A Flow Cytometry-Based Screen of Nuclear Envelope Transmembrane Proteins Identifies NET4/Tmem53 as Involved in Stress-Dependent Cell Cycle Withdrawal

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Abstract
Disruption of cell cycle regulation is one mechanism proposed for how nuclear envelope protein mutation can cause disease. Thus far only a few nuclear envelope proteins have been tested/found to affect cell cycle progression: to identify others, 39 novel nuclear envelope transmembrane proteins were screened for their ability to alter flow cytometry cell cycle/DNA content profiles when exogenously expressed. Eight had notable effects with seven increasing and one decreasing the 4N:2N ratio. We subsequently focused on NET4/Tmem53 that lost its effects in p53−/− cells and retinoblastoma protein-deficient cells. NET4/TMEM53 knockdown by siRNA altered flow cytometry cell cycle/DNA content profiles in a similar way as overexpression. NET4/TMEM53 knockdown did not affect total retinoblastoma protein levels, unlike nuclear envelope-associated proteins Lamin A and LAP2α. However, a decrease in phosphorylated retinoblastoma protein was observed along with a doubling of p53 levels and a 7-fold increase in p21. Consequently cells withdrew from the cell cycle, which was confirmed in MRC5 cells by a drop in the percentage of cells expressing Ki-67 antigen and an increase in the number of cells stained for β-galactosidase. The β-galactosidase upregulation suggests that cells become prematurely senescent. Finally, the changes in retinoblastoma protein, p53, and p21 resulting from loss of NET4/Tmem53 were dependent upon active p38 MAP kinase. The finding that roughly a fifth of nuclear envelope transmembrane proteins screened yielded alterations in flow cytometry cell cycle/DNA content profiles suggests a much greater influence of the nuclear envelope on the cell cycle than is widely held.

Introduction
Several proteins of the nuclear envelope are linked to human diseases ranging from muscular dystrophies to neuropathy, bone diseases, and progeroid aging syndromes [1,2]. These proteins include the intermediate filament A/C Lamin and several proteins integral to the nuclear membrane. Favorable molecular mechanisms to explain how mutations in nuclear envelope proteins produce pathology include loss of nuclear mechanical stability, alterations in gene expression, and cell cycle/stem cell maintenance defects (reviewed in [2,3,4]). However, the known functions of the proteins mutated in disease are insufficient to fully explain the pathologies observed without assistance from partner proteins that thus far have not been identified.

The first indication of a link between nuclear envelope diseases and the cell cycle came from studies with specific mutations in the nuclear envelope transmembrane protein (NET) Emerin linked to Emery-Dreifuss muscular dystrophy. It was reported that two disease-linked mutations prolonged S-phase from 12 h to 22 h when overexpressed in COS-7 cells [5]; however similar effects were not observed in all disease mutants and so this was not investigated in further detail. In C. elegans disruption of Emerin alone did not have a strong effect on the cell cycle, but when combined with disruption of a second NET, MAN1, it did [6]. Loss of Emerin has also been reported to interfere with retinoblastoma protein (pRb)-regulated genes in mouse and consequently with myogenic differentiation [7], and the same pRb-dependent cell cycle exit is disrupted in nuclear envelope-linked muscular dystrophy [8]. pRb is a tumor suppressor that regulates the cell cycle at the G1/S transition by regulating the E2F family of transcription factors [reviewed in [9]]. pRb also interacts with Lamin A [10], but this is thought to principally involve the nucleoplasmic and not the nuclear envelope pool of Lamin A because it operates in a complex with LAP2a, a soluble splice variant of the nuclear envelope protein LAP2 that is principally found in the nucleoplasm [11,12,13].

