Inhibition of the acetyltransferase NAT10 normalizes progeric and aging cells by rebalancing the Transportin-1 nuclear import pathway

Authors: Delphine Larrieu¹,²*, Emmanuelle Viré¹,³**, Samuel Robson¹,⁴**, Sophia Y. Breusegem², Tony Kouzarides¹ and Stephen P. Jackson¹*

* Correspondence: dl437@cam.ac.uk or s.jackson@gurdon.cam.ac.uk
** These authors contributed equally to the work

Affiliations:
¹ The Wellcome Trust/Cancer Research UK Gurdon Institute and Department of Biochemistry, University of Cambridge, CB2 1QH, United Kingdom.
² Current address: Cambridge Institute for Medical Research, Department of clinical biochemistry, University of Cambridge, CB2 0XY, United Kingdom
³ Current address: MRC Prion Unit at UCL, Institute of Prion Diseases, Queen Square House, Queen Square London, WC1N 3BG, United Kingdom
⁴ Current address: School of Pharmacy & Biomedical Science, University of Portsmouth, PO1 2UP, United Kingdom

One sentence summary: NAT10 inhibition normalizes HGPS through TNPO1
ABSTRACT

Hutchinson-Gilford progeria syndrome (HGPS) is an incurable premature ageing disease. Identifying deregulated biological processes in HGPS might thus help define novel therapeutic strategies. Recently, HGPS cells were shown to display abnormal nucleo-cytoplasmic transport and defects in the RanGTP gradient that mediates this. However, the mechanism behind these defects was not known. Here, we report that microtubule stabilisation in HGPS cells sequesters the non-classic nuclear import protein Transportin-1 in the cytoplasm, affecting the nuclear localisation of its cargos, including the nuclear pore protein NUP153. Consequently, nuclear Ran, nuclear anchorage of the nucleoporin Translocated Promoter Region (TPR) and chromatin organisation are disrupted, deregulating gene expression and inducing senescence. We show that inhibiting N-acetyltransferase 10 (NAT10) ameliorates HGPS phenotypes by rebalancing the nuclear to cytoplasmic ratio of Transportin-1. This restores nuclear-pore-complex integrity and nuclear Ran localisation, thereby correcting HGPS cellular phenotypes. We observed a similar mechanism in cells from normal aged individuals. This study identifies a nuclear import pathway affected in ageing and underscores the potential for NAT10 inhibition as a possible therapeutic strategy for HGPS, and perhaps also for pathologies associated with normal ageing.
INTRODUCTION

Hutchinson-Gilford progeria syndrome (HGPS) is a very rare, fatal premature ageing syndrome caused by a de novo heterozygous point mutation within exon 11 of the LMNA gene (1,2). LMNA encodes two type V intermediate filament proteins: lamin A and lamin C. Together with B-type lamins, these proteins form the nuclear lamina at the inner nuclear membrane, where they act as a scaffold to maintain nuclear architecture, nuclear pore complexes and chromatin organisation, and to regulate DNA replication and transcription. In HGPS, the mutation only affects lamin A and triggers the expression of a shorter, toxic form of the protein called progerin that acts as a dominant negative. Accumulation of progerin at the inner nuclear membrane alters nuclear lamina structure, causing abnormal nuclear morphology, aggregation of nuclear pore complexes, chromatin disorganisation due to loss of peripheral heterochromatin anchorage and transcriptional deregulation, ultimately leading to DNA damage accumulation and premature entry of HGPS cells into senescence (3-6). These cellular phenotypes are also observed in aged normal cells. In HGPS patients, progerin accumulation triggers segmental premature ageing, usually starting within the first year of life and leading to death at the average age of 14, mainly due to heart defects (7,8).

Although HGPS syndrome was first described over a century ago, the exact mechanisms by which progerin expression drives such striking cellular and organismal phenotypes are still being explored.

Various studies have reported disruption of nucleo-cytoplasmic transport in HGPS fibroblasts, due to an abnormal RanGTP gradient (9-11). Ran is a small GTPase that shuttles between the cytoplasm and the nucleus. Ran cycles between GDP-bound and GTP-bound states (12), with conversion between these states being catalysed by
regulators in the cytoplasm (RanGAP – Ran GTPase-Activating Protein) (13) and nucleus (RCC1- Regulator of Chromatin Condensation 1) (14,15). Therefore, Ran is predominantly in its GTP bound form in the nucleus, and in its GDP bound form in the cytoplasm. This creates a RanGTP gradient across the nuclear envelope (16,17) that allows active transport of protein cargoes through nuclear pore complexes by a family of carrier proteins called karyopherins. In HGPS, progerin expression was shown to induce defects in Ran nuclear localisation. Consequently, nuclear import of proteins requiring Ran-dependent active transport through nuclear pores appeared more strongly affected than that of proteins passively diffusing through nuclear pores in these cells. This was indeed the case for the nucleoporin translocated promoter region (TPR) (9,10), a polypeptide of ~265 kDa that forms a homodimer and is thus one of the largest nucleoporins. TPR is the last protein assembled at the nuclear pore complex and its anchorage in the nuclear envelope requires the nuclear pore protein NUP153 (18,19). Recently, we showed that disrupting NUP153 abundance or function affects Ran nuclear localisation, thus impairing nuclear import of 53BP1, involved in DNA damage repair. This contributed to DNA damage accumulation in ageing vascular smooth muscle cells (VSMCs) (20). Given that we had shown previously that Remodelin, a small-molecule inhibitor of N-acetyltransferase 10 (NAT10) protein normalises phenotypes of HGPS cells (21), we tested its effects on VSMCs. We observed that NAT10 inhibition restored NUP153 localisation and normalised nuclear Ran abundance in these cells through an uncharacterised mechanism (20). This raised the question as to why the abundance of nuclear Ran is altered in these cells and which aspect of nuclear transport machinery is disrupted, particularly as NUP153 can be imported by several import pathways (22-24).
Here, we show that the decrease of nuclear Ran abundance occurring in HGPS cells, as well as in cells from aged individuals, are linked to dysregulation of the non-classic transport pathway mediated by Transportin-1 (TNPO1), also called karyopherin β2 (25). Unlike classical importin proteins, which are general transporters, TNPO1 only mediates the nuclear import of a subset of proteins. Among these, the most extensively characterised is the RNA binding protein heterogeneous nuclear ribonucleoprotein 1 hnRNPA1 (26) that functions in several processes including mRNA biogenesis and promotion of transcription factor activity (27-29). More recently, nuclear pore complex protein NUP153 was characterised as a target for TNPO1-mediated nuclear import (24).

Accordingly, we observed that hnRNPA1, NUP153, TPR and nuclear Ran defects in HGPS cells were linked to deregulation of the TNPO1 nuclear-to-cytoplasmic ratio. Notably, we established that NAT10 inhibition restored the TNPO1 pathway in HGPS cells, thereby enhancing nuclear localisation of its cargos such as hnRNPA1 and NUP153. This allowed proper binding of TPR to nuclear pore complex, chromatin reorganisation, and global gene transcription re-balancing, collectively preventing premature entry of HGPS cells into senescence. NUP153 depletion did not affect the Transportin-1 pathway, supporting the notion that TNPO1 deregulation in HGPS is the cause and not the consequence of abnormal NUP153 nuclear localisation. This study thus provides mechanistic insight into how NAT10 inhibition reverses HGPS cellular phenotypes and provides further evidence that it may also be relevant in normal ageing.
RESULTS

Premature ageing and normal aged cells display nucleocytoplasmic transport defects.

