST-NRF2 plasmid. BamHI/AsuII digested pcDNA3.1 OST-NRF2 plasmid was digested with EcoRI/NotI enzyme combination and ligated as a control in these experiments.

For immunoprecipitation experiments NRF2, lamin A and progerin constructs were cloned into pcDNA3.1 (Life Technologies). pcDNA3.1 OST-NLS-mCherry was created by triple ligation of a 5' BamHI/3' AsuII digesting OneStrep (OST)-tag from pcDHblastMSCNard OST-LMNA (Addgene, #22661) with a 3' XhoI/5' AsuII NLS-mCherry PCR product from the pQXCN-Tetr-mCherry plasmid (Addgene, #59417) into BamHI/NotI digested pcDNA3.1 plasmid. pcDNA3.1 OST-NLS-mCherry-NRF2 plasmid was generated by introducing a 5'XhoI/3'NotI full length human NRF2 PCR fragment into this plasmid. pcDNA3.1 expressing NLS-mCherry-NRF2 with 9 additional N-terminal FLAG tag sequences was generated by replacing the OST-tag of pcDNA3.1 OST-NLS-mCherry-NRF2 with 9 FLAG tags. pcDNA3.1 OST-LaminA and OST-Progerin were generated by BamHI/EcoRI mediated cloning from previously published plasmids (Kubben et al., 2010)pcDNA3.1 expressing NLS-mCherry-NRF2 with 9 additional N-terminal FLAG tag sequences was generated by replacing the OST-tag of pcDNA3.1 OST-NLS-mCherry-NRF2 with 9 FLAG tags. pcDNA3.1 OST-LaminA and OST-Progerin were generated by BamHI/EcoRI mediated cloning from previously published plasmids (Kubben et al., 2010)pcDNA3.1 expressing NLS-mCherry-NRF2 with 9 additional N-terminal FLAG tag sequences was generated by replacing the OST-tag of pcDNA3.1 OST-NLS-mCherry-NRF2 with 9 FLAG tags. pcDNA3.1 OST-LaminA and OST-Progerin were generated by BamHI/EcoRI mediated cloning from previously published plasmids (Kubben et al., 2010)pcDNA3.1 expressing NLS-mCherry-NRF2 with 9 additional N-terminal FLAG tag sequences was generated by replacing the OST-tag of pcDNA3.1 OST-NLS-mCherry-NRF2 with 9 FLAG tags. pcDNA3.1 OST-LaminA and OST-Progerin were generated by BamHI/EcoRI mediated cloning from previously published plasmids (Kubben et al., 2010)