To determine if any of several newly identified nuclear envelope proteins play a role in the cell cycle, 39 novel confirmed NETs were screened for their ability to alter flow cytometry cell cycle/DNA content profiles when exogenously expressed. These NETs...
were identified in two recent proteomic analyses of liver and blood cells [14,15]. Seven of the NETs tested showed an increase in the 4N:2N ratio while one showed a decrease. To determine if pathways affected by these NETs involved the p53 master cell cycle regulator, these eight NETs were retested in p53−/− cells. The change in 4N:2N ratios still occurred in the absence of p53 for most NETs, but the effect of NET4/Tmem53 and NET39/Nchn was lost. NET4/Tmem53 was selected for a more detailed analysis of how it interacts with the p53 pathway. Knockdown of NET4/Tmem53 caused cell cycle withdrawal, apparently through a change in p53 protein levels, and significant loss of phosphorylated pRb in cells expressing NET4/Tmem53. However, the change in 4N:2N ratios still occurred in the absence of p53 for NET4/Tmem53, suggesting that some NETs that caused minor changes might also be p53 dependent. To better compare the results in the p53 positive and negative cells, the 4N:2N ratios from both cell lines were plotted for this set of NETs (Figure 3B). For NETs other than the two that lost their effects a similar pattern was observed between the two cell lines, although in some cases the 4N:2N ratio increase was slightly higher in the H116 p53−/− cell line. NET4/Tmem53, a previously uncharacterized protein with no known functional domains, was subsequently followed in more detail.

Some NETs depend on p53 and/or pRb for their effects

The cell cycle protein p53 is often referred to as a master regulator because it has a role in a large number of cell cycle pathways. The ability of the eight NETs identified in the initial screen to alter the flow cytometry DNA content profiles was re-examined in the HCT116 p53−/− cell line. The controls mRFP and NET51/C14orf1 had not yielded changes in the flow cytometry DNA content profile in the HEK293T cells and similarly yielded no significant changes in the p53−/− cells (Figure 3A). Correspondingly, NET11/Scepdh, NET31/Tmem209, Tmub1, Fam5c, Magt1, and Tmem126a that had exhibited increases in the 4N population in the HEK293T cells yielded similar changes in the p53−/− cells; so p53 does not appear to be involved in the potential effects of these NETs on the cell cycle. By contrast, in the p53−/− cells NET59/Nchn no longer exhibited a 4N increase and NET4/Tmem53 no longer exhibited a 4N decrease as had been observed in the HEK293T cells. Thus changes in the flow cytometry profiles from expression of NET59/Nchn and NET4/Tmem53 appear to be p53 dependent. To test if phosphorylated pRb levels were affected in the NET4/Tmem53 transfected cells. By contrast, in the p53−/− cells NET59/Nchn no longer exhibited a 4N increase and NET4/Tmem53 no longer exhibited a 4N decrease as had been observed in the HEK293T cells. Thus changes in the flow cytometry profiles from expression of NET59/Nchn and NET4/Tmem53 appear to be p53 dependent. To better compare the results in the p53 positive and negative cells, the 4N:2N ratios from both cell lines were plotted for this set of NETs (Figure 3B). For NETs other than the two that lost their effects a similar pattern was observed between the two cell lines, although in some cases the 4N:2N ratio increase was slightly higher in the H116 p53−/− cell line. NET4/Tmem53, a previously uncharacterized protein with no known functional domains, was subsequently followed in more detail.

Cell cycle effects dependent on p53 often involve changes in pRb [17] and links have previously been identified between pRb and nuclear envelope-associated proteins [10,11,12,13]. To determine if pRb also plays a role in the NET4/Tmem53-directed effects, flow cytometry cell cycle/DNA content profiles were determined for cells expressing NET4/Tmem53 or controls in HEK293T cells with normal or reduced levels of pRb, using siRNA oligos to knock down pRb. The reduction in the 4N:2N ratio caused by NET4/Tmem53 was lost in the pRb-depleted cells (Figure 4A). As pRb phosphorylation is crucial for its role in cell cycle progression [18,19,20], antibodies to a form of pRb phosphorylated at serine 780 were utilized to test if phosphorylated pRb levels were affected in the NET4/Tmem53 transfected cells. Because of low and variable transfection efficiencies this could not be assayed at a population level, thus transfected cells were stained with the antibodies and the levels of the phosphorylated pRb in the nucleoplasm were quantified by measuring the average pixel intensity (Figure 4B). Plotting these values revealed a significant loss of phosphorylated pRb in cells expressing NET4/Tmem53.

RNAi knockdown of NET4/Tmem53

Further study of the pathways through which NET4/Tmem53 affects cell cycle regulation would not be practical using exogenous expression because transfection efficiencies were too low (5−10%) to be able to quantify changes in pathway components by Western...
blot. The p53, p38 and p21 antibodies used in subsequent assays were tested by immunocytochemistry, but proved inadequate for quantification due both to a diffuse distribution throughout the cell body and cell-to-cell variation in intensities that appears to result from induction of stress pathways in some cells during transfection (data not shown). Therefore, to further elucidate the pathways through which NET4/Tmem53 affects the cell cycle, its knockdown was attempted.

