RNAi of FACE1 protease results in growth inhibition of human cells expressing lamin A: implications for Hutchinson-Gilford progeria syndrome

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Summary

FACE 1 is the endoprotease responsible for cleavage of prelamin A to lamin A. Transfection of HeLa cells with siRNA for human FACE 1 results in a strong phenotype. Protein and mRNA levels for FACE 1 are knocked down and cell division stops abruptly. Two populations of cells are detected. The first form aberrant mitotic spindles, arrest in mitosis and later enter apoptosis. The second show dramatic changes in nuclear morphology with extensive formation of lobulated nuclei and micronuclei. Using antibodies that specifically recognise prelamin A, but not lamin A, we show that prelamin A accumulates at the nuclear lamina in FACE1 silenced cells, whereas in control cells prelamin A is found in many small nuclear dots, but not at the nuclear lamina. In double knockdown experiments with FACE 1 and lamin A siRNAs, the results depend on which protein is knocked down first. FACE1 knockdown 24 hours prior to lamin A knockdown gives results similar to the single FACE1 knockdown. By contrast, lamin A knockdown 24 hours prior to FACE1 knockdown results in none of the changes described above. Silencing of FACE1 in HL60, a cell line that lacks lamin A, also has no effect. The combined results suggest that prelamin A is a poison in cells subjected to FACE 1 knockdown. Finally, we draw attention to similarities in phenotype between FACE1-silenced HeLa cells and fibroblasts from patients with Hutchinson-Gilford progeria syndrome containing prelamin A mutations that prevent cleavage by the FACE1 endoprotease.

Key words: RNAi, FACE1, Lamin A, siRNA, Progeria

Introduction

Mammals have three genes for nuclear lamins, the structural proteins of the nuclear lamina that underlies the inner nuclear membrane. The lamina associates with chromatin and has been implicated in the regulation of gene expression and in DNA synthesis (for a review, see Goldman et al., 2002). Lamin A and the two B lamins, B1 and B2, differ in expression during development and display different behaviours during disassembly of the nuclear envelope prior to cell division. Lamin A is expressed late in development and in some tissues even only postnatally (Broers et al., 1997; Röber et al., 1989). During mitosis, lamin A becomes soluble (Gerace and Blobel, 1980), whereas the B lamins remain bound to membrane vesicles (Goldman et al., 2002; Moir et al., 2000). All three lamins end with a CaaX motif that is subject to a series of post-translational modifications. After farnesylation of the cysteine, the three terminal residues are removed by a CaaX protease and the farnesylated cysteine becomes 0-methylated. Lamin A is subject to an additional and unique maturation step. The prelamin A is converted to mature lamin A by a proteolytic conversion. Protein chemical studies indicate cleavage between Tyr646 and Leu647 (Hennekes and Nigg, 1994; Sinensky et al., 1994; Weber et al., 1989). The reason for this maturation is not known, but it explains the solubilization of lamin A in mitosis. Lamin C is essentially a shortened lamin A form, which arises by alternative splicing. It has only six unique residues and ends at position 547. Mice lacking the lamin A gene develop normally, but their postnatal growth is severely retarded and they die of muscular dystrophy (Sullivan et al., 1999). Various reports show that the lamin A gene is connected to at least eight human diseases (reviewed by Burke and Stewart, 2002; Mounkes et al., 2003). Among these laminopathies is also the Hutchinson-Gilford progeria syndrome (HGPS).

HGPS is a rare genetic human disorder characterised by features reminiscent of marked premature aging. In a pioneering analysis Eriksson et al. (Eriksson et al., 2003) found a lamin A mutation in 20 out of 23 patients with classical HGPS. Of these, 18 harboured an identical de novo, not inherited, single base substitution G608G (GGC→GGT) within exon 11. This mutation activates a cryptic splice site within exon 11, resulting in a mutant protein lacking 50 internal amino acid residues (609-658) before the C-terminal end (658-664) with its CaaX motif. In addition, one patient showed a different substitution within the same codon, and one patient revealed a missense mutation in exon 2 (E145K). A simultaneously published, but less extensive study, reported the same mutation G608G in a patient suffering from mandibuloacral dysplasia with HGPS features (De Sandre-Giovannoli et al., 2003). Although occasional missense mutations in lamin A are found (Cao and Hegele, 2003; Chen et al., 2003; Eriksson et al., 2003; Novelli

et al., 2002), the major change in HGPS is the deletion of 50 amino acids resulting from a cryptic splice site, which has been analysed in detail (Eriksson et al., 2003). As this deletion removes the cleavage site for the conversion of prelamin A to lamin A, the mutated prelamin A may stay permanently in the farnesylated form.

Defective prelamin A processing has been clearly demonstrated in mouse knockouts of the ZMPSTE24 metalloproteinase (Bergo et al., 2002; Pendas et al., 2002). ZMPSTE24 is an orthologue of the yeast enzyme STE24, which is involved in the processing of mating pheromone a-factor, a short peptide ending in a farnesylated, O-methylated cysteine (Tam et al., 1998). Homozygote knockouts of the murine gene have a phenotype resembling that of HGPS patients including growth retardation and premature death from cardiac dysfunction and alopecia. Cells from such mice lack mature lamin A and show instead prelamin A (Bergo et al., 2002; Pendas et al., 2002). Thus murine ZMPSTE24, and its human orthologue FACE1, may indeed be the proteases involved in lamin A processing. In line with this view, the human enzyme expressed in yeast mutants complements a-factor synthesis (Agarwal et al., 2003; Tam et al., 1998). A study of four patients with mandibuloacral dysplasia associated with progeria identified one patient with defects in ZMPSTE24 in the absence of lamin A mutations (Agarwal et al., 2003). Interestingly recently seven out of nine patients with restrictive dermopathy showed a premature termination codon in one gene copy of FACE-1 (Navarro et al., 2004).

Here we report on the functional characterization of the human endoprotease FACE1 by siRNA mediated gene silencing. Silencing FACE1 in HeLa cells leads to accumulation of prelamin A at the nuclear lamina and to a halt in cell division. One population of FACE1-silenced cells arrest in aberrant mitosis and later enter apoptosis. The second population show abnormalities in nuclear morphology including micronuclei formation. By contrast, when FACE1 was silenced in HeLa cells lacking lamin A, mitotic arrest does not occur and the cells appear normal. The similarities seen between the results described here for FACE1-silenced HeLa cells and results of others on fibroblasts from Hutchinson-Gilford patients, where certain mutations in the lamin A molecule prevent cleavage by the FACE1 enzyme, are discussed.