Antibodies

The following antibodies were used for western blotting: α-lamin A/C goat polyclonal (SantaCruz, Sc-6215), α-NRF2 mouse monoclonal (R&D systems, MAB3925), α-OneStrep mouse monoclonal (IBA Tagnology, 2-1507-001), α-FLAG mouse monoclonal (Sigma, F3165), α-HSC70 rat monoclonal (Abcam, Ab19136), α-β-actin mouse monoclonal (Sigma, A2228), α-GAPDH mouse monoclonal (SantaCruz, Sc-32233), α-αtubulin rat monoclonal (Gentaur, #YSRTMCA77S) and α-Hemagglutinin mouse monoclonal (SantaCruz, sc-7392). For FACS the following antibodies were used: PE α-CD73 mouse monoclonal (BD, 5550257), FITC α-CD90 mouse monoclonal (BD, 555595) and APC α-CD105 mouse monoclonal (eBioscience, 17-1057-42). For immunofluorescence the following antibodies were used: α-progerin mouse monoclonal (Alexis Biochemicals, ALX-804-662), α-lamin B1 goat polyclonal (SantaCruz, Sc-6217), α-LAP2 rabbit polyclonal (SantaCruz, Sc-28541), α-HP1γ mouse monoclonal (Chemicon, MAB3450), α-trimethylated histone 3 lysine 27 rabbit polyclonal (Upstate, #07-449), α-Serine 139 phosphorylated H2AX mouse monoclonal (Millipore, #05-636), α-53BP1 rabbit polyclonal (Novus, NB100-304), α-NRF2 rabbit monoclonal (Abcam, 62352; Figure 2 and S2), α-NRF2 rabbit polyclonal (SantaCruz, Sc-722; Figure 3A-B), α-Serine 40 phosphorylated NRF2 rabbit polyclonal (Abcam, Ab76026), α-KEAP1 goat polyclonal
(SantaCruz, Sc-15246), α-MAF-F/G/K mouse monoclonal (SantaCruz, Sc-166548), α-CBP rabbit monoclonal (Cell Signaling, #7389). Secondary antibodies used for immunofluorescence detection were Alexa Fluor Donkey-anti-Mouse 568 and 647 (Invitrogen, #A10037, #A31571), Alexa Fluor Donkey-anti-Rabbit 568 and 647 (Invitrogen, #A10042, #A31573) and Alexa Fluor Donkey-anti-Goat 568 and 647 (Invitrogen, #A11057, #A21447). All IF stainings were performed after fixation in 4% paraformaldehyde (see below), with the exception of the α-NRF2 rabbit polyclonal antibody (SantaCruz, Sc-722; Figure 3A-B), which was used after standard methanol fixation (5 minutes at -20°C) (Voncken et al., 2005). All antibodies were used at dilutions recommended by the manufacturers.

**Immunofluorescence staining**

All steps for IF staining were performed at ambient temperature. 96 hours post-transfection cells were fixed by addition of 25μl formaldehyde per well (10 minutes), permeabilized for 10 minutes (PBS/0.5% triton-X 100), and washed once with PBS/0.05% Tween-20. Next, cells were incubated for 1 hour with primary antibodies (γH2AX, 1:1000, Millipore, #05-636; lamin B1, 1:500, SantaCruz, Sc-6217) diluted in blocking buffer (PBS, 0.05% tween-20, 5% bovine serum albumin). After two consecutive washes in wash buffer (PBS, 0.5% Tween-20), cells were incubated for 1 hour with secondary antibodies (Donkey-anti-Mouse 568, 1:500 Invitrogen, #A10037; Alexa Fluor Donkey-anti-Goat 647, Invitrogen, #A21447) together with 5μg/ml DAPI. After washing, cells could be stored for extended periods of time in PBS/0.05% Tween-20.

**One-STrEP pull-down**

293FT cells grown to 80% confluency in 15 cm tissue culture dishes were transfected with 1ml of Optimem reduced serum medium (Invitrogen) containing 40 μl X-tremeGENE HP DNA transfection reagent (Roche, #06366236001), 7 μg of pcDNA3.1 containing OST-tagged NRF2 orlamin A/Progerin bait, and 3 μg of the pcDNA 3.1 plasmid for either HA-tagged lamin A/progerin or FLAG-tagged NRF2 prey (see above). 18 hours post-transfection a pull-down of OST-tagged proteins was performed with streptactin matrix as described previously after 0.1% formaldehyde fixation (Figure 3D-E) (Kubben et al., 2010). For interaction experiments in figure 3F dermal fibroblasts expressing OST-lamin A or OST-progerin were used for purification of OST-lamin A and OST-progerin by OST-pull-down as previously described (Kubben et al., 2010), without the use of any fixation. Purified immobilized lamin proteins were next incubated with recombinant His6-tagged full length NRF2 (Origene, TP760529) in ChiP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH=8.1, 167 mM NaCl), and after 6 hours of incubation at 4°C, washed 6 times with ChiP dilution buffer.

