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Expression of disease-causing lamin A mutants impairs the formation of DNA repair foci

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Summary

A-type lamins are components of the nuclear lamina. Mutations in the gene encoding lamin A are associated with a range of highly degenerative diseases termed laminopathies. To evaluate sensitivity to DNA damage, GFP-tagged lamin A cDNAs with disease-causing mutations were expressed in HeLa cells. The inner nuclear membrane protein emerin was mislocalised upon expression of the muscular dystrophy mutants G232E, Q294P or R386K, which aberrantly assembled into nuclear aggregates, or upon expression of mutants causing progeria syndromes in vivo (lamin A del50, R471C, R527C and L530P). The ability of cells expressing these mutants to form DNA repair foci comprising phosphorylated H2AX in response to mild doses of cisplatin or UV irradiation was markedly diminished, unlike the nearly normal response of cells expressing wild-type GFP-lamin A or disease-causing H222P and R482L mutants. Interestingly, mutants that impaired the formation of DNA repair foci mislocalised ATR (for 'ataxia telangiectasia-mutated and Rad3-related') kinase, which is a key sensor in the response to DNA damage. Our results suggest that a subset of lamin A mutants might hinder the response of components of the DNA repair machinery to DNA damage by altering interactions with chromatin.

Key words: Nuclear lamina, Laminopathies, DNA repair, Phosphorylated H2AX, Emerin, ATR kinase

Introduction

Lamins are nuclear proteins that are components of a fibrous network underlying the inner nuclear membrane termed the nuclear lamina, and are also distributed throughout the interior of the nucleus (reviewed by Goldman et al., 2002). Lamins have been classified into two types based on biochemical properties and expression patterns. B-type lamins are expressed in most cells and are encoded by two separate genes, B1 and B2, whereas the A-type lamins have been detected primarily in differentiated cell types. The lamin A gene (*LMNA*) encodes lamin A and C transcripts, as well as germ-cell-specific lamin C2. Lamins belong to the intermediate filament family of proteins and have a short N-terminal head domain followed by an α-helical rod domain and a globular tail domain. The C-termini of lamins A, B1 and B2 bear a CAAX motif that is post-translationally modified and, in the case of lamin A, is subjected to a further maturation step by which prelamin A is proteolytically cleaved to give mature lamin A (reviewed by Stuurman et al., 1998).

Mutations in human *LMNA* cause several debilitating diseases, collectively termed laminopathies, that affect skeletal and cardiac muscle, adipose, bone and neuronal tissues, and also cause premature ageing syndromes. The majority of mutations cause autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD) (Bonne et al., 1999), whereas other mutations cause dilated cardiomyopathy with conduction system disease (DCM) (Fatkin et al., 1999), limb girdle muscular dystrophy (Muchir et al., 2000), or familial partial lipodystrophy (FPLD) (Shackleton et al., 2000; Cao and Hegele, 2000). Mutations in *LMNA* have also been linked to the relatively rare autosomal recessive disorders Charcot-Marie-Tooth disorder type 2 (De Sandre-Giovannoli et al., 2002) and mandibuloacral dysplasia (Novelli et al., 2002), and more recently to restrictive dermopathy (Navarro et al., 2004). Many of the above mutations are missense mutations that occur throughout the gene, although the FPLD mutations are clustered near the C-terminus. An interesting finding has been the linkage of mutations in *LMNA* to Hutchinson-Gilford progeria syndrome (HGPS) (Eriksson et al., 2003; De Sandre-Giovannoli et al., 2003) and to atypical Werner's syndrome (Chen et al., 2003). The most frequent mutation in HGPS is a nucleotide substitution (GGC to GGT) that does not cause an amino acid change (G608G) but activates a cryptic splice site that leads to a deletion of 50 amino acids near the C-terminus (residues 607-656), giving rise to a truncated protein termed lamin A del50 or progerin, which retains the C-terminus of prelamin A and is not proteolytically processed. A few missense mutations such as R471C and R527C that do not affect processing of the C-terminus of lamin A have also been linked to HGPS and atypical progerias (Eriksson et al., 2003; Cao and Hegele, 2003). Cells from patients expressing mutant lamins often exhibit a range of dominant-negative effects such as abnormal nuclear morphology, aberrant lamin assembly and altered gene regulation (reviewed by Wilson, 2000; Worman and Courvalin, 2002; Hutchison and Worman, 2004). HGPS cells in particular show serious defects in nuclear structure as well as early senescence (Eriksson et al., 2003; De Sandre-Giovannoli et al., 2003; Mounkes et al., 2003, Goldman et al., 2004), and recent reports indicate that HGPS cells and cells lacking functional Zmpste24, a metalloproteinase required for

prelamin A processing, are defective in DNA repair (Liu et al., 2005; Varela et al., 2005). Mice that lack intact *LMNA* develop severe muscle wasting similar to human EDMD by 3-4 weeks and die by 8 weeks after birth (Sullivan et al., 1999). Lamin-deficient cells have defective nuclear mechanics (Lammerding et al., 2004) and altered gene expression (Nikolova et al., 2004).