Materials and Methods

Silencing of FACE1 by siRNA

RNA interference mediated by duplexes of 21-nucleotide RNAs was performed in human HeLa cells (Elbashir et al., 2001; Harborth et al., 2001). The siRNA sequence used to target FACE1 was from position 271 to 291 relative to the first nucleotide of the start codon (GenBank accession number NM_005857). The siRNA targeting lamin A was from position 608 to 627 (NM_005572). For the mitotic protein Eg5 the siRNA has been described (Harborth et al., 2001). A siRNA sequence targeting firefly (*Photinus pyralis*) luciferase (accession number X 65324) from positions 153 to 175 was used as control (pGL2 siRNA). The 21-nucleotide RNAs were chemically synthesised by Dharmacon (Lafayette, CO) and delivered in salt free and deprotected form.

Cell culture and transfection of siRNA

The human HeLa SS6 and MCF7 cell lines were grown in DMEM containing 10% foetal calf serum, penicillin and streptomycin at 37°C with 5% CO_2. The human promyelocytic HL60 cell line was grown in RPMI1640 medium with 10 mM HEPES pH 7.0, 20% foetal calf serum and penicillin and streptomycin. Transfection with siRNA and Oligofectamine (Invitrogen, Karlsruhe, FRG; lot number 1122079) was as described (Elbashir et al., 2002). Cell growth was determined using an automated microscopy system (CellScreen, Innovartis, Bielefeld, FRG) by live cell imaging and by measuring the surface of the wells covered by the cells. HL60 cells were transfected using electroporation (Nucleofector Technology, Buffer V, Amaxa, Cologne, FRG).

Antibodies and indirect immunofluorescence microscopy

Primary antibodies were a goat prelamin A antibody (sc06214, batch number F0303, Santa Cruz Biotechnology, Santa Cruz, CA) that recognises an epitope in the 20 residues at the C-terminal end and the monoclonal α-tubulin antibody DM1A (Sigma-Aldrich, Taufkirchen). In addition we raised a rabbit antiserum against prelamin A using the synthetic peptide LLGNSSPRTQSPQNC (prelamin A residues 647-661) linked to ovalbumin. Prelamin A antibodies were purified on the immobilised peptide. We also used a rabbit antibody raised against the synthetic peptide CEDSGMEPRNEEEGNSEEI (residues 296-313) of FACE1 after affinity purification. A second rabbit FACE1 antibody was raised with the peptide corresponding to residues 58-73. Fluorescently labelled secondary antibodies were from Dianova (Hamburg, FRG).

Cells grown on glass coverslips were washed with PBS and then fixed either with –20°C methanol for 10 minutes (for the prelamin A goat antibody and tubulin antibody) or with 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 20 minutes followed by permeabilization with 0.2% Triton X-100 in PBS for 2 minutes at room temperature (for the prelamin A rabbit antibody and the FACE1 antibody). Fixed cells were washed briefly with PBS. Primary antibodies were then added and incubated for 1 hour at 37°C in a humid chamber. Coverslips were washed three times with PBS and incubated with the labelled secondary antibodies for 1 hour at 37°C. Cells were again washed three times in PBS and stained with 1 μM Hoechst 33342 (Sigma) to visualise the DNA and then were mounted on glass slides in Mowiol (Hoechst, Frankfurt, FRG).

To stain the endoplasmic reticulum (Terasaki et al., 1984) 1.5 μg/ml dihexyl oxacarbocyanine iodide in standard culture medium (DMEM) was added to living HeLa SS6 cells. Cells were incubated at 37°C with 5% CO_2 for 20 minutes. Cells were then fixed with paraformaldehyde and methanol for FACE1 immunofluorescence.

Western blotting

SDS gel electrophoresis was performed according to standard protocols. Proteins were transferred to nitrocellulose using the semi-dry transfer procedure (Kyhse-Andersen, 1984). Membranes were blocked in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH 7.4) containing 5% skimmed milk. The goat and rabbit prelamin A or rabbit FACE1 antibodies were diluted in the same buffer with 2.5% skim milk and applied for 1 hour at room temperature. Membranes were washed twice with TBST and once with TBST containing 0.5% Triton X-100. Affinity-purified horseradish peroxidase-conjugated rabbit anti-goat or swine anti-rabbit immunoglobulins were from Dako (Copenhagen, Denmark). They were diluted 1:10,000 in the blocking buffer with 2.5% skimmed milk and applied for 2 hours at room temperature. The bands were detected using ECL (Amersham Biosciences, Piscataway, NJ).

Branched DNA assay

The branched DNA assay (QuantiGene Discovery System, Genospectra, Fremont, CA) was used for quantification of mRNA

levels in cellular extracts as previously applied to quantify insulin receptor mRNA (Wang et al., 1997). It is a direct method for evaluation of mRNA levels, which requires neither RNA isolation and purification nor any amplification steps. The assay is based on specific cohybridization of the target mRNA and oligonucleotides predesigned by Genospectra. A probe set consists of three types of deoxyoligonucleotides: the capture extenders (CEs) are complementary to the target mRNA and to nucleotides which are immobilised in the capture plate, label extenders (LEs) also bind the target mRNA and the bDNA amplifier for subsequent enzymatic development and the blocking oligonucleotides (BLs). CEs, LEs and BLs are designed in a way to hybridise to a 300-500 nucleotide region along the target mRNA forming a DNA/RNA duplex.

The bDNA assay was performed according to the manufacturer's protocol. HeLa SS6 cells were grown in 96-well plates and transfected as described (Harborth et al., 2001), except that reagents were scaled down to one sixth of those used for 24 well plates. Cells were disrupted by application of lysis buffer containing CEs, LEs and BLs. 50 µl lysis buffer and 100 µl medium were applied and cells were incubated for 1 hour at 37°C. Cells lysates where transferred to the capture plates, plates were sealed and incubated at 52°C overnight. Capture plates were washed and incubated with an labelling/amplifier solution for 1 hour at 37°C. Subsequently plates were developed for 30 minutes at 52°C with the substrate solution. Emitted luminescence was measured with a luminometer (VICTOR Light, Perkin Elmer, Wellesley, MA). Target mRNA specific probes for lamin A, FACE1 and β-actin were used. In the data shown here cell extracts were harvested 24-96 hours post transfection and developed with the appropriate probes. Data represent levels of FACE1 mRNA and lamin A mRNA standardised with β-actin mRNA to control for cell numbers and then normalised using values obtained with control cultures transfected with GL2 siRNA as 100%.