**Western blotting**

Western blots were performed as described (Kubben et al., 2010; Zhang et al., 2015). Nuclear extracts were prepared as previously published (Andersen et al., 2002), with the addition of 0.033% Triton X-100 to buffer A (10 mM HEPES-KOH, 1.5 mM MgCl2, 10 mMKCl, 0.5 mM DTT). For OST detection PVDF membranes were blocked in 5% BSA/TBS-Tween, and subjected to an additional pre-blocking step with biotin blocking buffer (IBA TAGnology, #2-0501-002) according to manufacturer’s instructions.

**Microarrays and quantitative analysis of gene expression**

RNA samples, prepared as previously described (Pegoraro et al., 2009), of HGPS patients dermal fibroblast (HGADFN001, HGADFN003, HGADFN127 from Progeria Research Foundation; AG01972, AG06297, AG11513 from Coriell Cell Repository), and control dermal fibroblast cell lines (GM00038, GM05565 from Coriell Cell Repository; CRL-1474 from ATCC), were analyzed on Genechip human genome 133 (HG-U133 plus_2) microarrays (Affymetrix) at the Frederick Laboratory of Molecular Technology Microarray Facility. All microarray data are publically available under GEO accession number GSE69391. After normalization of intensity values to the median intensity of the arrays, ratios of the average HGPS versus control mRNA expression levels were calculated for each gene and plotted for non-NRF2 targets, NRF2 transcriptional targets, previously defined as genes that interact with NRF2 in ChiP-on-Chip experiments and show decreased mRNA expression upon genetic disruption of
NRF2 expression (Malhotra et al., 2010). Ratio expression profiles were statistically compared by the non-parametric two-sample Kolmogorov-Smirnov test. Analysis of mRNA expression levels of NRF2 target genes in HGPS patient and control cells, or upon induction of GFP-progerin in P1 cells, was carried out as previously described (Pegoraro et al., 2009) using primer combinations as indicated in Supplementary Table 1, and normalized to cyclophilin A. Geneset enrichment analysis (GSEA) was performed on mRNA expression datasets of wild-type and HGPS patient fibroblasts using the C3 transcription factor targets mySig database v3.0 (GSEA, Broad Institute) with the addition of an NRF2 signature set as described above (Malhotra et al., 2010), using standard settings as previously used (Fernandez et al., 2014). Gene signatures were considered enriched for false discovery rate (FDR) q-values < 0.01 and Family-Wise Error Rate (FWER) p-values < 0.05.

**Luciferase reporter assay**

Fibroblast were transfected in absence or presence of 1 µg/ml doxycycline according to the manufacturer’s instructions with X-tremeGENE HP DNA transfection reagent (Roche, #06366236001) and 9µg of the pGL-8xARE firefly luciferase reporter, which contains 8 copies of a minimal functional NRF2 responsive antioxidant responsive element (ARE) sequence as previously described (Wang et al., 2006), or 9µg of the pGL-0xARE plasmid, which does not contain ARE recognition sequences and serves as a negative control. Co-transfection with 1 µg pRL-TK (Promega) was used for normalization of transfection efficiencies. After 36 hours cells were harvested and both firefly and renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega).

**Reactive oxygen species detection**

To measure oxidative stress levels skin fibroblasts were grown in 384-wells clear bottom tissue culture plates (Perkin Elmer, #6007558), washed once with Optimem reduced serum medium (Invitrogen), and incubated with 20 µM of the cell permeate reagent 2’,7’-dichlorofluorescein diacetate (DCFDA) (Abcam, ab113851) at 37˚C, which becomes fluorescent upon oxidation by ROS. After 45 minutes the medium was replaced with regular tissue culture medium supplemented with the DNA stain DRAQ5 (1µM;Biostatus Limited). Fluorescent DCFDA and DRAQ5 co-staining were immediately imaged under 37˚C, 5%CO2 and 60% humidity life imaging conditions according to the manufacturer’s instructions using Perkin Elmer’s Opera system. The total cellular intensity of DCFDA was quantified by standard Acapella Image Analysis scripts as described above. Oxidative stress levels were quantified in iPSC based cell models through H2-DCFDA (Invitrogen, C6827 or Abcam, ab186028) based flow cytometry as per manufacturer’s instructions.

