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Research Paper

Identification of Markers for Cellular Stress and Senescence in Laminopathic Cells by Proteomic Analysis

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Lamins are components of the structural network in the nucleus and play a key role in nuclear organization and function. Mutations in the human lamin A gene cause a spectrum of genetic diseases that include muscular dystrophies, cardiomyopathy, lipodystrophy and premature ageing syndromes. Laminopathic cells display several abnormalities in nuclear morphology and function. We have carried out a comprehensive analysis of alterations in the protein profiles of HEK293T cells expressing a mutation in lamin A (Q294P) that causes Emery-Dreifuss muscular dystrophy, in comparison with cells expressing wild-type lamin A. Initially a total of 27 differentially expressed proteins were identified by quantitative two-dimensional gel electrophoresis followed by mass spectral analysis. Importantly, a 5-fold decrease in lamin B1 levels in mutant cells was observed by quantitative two-dimensional gel electrophoresis, which was supported by immunofluorescence analysis. Changes in levels of specific heat shock proteins, hnRNPs, DNA damage response proteins, metabolic enzymes and cytoskeletal proteins were also observed. Further detailed analysis of cell lysates by one-dimensional gel electrophoresis followed by high throughput mass spectrometry revealed 650 unique proteins from wild-type cells and 1494 unique proteins from mutant cells (with two or more than two peptide hits). Alterations in a number of proteins that are involved in chromatin structure, chromosome condensation, nucleosome assembly, epigenetic modifications, centromere organization, telomere length and DNA repair were observed. These data suggest that laminopathic cells exhibit characteristics of cellular stress and senescence, and provide new insights into the cellular basis of pathologies caused by laminopathic mutations.

Key Words: Lamin A; Lamin B1; Cellular Senescence; Chromatin Organization; Telomere Length; DNA Repair

Introduction

Lamins are structural proteins of the type V intermediate filament class of proteins which form a filamentous network or lamina that lies beneath the inner nuclear membrane and also extends into the interior of the nucleus in animal cells. The lamina plays an essential role in maintaining the integrity of the nuclear envelope and is involved in the organization of various nuclear functions such as DNA replication and transcription, as well as organization of chromatin. The nuclear lamina is able to cross-talk with the cytoskeletal organization via binding proteins in the nuclear membrane and thereby influences nuclear positioning and migration. Two major kinds of lamins are present in higher eukaryotes. The B-type lamins (B1 and B2) are expressed in all somatic cell types whereas the expression of A-type lamins (A and C) is observed in differentiated cells of most lineages (Dechat *et al.*, 2008; Parnaik, 2008). A- and B-type lamins form separate, interactive meshworks in the nucleus (Shimi *et al.*, 2008).

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Mutations in the human laminA gene cause a number of debilitating genetic diseases collectively termed laminopathies, which include muscular dystrophies, cardiomyopathy, lipodystrophy and progeria syndromes. Many laminopathies are autosomal dominant diseases and are characterized by tissue wasting and premature ageing phenotypes. At the cellular level, laminopathic cells display aberrant nuclear morphology, defective chromatin organization, impaired DNA repair, replication and transcription as well as defects in cellular differentiation and developmental pathways. Lamin mutations have been proposed to alter nuclear mechanics, impair gene regulation and cause enhanced proteosomal degradation (Parnaik *et al*., 2011; Butin-Israeli *et al*., 2012). Two-dimensional protein profiles of cells with reduced lamin levels or cells from patients with neuromuscular laminopathies have revealed alterations in heat shock proteins, hnRNPs, metabolic pathways and cytoskeletal organization (Chen *et al*., 2009; Foster *et al*., 2011; Magagnotti *et al*., 2012).

In our approach, we have compared the protein profiles of HEK293T cells expressing wild-type lamin A with cells expressing a lamin mutation Q294P that causes Emery-Dreifuss muscular dystrophy (EMD), by quantitative two-dimensional gel electrophoresis as well as one-dimensional gel electrophoresis followed by high throughput mass spectrometric analysis in order to obtain a more comprehensive list of altered proteins. Our data indicates a 5-fold decline in lamin B1 levels and significant alterations in markers for cellular stress and senescence in laminopathic cells compared with wild-type cells, in addition to changes in heat shock proteins, hnRNPs and components of metabolic pathways and cytoskeletal networks.

Materials and Methods

Cell Culture, DNA Transfection and Cell Sorting

The human embryonic kidney cell line HEK293T was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cells were transfected with a green fluorescent protein (GFP)-tagged lamin A construct by electroporation and maintained in the medium for 24 h. The two constructs that were used were wild-type lamin A and Q294P lamin mutant that causes EMD and have been described earlier (Manju *et al*., 2006). GFP-expressing cells were sorted in a fluorescence activated cell sorter (FACS) in order to obtain >99% cells expressing the GFP-tagged lamin. The sorted cells were centrifuged and the cell pellet was frozen in liquid nitrogen.