Apoptosis detection

To detect apoptotic cells a TUNEL test (In situ cell death detection kit, Roche, Mannheim, FRG) was used. Cells were fixed in –20°C methanol for 6 minutes and treated with PBS containing 0.1% Triton X-100 and 0.1% sodium citrate, pH 7.2 on ice for 2 minutes. The free 3′ ends of fragmented DNA were enzymatically labelled with FITC tagged deoxynucleotide triphosphates using deoxynucleotidyl transferase (TdT). Labelled DNA fragments were monitored by fluorescence microscopy.

Results

The endoprotease FACE1 is required for the processing of prelamin A in HeLa cells

Antibodies that specifically detect prelamin A, but not the processed lamin A, must recognise the C-terminal sequence of residues 647-664 (see Introduction). We have used two such antibodies: a commercial goat anti prelamin A antibody detecting an epitope in the 20 residues at the C terminal end and a rabbit antibody which we raised with a synthetic peptide corresponding to residues 647-661 and purified by antigen affinity chromatography. Both antibodies gave the same results.

In normal HeLa cells, as well as in cells transfected with GL2 siRNA (Fig. 1A, row 1), a low level of prelamin A is detected in multiple small dots in the nucleoplasm and no staining of the nuclear lamina is observed. By contrast, in cells that have been treated with FACE1 siRNA and then stained at different times after transfection (Fig. 1A, row 2) the prelamin A accumulated with time at the nuclear lamina. Similar results were also obtained with the FACE1 silenced human MCF7 cell line (data not shown). However, silencing with LMNA siRNA results in a reduction in intensity of the nuclear dots (Fig. 1A, row 3).

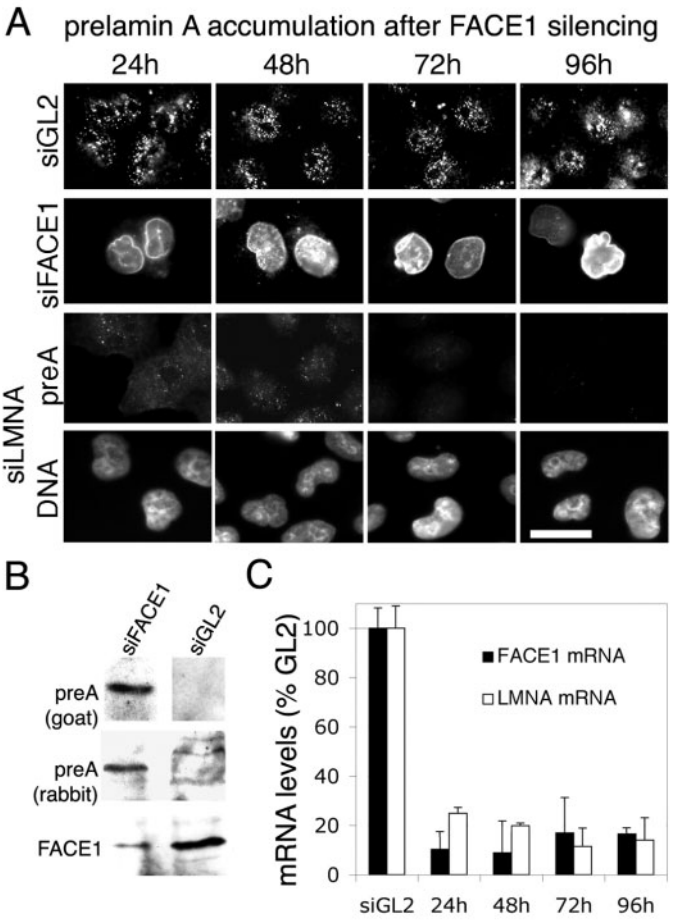

Fig. 1. Silencing of human FACE1 results in accumulation of prelamin A. (A) Human HeLa SS6 cells were immunostained with the antibody SC06214 recognising an epitope in the C-terminal region of unprocessed lamin A. This antibody detects prelamin A, but not lamin A. Transfection with an unspecific GL2 siRNA did not affect the normal localisation of prelamin A in subnuclear foci (row 1). Transfection with FACE1 siRNA results in progressive accumulation of unprocessed prelamin A in the nuclear lamina (row 2). Transfection of cells with lamin A siRNA targeting lamin A/C mRNA resulted in loss of prelamin A (row 3). Hoechst stain of the same cells (row 4). (B) Accumulation of prelamin A was confirmed by western blots of FACE1-silenced cells (siFACE1) and control populations (siGL2). Prelamin A accumulated in FACE1-silenced cells 48 hours after transfection, but was not detectable in extracts from cells transfected with the GL2 siRNA. (C) Silencing of FACE1 and lamin A in single siRNA experiments was confirmed by luminometric measurement of absolute mRNA levels via the branched DNA assay. Note that after 24 hours the mRNA is very much reduced for both targets and that these low levels are maintained at least until 96 hours after transfection. Bar, 20 µm.

A strong accumulation of prelamin A was also detected in western blots of extracts from FACE1-silenced HeLa cells (Fig. 1B). A specific signal with the appropriate molecular weight for prelamin A was detected only in extracts from the silenced cells, but not in extracts from cells that were transfected either with the control GL2 siRNA targeting firefly