Figure 1. Changes in flow cytometry cell cycle profiles for cells overexpressing NETs. HEK293T cells expressing mRFP-NET fusions were recovered by trypsinization and analyzed by flow cytometry at 48 h post-transfection. Data were analyzed using FlowJo software and histogram overlays are displayed as %Max, scaling each curve to mode = 100%. The red line is the mRFP expressing cells in the population while the blue line is the untransfected cells in the population (the majority of cells were not transfected). The transfected and untransfected populations were both set on the scale to 100 for the 2N population so that increases or decreases in the 4N peak reveal changes in the cell distribution. Arrows indicate significant changes in cell cycle profile between transfected and non-transfected cells.

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There are both long and short splice variants of NET4/TMEM53 (Figure 5A). To determine whether it would be better to design siRNA oligos to knock down one or both (the original screen used the shorter variant), long and short splice variants of NET4/TMEM53 with the GFP moiety at either terminus were cloned and then tested to determine if both could produce the flow cytometry cell cycle/DNA content ratio effect of the original mRFP construct. All constructs yielded the same effect (Figure 5B). Multiple siRNA oligos were then generated that should knock down both long and short splice variants. Although the HEK293T cells were ideal for the flow cytometry-based screen due to their generally high transfection efficiencies and comparatively stable DNA content, for subsequent more directed cell cycle experiments MRC5 primary fibroblasts [21] and the U2OS osteosarcoma cell line were used because they have comparatively more operational checkpoint machinery. Moreover, the primary fibroblasts can enter a state of senescence that is not possible for HEK293T cells.

Before proceeding, the ability of NET4/Tmem53-mRFP expression to alter flow cytometry DNA content profiles was evaluated in both MRC5 primary fibroblasts and the U2OS cells, confirming that a comparable effect to that observed in HEK293T cells occurred in the MRC5 and U2OS cells (Figure 5C).

Two siRNA oligos (si1 and si2) that should each in theory knock down both long and short splice variants (Figure 6A) effectively knocked down NET4/TMEM53 transcripts in MRC5 cells (Figure 6B). The si2 was slightly more effective than the si1 and therefore initially used in preference, though all relevant findings were subsequently verified with both oligos. As NET4/TMEM53 is an uncharacterized gene with no direct evidence for its full range of splicing possibilities, it is possible that additional splice variants exist that the two siRNA oligos do not knock down. Therefore, an esiRNA that should knock down any and all possible splice variants of NET4/Tmem53 was also tested and found to be effective in reducing NET4/TMEM53 transcripts in MRC5 cells (Figure 6B). Knockdown of NET4/TMEM53 transcripts was also successful in U2OS cells (Figure 6C).

Available NET4/Tmem53 antibodies could not be used to determine if the NET4/Tmem53 protein was also being knocked down because they recognized several closely migrating bands where NET4/Tmem53 protein is expected to migrate on Western blot. Nonetheless, the endogenous protein is very likely to be reduced because cells expressing NET4/Tmem53-GFP prior to addition of siRNA oligos for NET4/TMEM53 knockdown exhibited a loss of the fusion protein with both GFP and NET4/Tmem53 antibodies (Figure 6D and data not shown). This indirect approach typically indicates knockdown of the endogenous protein [22].

The effect of NET4/TMEM53 knockdown on flow cytometry cell cycle/DNA content profiles was next determined. An increase was observed in the population of cells with a 2N amount of DNA that was similar to the increase resulting from exogenous NET4/Tmem53 expression (Tables 1 and 2). A corresponding reduction was observed in the population of cells with 4N DNA content both with the knockdown and with overexpression. Both MRC5 and U2OS cell lines were subsequently used in parallel for most experiments to ascertain if any observed effects required the more reliable cell cycle checkpoints of primary cells (MRC5) and thus were not recapitulated in transformed cell lines such as the U2OS.