**Apoptosis and senescence associated β-galactosidase detection**

The FITC annexin V Apoptosis detection kit (BD, 556547) was used to determine cell apoptotic level according to the manufacturer’s instructions. Cell survival was measured by counting MSC cell number 72 hours after plating after trypan blue exclusion staining. Senescence associated β-galactosidase (SA-β-gal) staining was performed as described previously (Endisha et al., 2015). Briefly, cultured cells were washed in PBS and fixed at room temperature for 3 min in 2% formaldehyde and 0.2% glutaraldehyde. Fixed cells were stained with fresh staining solution for SA-β-gal activity at 37°C overnight, and counted for positivity (N>300).

**In vivo luminescence measurements**

MSC implantation experiments were performed as previously described (Pan et al., 2016; Zhang et al., 2015) on GC- and HGPS-iPSC-MSCs that express control or NRF2 targeting shRNA, control or caNRF2, or were vehicle or Oltipraz (20µM; 3 weeks) pretreated. In brief, 100µl of 1X10^6 MSCs transduced with lentiviruses expressing luciferase were injected into the midportion of the tibialis anterior (TA) muscle of immunodeficient mice (Pan et al., 2016; Zhang et al., 2015). 5 days after implantation, mice were anaesthetized and treated with D-luciferin. Then, photon emission was measured by the IVIS lumina system (PerkinElmer). Bioluminescence images were acquired at auto-set model. Photons were counted according to the digital false-color photon emission image of the mouse, and
the values were normalized by average cellular luciferase intensity before implantation. All animal experiments were conducted with the approval of the Institute of Biophysics, Chinese Academy of Science.

References


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<th>Reverse primer (5’-&gt;3’)</th>
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**Table S1. Primers used for quantitative real-time PCR analysis, related to main figure 4, main figure 6 and supplemental figure S1, S4 and S6.** Primer sequences for real-time PCR quantitative analysis of indicated human mRNAs in a 5’ to 3’ direction. CypA and 18S were used as housekeeping controls.
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<th>Progerin Value</th>
<th>Lamin B1 Value</th>
<th>γH2AX Value</th>
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<th>Lamin B1 Value</th>
<th>γH2AX Value</th>
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**Table S2. RNAi screening results of candidates, related to main figure 1.** Primary screening and verification results of candidates that lower induced GFP-progerin levels, as well as either prevent formation of defects in laminB1 or γH2AX. Progerin, laminB1, γH2AX, and combined laminB1+γH2AX parameters were quantified as described in the Experimental Procedures, and compared to negative control (non-targeting siRNA) and positive control (GFP-targeting siRNA) transfected GFP-progerin induced wild-type fibroblasts (96 hours), which values were arbitrarily set as indicated in the table at 1.00 and 2.00. Significant Z-scores, as defined by cut-off values as listed in Experimental Procedures, are indicated in bold and with asterisk. Underlined targets were selected for secondary verification with structurally distinct on-target plus siRNA pools.
### Gene Signatures Upregulated in WT vs. HGPS

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<th>Gene Signature</th>
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### Gene Signatures Upregulated in HGPS vs. WT

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**Table S4. Geneset Enrichment Analysis, related to main figure 2.** Geneset Enrichment Analysis of mRNA expression datasets for wild-type and HGPS human dermal fibroblasts using the C3 transcription factor targets mySig database v3.0 (GSEA, Broad Institute) with the addition of an NRF2 signature set (See Experimental Procedures). ES = Enrichment Score; NES = Normalized Enrichment Score; FDR = False discovery rate; FWER = Family-Wise Error Rate. All significantly enriched gene signatures with FDR Q-values < 0.01 and FWER P-value < 0.05 are listed.