Immunofluorescence Microscopy

HEK293T cells were seeded on 0.1% gelatin-coated glass coverslips for immunostaining. Cells were fixed in 3.5% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 6 min at room temperature. Cells were incubated with 0.5% gelatin in phosphate-buffered saline (PBS) for 1 h, followed by incubation with antibody to lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or lamin B1 (Abcam, Cambridge, MA, USA) for 1 h and then with Cy3-conjugated secondary antibody for 1 h at room temperature. Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) to stain nuclei and viewed under a Leica TCS SP8 confocal laser-scanning fluorescence microscope. Images were analyzed with Leica application software and assembled using Adobe Photoshop. Quantitative analysis of cells depleted in lamin B1 was carried out by visual inspection of n=100 cells.

Western Blot Analysis

Samples of transfected and untransfected HEK293T cells were lysed in Laemmli's sample buffer, boiled and electrophoresed through SDS-polyacrylamide gels. Gels were electroblotted onto nitrocellulose membrane filters and blocked for 1 h in 5% BLOTTO in Tris-buffered saline (TBS) containing 0.05% Tween-20. Filters were incubated with antibody to lamin A/C, which recognises both lamins A and C, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Millipore, Temecula, CA, USA) as loading control for 2 h, followed by species-specific peroxidase-conjugated secondary antibody in TBS containing 0.05% Tween-20 for 1 h. Bound antibody

was visualized using a chemiluminescence kit (Roche Applied Sciences, Indianapolis, IN, USA).

Two-Dimensional Gel Electrophoresis (2-DE)

The cell pellet was suspended in isotonic buffer (0.25 M sucrose, 1mM EDTA, 10mM Tris-Cl pH 7.5) followed by addition of lysis buffer (8.75 M urea, 2.5 M thiourea, 5% CHAPS, 50 mM DTT). After ultracentrifugation, carrier ampholyte (pH 3-10) was added to the supernatant. 200 µg of protein from the supernatant was then loaded onto a commercially available immobilized pH gradient (IPG) gel strip (pH 5-8; Bio-Rad Laboratories, Hercules, CA, USA) and isoelectric focusing (IEF) was performed. After the IEF run was completed, strips were equilibrated in buffer I (containing 6M urea, 0.375 M Tris pH 8.8, 2% SDS, 20% glycerol and 2% DTT), followed by a second incubation in buffer II which contained all the ingredients of buffer I except that DTT was replaced with 2.5% iodoacetamide. Strips were then transferred onto a 12% SDS-PAGE gel and SDS-PAGE was performed. Proteins were stained with colloidal Coomassie Brilliant Blue CBBG250. Spot analysis of the 2-D gels was done using PDQuest Version 8.0.1 software (Bio-Rad Laboratories, Hercules, CA, USA).This software computes the intensity of each spot and normalizes it to the total intensity of all the spots in the gel (excluding streaks). This was done for 3 sets of gels with three biological replicates of cell lysates processed simultaneously.

In-Gel Digestion and Extraction of Tryptic Peptides

Bands excised from 1-DE or 2-DE gels were destained, washed and dried. The dried gel pieces were washed with digestion buffer (40 mM ammonium bicarbonate and 10% acetonitrile) and 40 µl of trypsin buffer (13 ng/µl of trypsin in digestion buffer) was added to the gel pieces. The samples were incubated overnight at 37ºC. Peptides were extracted from the gel matrix by incubating the gel pieces with extraction buffer (50% acetonitrile and 0.3 % trifluroacetic acid (TFA)) and the supernatant was collected and dried. The dried peptide sample was reconstituted in 5% acetonitrile and 0.1% TFA and purified by a zip-tip.

LC MS/MS Analysis

Nanoflow electrospray ionization tandem mass spectrometric analysis of peptide samples was carried out using LTQ-OrbitrapVelos (Thermo Scientific, Bremen, Germany) interfaced with nanoflow LC system (EASY nLC Proxeon, now Thermo Scientific). The chromatographic capillary column used was reversed phase BioBasic C-18 analytical column (particle size 5 µm, pore size 300Å; 5µ X 10 cm) from Thermo Scientific. The peptides were eluted using a linear gradient of 5-30% acetonitrile over 65 min. Mass spectrometric analysis was carried out in a data dependent manner with full scans acquired using the Orbitrap mass analyzer at a mass resolution of 60,000 at 400 m/z. For each MS cycle, twenty most intense precursor ions from a survey scan were selected for MS/MS and fragmentation detected at a mass resolution of 15,000 at m/z 400. The fragmentation was carried out using collision-induced dissociation with 35% normalized collision energy. The automatic gain control for full FT MS was set to 1 million ions and for MS/MS was set to 0.1 million ions with a maximum time of accumulation of 500 ms, respectively. For accurate mass measurements, the lock mass option was enabled. For protein identification the MS and MS/MS data was searched on Proteome Discoverer (Thermo Fisher Scientific, Beta Version 1.3.0.339) based on the workflow with spectrum selector and reporter ion quantifier. MS/MS search was carried out using SEQUEST search algorithm, against the IPI Human database. Search parameters included trypsin as the enzyme with 1 missed cleavage allowed; oxidation of methionine was set as a dynamic modification while alkylation at cysteine was set as static modifications. Precursor and fragment mass tolerance were set to 20 ppm and 0.1 Da, respectively. The peptide and protein data were extracted using high peptide confidence and top one peptide rank filters. The false discovery rate (FDR) was calculated by enabling the peptide sequence analysis using a decoy database. High confidence peptide identifications were obtained by setting a target FDR threshold of 1% at the peptide level. Complete dataset and identified peptide sequences are available upon request.