We found by immunofluorescence staining that, as expected (30), most Ran was in the nuclei of interphase fibroblasts from a normal 20-year-old individual (Normal Fibro, Fig. 1A). By contrast, and in line with previous reports (9,10), we observed that Ran localization was abnormal in fibroblasts from an HGPS patient (HGPS 11513), with most Ran being cytoplasmic (Fig. 1A). Because evidence suggests that HGPS might recapitulate various aspects of normal ageing (31), we also looked at Ran sub-cellular localisation in fibroblasts from healthy individuals between 81 and 96 years-of-age (AG11240, AG04059, AG05248 and AG09602). Similarly to HGPS cells, nuclear Ran appeared to be depleted to various extents in cells from aged individuals (Fig. 1A). This was not associated with decreased Ran protein abundance in HGPS or in fibroblasts from aged individuals (Fig. 1B), suggesting that changes of Ran sub-cellular localisation reflected dysregulated RanGTP gradient formation across the nuclear envelope, rather than changes of Ran expression. As reported previously (10), decreased nuclear Ran in HGPS cells was also associated with decreased nuclear staining intensity of the nucleoporin TPR (Fig. 1C, D) as well as in cells from aged normal individuals (Fig. S1A, B). These results confirmed previous findings on HGPS cells and also suggested that a similar nucleo-cytoplasmic transport defect may occur during normal ageing.

Inhibition of NAT10 re-balances nucleocytoplasmic transport.

Because TPR requires the nuclear pore complex protein NUP153 to anchor it to the nuclear pore-complex basket (18), we assessed whether NUP153 was present at the
nuclear periphery by immunofluorescence in normal and in HGPS fibroblasts. We observed that NUP153 nuclear intensity was significantly decreased in HGPS cells compared to controls, in a manner that correlated with decreased nuclear TPR staining (Fig. 1E, F and Fig. S2A). Because we had previously reported that inhibition of N-acetyltransferase protein NAT10 reverses abnormal phenotypes of HGPS cells as well as aged smooth-muscle cells (20,21), we investigated whether NAT10 inhibition affected NUP153 and TPR staining. Indeed, when we treated HGPS cells with the NAT10 inhibitor Remodelin (21), this increased nuclear staining intensity of NUP153 and TPR (Fig. 1E, F). To assess whether this reflected an enhanced interaction between NUP153 and TPR proteins after Remodelin treatment, we performed a proximity ligation assay (PLA) that allows protein-protein interaction detection and quantification (32). In this assay, we used primary antibodies against TPR and NUP153, followed by incubation with a pair of secondary antibodies coupled to fluorescent labelled oligonucleotides. Following an enzymatic ligation and amplification reaction, proteins that are in close enough proximity (~30 nm) lead to the production of a fluorescent signal. While interaction between NUP153 and TPR was clearly detected in normal fibroblasts (Fig. 1G, green dots) it was almost completely absent in untreated HGPS cells, in accord with these proteins being abnormally localised. However, upon Remodelin treatment, the interaction between NUP153 and TPR was greatly enhanced (Fig. 1G). This suggested that NAT10 inhibition promoted normal nuclear localisation and anchorage of both NUP153 and TPR at the nuclear periphery of HGPS cells.

We next looked at the effect of NAT10 inhibition on Ran sub-cellular localisation in HGPS cells. Strikingly, short-interfering RNA (siRNA) mediated depletion of NAT10 (siNAT10), or NAT10 inhibition by Remodelin essentially completely abolished Ran
nuclear localisation defects in HGPS cells (Fig. 1H, I, Fig. S2B). In line with nuclear transport factor 2 (NTF2) mediating Ran nuclear import (33,34), siRNA depletion of NTF2 in normal fibroblasts decreased Ran nuclear intensity, similar to that observed in untreated HGPS cells (Fig. 1H, I).

**Ran disruption modifies chromatin compaction and triggers senescence.**

Because many cellular processes are disrupted in HGPS, we explored which might be associated with decreased nuclear Ran abundance. One of the phenotypes observed in HGPS cells is chromatin disorganisation and loss of heterochromatin marks including histone H3K9me3 (35,36). To assess whether disrupting Ran could affect chromatin state at the global level, we transfected non-progeric human cells with NTF2 siRNA (siNTF2; Fig. 2A) and looked at the effect on global chromatin compaction as measured by using DNA digestion by micrococcal nuclease (MNase). This established that disruption of nuclear Ran localisation led to global chromatin decompaction (Fig. 2B) and suggested that, by affecting the bi-directional transport of many large cargo proteins, Ran deregulation might have broad effects on chromatin organisation, as observed in HGPS (35,36). Chromatin relaxation was also observed – albeit to a lesser extent – upon TPR depletion, reflecting the known role of TPR and nuclear pore complexes in chromatin organisation at the nuclear periphery (37,38).

We then assessed the effect of NTF2 depletion on the heterochromatin mark H3K9me3 in normal fibroblasts, as well as in HGPS cells. We observed a marked decrease of H3K9me3 staining intensity upon NTF2 depletion in normal fibroblasts (Fig. 2C, D), similar to the basal level observed in HGPS cells (siCT). NTF2 depletion did not affect H3K9me3 further in HGPS cells. Moreover, we observed a similar decrease of
H3K9me3 in high passage number normal fibroblasts (P61), suggesting that the perturbation observed in HGPS cells resembles what happens when normal cells reach senescence. In addition to its effect on chromatin, disrupting nuclear Ran (siNTF2) also promoted the entry of normal fibroblasts into senescence as measured by senescence-associated β-galactosidase staining (Fig. 2E, F). However, depleting NTF2 in HGPS cells that already had nuclear Ran defect did not significantly further increase the proportion of senescent cells. Collectively, these results suggested that deregulation of nuclear Ran observed in HGPS cells might, by globally affecting nucleocytoplasmic transport, contribute to downstream phenotypes of chromatin disorganisation and premature entry into senescence.

**NAT10 inhibition reverses Transportin-1 nuclear import pathway defects in HGPS cells.**

We showed recently that NUP153 disruption can affect nuclear Ran abundance (20). In light of this, and because NTF2 abundance or sub-cellular localisation do not appear to be affected in HGPS cells (Fig. S3A), we hypothesized that Ran gradient deregulation occurring in HGPS might reflect the loss of NUP153 nuclear import. This led us to investigate which upstream pathway(s) could affect NUP153 nuclear import in HGPS cells, especially since NUP153 can be imported by several proteins. However, recent evidence suggests that, in interphase cells, NUP153 is imported into the nucleus by the non-classic nuclear transport receptor Transportin-1 (TNPO1) (24), a karyopherin β protein that promotes nuclear import of only a specific subset of proteins, including NUP153 and hnRNPA1. We therefore assessed TNPO1 sub-cellular localization in normal and HGPS fibroblasts by immunofluorescence staining. While the nuclear-to-cytoplasmic ratio of TNPO1 was ~1 in normal fibroblasts, TNPO1 was depleted from
the nucleus of HGPS cells (Fig. 3 A-B), similarly to what we had observed for Ran (Fig. 1). To assess whether changes in the nuclear-to-cytoplasmic ratio of TNPO1 in HGPS cells had an impact on its nuclear import function, we assessed the sub-cellular localization of hnRNPA1, one of the best-described TNPO1 cargo proteins. In accord with TNPO1 pathway deficiency in HGPS cells, we observed a marked decrease of nuclear hnRNPA1 in such cells (Fig. 3A). Similar defects were observed for the Ewing Sarcoma protein EWS, another characterised TNPO1 cargo (39) (Fig. S3B, C). By contrast, the localization of NAT10, whose sub-cellular distribution is not detectably affected in HGPS (Fig. S4A and as described in (21)), was not altered upon interfering with TNPO1 function via expression of MBP-M9M, a peptide binding to TNPO1 and competing with its natural substrates (40) (Fig. S4B). These results thus highlight Tranportin-1 as a new pathway deregulated in HGPS.