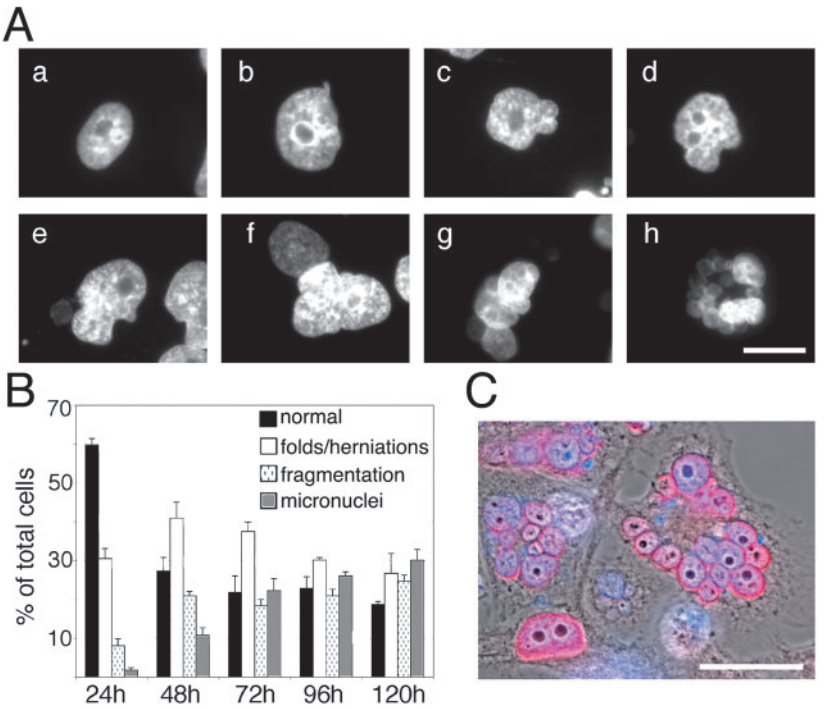

Fig. 2. Silencing of FACE1 causes abnormalities in nuclear morphology. (A) Control HeLa cells (a) show a normal nuclear morphology whereas FACE1-silenced HeLa cells display a range of abnormal nuclear morphologies (b-h) when stained with Hoechst dye. The abnormalities included minor effects such as herniations in the nuclear periphery (b) and folds (c-f), or more severe effects such as lobules (f) and fragments (g) as well as formation of micronuclei (h). The number of cells with severe abnormalities increased significantly at later times after transfection. (B) Comparative statistics. Cells were divided into four classes. 'Normal' nuclei have a morphology as in a; 'folds and herniations' as in c-f, fragmentation as in g; and micronuclei as in h. The percentage of normal nuclei decreased from 60% at 24 hours to 20% at 72 hours. Folds and herniations reached a maximum (>40%) 48 hours after transfection and then decreased slowly. The number of cells with micronuclei increased throughout the observation time and reached a maximum (>30%) 120 hours post transfection. (C) Cells treated with FACE1 siRNAs in-phase superimposed with prelamin A stain (red) and Hoechst DANN stain (blue) to show micronuclei. Bars, 15 μm.

(*Photinus pyralis*) luciferase (Fig. 1B) or with lamin A siRNA (data not shown). The level of FACE1 endoprotease was strongly reduced after silencing with FACE1 siRNAs (Fig. 1B).

Successful silencing of FACE1 by transfection of FACE1 siRNA was confirmed by the novel branched DNA (bDNA) assay (Fig. 1C). This assay allowed direct luminometric measurement of mRNA levels from cell extracts by specific immobilization of target mRNAs and labelling with DNA probes. Samples were derived from FACE1, LMNA or GL2 siRNA-transfected HeLa cells. mRNA levels from GL2 cells were used for normalization of values obtained from silenced cell populations. The bDNA assay showed a reduction of about 80% in the FACE1 and LMNA mRNA levels 24 hours after transfection. Thus, this new method appears to be an appropriate technique for quantification of mRNA knockdown in RNAi experiments.

The combined results suggest that after loss of the endoprotease, FACE1 cells were unable to process farnesylated prelamin A by cleavage of the C-terminal 18 amino acids, and that in consequence prelamin A may be incorporated in place of the normal, processed lamin A into the nuclear lamina.

FACE1 knockdown causes changes in nuclear morphology as well as aberrant mitosis

Two populations could be distinguished in cells treated with FACE1 siRNA. The first population (Fig. 2) showed changes in nuclear morphology with many cells displaying micronuclei. The second population (Fig. 3) was formed by cells that arrested in aberrant mitosis and later underwent apoptosis.

Accumulation of prelamin A in HeLa cells led to dramatic changes in the nuclear architecture with increasing time after transfection with FACE1 siRNA (Fig. 2). These changes included folds in the nuclear envelope, herniations and crevices extending into the nuclear periphery as well as lobulation and increasing fragmentation of nuclei and formation of micronuclei. All effects could be detected early after siRNA transfection (24 hours), but the percentage of cells displaying more severe effects increased with time post transfection (see Fig. 2B). Micronuclei were observed in 30% of the cells 120 hours post transfection (Fig. 2C).

A very striking change observed after knockdown of the FACE1 endoprotease was the accumulation of cells in aberrant mitosis (Fig. 3). Cells were arrested in early metaphase and displayed disturbed spindle architecture and abnormal chromosome congression (Fig. 3A, row 1). Analysis of FACE1-silenced cells by the TUNEL assay showed that the cells in this population underwent apoptosis following the aberrant mitotic arrest (Fig. 3B, row 1). Quantification of cells in mitotic arrest and apoptosis are shown in Fig. 3C and D. After 24 hours, 25% of the cells were arrested in mitosis and after 48 hours this value increased to a maximum of 35% (Fig. 3C). At later times, the percentage of cells arrested in aberrant mitosis decreased. The percentage of cells that were apoptotic reached a maximum 72 hours post transfection with FACE1 siRNA (Fig. 3D).

Measurements of cell growth were made with the CellScreen System for the FACE1 siRNA transfected cells, as well as for GL2 and LMNA transfected cells (Fig. 3E). Cells transfected with FACE1 siRNA stopped growing abruptly. LMNA silenced cells showed a small reduction in growth rate (see also Elbashir et al., 2002) when compared to the GL2 control, but the change in growth rate was minor in comparison to that seen with the FACE1-silenced cells.

The phenotypic changes after FACE1 silencing are due to the increase in prelamin A

Double RNAi experiments were performed to see whether the phenotypic changes were due to the accumulation of prelamin