Results and Discussion

Localization of GFP-Tagged Lamins

HEK293T cells transiently expressing GFP-tagged wild-type lamin A or mutant Q294P were initially characterized by immunofluorescence assays with antibodies to lamins. GFP-tagged wild-type lamin A was located at the nuclear periphery in a typical pattern, and it colocalized with lamin A/C and lamin B1as expected (Fig. 1A, B). However, GFP-tagged lamin mutant Q294P was distributed in aggregates throughout the nucleus, and its expression resulted in aberrant nuclear morphology and disruption of the endogenous lamin A network, consistent with our earlier observation in other cell types (Manju *et al.*, 2006). Importantly, we made the novel observation that endogenous lamin B1 was depleted in 88% of cells expressing lamin A mutant Q294P compared to <5% of cells expressing wild-type lamin A (Fig. 1A,B). Western blot analysis confirmed that GFP-tagged wild-type and mutant lamin A proteins were expressed at equal levels in cell lysates that were subsequently used for 1-D and 2-D gel electrophoresis (Fig. 1C). Since lamin cleavage is a marker for apoptosis, we also checked for the presence of lamin fragments in the western blot. Very low levels of lamin cleavage products were detected in samples of transfected cells, indicating a negligible number of apoptotic cells in the lysates.

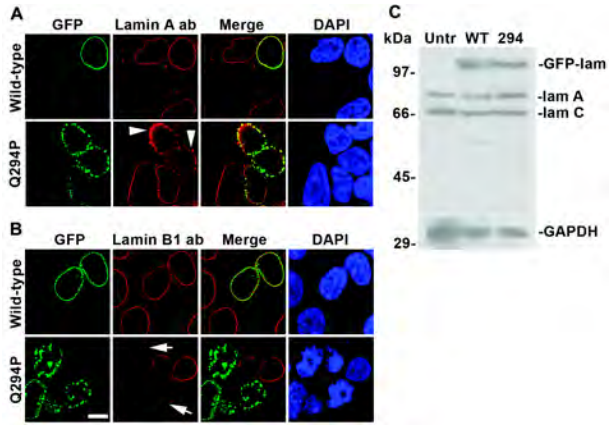

Fig. 1: Expression of GFP-tagged lamin A in HEK293T cells. (A, B) Cells were transiently transfected with GFP-lamin A wild-type or Q294P constructs, stained with anti-lamin A/C antibody or lamin B1 antibody and counterstained with DAPI. (C) Lysates of FACS-sorted cells expressing GFP-lamin A wild-type or Q294P and untransfected cells were analyzed by western blotting with antibodies to lamin A/C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Cells showing disruption of endogenous lamin A network (arrowheads) and depletion of lamin B1 (arrows) are indicated. Bar, 10 µm

Two-Dimensional Gel Electrophoresis Profile

In order to identify changes in the proteome upon expression of a laminopathic mutant, we initially compared protein profiles of HEK293T cells expressing GFP-tagged wild-type lamin or mutant Q294P by 2-D gel electrophoresis. GFP-expressing cells were sorted by FACS and the total cell lysates of the sorted cells were analysed. For 2-D electrophoresis, first dimension isoelectric focussing was carried out on an IPG strip with pI values in the range of 5-8, followed by SDS-PAGE separation. Proteome maps were compared using PDQuest software and differentially expressed spots were excised and identified by analysis on an LTQ-OrbitrapVelos mass spectrometer, with protein identification being carried out by SEQUEST. Quantitative analysis of protein levels was performed with three biological replicates. Forty-two differentially expressed spots were obtained, of which 27 spots gave definitive IDs by mass spectral analysis whereas the remaining spots could not be identified with certainty by mass spectral analysis. The positions of the spots are shown in a representative gel in Fig. 2 and the list of identified protein IDs is given in Table 1. The fold change in protein levels in the lamin mutant sample is indicated. Several proteins were downregulated in cells expressing mutant lamin Q294P and these are described below.

Heat Shock Proteins (HSPs): A number of HSPs were found to be downregulated in cells expressing lamin mutant Q294P. These include mitochondrial HSP75 (TRAP1), HSPA5, HSP90-β and HSP71-2 (HSPA8). While the main function of HSPs is to prevent accumulation of denatured or aggregated proteins, certain HSPs have specific functions. HSPA8 is a target of tumour suppressor protein p53 and it is transcriptionally repressed upon genotoxic stress (Bansal *et al.*, 2011) and also plays a role in maintaining myofibrillar integrity under mechanical

stress (Hishiya *et al*., 2010). TRAP1 knockdown has been shown to activate the endoplasmic reticulum-resident caspase-4 in response to stress, thereby inducing cell death in human cells (Takemoto *et al*., 2011).