We also observed loss of nuclear TNPO1 in normal cells from aged individuals (Fig. 3C), with no apparent change in abundance of TNPO1 or hnRNPA1 proteins, consistent with the HGPS cells (Fig. 3D). As NAT10 inhibition normalized NUP153 and TPR nuclear accumulation together with nuclear Ran abundance in HGPS cells (Fig. 1), we assessed its effects on the upstream Transportin-1 pathway. Both Remodelin treatment and NAT10 depletion (siNAT10) reverted the abnormal nuclear-to-cytoplasmic ratio of TNPO1 in HGPS cells to a level comparable to that of normal fibroblasts (Fig. 3 A-B). Taken together, these results suggested that the Transportin-1 pathway is affected in HGPS cells as well as in normal cells from aged individuals, leading to defects in the nuclear import of TNPO1 and its cargos, including hnRNPA1 and NUP153, thereby contributing to downstream defects of the RanGTP gradient. We conclude that NAT10 inhibition results in global normalisation of Transportin-1 sub-cellular localisation and
function in HGPS cells, thereby alleviating downstream phenotypes. Although TNPO1 depletion itself reduces proliferation of normal RPE-1 cells (Fig. S5A), the Transportin-1 pathway defects observed in HGPS and in ageing cells do not appear to be a mere consequence of slower cell growth. Indeed, mimicking reduced cell growth through serum starvation of normal cells (RPE-1 and normal fibroblasts) did not discernibly affect the Transportin-1 pathway, nor the subcellular localisation of Ran or H3K9me3 (Fig. S5B-D) that are strongly affected in HGPS cells.

**Modulation of microtubule stability by NAT10 inhibition affects the Transportin-1 pathway.**

We previously observed that HGPS fibroblasts display a more stable microtubule network than control cells (21), and that this contributes to abnormal HGPS cellular phenotypes. We also showed that NAT10 inhibition improved these phenotypes, at least in part by targeting and destabilizing microtubules, thus releasing external forces on the nucleus to normalize nuclear shape (21). This is in accord with a pool of NAT10 being associated with the cytoskeleton, as observed by subcellular fractionation and immunofluorescence (Fig. S6A-B). To assess whether there was a link between accumulation of TNPO1 in the cytoplasm of HGPS cells and microtubule stability, we first performed a PLA assay between TNPO1 and alpha-Tubulin. This established that, despite similar TNPO1 and alpha-Tubulin staining intensities observed by immunofluorescence (Fig. S7A) and similar protein abundance observed by western blotting (Fig. S7B), the level of interaction between TNPO1 and alpha-Tubulin was significantly higher in HGPS than in normal fibroblasts, whether measured per cell or per set area of cytoplasm (Fig. 4A-B). The reason for measuring the number of PLA spots within a set area was to account for the fact that senescent cells are bigger, which
could have biased the results. However, there was a significant increased number of PLA spots even within the set area of cytoplasm (Fig. 4B right panel), showing that the increased interaction was not a reflection of the increased cell area. The interaction between TNPO1 and alpha-Tubulin was confirmed by immunoprecipitation using extracts of normal fibroblasts, for which we could obtain enough cells for such studies (Fig. S7C).

Next, to understand whether the effects of NAT10 inhibition on TNPO1 was associated with microtubule stabilization, we treated normal and HGPS fibroblasts with the microtubule stabilizing or destabilizing agents tubacin or nocodazole, respectively (Fig. 4C). Notably, mimicking microtubule stabilization, as occurs in HGPS cells, by treating normal fibroblasts with tubacin led to significant defects in both TNPO1 and hnRNPA1 nuclear import (Fig. 4C-D). By contrast, destabilizing microtubules in HGPS cells or in normal cells from aged individuals with nocodazole enhanced TNPO1 nuclear localization, and presumably by promoting TNPO1 transport function, increased abundance of nuclear hnRNPA1 (Fig. 4C-D and Fig. S8). These results suggested that the Transportin-1 mediated import pathway can be altered by microtubule stability, and that by modulating this, NAT10 inhibition re-balanced the TNPO1 nuclear-to-cytoplasm ratio and enhancing nuclear import of TNPO1 cargo proteins.

**NAT10 inhibition-mediated normalisation of HGPS cells requires TNPO1.**

To determine whether the normalization of heterochromatin markers and the decrease of senescence observed upon NAT10 inhibition in HGPS cells depended on Transportin-1, we treated normal or HGPS fibroblasts with siRNAs to deplete NAT10 alone or in combination with NUP153 or TNPO1 depletion. As we observed previously
(20), NUP153 depletion in normal cells led to loss of nuclear Ran, probably because it affects the structure of nuclear pore complexes that are important for RanGTP gradient formation. However, NUP153 depletion did not affect the Transportin-1 pathway, as observed by the normal sub-cellular localisation of TNPO1 and its downstream target hnRNPA1 (Fig. S9). Cells impaired in nuclear Ran due to TNPO1 depletion (siTNPO1 Fig. 5A, see cells highlighted by arrows and Fig. S10) or as observed in HGPS cells (siCT, Fig. 5B), also displayed significantly reduced levels of the heterochromatin marker H3K9me3 (Fig. 5C). This is in accordance with the chromatin decompaction that we observed upon Ran deregulation (Fig. 2B, C) and suggested that TNPO1 depletion was sufficient to cause this phenotype. Furthermore, co-depletion of NUP153 and NAT10 in normal fibroblasts (siNUP153+siNAT10) produced similar H3K9me3 phenotypes compared to siNUP153 alone (Fig. 5A, C), identifying NUP153 as a critical mediator of NAT10-dependent cellular rebalancing. While NAT10 depletion alone restored nuclear Ran and H3K9me3 staining in HGPS cells, this did not happen when NUP153 or TNPO1 was co-depleted with NAT10 (Fig. 5B, C). Similarly, while NAT10 depletion strongly decreased the proportion of cells positive for senescence-associated β-galactosidase in HGPS cells, this was not the case when NUP153 or TNPO1 was co-depleted with NAT10 (Fig. 5D-E). These results indicated that normalization of phenotypes caused by NAT10 depletion in HGPS cells indeed occurred via restoration of TNPO1 pathway function (thus, when TNPO1 or its cargo NUP153 are absent, NAT10-dependent phenotypic rescue does not occur). TNPO1 or NUP153 depletion in normal fibroblasts also promoted senescence, implying that the Transportin-1 pathway is important to maintain cellular homeostasis under such settings.

TNPO1 is required for NAT10 inhibition-mediated gene expression modulation.
In light of the above observations, we hypothesized that enhancing the Transportin-1 pathway in HGPS cells via inhibiting NAT10 might have an impact on gene expression. To test this idea, we performed global gene-expression analysis on HGPS cells compared to normal fibroblasts, after 8 weeks of chronic Remodelin treatment (media was supplemented with fresh Remodelin every 3 days). Ensuing “volcano plots” of each individual differential expression analysis (Fig. 6A) show log10 adjusted p-values plotted against log2 fold gene-expression changes. Significant genes (based on a fold-change threshold of 2-fold up or down and an adjusted p-value threshold of 0.05) are indicated by the red (increased expression) and blue (decreased expression) regions of the plots. As expected and as previously reported (31,35,41), we observed deregulation of gene expression in HGPS cells compared to normal fibroblasts (Fig. 6A left panel and Fig. 6B row 1), with 137 and 221 genes whose expression significantly increased or decreased respectively. Notably, chronic long-term treatment with Remodelin had very little effect on gene expression in normal fibroblasts, with only 17 genes whose expression significantly increased or decreased (Fig. 6A middle panel). In marked contrast, Remodelin had a strong effect on HGPS cells (Fig. 6A right panel), modifying the expression of 652 genes (Table S1). Strikingly, these Remodelin-induced gene-expression changes in HGPS cells were largely reciprocal to the gene expression differences between HGPS cells and normal cells (Fig. 6B; compare row 2 with row 1). Thus, long-term Remodelin treatment had a strong rebalancing effect on the expression of most genes deregulated in HGPS cells.