**Table 1. Percentage of cells in each cell cycle phase by flow cytometry upon exogenous expression of NET-mRFP fusions.**

| NET        | mRFP negative | mRFP positive |
|------------|---------------|---------------|
|            | % G1 | % S | % G2/M | % G1 | % S | % G2/M |
| untransfected | 60  | 14 | 21   | -   | -   | -      |
| mRFP        | 61  | 14 | 20   | 62  | 13 | 20     |
| NET4/Tmem53 | 62  | 14 | 20   | 74  | 8  | 14     |
| NET11/Sccpdh| 60  | 14 | 20   | 37  | 18 | 38     |
| NET31/Tmem209| 58  | 15 | 21   | 39  | 15 | 38     |
| NET51/C14orf1| 62  | 16 | 18   | 60  | 17 | 19     |
| NET59/Nichn | 59  | 15 | 22   | 37  | 19 | 35     |
| Fam3c       | 62  | 10 | 18   | 44  | 10 | 30     |
| Magt1       | 65  | 12 | 19   | 53  | 14 | 26     |
| Tmub1       | 63  | 13 | 19   | 50  | 14 | 31     |
| Tmem126a    | 65  | 13 | 14   | 53  | 17 | 26     |

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**Figure 2. The changes in flow cytometry profile of cells expressing NETs are not caused by aberrant nuclear morphology of cells.** Images of HEK293T cells expressing NET-mRFP shows nuclear morphology depicted by DAPI staining (upper panel) compared to mRFP signal (lower panel) indicating cells expressing NETs. doi:10.1371/journal.pone.0018762.g002
line that has stable p53- and pRb-dependent checkpoints but is defective for p16INK [23,24].

NET4/TMEM53 knockdown causes premature senescence in primary fibroblasts but only a cell cycle delay in the transformed U2OS cells

When MRC-5 cells were transfected with siRNA oligos or esiRNA for NET4/TMEM53 the number of cells per dish appeared to be lower than in cultures transfected with the scrambled control siRNA oligo, yet there did not seem to be an increase in apoptotic cells. In order to establish the cause of this difference, cell proliferation was assessed using antibodies to the nuclear protein Ki-67 that is present only in proliferating cells [25,26]. In cells knocked down for NET4/TMEM53 (Figure 7A, upper graph) there was a notable decrease in the frequency of Ki-67 positive cells from 57.6% in the control scrambled oligo transfected cells to 26.9% for the NET4/TMEM53 si2 transfected cells and 10.9% for the NET4/TMEM53 si1 transfected cells. The esiRNA also confirmed the phenotype (39.6% Ki-67 positive cells), further indicating its specificity to the NET4/Tmem53 knockdown.

The marked reduction in cell proliferation upon NET4/TMEM53 knockdown could indicate a permanent arrest or a temporary arrest from which cells could subsequently recover. ß-
galactosidase activity, a characteristic feature of senescent cells [27], was therefore assessed in MRC5 primary fibroblasts knocked down for NET4/TMEM53. At 72 h post transfection, the percentage of β-galactosidase positive cells was 3 times higher in cell cultures transfected with NET4/TMEM53 siRNA oligos or esiRNA as compared to untransfected cultures or those transfected with the control scramble oligo (Figure 7A, lower graph). This suggests a tendency of NET4/Tmem53 deficient MRC-5 cells to withdraw from the cell cycle due to premature senescence, which is a programmed cell response to many extra- and intracellular stresses with features similar to proliferative senescence and leading to permanent cell cycle exit.

By contrast, when NET4/TMEM53 siRNA oligo si2 was transfected into the transformed osteosarcoma U2OS cell line no decrease of Ki-67 expression or increase of β-galactosidase was detected (data not shown), indicating that these cells do not fully exit the cell cycle. This difference is likely due to the absence in U2OS cells of p16INK, as this kinase has been reported to be important for establishment of senescence [23,24]. Confirming these reports, transcript levels of p16INK were undetectable in

Table 2. Percentage of cells in each cell cycle phase by flow cytometry for knockdown of NET4/TMEM53 and controls in U2OS cells.

|          | %G1 | %S | %G2/M |
|----------|-----|----|-------|
| untransfected | 58  | 10 | 32    |
| scramble   | 59  | 11 | 30    |
| si2        | 65  | 12 | 22    |

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U2OS cells and strongly upregulated in senescent MRC5 cells (Figure 7B). Knockdown of NET4/TMEM53 also induced p16INK expression in MRC5 cells, albeit less so than by full senescence. doi:10.1371/journal.pone.0018762.g007