RNA Processing Factors: Our data showed that heterogeneous ribonuclear proteins hnRNP-H3, hnRNP C1, hnRNP-L and hnRNP-K were down regulated or absent in cells expressing mutant lamin. In addition to their role in pre-mRNA processing events and mRNA export, certain hnRNPs like hnRNP-A18, B1, C1/C2 and K also play a key role in coordinating repair pathways following exposure to ionizing radiation (Haley *et al*., 2009). HnRNP C1/C2 is a major component of the nuclear matrix and cells with reduced hnRNP C1/C2 expression are sensitized to cellular stress (Hossain *et al*., 2007). Far upstream element-binding protein 2 (KHSRP), which is undetectable in cells expressing lamin mutant Q294P, is a multifunctional RNA binding protein that promotes the rapid decay of AU-rich element (ARE)-containing mRNAs, which include genes involved in cell proliferation, stress response and cancer and is a downstream target of BRCA1-mediated signalling upon genotoxic stress (Santarosa *et al*., 2010).

Cytoskeletal Proteins: We observed a significant depletion in levels of the cytoskeletal proteins vimentin, keratin type II-1 and β-tubulin in cells expressing lamin mutant Q294P. The lamina is linked to the cytoskeleton via interactions with nuclear membrane proteins termed nesprins and SUN (Sad1/UNC-84 homology) domain proteins and disruption of this linkage can cause defects in nuclear positioning and migration during development (Starr and Han, 2003). Changes in abundance of cytoskeletal proteins have been reported in lamin-depleted cells and laminopathic cells (Chen *et al*., 2009; Foster *et al*., 2011; Magagnotti *et al*., 2012). Changes in cytoskeletal organization are likely to affect proteins involved in intracellular transport and signalling, like zymogen granule protein 16B and guanine nucleotide binding protein subunit β2 which were reduced in the mutant cells.

Metabolic Enzymes: Levels of key enzymes of metabolic pathways such as α-enolase, L-lactate dehydrogenase B chain, pyruvate dehydrogenase E1 component subunit β and cytochrome b-c1-2, and enzymes of carbohydrate metabolism such as α-amylase and α-glucosidase were reduced in cells expressing the lamin mutant Q294P. A decrease in levels of proteins involved in energy metabolism has

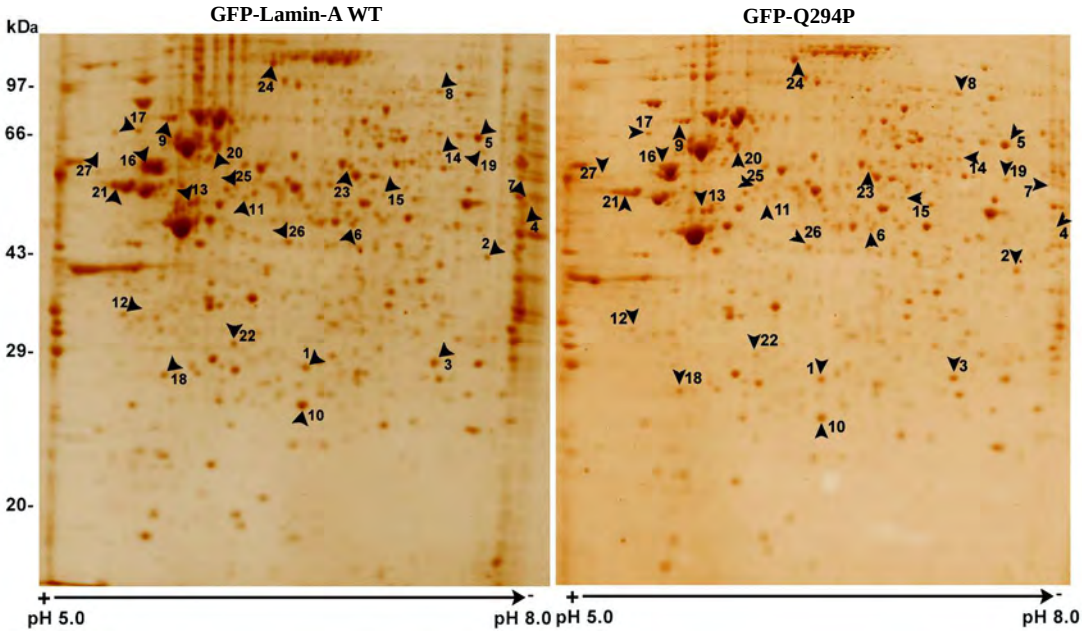

Fig. 2: 2-DE profiles of proteins from cells expressing GFP-tagged lamin A wild-type or Q294P. Whole cell lysates of HEK293T cells expressing GFP-lamin A wild-type (Left panel) or Q294P (Right panel) were analyzed by 2-DE followed by spot alignment. Differentially expressed proteins (indicated by arrowheads) were quantitated and then eluted from the GFP-lamin A wild-type 2-D gel and analyzed by mass spectrometry

Table 1: Identification of differentially expressed proteins by 2-DE in mutant vs wild-type cells