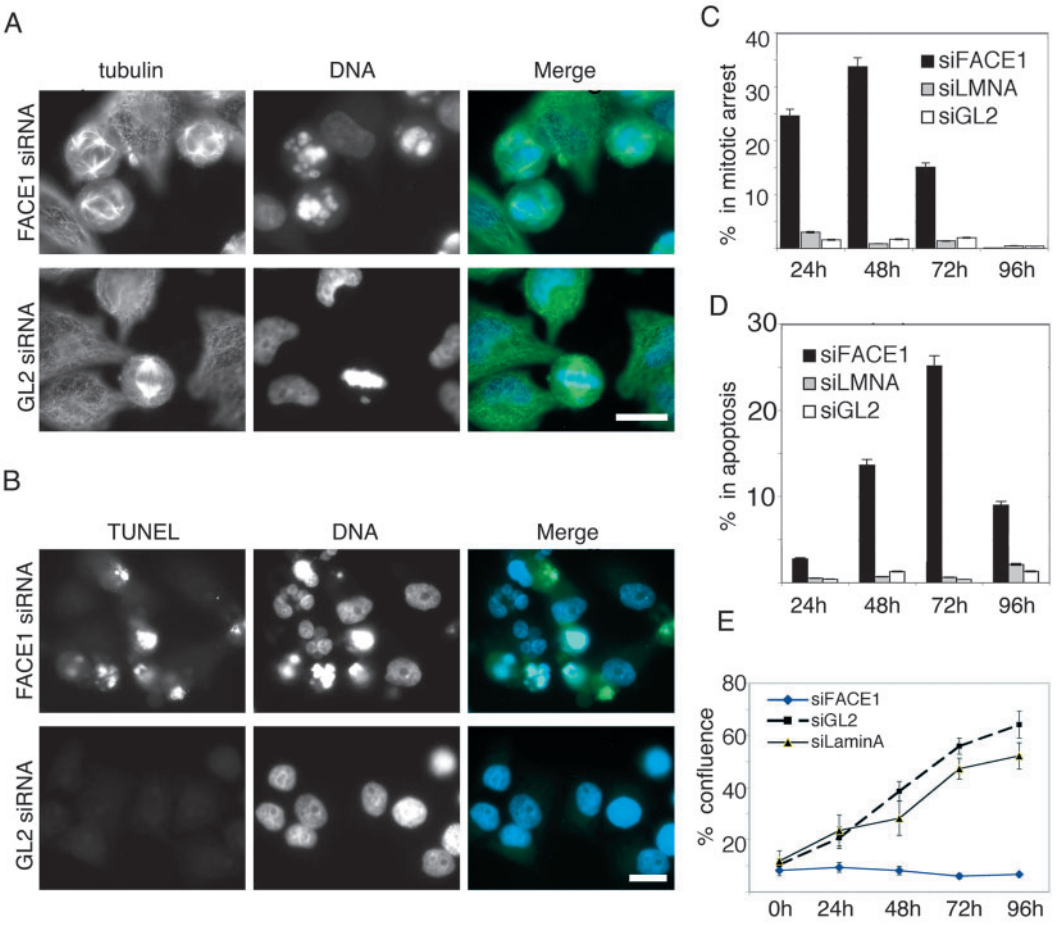

Fig. 3. Aberrant mitosis and apoptosis are consequences of FACE1 knockdown. (A) RNAi mediated knockdown of FACE1 led to mitotic arrest of HeLa SS6 cells within 24 hours of transfection. Arrested cells (top) displayed abnormal mitotic spindle architecture and disturbed chromosome congression. Control cells (bottom) transfected with GL2 siRNA showed normal mitosis. Cells were stained with tubulin antibody and with Hoechst dye. (B) TUNEL assay on FACE1-silenced (top) and control cells (bottom) 72 hours after siRNA transfection shows that the mitotically arrested cells have entered apoptosis. (C) Quantification of mitotic arrest after FACE1 silencing. The fraction of cells showing mitotic arrest increased with time after transfection, reaching a maximum of 35% of the total cell population at 48 hours. Cells transfected with either GL2 or with lamin A siRNA did not arrest in mitosis. (D) Quantification of apoptosis after FACE1 silencing. The percentage of apoptotic cells reached a maximum of 25% 72 hours after transfection and then decreased. Apoptosis was observed only in FACE1-silenced cells, but not in control (GL2 siRNA) or in lamin A siRNA-transfected cells. (E) Cell growth measured with the CellScreen apparatus was reduced dramatically after silencing with FACE1 siRNA. Bar, 15 μm.

A. Mitotic arrest and the changes in nuclear morphology were not observed when lamin A was silenced 24 hours before the FACE1 knockdown (Fig. 4A, top). Thus, when cells were stained with tubulin antibodies, normal mitotic spindles were seen and mitotic cells with abnormal multipolar spindles were not found. By contrast, if FACE1 was silenced 24 hours before lamin A was silenced, results were similar to those reported above for FACE1 silencing alone in that accumulation of cells with aberrant mitotic arrest was observed 48 hours after the first transfection (Fig. 4A, bottom). These data strongly suggest that the accumulation of prelamin A is the cause of the effects seen in HeLa cells.

The bDNA assay was used to confirm that both FACE1 and lamin A were knocked down when a double RNAi experiment was performed. In the double knockdown experiment leading to normal mitosis, i.e. knockdown of lamin A followed 24 hours later by knockdown of FACE1 (LMNA-FACE1), mRNA levels are reduced to 20% when assayed 24 hours after the second transfection (*, Fig. 4B). When FACE1 is knocked down 24 hours prior to LMNA (FACE1-LMNA), again a reduction in mRNA levels for both proteins is seen with the FACE1 mRNA more strongly reduced than the LMNA mRNA.

Other double RNAi experiments were also done using control GL2 siRNAs followed 24 hours later by transfection with either FACE1 or with lamin A siRNAs. As can be seen from Fig. 4B, use of the GL2-FACE1 combination results in knockdown of FACE1 but not of lamin A mRNA, whereas use of the GL2-LMNA combination knocks down lamin A but not FACE1. In other double RNAi experiments (data not shown) HeLa cells were transfected with GL2 siRNAs followed 24 hours later with siRNAs for the Eg5 motor protein, or with GL2 siRNAs followed by β-actin siRNAs. The phenotype obtained with the GL2-Eg5 was the same as those seen when cells are transfected only with Eg5 siRNAs. Likewise the phenotype obtained with the GL2-β-actin combination was identical to

that seen with β-actin in a single RNAi transfection experiment (Harborth et al., 2001). The double RNA experiments in Fig. 4, as well as the double RNAi experiments with Eg5 and β-actin suggest that the consecutive use of two different RNAi transfections will become a very useful tool in RNAi technology.

Additional confirmation that the phenotypic changes after FACE1 silencing are due to the increase in prelamin A, comes from the HL60 cell line. This human promyelocytic cell line does not express the lamin A gene. When HL60 cells were transfected by electroporation with FACE1 siRNA, no growth retardation, mitotic arrest or effects on nuclear architecture were observed (data not shown).