Next, we asked whether the gene expression changes mediated by NAT10 inhibition would still occur in the absence of TNPO1. To address this question, we performed a separate analysis following short term depletion of NAT10 and/or TNPO1 by siRNA.
We used NAT10 siRNA (instead of Remodelin) to enable rigorous comparison to siRNA-mediated depletion of TNPO1. Due to the shorter timeframe of siRNA experiments, we applied a lower threshold (1.5-fold up or down) in this analysis, which increased the number of deregulated genes in control (siCT) HGPS cells (Fig. 6C, left panel) compared to earlier experiments with Remodelin (Fig. 6A, left panel). Similar to Remodelin treatment, NAT10 depletion modified the expression of more genes in HGPS cells than it did in normal fibroblasts (Fig. 6C, middle and right panel and Table S2). It did, however, have a stronger impact on normal fibroblasts than Remodelin. Indeed, NAT10 depletion via siRNA abolishes all functions of NAT10 such as its RNA helicase activity and its role in ribosome biogenesis (42). Even though we did observe a rebalancing effect on some HGPS deregulated genes (Fig. 6D), short-term NAT10 depletion via siRNA treatment did not have comparable effects to Remodelin treatment on global gene expression rebalancing (Fig. 6D row 2 vs row 1). This suggests that long-term NAT10 inhibition is required to observe global rebalancing of HGPS gene expression profile.

To assess whether NAT10 depletion-mediated gene expression changes were occurring through TNPO1, we compared NAT10 depletion alone with co-depletion of NAT10 and TNPO1 (Fig. 7A). We observed that 70% of the genes whose expression significantly increased or decreased upon NAT10 depletion alone in normal fibroblasts were not modified anymore upon co-depletion with TNPO1 (Fig. 7B, red only genes, Tables S3-4), despite similar level of NAT10 protein knock-down (Fig. 7A). In HGPS cells, this number was reduced to 40% (Fig. 7C), likely a result of the deregulation of additional pathways. However, since we observed that the TNPO1 pathway was required for the NAT10 inhibition-mediated phenotypic changes in HGPS cells, we
hypothesized that this specific set of genes (Fig. 7C, red only genes) would be mainly responsible for the HGPS cellular rescue. Gene ontology analysis of those genes significantly affected by NAT10 depletion only (Table S5) and not by co-depletion with TNPO1 in HGPS cells showed an enrichment for various cellular components (CC, Fig. 7D) including cell substrate junction and focal adhesion, extracellular matrix or endoplasmic reticulum for genes whose expression was decreased (blue), or Golgi and small ribosomal subunits for genes whose expression was increased (red). When plotting this specific set of genes that we refer to as “siNAT10 only” on a heatmap (Fig. 7E row 1 – corresponding to Fig. 7C, red only genes), we observed that the expression of about 40% of this specific set of genes was rebalanced towards the expression observed in normal fibroblasts (Fig. 7E compare genes with opposite colours in row 2 vs row 1).

Then, to assess whether the TNPO1 pathway was affected in HGPS, we compared the effect of TNPO1 depletion on gene expression profiles in normal fibroblasts (Fig. 7F, row 1) with gene expression levels in HGPS compared to normal fibroblasts (Fig. 7F, row 2). This showed that 58% of the genes whose expression significantly increased or decreased upon TNPO1 depletion in normal fibroblasts showed a similar trend in control HGPS cells (Fig. 7F, compare genes that appear blue or red in both rows 1 and 2). TNPO1 depletion can be expected to have differential effects compared to a change of TNPO1 sub-cellular localisation as observed in HGPS, where the protein is still expressed (Fig. 7A and TNPO1 gene indicated by a * on the heatmap Fig. 7F).

Moreover, short-term TNPO1 depletion by siRNA might not recapitulate the effects of long-term decreased nuclear abundance of TNPO1 observed in HGPS cells. These results however support our hypothesis that the TNPO1 pathway is affected in HGPS
and that NAT10 inhibition mediates gene expression changes, at least in part through TNPO1.

Collectively, these results suggested that NAT10 inhibition enhances global HGPS cell fitness by restoring TNPO1 function, thus rebalancing nucleo-cytoplasmic transport, restoring normal chromatin organization and control of gene expression, and thereby preventing premature entry into senescence.
DISCUSSION

TNPO1 dysfunction in HGPS drives nuclear Ran defects.

In this study, we established that the Transportin-1 pathway of nuclear import is mislocalized in cells derived from patients with Hutchinson-Gilford progeria syndrome. Furthermore, we found that defective nuclear import of TNPO1 cargo proteins including NUP153 (24) and hnRNPA1 (26) appeared to contribute to downstream phenotypic defects in HGPS cells, including abnormal nuclear pore assembly, chromatin disorganisation, gene expression changes and premature entry into senescence. While previous studies had reported RanGTP gradient defects in HGPS cells (9,10), the mechanism behind it was still unclear. Given the known link between oxidative stress and nucleocytoplasmic transport (as reviewed in (43)), and as oxidative stress is higher in HGPS cells (11), it was thought to be the main cause of decreased nuclear Ran abundance in progeria. However, oxidative stress has been shown to affect classical Importin nuclear import pathways (44), whereas we found here that the TNPO1 pathway appeared to be most affected in HGPS. Therefore, based on our findings herein, we suggest that depletion of Ran from the nucleus in HGPS cells is a consequence of dysfunctional TNPO1, rather than a direct effect of progerin expression, oxidative stress or NUP153 deregulation. In addition, by showing that NAT10 inhibition restores TNPO1 dependent nuclear import by releasing TNPO1 from the cytoplasm of HGPS cells, we have provided insights into how NAT10 inhibition, through its effects on microtubule stability, ameliorates cellular defects of HGPS cells. As described previously (21), NAT10 inhibition does not affect progerin expression or localisation, suggesting that its effects on HGPS cells occur through a progerin-independent pathway.
It is still unclear how NAT10 activity might increase during normal and premature ageing. However, a recent study suggests that NAT10 activity is modulated by autoacetylation and that sirtuin proteins can mediate NAT10 deacetylation (45). Sirtuin proteins have been linked to various age-related pathologies (reviewed in (46)), with their decreased activity being associated with senescence. In this context, reactivation of SIRT1 has been shown to extend health and lifespan in an HGPS mouse model (47). Therefore, we speculate that decreased sirtuin activity as occurs in aging might lead to increased NAT10 acetylation and activity, therefore contributing to increased microtubule stability. Whether the acetylation of other reported NAT10 substrates such as p53 (48), the upstream binding factor (UBF) (49) and rRNA (50) is also affected in HGPS is not known.

Microtubule stability modulates nucleo-cytoplasmic transport.

Based on our findings, we propose the following model depicted in Fig. 8 with NUP153 as an example of a TNPO1 cargo protein. 1) In normal cells, microtubules bind to the nuclear pore complex (51) and TNPO1 binds to specific cargo proteins in the cytoplasm. 2) The TNPO1-cargo protein complex is translocated into the nucleus through the nuclear pore. 3) Once in the nucleus and upon RanGTP binding to TNPO1, the NUP153 cargo dissociates from TNPO1 and is incorporated as a component of the NPC basket structure, responsible for anchoring TPR (18,19), the last nucleoporin to be assembled (52). The resulting mature NPC is then fully functional for nucleocytoplasmic transport and other NPC-dependent roles. 4) During the progression of HGPS and during ageing of normal cells, increased microtubule network stability appears to sequester TNPO1 in the cytoplasm. 5) As a consequence, TNPO1 cargos are not imported properly into the nucleus and one of these, NUP153, does not incorporate efficiently into nuclear
pore complexes. This results in decreased nuclear Ran abundance, as well as defects of TPR anchorage at nuclear pore complexes. Consequently, during the progression of HGPS and upon normal-cell ageing, there is progressive chromatin disorganisation and decompaction, global gene expression changes and premature entry into senescence. We speculate that the global deregulation of gene expression observed in HGPS cells is probably a consequence of both a direct effect of abnormal nuclear architecture on chromatin organisation, and of nucleo-cytoplasmic transport defects affecting the nuclear import of various proteins, including TPR that directly binds chromatin (38) and hnRNPA1 that modulates the activity of transcription factors (27-29). 6) By destabilising the microtubule network (21), NAT10 inhibition releases TNPO1 from the cytoplasm in HGPS or in normal cells from aged individuals, enhancing its nuclear translocation together with ensuing nuclear import of its cargos such as NUP153 and hnRNPA1. This then substantially normalizes normal chromatin organisation, Ran mediated nucleo-cytoplasmic transport and global transcription patterns. In support of this model, wherein NAT10 inhibition ameliorates HGPS phenotypes via the Transportin-1 pathway, we found that Remodelin had little effect on HGPS phenotypes when they had been depleted of TNPO1.