Spot	Protein name	Accession no	Score	Coverage %	Peptides	MW (kD)	pI	Fold change[a]
1	Zymogen granule protein 16B	IPI00060800	673.5	29.81	5	22.72	7.39	(-) 1.37 ± 0.32
2	Cytochrome b-c12	IPI00305383	136.7	15.67	6	48.41	8.63	(-) 1.76 ± 0.53
3	Guanine nucleotide binding protein subunit β2	IPI00848226	323.7	51.42	12	35.05	7.69	(-) 1.41 ± 0.26
4	Heat shock protein 75 (TRAP1)	IPI00030275	1532.8	59.38	40	80.05	8.21	(-)1.38 ±0.06
5	HnRNP-L	IPI00027834	808.5	37.18	16	64.09	8.22	(-)1.88 ± 0.06
6	α-amylase 1	IPI00300786	573.4	49.90	18	57.73	6.93	ND[b] in M
7	Keratin, type II-1	IPI00220327	885.1	24.38	31	65.99	8.12	ND in M
8	Far upstream element-binding protein 2(1)	IPI00479786	1450.4	55.56	34	73.07	7.31	ND in M
9	Heat shock protein 90-β	IPI00414676	169.7	28.31	19	83.21	5.03	(-)2.90 ± 0.94
10	L-lactate dehydrogenase B chain	IPI00219217	2122.9	46.11	15	36.61	6.05	(-)2.50 ± 0.22
11	HnRNP-H3	IPI00908896	103.8	27.27	10	47.05	6.34	(-)1.41 ± 0.05
12	HnRNP-C1	IPI00216592	214.5	30.72	13	32.31	5.08	(-)1.14 ±0.06
13	HnRNP-K	IPI00514561	613.1	23.13	15	47.52	5.63	(-)4.88± 2.61
14	Prelamin-A	IPI00021405	837.1	37.50	25	74.09	7.02	ND in M
15	Heat shock protein 71-2 (HSPA8)	IPI00939595	53.2	8.52	3	53.48	5.86	ND in M
16	Vimentin	IPI00418471	3440.0	59.87	30	53.61	5.12	(-)3.67 ± 0.44
17	Heat shock protein A5	IPI00003362	189.8	29.77	24	72.37	5.16	(-)2.31 ± 1.06
18	Nucleophosmin (3)	IPI00658013	160.7	15.06	4	28.38	4.79	(-)2.20 ± 0.77
19	Radiation sensitive 23B	IPI00956313	331.2	39.11	19	58.29	8.00	ND in M
20	LaminB1	IPI00217975	1186.6	45.73	31	66.36	5.16	(-)4.68± 1.61
21	Tubulin-β	IPI00645452	3968.8	66.67	24	47.73	4.81	(-)2.37 ± 0.84
22	Pyruvate dehydrogenase E1β (2)	IPI00549885	325.5	21.70	8	37.17	5.90	(-)1.51±0.22
23	Aminoimidazole-4-carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase -like	IPI00925601	836.7	53.13	24	64.48	6.71	(-)1.49±0.30
24	α-glucosidaseAB-like	IPI00383581	170.7	7.14	6	112.85	6.06	ND in M
25	T-complex protein 1	IPI00027626	1002.4	51.60	19	58.00	6.68	(+)1.22± 0.11
26	Pontin (1)	IPI00021187	1806.2	66.01	22	50.20	6.42	(+)1.87± 0.16
27	Protein disulfide isomerase	IPI00010796	2690.7	53.74	24	57.10	4.87	(+)1.10 ± 0.02

[a]Depicts down regulation (-) or upregulation (+) of protein levels in lamin mutant cells with respect to lamin wild-type cells. Isoform numbers or alternate names are shown in brackets; [b]ND: Not detectable (below detection limit of software)

also been observed in cells from laminopathic patients (Magagnotti *et al*., 2012), while downregulation of transcripts for glycolysis genes has been documented in limb-girdle muscular dystrophy and lipodystrophy patients with mutations in lamin A (Boschmann *et al*., 2010). The enzyme amino-imidazole-4-carboxamide ribonucleotide trans-formylase/inosine monophosphate cyclohydrolase, which is involved

in folate metabolism and is reduced in mutant cells, is an important target for antineoplastic agents (Wolan *et al*., 2003).

DNA Damage Response (DDR) Proteins: Proteins directly involved in DDR that were depleted in cells expressing lamin mutant Q294P included radiation sensitive 23B (RAD23B) which has been shown to play an important role in cell survival after UV irradiation (Renaud *et al*., 2011), and nucleophosmin, a phosphoprotein belonging to the nucleoplasmin family of chaperones which directly interacts with p53 leading to its stabilization. Downregulation of nucleophosmin reduces p53 stabilization in response to genotoxic stress thereby impairing p53-dependent cell cycle arrest (Colombo *et al*., 2002). Mutations in lamin A have been shown to impair the cellular response to DNA damage (Liu *et al*., 2005; Manju *et al*., 2006).