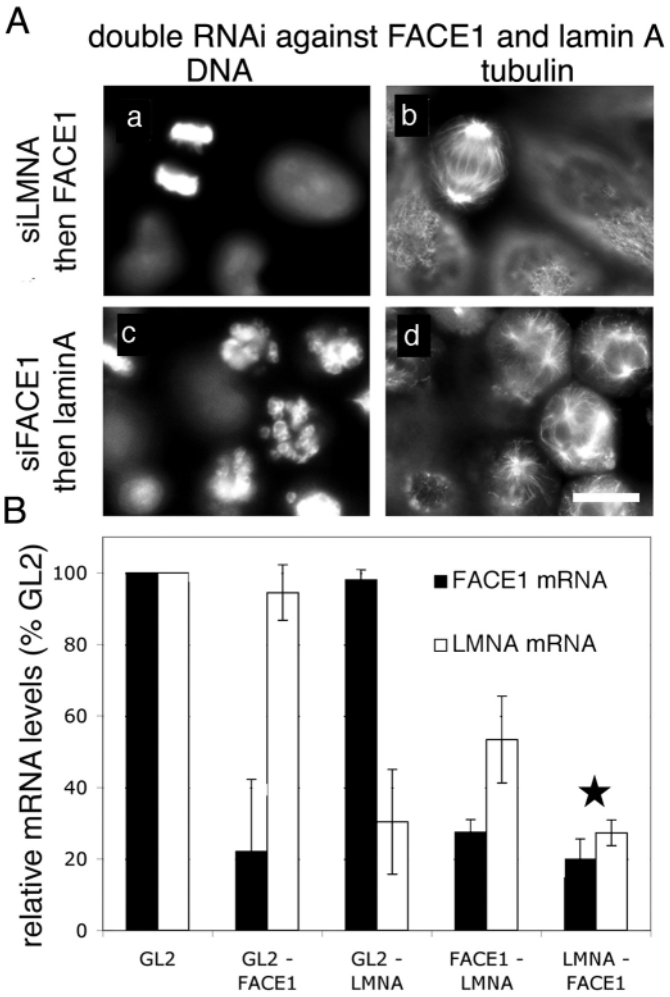

Fig. 4. Double RNAi suggests prelamin A is responsible for phenotypic changes after FACE1 silencing. (A) Silencing of lamin A prior to FACE1 prevented phenotypic changes. Human HeLa SS6 cells were transfected with the siRNA specific for the first target and after 24 hours with the second siRNA. Cells were stained for tubulin and for DNA 24 hours after the second transfection. (a,b) Knockdown of lamin A before FACE1 did not cause abnormal mitosis, apoptosis or changes in the nuclear morphology. (c,d) Silencing of FACE1 prior to silencing of lamin A led to aberrant mitotic arrest. (B) Successful transfection in the double knockdown experiments was confirmed by the branched DNA assay. Data represent the relative mRNA levels 24 hours after the second transfection for the LMNA-FACE1 combination (★) and for the FACE1-LMNA combination. Double siGL2 transfection was used for normalisation (GL2). That the second transfection was effective was further confirmed by transfecting either FACE1 or lamin A siRNA 24 hours after siGL2 delivery (GL2-FACE1 and GL2-LMNA). Bar, 10 μm.

Location of FACE1 in HeLa cells

The immunofluorescence results in Fig. 5 show that in HeLa cells FACE1 is located in spots at the nuclear membrane and in the endoplasmic reticulum. Staining of the endoplasmic reticulum was confirmed by first staining live cells with 3,3' dihexyloxacarbocyanine iodide and then with FACE1 antibodies. In cells transfected with FACE1, siRNA staining at the nuclear membrane and in the endoplasmic reticulum is very strongly reduced 48 hours after transfection.

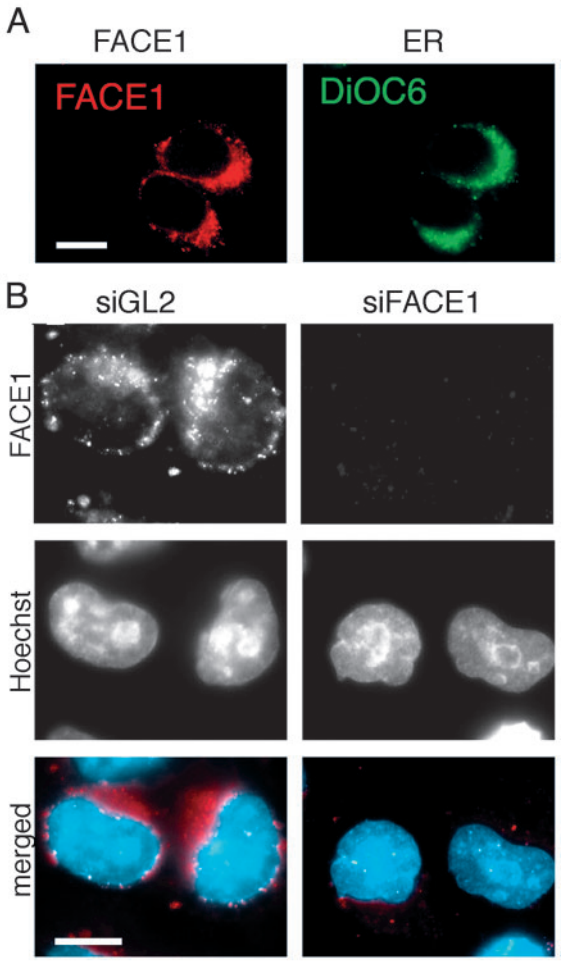

Fig. 5. Immunofluorescence detection of FACE1 in the endoplasmic reticulum and the nuclear periphery. (A) HeLa cells were stained with dihexyloxacarbocyanine iodide to label the endoplasmic reticulum and then with the rabbit FACE1 antibody against residues 296-313. FACE1 is seen in the endoplasmic reticulum identified by $DiOC_6$ staining and is also seen in spots at the nuclear periphery. (B) Immunofluorescence with the FACE1-specific antibody 48 hours after transfection of FACE1 or GL2 siRNA demonstrates silencing of FACE1 in cells transfected with FACE1 siRNA, but not in cells transfected with GL2 siRNA. Bar, 10 μm.

Discussion

Here we have successfully silenced the human endoprotease FACE1 by RNA interference using the siRNA technique (Elbashir et al., 2001; Harborth et al., 2001). To document silencing after transfection of target specific siRNA a novel technique was introduced. The new bDNA assay (Wang et al., 1997) allowed quantitative determination of the absolute mRNA levels without the pitfalls of RNA extraction, purification and amplification steps as required in real-time PCR approaches. Thus FACE1 and lamin A knockdown could be confirmed on the quantitative mRNA level as well as on the protein level using indirect immunofluorescence microscopy and western blotting.