In addition to helping to explain the mechanistic basis for HGPS cell dysfunction, our study highlights how specific components of the nucleo-cytoplasmic transport are affected by a range of factors, in this case cytoskeletal integrity. This correlates with a recent study showing that mechanical forces can modulate nuclear pore opening and conformation, thereby modifying nucleo-cytoplasmic transport efficiency (53). Finally, our study underscores the potential for NAT10 inhibition as a possible therapeutic strategy for HGPS, and perhaps also for certain pathologies associated with aging.
MATERIAL AND METHODS

Cell culture and transfections. Normal skin primary fibroblasts from young healthy individual (GM03440, 20 years old), from Hutchinson-Gilford progeria syndrome (AG11498: 14 years old and AG11513: 8 years old) and from aged individuals (AG11240: 81 years old female, AG04059: 96 years old male, AG05248: 87 years old male, AG09602: 92 years old female) were purchased from Coriell Cell Repositories and used in passage matched-cells (unless indicated otherwise on the figure) between passage number 9-25. Cells were grown in Dulbecco’s modified Eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (BioSera), 2mM L-glutamine, 100U per ml penicillin, 100 µg ml⁻¹ streptomycin. Normal Retinal Pigmented Epithelial Cells (RPE-1) were grown in DMEM:F12 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (BioSera), 2mM L-glutamine, 100U per ml penicillin, 100 µg ml⁻¹ streptomycin. For serum starvation experiments, the same medium was used without any fetal bovine serum. The following siRNA duplexes were obtained from Life Sciences: NAT10 stealth RNAi: GAGCAUGGACCUCUCUGAAUA, and as control siRNA, stealth RNAi negative control duplexes were used. TNPO1 siRNA were from Sigma Aldrich: GCAAAGAUGUACUCGUAAG, GUAUAGAUGCAGCUCUUA and GUAAAUACCAGCAUAAGAA. Smart pool NUP153 and NTF2 small interfering RNA (siRNA) oligonucleotides where purchased from Dharmacon (Lafayette, USA). siRNA transfections were carried out using Lipofectamine RNAiMax (Life Sciences), following the manufacturer’s instructions. Cells were analysed 48 to 72 h after transfection. U2OS human osteosarcoma cells were grown in DMEM supplemented as above. Plasmid encoding the TNPO1 inhibitor Myc-MBP-M9M (40) was transfected
using Lipofectamine 2000 (Life Sciences), following the manufacturer’s instructions. Cells were analysed 24h after transfection.

**Cell proliferation.** Non-transformed human RPE-1 cells were plated at equivalent low densities and transfected with control (siCT) or TNPO1 siRNA (siTNPO1) as explained above. Six days later, cells were fixed and stained with crystal violet staining solution (0.5% Crystal Violet, 20% methanol).

**Cell growth inhibition.** RPE-1 cells or normal fibroblasts were either kept in normal medium supplemented with 10% fetal bovine serum (FBS) or in medium without serum to arrest cell proliferation. After 4 days of serum deprivation, cells were processed for immunofluorescence.

**Drug treatments.** Remodelin was synthetized as described previously (21) incubated at 1 µM for at least 5 days, renewing the medium every 3 days. Long-term Remodelin treatment for microarray experiments was performed by keeping and passaging cells in medium containing 1 µM Remodelin or DMSO only for 12 population doublings. For immunofluorescence assays, Nocodazole and Tubacin were added to the medium for 16h at 100ng/ml and 10µM respectively.

**Senescence assay.** Senescence was assessed 72h after siRNA transfection using the Senescence β-Galactosidase Staining Kit #9860 from Cell Signaling. Blue cells were counted using the Cell counter plugin from Image J.
**Immunoblotting.** Total cell extracts were prepared by scraping cells in SDS lysis buffer (4% SDS, 20% glycerol, and 120 mM Tris-HCl, pH 6.8), boiling for 5 min at 95°C, followed by 10 strokes through a 25-gauge needle. Before loading, lysates were diluted with a solution of 0.01% bromophenol blue and 200 mM DTT and boiled for 5 min at 95°C. Proteins were resolved by SDS-PAGE on 4-12% gradient gels (NUPAGE, Life Sciences) and transferred onto nitrocellulose membrane (Protran; Whatman). Secondary antibodies conjugated to IRDye 800CW were from LI-COR Biosciences. Detection was performed with an imager (Odyssey; LI-COR Biosciences).

**Subcellular fractionation.** Cells were harvested with trypsin-EDTA and centrifuged at 500xg for 5 min. Cells were washed once with 1ml ice-cold PBS and centrifuged at 500xg for 5min. Supernatant was removed and 20µl of packed cell volume was processed for subcellular fractionation using the kit for cultured cells from ThermoFisher (Cat number 78840), using the manufacturer’s instructions. Subcellular fractions were then processed for immunoblotting as described above. Protein compartmentalisation in chromatin and cytoskeletal fractions was assessed by detection of the Histone H3 and alpha-Tubulin proteins.

**Immunofluorescence.** Cells were washed with PBS and fixed for 20 min with 2% PFA in PBS. Cells were permeabilised for 5 min with PBS/0.2% Triton X-100, and blocked with PBS/0.2% Tween 20 (PBS-T) containing 5% BSA. Coverslips were incubated for 1 h with primary antibodies and for 30 min with appropriate secondary antibodies coupled to Alexa Fluor 488, 594 or 647 fluorophores (Life Technologies), before being incubated with 2µg/ml DAPI. Pictures were acquired with a FluoView 1000 confocal microscope (Olympus) using the same laser power for matching images and antibodies.
All the immunofluorescence experiments were performed at least 3 times independently and the pictures shown in the figures are representative images of the 3 experiments. Image analyses were carried out with the Volocity software (PerkinElmer) using the following protocol: Find objects (DAPI). Subpopulation: Fill holes in objects. Exclude touching edge of image. Exclude objects by size: <100µm². Measure. The fluorescence intensity in each channel was then recorded.

**Proximity Ligation Assay (PLA).** The PLA assays were carried out using the Duolink® In Situ Red Starter Kit Mouse/Rabbit (DUO9101, Sigma Aldrich) following manufacturer’s instructions. Image analysis was carried out with the Volocity software (PerkinElmer) using the following protocol: Find objects (DAPI). Exclude objects >1000µm² and <20µm². Fill holes in objects. Exclude touching edge of image. Find spots using intensity. Compartmentalize: Divide spots – Between nuclei. Select ROI and record the number of PLA spots.

**Antibodies.** The following antibodies were used as indicated:

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Micrococcal nuclease digestion sensitivity assay. As this assay requires a lot of cells, we could not perform it with HGPS cells and thus used human osteosarcoma U2OS cells instead. 1x10^6 cells were trypsinized, harvested, and washed once with 1 ml of 1x RSB buffer (10 mM Tris, pH 7.6, 15 mM NaCl, and 1.5 mM MgCl_2). After centrifugation (300 x g), the cell pellet was resuspended in 1 ml of 1x RSB buffer with 1% Triton-X 100 and homogenized by five strokes with a loose-fitting glass pestle to release nuclei. Nuclei were collected by centrifugation (13,000 x g) and washed twice with 1 ml of buffer A (15 mM Tris, pH 7.5, 15 mM NaCl, 60 mM KCl, 0.34 M sucrose, 0.5 mM spermidine, 0.15 mM spermine, 0.25 mM PMSF, and 0.1% β-mercaptoethanol). Nuclei were resuspended in Buffer A and aliquoted into 100 µl aliquots. 1.2 µl of 0.1 M CaCl_2 was added to each aliquot and nuclei were digested by addition of 0.25 µl of 200 U/ml MNase (Sigma-Aldrich) and incubated at 30°C. Each aliquot was put back on ice at different time points and digestion was immediately stopped by addition of 3 µl EDTA. DNA was purified using the Qiagen PCR purification kit and 1500 ng of DNA was analysed on a 1.5% agarose gel. Digestion profiles were analysed using ImageJ and values were adjusted relative to the global intensity of each lane to compensate for DNA loading variations.