Lamins: We observed a ~5-fold depletion of lamin B1 in cells expressing lamin mutant Q294P which was consistent with our immunofluorescence data. Decline in expression of lamin B1 has been observed in cells expressing lamin A progeria mutations (Scaffidi and Misteli, 2005; Taimen *et al*., 2009). Although A- and B-type lamins form separate meshworks in the nucleus, these have been shown to interact closely and influence their respective functions and assembly (Shimi *et al*., 2008; Tripathi *et al*., 2009). Lamin B1 depletion results in genome-wide changes in chromatin organization and gene expression (Sadaie *et al*., 2009; Shah *et al*., 2013) and impaired DNA repair (Butin-Israeli *et al*., 2013). We also observed a decrease in levels of isoform A of prelamin A/C in the mutant cells.

The proteins identified by 2-DE profiling that were upregulated in cells expressing mutant lamin Q294P include the chaperonin T-complex protein 1which is also overexpressed in colorectal cancer (Coghlin *et al*., 2006), and the ATPase pontin (also known as RuvBL1) which belongs to the family of AAA+ ATPases that are involved in chromatin remodelling, DNA damage repair and biogenesis of telomerase (Grigoletto *et al*., 2011). Quantitative analysis showed that the levels of protein disulfide isomerase were increased to a small extent only.

Changes in expression of protein chaperones, RNA processing proteins, cytoskeletal proteins and metabolic enzymes have been frequently observed in 2-DE analysis of different cell types misexpressing lamin A. In HeLa cells depleted of lamin A protein by RNA interference, hnRNP proteins like hnRNP-K and hnRNP-C1/C2 and the cytoskeletal proteins keratin and actin were also downregulated (Chen *et al*., 2009), whereas the protein profile of a colorectal cell line (SW480) depleted of lamin A showed decrease in nucleophosmin, HSPD1 and HSPA9, and upregulation of β-actin and vimentin (Foster *et al*., 2011). In a proteomic study with skin fibroblasts from patients with neuromuscular laminopathies, proteins downregulated in the patients cells which were also reduced in our lamin mutant Q294P samples included α-enolase, HSP90, hnRNP C1/C2, L-lactate dehydogenase B chain, vimentin and β-tubulin (Magagnotti *et al*., 2012). It is interesting to note that a few proteins which were downregulated in cells with lamin A mutations or lamin depletion were also found to be altered in malignant tumour samples. Proteomic analysis of a malignant tumour clone of mouse fibrosarcoma cells showed that expression of proteins like lamin A/C, far upstream element-binding protein 2, guanine nucleotide binding protein subunit β2 and hnRNP-K was reduced considerably in these cells (Kuramitsu *et al*., 2010).

High Throughput Proteomic Analysis

Previously reported 2-DE protein profiles of cells depleted of lamin A or expressing laminopathic mutations have revealed 10-50 differentially expressed proteins with identifiable IDs by mass spectral analysis (Chen *et al*., 2009; Foster *et al*., 2011; Magagnotti *et al*., 2012). In our 2-DE analysis of cells expressing GFP-tagged wild-type lamin A or mutant Q294P, 27 proteins could be identified that showed differences in expression. The low number of proteins that have been identified is probably due to limitations of the technique. Hence we carried out a high throughput analysis of whole cell lysates by 1-DE followed by mass spectral analysis. Whole cell lysates from FACS-sorted HEK293T cells expressing either GFP-tagged wild-type lamin A or mutant Q294P were used for 1-DE analysis. The mass

spectral analysis of the tryptic peptides was carried out on an LTQ-OrbitrapVelos mass spectrometer and the MS/MS spectra were analyzed using SEQUEST software. 1-DE analysis was carried out with samples from five biological replicates and the protein IDs from all the rounds were pooled together for analysis. After removing duplicates, a total of 7742 protein IDs were identified from GFP-tagged wild-type lamin A expressing cells and 9660 protein IDs from mutant Q294P cells. From these, we obtained a total of 2119 protein IDs which were unique to GFP-tagged wild-type lamin A cells of which 996 proteins showed two or more than two peptide hits. Similarly for cells expressing GFP-tagged mutant Q294P, a total of 4038 unique protein IDs were obtained out of which 2160 proteins showed two or more than two peptide hits. After removal of uncharacterized protein IDs (~25% of total), 650 unique proteins from GFP-tagged wild-type lamin A cells and 1494 unique proteins from GFP-tagged mutant Q294P cells were obtained. The Venn diagram representation of this analysis is shown in Fig. 3.