In control HeLa cells, only small amounts of prelamin A are present. Prelamin A is located to spots within the nuclei, but not to the nuclear lamina (Fig. 1A). Similar results have been reported for control fibroblasts (Goldman et al., 2004). Western blotting of control cells with prelamin A specific antibodies shows no band at the prelamin A position in control cells (Fig. 1B). By contrast, in HeLa and MCF7 cells RNAi of FACE1 results in accumulation of relatively large amounts of unprocessed prelamin A at the nuclear membrane both by immunofluorescence (Fig. 1A) and by immunoblotting (Fig. 1B) within 24-48 hours after transfection. These findings support results on the ablation of the murine gene. Ablation of ZMPSTE24 showed an accumulation of prelamin A owing to defective processing of prelamin A (Bergo et al., 2002; Pendas et al., 2002). They further establish that prelamin A is a substrate of ZMPSTE24 and that its accumulation results in HGPS-related defects, but they do not show whether FACE1 has other substrates in addition to prelamin A (Bergo et al., 2002; Pendas et al., 2002). Our results with the HeLa cell system are therefore of particular interest. They show that the loss of FACE1 leads only to the strong phenotypes when the cells express the lamin A gene. When HeLa cells are subjected to an RNAi knockdown of lamin A (Elbashir et al., 2001; Elbashir et al., 2002; Harborth et al., 2001), such cell populations behave normally when FACE1 is subsequently downregulated by RNAi (Fig. 5A). We have confirmed by the branched DNA assay that such cells indeed have a strongly reduced level of FACE1 mRNA (Fig. 1C). Thus it seems that at least in normal HeLa cells, prelamin A is an important substrate of FACE1 and that other substrates, if they exist, are not of immediate importance for cellular growth. The finding that HL60, a cell line that does not express the lamin A gene, shows no effects on FACE1 silencing supports this conclusion.

A variety of interesting phenotypic changes have been observed in the siRNA-transfected HeLa cells in addition to the accumulation of unprocessed lamin A at the nuclear membrane (Figs 1-5). Two cell populations are detected after silencing with FACE1 siRNA. The first population shows changes in nuclear morphology. Nuclei lose their shape, develop folds and lobulation and show progressive fragmentation (Fig. 2). Presumably these changes are due to differences between how prelamin A and lamin A incorporate into the nuclear lamina. The second population is characterised by multipolar spindles and arrest in mitosis (Fig. 3A). The percentage of cells in mitotic arrest reaches a maximum of 31% at 48 hours (Fig. 3C). These cells become apoptotic with the percentage of cells in apoptosis reaching 25% at 72 hours (Fig. 3D). The cause of disturbed spindle architecture and chromosome congression in metaphase is unclear, but presumably results from changes in lamina stability and protein/DNA interactions. Farnesylated, 0-methylated prelamin A could have similar membrane anchoring or hydrophobic interaction properties as the B type lamins (i.e. lamin B1 and lamin B2) and therefore, like B lamins, might remain connected to structures of the nuclear envelope when cells enter mitosis. By contrast, mature lamin A is phosphorylated at the beginning of mitosis, depolymerises, and is soluble in the cytoplasm until it is reassembled on the inner face of the nuclear envelopes in the daughter cells (for reviews, see Goldman et al., 2002; Moir et al., 2000).

Fibroblasts from both ZMPSTE24 deficient mice (Pendas et al., 2002) and from HGPS patients (Eriksson et al., 2003) show abnormalities in nuclear morphology and in this respect are therefore similar to the FACE1-silenced HeLa cells studied here. Fibroblasts from HGPS patients show the same progression of changes in nuclear morphologies that we observed upon FACE1 silencing, starting with folds and herniations, fragmentation of nuclei and ending with micronucleated cells. In addition, a high percentage of cells are apoptotic at late passages of HGPS-derived fibroblasts (Bridger and Kill, 2004). The most common HGPS mutation was originally identified as an ablation of the FACE1 cleavage site in prelamin A (Eriksson et al., 2003). Accumulation of prelamin A at the nuclear membrane has been noted in cells from a HGPS patient with this mutation (Goldman et al., 2004), thus paralleling our results with FACE1 silenced HeLa cells.

The rapid effects on mitosis of FACE1 negative silenced cells indicate a fast intracellular turnover of the protease, since already 24 hours after siRNA delivery a high percentage of cells displayed silencing specific defects and had begun to accumulate prelamin A (Fig. 1A, Fig. 4). The results from the CellScreen measurements (Fig. 3E) show that there is a dramatic arrest in cell growth in the FACE1 siRNA-treated cells.

Relatively little is known about consequences of mutations in the FACE1 gene, which maps to chromosome 1p34. Agarwal and colleagues described a Belgian family with mutations in FACE1 (Agarwal et al., 2003). A frameshift mutant introducing an early termination codon provides an inactive enzyme and a missense mutation in a highly conserved region (W340R) yields reduced enzymatic activity. Individuals who were heterozygous for one of these two mutations seem normal, whereas a patient with both mutations had mandibuloacral dysplasia with progeroid appearance and generalised lipodystrophy and died aged 24 years. Recently, the lamin A and FACE1 genes were sequenced from nine patients with restrictive dermopathy (RD) (Navarro et al., 2004). Two had a heterozygous splicing mutation in the lamin A gene, leading to the complete or partial loss of exon 11 in the mRNAs that resulted in a truncated prelamin A. In the other seven patients, a unique heterozygous one base pair insertion in exon 9 led to the creation of a premature termination codon in FACE1. In cells grown in culture from patients with FACE1 mutations, loss of normal lamin A expression was observed and in the first three cell divisions up to 50% of cells had nuclei that were abnormal in shape or size. The authors suggest that the FACE1 mutation identified in their study is a necessary but not sufficient genetic defect to cause restrictive dermopathy, as

in four families the same mutation was present in one of the parents who was free of disease (Navarro et al., 2004). They further suggest that a second gene may be mutated in patients with restrictive dermopathy.

In conclusion, in HeLa cells expressing lamin A, the knockdown of the endoprotease FACE1 has deleterious effects. As these do not occur after lamin A expression is suppressed by RNAi, we propose that the large increase in unprocessed prelamin A may be the direct cause for the lethal phenotype. Although we do not yet understand the cell biological effects in detail, the presence of the permanently farnesylated/0-methylated C-terminal cysteine could have severe effects on lamin A assembly, organisation and function that appear related to those postulated for HGPS patients (Goldman et al., 2004). The HeLa system established here should be very useful for further detailed studies.

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References

Agarwal, A. K., Fryns, J. P., Auchus, R. J. and Garg, A. (2003). Zinc metalloproteinase, ZMPSTE24, is mutated in mandibuloacral dysplasia. *Hum. Mol. Genet.* **12**, 1995-2001.

Bergo, M. O., Gavino, B., Ross, J., Schmidt, W. K., Hong, C., Kendall, L. V., Mohr, A., Meta, M., Genant, H., Jiang, Y. et al. (2002). Zmpste24 deficiency in mice causes spontaneous bone fractures, muscle weakness, and a prelamin A processing defect. *Proc. Natl. Acad. Sci. USA* **99**, 13049-13054.