Microarray. Three independent biological replicates were used for each condition. RNA extracted from cells was purified using Direct-zol RNA MiniPrep kit (Zymo Research, cat. no. R2072) and treated with DNaseI (Zymo), according to manufacturers’ instructions. RNA quality was assessed using the Tapestation (Agilent Technologies) and Qubit (Invitrogen). Quality analysis of total RNA, hybridisation and data capture was performed at the Cambridge Genomic Services. Illumina HumanHT-12 v4 beadchips were used for gene expression screening. Results were analysed using the
Lumi (54) Bioconductor package in R. Probe-level data were combined to give gene-level data by taking the mean of all probes for a single gene. Differentially expressed genes were identified in each cell line using limma (55), using a 2-way factorial design with disease state as the first factor (HGPS vs normal fibroblasts) and treatment as the second factor (Remodelin vs DMSO, or siNAT10/siTNPO1/both vs siCT). Differentially expressed genes were calculated by performing pairwise comparisons between the four groups (Treated Normal, Untreated Normal, Treated HGPS, Untreated HGPS) for each treatment. All resulting p-values were corrected for multiple testing by using the Benjamini and Hochberg False Discovery Rate correction (52). Genes were defined as showing significantly altered expression if they showed a fold change greater than some threshold value (2-fold up or down for Remodelin, 1.5-fold up or down for siRNA) compared to control, with an adjusted p-value lower than 0.05.
SUPPLEMENTARY MATERIALS

Fig. S1: TPR nuclear localisation is impaired in normal cells from aged individuals and normalised by NAT10 inhibition.

Fig. S2: Nuclear import is affected in HGPS.

Fig. S3: TNPO1 dependent protein cargos are affected in HGPS cells.

Fig. S4: NAT10 nuclear import is not dependent on TNPO1.

Fig. S5: Cell proliferation inhibition does not affect the Transportin-1 pathway.

Fig. S6: NAT10 localises both in the nucleus and in the cytoskeleton.

Fig. S7: TNPO1 accumulates at the microtubule network of HGPS cells.

Fig. S8: TNPO1 pathway is defective in HGPS cells and in normal cells from aged individuals, and this is rescued by microtubule destabilization.

Fig. S9: NUP153 depletion does not affect the Transportin-1 pathway.

Fig. S10: Efficiency of protein depletions.

Table S1: List of genes modified by Remodelin treatment

Table S2: List of genes modified by NAT10 depletion

Table S3: List of genes modified by TNPO1+NAT10 depletion

Table S4: List of genes modified by TNPO1 depletion

Table S5: Gene ontology analysis of “siNAT10 only” genes


Acknowledgments: We thank Dr Raphael Rodriguez (Curie Institute, Paris) for Remodelin synthesis, and Dr Yuh Min Chook for providing us with the plasmid encoding Myc-MBP-M9M. We thank Alison Schuldt and Kate Dry for critical reading of the manuscript. We thank all members of the Jackson lab, particularly J. Forment for discussions and comments. **Funding:** Research in the Jackson laboratory is funded by Cancer Research UK (CRUK) program grant C6/ A18796 and a Wellcome Trust Investigator Award (206388/Z/17/Z). Institute core funding is provided by CRUK (C6946/A24843) and the Wellcome Trust (WT203144). S.P.J. receives his salary from the University of Cambridge, UK. D.L was funded by a Project Grant from the Medical Research Council, UK MR/L019116/1 and is now funded, together with S.B., by a Wellcome Trust Henry Dale fellowship (86609). E.V., S.R. and the T.K. laboratory were supported by a grant from Cancer Research UK (Grant Reference RG17001) in addition to benefiting from core support from the Wellcome Trust (WT203144) and Cancer Research UK (C6946/A24843). TK is supported by a Cancer Research UK Gibbs Fellowship. **Author contributions:** DL designed, carried out and analysed all experiments, except the microarray and wrote the paper. EV carried out the microarray experiment and commented on the manuscript. SR analysed all microarray data, generated the corresponding graphs and legends. SB helped with revisions of the paper by repeating western blots. SPJ supervised the study and helped writing and editing the manuscript. TK commented on the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** The raw data and processed differential expression tables from the microarray are available from ArrayExpress under accession number E-MTAB-6651 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6651/).

**FIGURE LEGENDS**
Figure 1: Nucleocytoplasmic transport is affected in aging and enhanced by NAT10 inhibition. (A) Representative immunofluorescence images from three independent experiments showing subcellular localisation of Ran in skin fibroblasts from a 20-year-old normal male (Normal fibro), aged individuals (AG11240, a 81-year-old female; AG04059, a 96-year-old male; AG05248, a 87-year-old male; AG09602, a 92-year-old female) or a 8-year-old HGPS patient (HGPS 11513). Scale bar: 50µm. (B) Representative Western blot from three independent experiments showing the abundance of the indicated proteins in extracts of young, aged and HGPS cells. (C) Representative immunofluorescence images from three independent experiments showing nuclear Ran and TPR in HGPS cells as compared to normal cells. Scale bar: 50µm. (D) Quantification of TPR nuclear intensity in the indicated cells. (Mann Whitney test, number of nuclei n>100 from three independent experiments. Error bars indicate s.e.m. *p=0.016). (E) Representative immunofluorescence images from three independent experiments showing localization of NUP153 and TPR in normal fibroblasts (Normal Fibro) and fibroblasts from HGPS patients (HGPS 11513), in the absence (untreated) or presence of the NAT10 inhibitor Remodelin. Scale bar: 50µm. (F) Quantification of NUP153 and TPR nuclear intensity from experiments as shown in (E) (Mann Whitney test, number of nuclei n>70 from three independent experiments. Error bars indicate s.e.m. P values from left to right: *p=0.0111, ***p=0.0002, *p=0.0163, ***p=0.0007). (G) Proximity ligation assay (PLA) showing the interaction of TPR and NUP153 in normal and HGPS fibroblasts in the absence (untreated) or presence of the NAT10 inhibitor Remodelin (quantified in the right panel as the mean number of dots per nucleus; number of nuclei n>50 from three independent experiments). Scale bar: 50µm. (H) and (I) Ran nuclear localisation in HGPS cells upon depletion of NTF2 (siNTF2) or NAT10 (siNAT10) or upon NAT10 inhibition
(Remodelin). Scale bar: 50µm. (Mann Whitney test, number of nuclei n>70 from three independent experiments. Error bars indicate s.e.m. P values from left to right: **p=0.0043, ****p<0.0001, ns: non significant, ****p<0.0001, **p=0.0029).