In our 2-DE protein profiles, we had observed significant reduction in levels of lamin B1 and DDR proteins. Decline in expression of lamin B1 has been associated with cellular senescence in several human and murine fibroblast cell lines (Shimi *et al*., 2011; Freund *et al*., 2012). Cellular senescence generally involves arrest of cellular proliferation and has been linked to cancer and ageing. Stimuli that induce cellular senescence include DNA damage, disorganization of chromatin and shortened telomeres (Campisi and d'Adda di Fagagna, 2007). Although laminopathic mutations have been shown to have

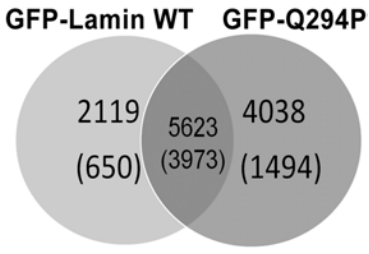

Fig. 3: Mass spectrometric analysis of proteins from cells expressing GFP-tagged lamin A wild-type or Q294P. Venn diagram representation of numbers of proteins identified by 1-DE and mass spectrometry; numbers in brackets represent IDs of characterized proteins with two or more peptide hits

widespread effects on chromatin organization, changes in levels of only a few chromatin proteins like heterochromatin protein 1 (HP1) and methylated histones have been reported (Goldman *et al*., 2004; Columbaro *et al*., 2005; Chaturvedi and Parnaik, 2010). In our data, a number of proteins or protein isoforms involved in chromatin structure, chromosome condensation, nucleosome assembly, epigenetic modifications and centromere organization were detectable exclusively in either wild-type lamin cells or lamin mutant Q294P cells. These proteins are listed in Tables 2 and 3. Alterations in expression of centromeric proteins are consistent with the aberrant localization of centromeres observed in HGPS cells (Taimen *et al*., 2009). Changes in expression of histone methyl transferases, acetyltransferases and deacetylases are compatible with alterations in gene expression due to histone modifications observed in cells depleted of lamin B1 (Sadaie *et al*., 2013; Shah *et al*., 2013).

Telomere function requires a normal nuclear lamina and dysfunctional, shortened telomeres have been observed in HGPS and lamin A-deficient cells (Taimen *et al*., 2009; Gonzalez-Suarez *et al*., 2009; Decker *et al*., 2009). Our data showed that only lamin mutant cells expressed detectable levels of PIN2/TRF-1 telomerase inhibitor, whose overexpression leads to shortened telomeres (Zhou *et al*., 2003). Earlier studies have shown that laminopathic cells exhibit a delayed response to DNA damage and increased double-strand breaks (Liu *et al*., 2005; Manju *et al*., 2006; Gonzalez-Suarez *et al*., 2009), which have been attributed to decreased levels of ATR kinase and p53 binding protein (Gonzalez-Suarez *et al*., 2011; Muralikrishna *et al*., 2012). In the present analysis, additional DNA repair proteins such as replication protein A, DNA mismatch repair protein Msh 3, DNA repair protein XRCC3 and Werner's helicase interacting protein 1 were not detected in lamin mutant cells while certain other proteins involved in DNA repair like histone H2AX, which is phosphorylated at repair foci upon DNA damage (Redon *et al*., 2002), and double-strand break repair protein MRE11A, MMS19 nucleotide excision repair protein, UV excision repair protein RAD23-A and DNA repair proein RAD51-1were detected only in

lamin mutant cells (Tables 2, 3). These alterations are likely to contribute to the impaired DDR reported previously in laminopathic cells (Liu *et al*., 2005; Manju *et al*., 2006; Gonzalez-Suarez *et al*., 2009).

In addition to the high throughput data which has been tabulated in this paper, we observed changes in categories of proteins that were also altered in 2-DE protein profiles and these included hnRNPs, HSPs and cytoskeletal proteins. A number of changes were also observed in components of the ubiquitin-proteasome system, which is consistent with our previous findings of upregulation of specific ubiquitin ligases and increased proteasomal degradation of key proteins in laminopathic cells (Chaturvedi and Parnaik, 2010; Chaturvedi *et al*., 2012; Muralikrishna *et al*., 2012). We also observed changes in proteins involved in mitogen-activated protein kinase (MAPK) signalling in both wild-type lamin and mutant cells but did not detect MAPK kinase 6 which causes continual p38 MAPK activity and induces senescence (Freund *et al*., 2011); moreover, decline of lamin B1 upon DNA damage has been reported to be independent of this pathway (Freund *et al*., 2012). On the other hand, under conditions of oxidative stress, p38 MAPK signalling has been reported to

Table 2: High throughput analysis of proteins that are detectable only in lamin wild-type cells

Accession no	Protein name	Score	Coverage %	Peptides	MW kDa	pI
Chromatin organization						
IPI00890837	Structural-maintenance-of-chromosomes (SMC) protein 1 (1)	71.94	11.82	18	226.2	7.30
IPI00411592	Chromodomain-helicase-DNA-BP3 (2)	104.4	7.68	11	222.7	7.52
IPI00009286	Histone-lysine N-methyl transferase, mixed-lineage-leukemia (MLL) (1)	21.87	1.97	5	431.5	9.09
IPI00645162	SMC protein 1 (3)	9.51	7.35	4	85.9	6.70
IPI00893552	Bromodomain-containing protein 2	8.86	5.71	3	67.2	9.09
IPI00020985	Histone acetyltransferase p300	15.29	1.66	3	264.0	8.50
IPI00329216	DNA (cytosine-5)-methyltransferase 3A (1)	22.35	4.61	3	101.8	6.57
IPI00410716	Bromodomain-containing protein 3 (2)	13.95	5.94	3	60.9	9.39
IPI00552442	Centromere protein P	18.35	18.40	3	33.1	6.27
IPI00445741	SMC-associated protein 1	5.37	2.45	2	104.8	6.11
IPI00793392	MLL1/MLL subunit C17orf49 (3)	5.15	31.88	2	13.9	7.24
IPI00552142	Centromere protein I (1)	4.77	3.31	2	86.7	8.76
IPI00376404	Nucleosome-remodeling factor BPTF (4)	8.25	1.48	2	322.0	6.38
IPI00018689	Centromere protein Q	8.46	9.70	2	30.6	9.42
DNA repair						
IPI00329605	DNA mismatch repair protein Msh3	21.22	6.95	5	127.3	8.02
IPI00017373	Replication protein A 14 kD subunit	9.06	29.75	3	13.6	5.08
IPI00023073	DNA repair protein XRCC3	14.30	7.51	2	37.8	8.48
IPI00642145	Replication protein A 32 kD subunit-like	7.89	56.73	2	11.1	6.27
IPI00645459	Werner's helicase interacting protein 1 (3)	5.65	5.39	2	49.5	8.28