Bridger, J. M. and Kill, I. R. (2004). Aging of Hutchinson-Gilford progeria syndrome fibroblasts is characterised by hyperproliferation and increased apoptosis. *Exp. Gerontol.* **39**, 717-724.

Broers, J. L., Machiels, B. M., Kuijpers, H. J., Smedts, F., van den Kieboom, R., Raymond, Y. and Ramaekers, F. C. (1997). A- and B-type lamins are differentially expressed in normal human tissues. *Histochem. Cell Biol.* **107**, 505-517.

Burke, B. and Stewart, C. L. (2002). Life at the edge: the nuclear envelope and human disease. *Nat. Rev. Mol. Cell Biol.* **3**, 575-585.

Cao, H. and Hegele, R. A. (2003). LMNA is mutated in Hutchinson-Gilford progeria (MIM 176670) but not in Wiedemann-Rautenstrauch progeroid syndrome (MIM 264090). *J. Hum. Genet.* **48**, 271-274.

Chen, L., Lee, L., Kudlow, B. A., Dos Santos, H. G., Sletvold, O., Shafeghati, Y., Botha, E. G., Garg, A., Hanson, N. B., Martin, G. M. et al. (2003). LMNA mutations in atypical Werner's syndrome. *Lancet* **362**, 440-445.

De Sandre-Giovannoli, A., Bernard, R., Cau, P., Navarro, C., Amiel, J., Boccaccio, I., Lyonnet, S., Stewart, C. L., Munnich, A., le Merrer, M. et al. (2003). Lamin a truncation in Hutchinson-Gilford progeria. *Science* **300**, 2055.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-498.

Elbashir, S. M., Harborth, J., Weber, K. and Tuschl, T. (2002). Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26**, 199-213.

Eriksson, M., Brown, W. T., Gordon, L. B., Glynn, M. W., Singer, J., Scott, L., Erdos, M. R., Robbins, C. M., Moses, T. Y., Berglund, P. et al. (2003). Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature* **423**, 293-298.

Gerace, L. and Blobel, G. (1980). The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell* **19**, 277-287.

Goldman, R. D., Gruenbaum, Y., Moir, R. D., Shumaker, D. K. and Spann, T. P. (2002). Nuclear lamins: building blocks of nuclear architecture. *Genes Dev.* **16**, 533-547.

Goldman, R. D., Shumaker, D. K., Erdos, M. R., Eriksson, M., Goldman, A. E., Gordon, L. B., Gruenbaum, Y., Khuon, S., Mendez, M., Varga, R. et al. (2004). Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. *Proc. Natl. Acad. Sci. USA* **101**, 8963-8968.

Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T. and Weber, K. (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell Sci.* **114**, 4557-4565.

Hennekes, H. and Nigg, E. A. (1994). The role of isoprenylation in membrane attachment of nuclear lamins. A single point mutation prevents proteolytic cleavage of the lamin A precursor and confers membrane binding properties. *J. Cell Sci.* **107**, 1019-1029.

Kyhse-Andersen, J. (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**, 203-209.

Moir, R. D., Spann, T. P., Lopez-Soler, R. I., Yoon, M., Goldman, A. E., Khuon, S. and Goldman, R. D. (2000). Review: the dynamics of the nuclear lamins during the cell cycle – relationship between structure and function. *J. Struct. Biol.* **129**, 324-334.

Mounkes, L., Kozlov, S., Burke, B. and Stewart, C. L. (2003). The laminopathies: nuclear structure meets disease. *Curr. Opin. Genet. Dev.* **13**, 223-230.

Navarro, C. L., de Sandre-Giovannoli, A., Bernard, R., Boccaccio, I., Boyer, A., Genevieve, D., Hadj-Rabia, S., Gaudy-Marqueste, C., Smitt, H. S., Vabres, P. et al. (2004). Lamin A and ZMPSTE24 (FACE-1) defects cause nuclear disorganization and identify restrictive dermopathy as a lethal neonatal laminopathy. *Hum. Mol. Genet.* **13**, 2493-2503.

Novelli, G., Muchir, A., Sangiuolo, F., Helbling-Leclerc, A., D'Apice, M. R., Massart, C., Capon, F., Sbraccia, P., Federici, M., Lauro, R. et al. (2002). Mandibuloacral dysplasia is caused by a mutation in LMNA-encoding lamin A/C. *Am. J. Hum. Genet.* **71**, 426-431.

Pendas, A. M., Zhou, Z., Cadinanos, J., Freije, J. M., Wang, J., Hultenby, K., Astudillo, A., Wernerson, A., Rodriguez, F., Tryggvason, K. et al. (2002). Defective prelamin A processing and muscular and adipocyte alterations in Zmpste24 metalloproteinase-deficient mice. *Nat. Genet.* **31**, 94-99.

Röber, R. A., Weber, K. and Osborn, M. (1989). Differential timing of nuclear lamin A/C expression in the various organs of the mouse embryo and the young animal: a developmental study. *Development* **105**, 365-378.

Sinensky, M., Fantle, K., Trujillo, M., McLain, T., Kupfer, A. and Dalton, M. (1994). The processing pathway of prelamin A. *J. Cell Sci.* **107**, 61-67.

Sullivan, T., Escalante-Alcalde, D., Bhatt, H., Anver, M., Bhat, N., Nagashima, K., Stewart, C. L. and Burke, B. (1999). Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *J. Cell Biol.* **147**, 913-920.

Tam, A., Nouvet, F. J., Fujimura-Kamada, K., Slunt, H., Sisodia, S. S. and Michaelis, S. (1998). Dual roles for Ste24p in yeast a-factor maturation: NH2-terminal proteolysis and COOH-terminal CAAX processing. *J. Cell Biol.* **142**, 635-649.

Terasaki, M., Song, J., Wong, J. R., Weiss, M. J. and Chen, L. B. (1984). Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes. *Cell* **38**, 101-108.

Wang, J., Shen, L., Najafi, H., Kolberg, J., Matschinsky, F. M., Urdea, M. and German, M. (1997). Regulation of insulin preRNA splicing by glucose. *Proc. Natl. Acad. Sci. USA* **94**, 4360-4365.

Weber, K., Plessmann, U. and Traub, P. (1989). Maturation of nuclear lamin A involves a specific carboxy-terminal trimming, which removes the polyisoprenylation site from the precursor; implications for the structure of the nuclear lamina. *FEBS Lett.* **257**, 411-414.