NET4/TMEM53 knockdown results in alteration of levels of several cell cycle regulators

To further understand the pathways mediating the effect of NET4/Tmem53 on the cell cycle, levels of various cell cycle regulators before and after NET4/Tmem53 depletion were quantified by Western blot. In both MRC5 and U2OS cells the p53 protein level was increased ~2-fold upon loss of NET4/Tmem53 while p21 levels increased ~7-fold (Figure 8A,B). Total levels of pRb remained relatively unchanged, but pRb became hypophosphorylated when NET4/Tmem53 was knocked down and phosphorylated p38MAP kinase levels increased (Figure 8A,B). It should be noted that although total pRB levels were unchanged on average over several experiments with NET4/TMEM53 knockdown, there was reasonable variability in the levels of total pRB among experiments. Thus the ratio of phosphorylated pRB to total pRB was likely also somewhat variable, but levels of the phosphorylated pRB were notably reduced in all experiments. To confirm that the effect observed was specific for NET4/TMEM53 knockdown the experiment was repeated using oligos si1, si2, and the esiRNA. The same result was observed with p53 and p21 increasing upon loss of NET4/Tmem53 (Figure 8C).

By contrast no changes were observed in levels for other NE proteins previously shown to contribute to cell cycle regulation Emerin, LAP2 (both soluble and transmembrane splice variants alpha and beta), or Lamin A (Figure 9A). Neither were differences observed in cyclins A, C, D, or E (Figure 9A,B). Cyclin B levels may have been slightly reduced; however, considerable variability was observed between all experiments and so no clear conclusion could be drawn (data not shown).

NET4/Tmem53 effects on cell cycle protein levels are mediated by the p38MAP kinase

The p38MAP kinase that was upregulated by NET4/TMEM53 knockdown mediates cell response to range of stresses, such as UV irradiation, osmotic shock, heat shock, starvation and cytokines [28]. p38 phosphorylates and stabilizes p33, which activates transcription of the cdk inhibitor p21 that subsequently blocks the cell cycle [17]. As this study has shown that p21 is also upregulated by NET4/TMEM53 knockdown, it seemed likely that the effects on cell cycle proteins p53, pRB and p21 are mediated by the p38MAP kinase.

To further test if the increases in p53 and p21 were dependent on p38MAP kinase, a specific p38 inhibitor SB203580 (Calbio-
chem) was added to the cultures 24 h after transfection with the siRNA oligos. The cells were harvested 48 h later and levels of p53, p21, and phosphorylated p38 measured by Western blot. In all cases the increases resulting from knockdown of NET4/TMEM53 were lost when p38MAP kinase was inhibited (Figure 10A,B). Moreover, the NET4/TMEM53 knockdown-mediated decrease in Ki-67 positive cells was partially abrogated and the increase in ß-galactosidase staining cells was completely abrogated (Figure 10C,D). Thus, p38MAP kinase appears to be activated by NET4/TMEM53 knockdown and mediates most of the NET4/Tmem53 effects on the cell cycle.

Figure 8. NET4/TMEM53 knockdown alters levels of several cell cycle proteins. Protein lysates were recovered from both MRC5 and U2OS cells that were either untransfected, transfected with a scrambled siRNA, or transfected with the NET4/TMEM53 si2 oligo. (A) Western blots reacted with antibodies to pRb phosphorylated on serine 780, total pRb, p53, phosphorylated p38MAP kinase, p21, and actin as a loading control. Note that some variation in total pRb levels was observed between experiments, causing the ratio of phosphorylated pRb to total pRb to be also somewhat variable. Because the phosphorylated form migrates slightly differently on SDS-PAGE the change in the spread of the band gives a greater appearance of such variability than is actually measured upon quantification. (B) Quantification of levels of each protein normalized to actin using a LI-COR Odyssey and fluorescent secondary antibodies. Averages from three experiments are shown with standard errors. (C) To ensure that results reflected effects of NET4/TMEM53 knockdown and not off-target effects both siRNA oligos and the esiRNA were tested for effects on p53 and p21 levels. Similar results were observed with all NET4/TMEM53 knockdowns. doi:10.1371/journal.pone.0018762.g008