Table 3: High throughput analysis of proteins that are detectable only in lamin mutant cells

Accession no	Protein name	Score	Coverage %	Peptides	MW kDa	pI
Chromatin organization						
IPI00218500	Mixed-lineage-leukemia (MLL) (14P-18B)	56.21	3.46	8	427.5	9.14
IPI00023860	Nucleosome assembly protein 1-like 1	47.78	21.99	7	45.3	4.46
IPI00173901	Histone-lysine N-methyltransferase, H3K36 and H4K20 (2)	13.82	3.05	5	267.2	8.51
IPI00254408	Nucleosome-remodeling factor BPTF (2)	14.56	2.57	5	324.9	7.08
IPI00171252	Histone-lysine N-methyltransferase EZH2 (3)	13.52	7.50	4	81.0	7.12
IPI00376481	Centromere protein V (3)	51.09	22.43	4	29.7	9.73
IPI00953599	Histone acetyltransferase MYST2-like	22.48	13.12	4	51.4	8.92
IPI00010158	Chromatin accessibility complex protein 1	13.22	34.35	3	14.7	5.10
IPI00010388	Major centromere autoantigen B	7.01	4.84	2	65.1	4.55
IPI00014266	Bromodomain-containing protein 3 (1)	18.16	3.72	2	79.5	9.36
IPI00031519	DNA (cytosine-5)-methyltransferase 1(1)	5.38	2.21	2	189.4	8.02
IPI00440728	Bromodomain-containing protein 4 (2)	4.82	3.05	2	80.4	8.62
IPI00514649	Histone deacetylase 1	5.00	17.54	2	24.5	8.31
IPI00619925	Centromere-associated protein E (3)	16.18	1.01	2	301.6	5.57
IPI00645124	Histone deacetylase 8 isoform 2	9.10	9.79	2	31.9	5.97
IPI00788136	Centromere protein O (2)	19.82	9.86	2	33.0	7.93
IPI00807400	Structural-maintenance-of-chromosomes (SMC) protein 1B (2)	8.50	2.15	2	135.8	7.93
IPI00893348	Centromere protein M	7.09	30.14	2	8.1	8.54
IPI00922333	SMC 1-like protein	5.96	4.65	2	59.1	9.23
IPI00941101	Histone-lysine N-methyltransferase SUV39H1	7.99	6.07	2	47.9	8.03
IPI00945286	Histone-lysine N-methyltransferase EZH2 (5)	12.11	4.17	2	79.6	7.23
Telomere length						
IPI00297357	PIN2/TRF1-interacting telomerase inhibitor 1(1)	16.59	17.68	4	37.0	9.60
DNA repair						
IPI00219037	Histone H2AX	423.7	53.15	8	15.1	10.7
IPI00940798	Double-strand break repair protein MRE11A (1)	43.68	10.88	6	80.5	5.90
IPI00941928	MMS19 nucleotide excision repair protein homolog (1)	28.04	7.38	5	113.2	6.35
IPI00008219	UV excision repair protein RAD23-A	13.64	10.74	3	39.6	4.58
IPI00220649	DNA repair protein RAD51-1(2)	12.19	20.25	3	26.3	5.50

increase lamin B1 levels and lead to nuclear shape changes and senescence (Barascu *et al*., 2012). A few antioxidant proteins were also differentially detected in wild-type lamin and mutant cells. Thus our study has generated an exhaustive dataset that can be analyzed in detail in future studies, to gain insights into the functional importance of identified altered proteins in laminopathic disease processes.

In summary, our major finding from protein profiling by quantitative 2-DE and high throughput proteomic analysis is that expression of the EMD lamin A mutation Q294P in cells leads to decline in lamin B1 levels accompanied by changes in expression of markers that are associated with cellular stress and senescence as well as DDR, cell death and other stress-related processes.Our study provides new information on changes in a number of proteins or protein isoforms that are involved in chromatin organization, DNA repair and telomere length that have not been reported in earlier proteomics studies on cells misexpressing lamin A. These results have important implications for understanding the basis of tissue wasting and premature aging phenotypes in laminopathies.

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