Figure 2: Disruption of Ran induces chromatin relaxation and senescence. (A) and (B) Analysis of chromatin relaxation by MNase assay in U2OS cells upon TPR depletion (siTPR) or disruption of Ran nuclear import by NTF2 depletion (siNTF2). Scale bar: 30µm. Digested chromatin was analysed on an agarose gel and quantified by using ImageJ. The graph is a representative figure of three independent experiments and represents the relative bands intensity (y axis) of the digested nucleosomes that migrate further from the well (x axis) as the chromatin is more relaxed, and thus more accessible to the MNase enzyme. (C) Representative immunofluorescence images from two independent experiments showing H3K9me3 staining in young (Normal Fibro, passage number (P) 19) or old (Normal Fibro P61) fibroblasts, as well as in HGPS cells, upon transfection with a control (siCT) or NTF2 siRNA (siNTF2) to disrupt the RanGTP gradient. (D) Quantification of H3K9me3 nuclear intensity from images as shown in C (Mann Whitney test, number of nuclei n>50 from two independent experiments. Error bars indicate s.e.m. P values from left to right: *p=0.0159, *p=0.014). (E) Senescence associated β-galactosidase staining (blue cells) upon transfection with a control (siCT) or NTF2 (siNTF2) siRNA in normal (Normal fibro) and HGPS (HGPS 11513) fibroblasts at the indicated passage number (P). (F) Quantification of blue cells from experiments as shown in (C). Number of cells n>200 from three independent experiments. (Mann-Whitney test, P values from left to right: ***p=0.0003, ***p=0.0002. Error bars indicate s.e.m.).
Figure 3: The Transportin-1 pathway is defective in HGPS cells and rescued by NAT10 inhibition. (A) Representative immunofluorescence images from three independent experiments showing the subcellular localization of TNPO1 and its downstream target hnRNPA1 in normal or HGPS fibroblasts in the absence (untreated) or presence of the NAT10 inhibitor Remodelin. Scale bar: 50µm. (B) Zoom-in images from panel (A) showing TNPO1 nuclear vs cytoplasmic subcellular localisation (scale bar: 50µm) as quantified in the bottom panel as nuclear-to-cytoplasmic ratio (N:C). Nuclei are outlined with white dotted lines. Box plots represent median (line), 10-90 percentiles (whiskers) and outliers (dots). (Mann Whitney test, number of nuclei n>50 from three independent experiments. P values from left to right: ****p<0.0001, ***p=0.0003, ****p<0.0001, ns: non significant. Bars represent s.e.m.). (C) and (D). Representative immunofluorescence images from three independent experiments showing TNPO1 subcellular localisation in other HGPS fibroblasts (HGPS 11498) and in cells from normal aged individuals (AG11240 and AG059) (scale bar: 50µm). Overall TNPO1 and hnRNPA1 abundance is observed by western blotting (D) (representative image from three independent experiments).

Figure 4: Microtubule reorganisation modulates TNPO1 localisation and function. (A) Proximity ligation assay (PLA) was performed using anti TNPO1 and alpha-tubulin antibodies. Representative immunofluorescence images from three independent experiments showing TNO1 and alpha-tubulin interaction. Each dot represents an interaction between one molecule of TNPO1 and one molecule of alpha-tubulin. Scale bars: 20µm. (B) Quantification of the PLA assay as shown in (A), representing both the total number of dots per cell (left graph) or the average number of spots in a set area of cytoplasm (Mann Whitney test, number of nuclei n>46 from three independent experiments).
Representative immunofluorescence images from three independent experiments showing the effect of the destabilization (nocodazole) or stabilization (tubacin) of the microtubule network on the nuclear localization of TNPO1 and its downstream target hnRNPA1. Scale bar: 50µm. (D) Quantification of the TNPO1 nuclear-to-cytoplasmic ratio (N:C; top panel) and of hnRNPA1 nuclear intensity (bottom panel). Box plots represent median (line), 10-90 percentiles (whiskers) and outliers (dots). (Mann Whitney test, number of nuclei n>50 from three independent experiments. Top graph p values from left to right: **p=0.0011, ***p=0.0002, ***p=0.0002, ns: non significant. Bottom graph p values from left to right: ***p=0.0002, ***p=0.0001, ***p=0.0001, **p=0.0023, ns: non significant. Bars represent s.e.m.).

Figure 5: TNPO1 is required for cellular normalisations mediated by NAT10 inhibition. (A) and (B) Representative immunofluorescence images from three independent experiments showing Ran and H3K9me3 staining in normal (A) or HGPS fibroblasts (B) after transfection with the indicated siRNA (see Fig. S10 for protein knock down efficiency). Scale bar: 50µm. (C) Quantification of H3K9me3 and Ran nuclear intensity from the cells as shown in A and B. (Unpaired t test, number of cells n>150 from three independent experiments. Error bars indicate s.e.m. P values from left to right: H3K9me3 Normal Fibroblasts: *p=0.0195, **p=0.0087, *p=0.0315, *p=0.0197, **p=0.0064. H3K9me3 HGPS: **p=0.0097, *p=0.03, *p=0.0393. Ran Normal Fibroblasts: *p=0.025, **p=0.0075, *p=0.0443, *p=0.011, *p=0.015. Ran HGPS: *p=0.0342, *p=0.0407, *p=0.0368. (D) Quantification of senescence associated β-galactosidase (SA-β-Gal) staining (blue; E) in cells that had been treated with the indicated siRNAs. (Unpaired t test, number of cells n>200 from three
independent experiments. Error bars indicate s.e.m. P values from left to right: *p=0.0107, *p=0.049, ***p=0.0001, ****p<0.0001, ns: non significant).

**Figure 6: Effects of NAT10 inhibition or depletion on gene expression changes.** (A) Volcano plots from the microarray analysis showing the number of genes whose expression was decreased (blue) or increased (red) under the indicated conditions. The log10 of the adjusted p-value is used such that the more significant a gene is, the higher it will be on the y-axis. The log2 of the fold change is used such that genes with increased or decreased expression have equal weighting on the figure. Using three biological replicates, genes were classed as showing gene expression changes if they were altered by 2-fold (up or down) or greater with an adjusted p value less than or equal to 0.05. For both normal and HGPS fibroblasts, Remodelin was used as a chronic treatment over eight weeks (middle and right panels). (B) Heat-maps of genes presented in (A) showing the effect of Remodelin treatment on gene expression in HGPS cells. (C) Volcano plots from the microarray analysis as presented in (A) and using three biological replicates, showing the number of genes whose expression was decreased (blue) or increased (red) by 1.5-fold or greater under the indicated conditions. NAT10 depletion (siNAT10) was transient (5 days). (D) Heat-maps of genes presented in (C) showing the effect of NAT10 depletion on gene expression in HGPS cells.

**Figure 7: NAT10 modulates gene expression through TNPO1.** (A) Representative Western blotting analysis from 2 independent experiments showing the efficiency of NAT10 (siNAT10) and TNPO1 (siTNPO1) depletion. (B) and (C) Venn diagrams from the microarray analysis showing the overlap between genes whose expression was significantly increased or decreased upon siNAT10 alone or together with siTNPO1
(siNAT10+siTNPO1). (D) Gene ontology analysis showing cellular component terms enrichment (CC) from genes whose expression was significantly decreased (blue) or increased (red) only upon siNAT10 in HGPS cells. (E) Heat-maps of siNAT10 specific genes presented in (C) and (D), showing the gene expression changes for genes modified by siNAT10 only (compared to siCT) in HGPS cells (row 1) and their expression in HGPS cells compared to normal fibroblasts (row 2). (F) Heat-maps of genes whose expression was significantly modified by TNPO1 depletion (siTNPO1) in normal fibroblasts (row 1), and their expression in HGPS cells compared to normal fibroblasts (row 2). TNPO1 is indicated by a *. Red indicates increased gene expression, whilst blue represents decreased gene expression. All these analyses were performed using three biological replicates.

Figure 8: Model for how NAT10 inhibition rescues HGPS phenotypes. See main text for more details. 1) In normal cells, TNPO1 binds to its cargo proteins in the cytoplasm. 2) The TNPO1-cargo protein complex is translocated into the nucleus through the nuclear pore. 3) Once in the nucleus and upon RanGTP binding to TNPO1, the NUP153 cargo dissociates from TNPO1 and is incorporated into the nuclear pore complex, allowing TPR anchorage. Mature, fully functional nuclear pore complex regulates nucleocytoplasmic transport and other NPC-dependent roles. 4) In HGPS and aging, increased microtubule network stability sequesters TNPO1 in the cytoplasm. 5) TNPO1 cargos such as NUP153 are not imported properly into the nucleus. This results in decreased nuclear Ran abundance and defects of TPR anchorage at nuclear pore complexes. This leads to chromatin disorganisation, global gene expression changes and premature entry into senescence. 6) By destabilising the microtubule network, NAT10 inhibition releases TNPO1 from the cytoplasm, enhancing its nuclear...
translocation together with ensuing nuclear import of its cargos such as NUP153 and hnRNPA1. This then substantially normalizes normal chromatin organisation, RanGTP mediated nucleo-cytoplasmic transport and global transcription patterns.
PLA TNPO1 + alpha Tubulin

A

Normal Fibro  HGPS 11513

B

Mean number of spots/cell

Number of spots/set area of cytoplasm

Normal HGPS Fibro  11513

C

DAPI  hnRNPA1  TNPO1  Merge

Normal Fibroblasts

Untreated

Nocodazole

Tubacin

HGPS 11513

Untreated

Nocodazole

Tubacin

D

TNPO1 N:C ratio

Mock treated  Nocodazole  Tubacin

Normal HGPS Fibro  11513

hnRNPA1 mean nuclear intensity (A.U.)