In addition to providing structure and shape to the nucleus, the lamins are involved in organising several nuclear functions such as DNA replication (Moir et al., 2000a), RNA polymerase II (pol II) transcription (Spann et al., 2002; Kumaran et al., 2002) and mitotic assembly and disassembly. Lamin-chromatin interactions have also been proposed to have an important role in nuclear organisation (reviewed by Goldman et al., 2002). During the late stages of apoptosis, the lamins are proteolytically cleaved by caspases, which helps to disassemble the lamina and allows nuclear condensation and fragmentation to proceed (reviewed by Cohen et al., 2001). Ectopic expression of various lamin A mutants upon transfection in cultured cells has been shown to affect normal lamin assembly and alter the localisation of the inner nuclear membrane protein emerin, which is involved in lamin-chromatin interactions (Östlund et al., 2001; Vigoroux et al., 2001; Raharjo et al., 2001; Holt et al., 2003). Certain mutants also hinder muscle differentiation and promote cell death upon serum withdrawal (Favreau et al., 2004; Mariappan and Parnaik, 2005). Expression of lamin A del50 leads to strongly dominant-negative effects on nuclear architecture, and blocks progression of cells through S phase (Goldman et al., 2004). Although certain lamin A mutants cause cell division defects, it is not clear whether cells expressing lamin mutants are affected in their response to DNA-damaging agents.

The initial response to DNA damage involves the chromatin-dependent activation of complex checkpoint signalling pathways that delay the cell cycle and repair the defects. The major kinases that serve as damage sensors and regulate cell-cycle checkpoints and DNA repair by phosphorylation of key substrates are ATM (for 'ataxia telangiectasia mutated') and ATR (for 'ATM and Rad3 related') kinases that belong to the phosphoinositide 3-kinase-related family of serine/threonine protein kinases (reviewed by Zhou and Elledge, 2000; Abraham, 2001; Bartek et al., 2004). We have examined the early response of cells expressing disease-causing lamin A mutants to DNA damage induced by cisplatin or UV, which primarily cause replicational stress as a result of stalled replication forks in S phase. A wide range of disease-causing mutants have been analysed and have been linked to progeroid syndromes and autosomal dominant forms of EDMD, DCM and FPLD. We observe that several mutants impair the formation of phosphorylated H2AX at DNA repair foci and hinder the recruitment of 53BP1 to repair sites. These mutants disrupt emerin localisation and, importantly, also mislocalise ATR kinase in untreated cells. Hence, lamin mutants are likely to affect the response of the DNA repair machinery to DNA damage by altered interactions with chromatin.

Results

Localisation of lamin A mutants

The intracellular localisation of mutant lamin A proteins was determined by transient transfection of fusion constructs bearing the GFP tag fused to the N-termini of the constructs into HeLa cells. The assembly of wild-type GFP-lamin A into a typical nuclear rim pattern has been well documented (Broers et al., 1999; Moir et al., 2000b). The mutations studied were E203G, H222P, G232E, Q294P, R386K, R471C, R482L, R527C, L530P and lamin A del50 (see Fig. 1A for positions of mutations). In addition to the lamin A del50, R471C and R527C mutations that cause human progeroid syndromes, mice homozygous for the L530P mutation show strong progeroid symptoms (Mounkes et al., 2003), although in humans this mutation leads to AD-EDMD (Bonne et al., 1999). H222P, G232E, Q294P and R386K have been identified in AD-EDMD (Bonne et al., 2000). E203G is mutated in DCM (Fatkin et al., 1999), and the R482L mutation is found in patients with FPLD (Shackleton et al., 2000).