Figure 9. NET4/TMEM53 knockdown did not affect levels of other nuclear envelope proteins linked to the cell cycle or cyclins. (A) Protein lysates were recovered from U2OS cells treated as in Figure 8 and reacted on Western blots with antibodies to the various proteins. This experiment was repeated 3 times and a representative Western blot is shown. (B) Cyclin levels were quantified from three separate experiments analyzed by LI-COR using fluorescent secondary antibodies and are plotted normalized to the actin control. Standard errors are shown. doi:10.1371/journal.pone.0018762.g009
Discussion

This flow cytometry-based screen identified several NETs with likely cell cycle functions. These NETs are generally uncharacterized proteins, many of which have conserved domains of unknown function. However, NET59/Ncln has separately been linked to a pathway that could intersect with cell cycle regulation: this NET interacts with Nomol1 that binds Smad proteins and thus intersects with TGFβ signaling pathways [29]. The NET MAN1 has also been found to interact with Smad proteins [30,31] while the NET Emerin interacts with β-catenin [32] and disruption of these two NETs yields cell cycle defects [6]. Thus, combined signaling/cell cycle effects appear to be a common trait among NETs and it is likely that further analysis of the other NETs identified in this screen will yield some similar intersections of nuclear envelope proteins with cell cycle pathways. That these 8 NETs represent roughly 20% of the proteins tested in the flow cytometry cell cycle/DNA content screen and that several recent studies have found NETs associated with centrosomes or the mitotic spindle [33,34,35] suggest that a great many nuclear envelope proteins will be found to contribute to cell cycle regulation.

Here, additionally, NET4/Tmem53 has been demonstrated to influence cell cycle regulation, affecting maintenance of the proliferative state of cells. Although the mechanism by which NET4/Tmem53 expression levels activate p38MAP kinase has thus far been elusive, it is clear that the effect of NET4/Tmem53 on proliferation is through p38MAP kinase-dependent upregulation of p53 and p21 with subsequent reduction in levels of phosphorylated pRb. Thus unlike Lamin A and LAP2α that exert effects directly on pRb to influence cell proliferation [11,12,13], NET4/Tmem53 acts upstream through stress signaling pathways.

NET4/Tmem53 was originally identified in a proteomic analysis of liver nuclear envelopes [14] and high-resolution microscopy on tagged exogenously expressed protein suggests that it resides in the outer nuclear membrane [16]. Antibodies generated to NET4/Tmem53 were previously shown to principally stain the nuclear periphery in liver tissue cryosections and nuclear envelope versus microsomal fractions isolated from rodent liver [16]; however, despite reactivity on tissue sections, these antibodies recognize several additional bands at the expected molecular weight of NET4/Tmem53 in cultured cells and so could not be exploited in the current study. The indication of NET4/Tmem53 as an outer nuclear membrane protein is not inconsistent with NE functions on the cell cycle. For example, Emerin, originally thought to reside only in the inner nuclear membrane [36], affects signaling pathways and has recently been shown to function also in the outer nuclear membrane and ER [37].

A potential functional relevance for NET4/Tmem53-induced cell cycle regulation is suggested by observations that NET4/Tmem53 is highly expressed in liver cells and adipocytes according to the BioGPS transcriptome database [38,39]. Recent studies have shown that the differentiations of mesenchymal stem cells (MSCs) is influenced by mechanical strain. In response to the application of physical strain MSCs are prevented from differentiating along an adipogenic lineage and instead are redirected towards an osteoblast lineage [40]. This gives rise to the possibility the physical stress provides a homeostatic mechanism for mesenchymal lineage selection. Since it has already been shown the NE plays a key role in propagating stress signaling and sensing [41], it is important to understand which NE proteins interact with stress signaling pathways and how they might influence stem cell self renewal and differentiation. We believe that our data suggests that NET4/Tmem53 has such a role. Future studies will focus on putative regulation of adipogenic stem cells by NET4 and its possible involvement in Laminopathies such as Dunnigan-type familial partial lipodystrophy (FPLD; [42,43]) or mandibuloacral dysplasia (MADA; [44]).

Methods

Plasmid construction

NET expressing plasmids were generated from IMAGE clones as previously described [14,15,16]. All were under regulation of the CMV promoter with their carboxyl-termini fused to monomeric red fluorescent protein (mRFP).