Mock treated  Nocodazole  Tubacin

Normal Fibro  HGPS 11513

***  **  ns
A

HGPS vs Normal Fibroblasts

Normal Fibroblasts + Remodelin

HGPS + Remodelin

B

1: HGPS vs Normal Fibroblasts

2: HGPS + Remodelin vs HGPS NT

C

siCT HGPS vs Normal Fibroblasts

HGPS siNAT10 vs siCT

Normal Fibroblasts siNAT10 vs siCT

D

1: siCT HGPS vs Normal Fibroblasts

2: HGPS siNAT10 vs siCT
SUPPLEMENTARY MATERIALS

NAT10 inhibition normalizes HGPS cells by rebalancing the Transportin-1 nuclear import pathway

Authors: Delphine Larrieu\(^1,2\)*, Emmanuelle Viré\(^1,3\)^\(^\text{P}\), Samuel Robson\(^1,4\)^\(^\text{P}\), Sophia Y. Breusegem\(^2\), Tony Kouzarides\(^1\) and Stephen P. Jackson\(^1\)*

* Correspondence: dl437@cam.ac.uk or s.jackson@gurdon.cam.ac.uk

\(^\text{P}\) These authors contributed equally to the work

Affiliations:

1 The Wellcome Trust/Cancer Research UK Gurdon Institute and Department of Biochemistry, University of Cambridge, CB2 1QN, United Kingdom.

2 Current address: Cambridge Institute for Medical Research, Department of clinical biochemistry, University of Cambridge, CB2 0XY, United Kingdom

3 Current address: MRC Prion Unit at UCL, Institute of Prion Diseases, Queen Square House, Queen Square London, WC1N 3BG, United Kingdom

4 Current address: School of Pharmacy & Biomedical Science, University of Portsmouth, PO1 2UP, United Kingdom

One sentence summary: NAT10 inhibition normalizes HGPS through TNPO1
Figure S1: TPR nuclear localisation is impaired in normal cells from aged individuals and normalised by NAT10 inhibition. (A) Representative immunofluorescence pictures from three independent experiments showing nuclear localization of TPR in the indicated cells in the absence (top) or presence (bottom) of Remodelin. Scale bar: 50µm. (B) Quantification of TPR nuclear intensity from three independent experiments as shown in A (Unpaired t test, bars represent s.e.m. P values from left to right: *p=0.0462, **p=0.0086, *p=0.0485, *p=0.024, *p=0.049).
Figure S2: Nuclear import is affected in HGPS. (A) Representative immunofluorescence images (left) and quantification (right) of NUP153 (green) and TPR (red) staining from three independent experiments in normal fibroblasts or fibroblasts from HGPS patient 11498 (Unpaired t test, P values from left to right: *p=0.0354, *p=0.023) Bars represent s.e.m. (B) Representative immunofluorescence images (left) and quantification (right) of Ran from three independent experiments in normal fibroblasts or fibroblasts from HGPS patient 11498, in untreated cells or upon NAT10 inhibition by Remodelin (Unpaired t test, P values from left to right: **p=0.0092, **p=0.043). Bars represent s.e.m. Scale bars: 50µm.
Figure S3: TNPO1 dependent protein cargos are affected in HGPS cells. (A) Representative immunofluorescence images from three independent experiments showing the subcellular localisation of the NTF2 protein responsible for nuclear import of Ran. Scale bar: 50µm. (B) Representative immunofluorescence images from three independent experiments showing nuclear staining intensity of TNPO1 cargos including NUP153 and the Ewing sarcoma protein EWS in HGPS cells compared to controls. Scale bar: 50µm. (C) Quantification of EWS nuclear intensity in the indicated conditions (Unpaired t test from three independent experiments, bars represent s.e.m. P values from left to right: **p=0.0016, **p=0.0031).
Figure S4: NAT10 nuclear import is not dependent on TNPO1. (A) Representative immunofluorescence images from three independent experiments showing NAT10 (green) and Ran (red) staining in both normal and HGPS fibroblasts. Scale bar: 50µm. (B) Human osteosarcoma U2OS cells were transfected with the plasmid encoding the TNPO1 inhibitor Myc-MBP-M9M (40) (red) to assess the effects on NAT10 nuclear localization (green). Scale bar: 20µm.
**Figure S5: Cell proliferation inhibition does not affect the Transportin-1 pathway.**

(A) Crystal violet staining of non-transformed human RPE-1 cells transfected with control (siCT) or TNPO1 siRNA (siTNPO1). Representative wells from three independent experiments are shown. (B) and (C) RPE-1 cells were either kept in normal or in medium without serum to arrest cell proliferation. Representative immunofluorescence images from three independent experiments showing staining of TNPO1 and its downstream cargo hnRNPA1 (B) as well as Ran and the heterochromatin marker H3K9me3 (C). (D) Normal fibroblasts were either kept in normal or in medium without serum to arrest cell proliferation. Representative immunofluorescence images from two independent experiments showing staining of TNPO1 and its downstream cargo hnRNPA1. Scale bars: 30µm.
Figure S6: NAT10 localises both in the nucleus and in the cytoskeleton. (A) Representative western blotting from two independent subcellular fractionations showing the abundance of the indicated proteins in the chromatin and cytoskeletal fractions of normal fibroblasts and HGPS cells in the absence (non treated, NT) or presence of Remodelin. (B) Representative immunofluorescence images from three independent experiments showing the subcellular localisation of NAT10 upon low (middle image) or high exposure (right image). Scale bar: 10µm.
Figure S7: TNPO1 accumulates at the microtubule network of HGPS cells. (A) Representative immunofluorescence images from three independent experiments showing the signal coming from the anti-TNPO1 (green) and anti-tubulin (red) antibodies used in the PLA assay. Scale bars: 50µm. (B) Representative Western blotting from three independent experiments showing the global abundance of the indicated proteins in normal vs HGPS cells. (C) Immunoprecipitation of TNPO1 in normal fibroblasts.
Figure S8: TNPO1 pathway is defective in HGPS cells and in normal cells from aged individuals, and this is rescued by microtubule destabilization. Representative immunofluorescence images (left) and quantification (right) from three independent experiments showing nuclear staining intensity of TNPO1 (red) and hnRNPA1 (green) in HGPS (HGPS11498) and in normal cells from aged individual (AG04059) treated with the indicated molecules. Scale bar: 50µm. (Unpaired t test, P values from left to right: ***p=0.0001, **p=0.0069, **p=0.0081, *p=0.0128, *p=0.0420, *p=0.0253. Bars represent s.e.m.).
**Figure S9: NUP153 depletion does not affect the Transportin-1 pathway.** Normal Fibroblasts were transfected with the indicated siRNA before being stained for hnRNPA1 (green) and TNPO1 (red). Representative immunofluorescence images (left) from two independent experiments showing the effects of TNPO1 (siTNPO1) or NUP153 depletion (siNUP153) on the nuclear staining intensity of hnRNPA1. Scale bar: 50 µm. Right: Quantification of hnRNPA1 nuclear intensity from two independent experiments in the indicated siRNA (Unpaired t test, P value: ***p=0.0004. Bars represent s.e.m.).
Figure S10: Efficiency of protein knock-down. The efficiency of the indicated siRNA was assessed by staining for the corresponding proteins. Representatives immunofluorescence images from three independent experiments are shown. Scale bar: 50 μm.