Appropriate expression of the mutant proteins was confirmed by western blot analysis of transfected cell lysates with antibodies to GFP. None of the constructs yielded abnormally sized proteins. Lamin A del50 gave a product that was ~5 kDa smaller than the other constructs, as expected (see Fig. 1B). Each mutant protein migrated as a doublet and, except for lamin A del50, the faster migrating band representing processed lamin A was more prominent. Microscopic analysis of transfected cells indicated that the progeroid constructs (R471C, R527C, L530P and lamin A del50) as well as E203G, H222P and R482L were predominantly localised to the nuclear periphery (Fig. 1C and a summary of the data is given in Table 1). However,

Table 1. Summary of effects of ectopically expressed lamin A mutants

Mutant	Disease	Nuclear location/morphology	Emerin	ATR	γ-H2AX[§]
E203G	DCM	Peripheral*/normal	Disrupted	Mislocalised	Low
H222P	AD-EDMD	Peripheral/normal	Normal	Normal	Normal
G232E	AD-EDMD	Aggregates/distorted	Disrupted	Mislocalised	Low
Q294P	AD-EDMD	Aggregates/distorted	Disrupted	Mislocalised	Low
R386K	AD-EDMD	Aggregates/distorted	Disrupted	Mislocalised	Low
R471C	HGPS	Peripheral/few lobulated[†]	Disrupted	Mislocalised	Low
R482L	FPLD	Peripheral/normal	Normal	Normal	Normal
R527C	HGPS	Peripheral/few lobulated[†]	Disrupted	Mislocalised	Low
L530P	AD-EDMD/HGPS	Peripheral/few lobulated[†]	Disrupted	Mislocalised	Low
Del50	HGPS	Peripheral/lobulated[‡]	Disrupted	Mislocalised	Low

*With small aggregates at the periphery in ~40% of transfected cells.
[†]10-15% of transfected nuclei, rest normal.
[‡]60% of transfected nuclei.
[§]After treatment with cisplatin for 4 hours or UV for 30 minutes.