Cell culture and transient transfection

HEK293T (from ATCC; [45]), U2OS (from ATCC; [24]), MRC5 (from the EUACC; [21]) and HCT116 p53−/− (kind gift of B. Vogelstein, Johns Hopkins University; [46]) cells were maintained in high glucose DMEM (Invitrogen) supplemented
with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin sulfate (Invitrogen). For U2OS siRNA assays cells were seeded at 30% confluency and the next day transfected using Dharmafect transfection reagent 1 according to the manufacturer recommendations (Dharmacon RNA Technologies). MRC5 cells were trypsinized at ~50% confluency and 3×10⁶ cells were aliquoted for nucleofection using Kit R, Program V-020 (Lonza). The cells were transfected with either 8 μg of siRNA oligo or 12 μg of esiRNA (esiRNA human TMEM53, Sigma) and plated to 10 cm dishes with several coverslips. At 48 or 72 h coverslips were removed and fixed for immunocytochemistry and the remaining cells on the plate were harvested by trypsinization and lysed in sample buffer for SDS-PAGE and Western blotting. Alternatively they were lysed in Trizol reagent (Sigma) for isolation of RNA. HEK293T cells were either nucleofected using Kit V Program Q-001 (Lonza) or were transfected using Fugene 6 or HD (Roche). For microscopy, cells were generally plated on cover slips in 24 well dishes after nucleofection or were first plated and then transfected using Fugene. In this case, plating was at ~10% confluency so that cells did not reach confluency before 30 h post-transfection when they were fixed. Then, roughly 12 h after plating, DNA was transfected using Fugene. For p38MAP kinase inhibitor experiments the specific inhibitor SB203580 (Calbiochem) was added to cultures at 10 μM concentration 12 h after transfection with siRNA oligos.

Cell cycle assay
Plasmids encoding different NETs fused to mRFP were transfected into HEK293T cells using Fugene 6 transfection reagent (Roche). At 48 h post-transfection, the DNA stain Hoechst 33342 (Molecular Probes) was added to the cells at a final concentration of 5 μg/ml and left to incubate at 37°C for a period of 30 min to 60 min. Cells were harvested by trypsinization, trypsin was inactivated with serum and cell pellets were collected by centrifugation at 250×g for 5 min at RT, washed once in PBS and resuspended in 1 ml of PBS. Cells were immediately analyzed on an LSR II flow cytometer (BD Bioscience, UK) equipped with 488 nm and 350 nm lasers and appropriate filters. Cells with fragmented DNA that might be undergoing necrosis or apoptosis and cell aggregates were excluded from analysis by application of electronic gates. Cell cycle analysis was carried out on the live singlets gate using FlowJo software (TreeStar, Inc.). At least 10,000 cells were scored for the total live singlets and 1,000 cells for the mRFP positive live singlets. Each experiment was repeated at least 2 times for negative results and 3 times for those with a positive effect on the cell cycle. For further confirmation NETs that showed an effect on the cell cycle in 3 independent experiments (NET4/Tmem53, NET11/Scppdh, NET31/Tmem209, NET59/Ncln, Tmub1, Fam3c, Magt1 and Tmem126a) were repeated a 2nd time where at least 20,000 mRFP positive intact singlets were counted. Data are displayed in the form of histogram overlays using %Max option, which scales each population curve to mode = 100%.

Antibodies
Antibodies to the following proteins were used: Ki-67 (610968, BD Transduction Lab), total Rb (4H1 9309, Cell signaling), phospho-Rb (9307, Cell Signaling), p21 (556430, BD Transduction lab), p53 rabbit (9282, Cell signaling), p53 mouse (NCL-p53-DO1, Leica), p38 total (9212, Cell Signaling), active p38 (V3281 Anti-active MAPK Family Sampler, Promega), cyclin E mAb clone HE12 (32-1600, Invitrogen), cyclin A mAb clone Cy-A1 (4710, Sigma), cyclin D (2922, Cell Signaling), cyclin B (SC245, Santa Cruz), LAP-2 (60-1002, Millipore), Lap2α previously described in [47], Lamin A and B1 (3262 and 3931) previously described in [48]. All fluorophore-conjugated secondary antibodies used for immunofluorescence were minimally cross-reactive from donkey (Jackson ImmunoResearch) or goat (Molecular Probes). For Western blotting IR800 conjugated goat anti-rabbit antibodies (LI-COR Biosciences) were used.

Immunofluorescence microscopy
Cells transfected with NETs were fixed for 7 min in 3.7% formaldehyde, permeabilized for 6 min in 0.1% Triton X-100, blocked with 3% BSA in PBS, and reacted for 40 min at RT with antibodies to Ki-67 or phospho-Rb. After washing, appropriate fluorophore-conjugated secondary antibodies were added for 30 min at RT and washed. Cells were also stained with Hoechst 33342 (Molecular Probes) to visualize nuclei and mounted in fluoromount G (EM Sciences). For β-galactosidase assays, histochemical staining at pH 6.0 was performed as described in [27].

Images were obtained using a Nikon TE-2000 microscope equipped with a 1.43 NA 100× objective, Sedat quad filter set, PIFOC Z-axis focus drive (Physik Instruments), and CoolSnapHQ High Speed Monochrome CCD camera (Photometrics) run by IPLab image acquisition software. Micrographs were saved from source programs as .tif files and prepared for figures using Photoshop 8.0.

Quantification of grey levels for NETs was performed using.tif images from IPLab imported into Image Pro Plus. Cells fixed and stained with phospho-Rb antibodies were imaged using identical settings. Average grey scale values [pixel] intensity were measured for at least 40 mRFP positive cells and as many untransfected cells on the same coverslips.

Quantitative Western blotting
Cells were scraped and lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 0.2% NP-40 in the presence of protease inhibitor cocktail (Roche 11 873 580 001) by heating at 65°C for 2 min and sonication in a sonibath at 4°C. Protein concentrations were determined using the Bradford Method (BioRad). An equal volume of protein sample buffer (100 mM Tris pH 6.8, 4 mM Urea, 2% SDS, 50 mM DTT and 15% sucrose) was added and the samples were boiled at 100°C for 5 min then sonicated in a sonibath with high frequency for 10 min with 30 sec interval on/off. Equal amounts of protein were resolved by SDS-PAGE and transferred to Nitrocellulose membrane (LI-COR Biosciences). Membranes were blocked in PBS, 5% milk, 0.2% tween-20. Primary antibodies were diluted in this buffer and allowed to incubate overnight at 4°C. Secondary antibodies IR800 conjugated goat anti-rabbit (LI-COR Biosciences) were added at concentration 1/5000 at RT for 2 h. Visualization and quantification were performed using a LI-COR Odyssey and software (Odyssey 3.0.16) using median background subtraction. A minimum of three independent blots was run for each NET and control. The averages from all three are presented in figures with standard error shown.

siRNA oligos
NET4/TMEM53 oligo si1: 5’-AGAAGUGGGUGUGGAGG-GAGG-3’, NET4/TMEM53 oligo si2: 5’-UAGUAAGUGG-GUAGUCAGG-3’. Scramble control oligo 5’-UCGAAGU- AUUCCGGGUACG-3’. Sigma NET4/TMEM53 esiRNA EH-U027971.

pRb siRNA oligo: GGCUCUUAAGUUUCGCUAG [49].
RT-PCR analysis

Cells were lysed on tissue culture plates with Tri-Reagent (Sigma), and total RNA was extracted according to the manufacturers' instructions. RT-PCR reactions were carried out with 100 ng of total RNA using the Titan one tube RT-PCR system (Roche) in accordance with the manufacturer's instructions, except that the dNTP concentration was increased to 500 mM and MgCl₂ increased to 3 mM. Typical reaction conditions were 30 min reverse transcription at 50°C; 2 min denaturation at 94°C; then 24 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 45 s. Pepsidylprolyl isomerase A (PPIA) was used as a loading control and reactions were repeated at least three times.

The human primer sets used were: NET4/TMEM53 5’-AAGCTGCTCGACCTGCTC3’-3’ and 5’-CAGAGGGCTGTT-GTATGAA-3’; PPIA 5’-CACCGTGTTTCTGACATGG-3’ and 5’-TCGAGTGTGTCGACAGTCGACG-3’.

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Author Contributions

Conceived and designed the experiments: NK VS CJH MV ECS. Performed the experiments: NK VS MW DGB. Analyzed the data: NK VS CJH MV. Contributed reagents/materials/analysis tools: CJH VP MV. Wrote the paper: ECS.

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