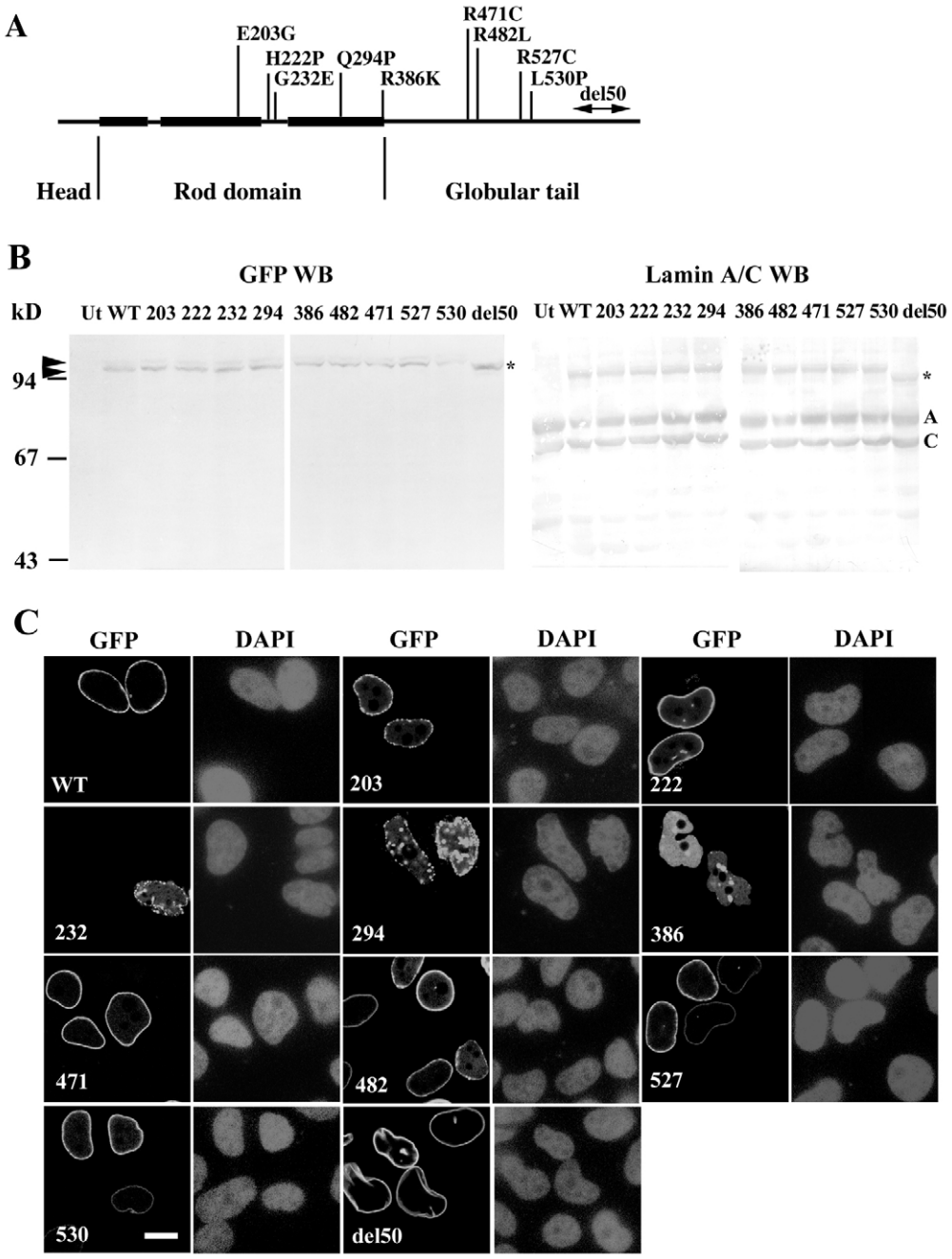


Fig. 1. Assembly properties of GFP-tagged lamin A constructs. (A) Domain structure of the lamin A protein indicating disease-causing mutations that have been analysed in this study. (B) Western blot analysis of HeLa cell lysates expressing GFP-tagged lamin constructs, probed with anti-GFP and anti-lamin A antibodies. Unprocessed and processed forms of lamins are indicated by arrowheads, lamin A del50 is marked by asterisks, and A and C indicate lamin A and lamin C. Molecular mass markers indicated on the left are: phosphorylase b, 94 kDa; albumin, 67 kDa; and ovalbumin, 43 kDa. (C) Immunofluorescence analysis of GFP-tagged lamin A constructs transiently transfected into HeLa cells and counterstained with DAPI. Ut, untransfected cells; WT, wild-type GFP-lamin A; del50, lamin A del50; numbers represent positions of altered amino acid residues indicated in A. Bar, 10 μm.

expression of lamin A del50 caused extensive changes in nuclear morphology, resulting in lobulated nuclei in ~60% of cells, as reported earlier (Goldman et al., 2004), whereas the number of lobulated nuclei in the other progeroid mutants was only 10-15%. By contrast, expression of G232E, Q294P and R386K gave rise to large intranuclear aggregates indicative of defective assembly, with variable diffuse nucleoplasmic staining, as observed earlier with FLAG-tagged R386K (Östlund et al., 2001); the nuclear morphology was also distorted in many cells. As these assays were done with transiently transfected cells, a small fraction of cells (~10%) showing high levels of expression of the less disruptive lamin constructs, such as H222P, R482L and including wild-type lamin A, also displayed small aggregates and striated or tubular patterns of expression. To minimise overexpression of the lamin construct, cells were analysed by 24 hours of transfection. At this time point, the expression of GFP lamin A was only 20-30% of endogenous lamin A (Fig. 1B). Similar patterns of localisation were obtained when mutant lamin A proteins were expressed in the C2C12 mouse myoblast cell line (data not shown).

Alterations in localisation of emerin

Emerin is an integral nuclear membrane protein that is an important binding partner for lamin A/C (Clements et al., 2000; Vaughan et al., 2001), and expression of certain lamin A mutants in transfected cells can alter its localisation (Östlund et al., 2001; Raharjo et al., 2001; Vigoroux et al., 2001; Holt

et al., 2003). Mutant lamins also bind less efficiently to emerin in in vitro binding assays (Raharjo et al., 2001). We observed that expression of G232E, Q294P or R386K mutants resulted in extensive disruption of emerin staining at the nuclear rim (Fig. 2A). Furthermore, emerin staining was reduced in a significant majority of cells expressing E203G, R471C, R527C, L530P or lamin A del50. In the case of E203G, cells with small aggregates of mutant lamin at the periphery showed mislocalisation of emerin. These effects on emerin localisation were observed in only a small percentage of cells expressing the H222P and R482L mutants, or wild-type GFP-lamin A, and a quantitative analysis of these data is shown in Fig. 2B.

Decreased formation of γ-H2AX foci

An early response to various types of DNA damage is phosphorylation of histone H2AX, a variant form of the histone H2A, to give intranuclear γ-H2AX foci; this is mediated by ATM, ATR and DNA-dependent protein kinases (reviewed by Redon et al., 2002). We have tested the ability of HeLa cells expressing lamin A mutants to respond to DNA damage by quantitating the formation of DNA repair foci labelled with an antibody specific for γ-H2AX. DNA damage was induced after transient transfection of lamin constructs by treatment of cells with cisplatin or UV irradiation, which cause replicational stress. Conditions were standardised so that events occurring prior to apoptosis and lamin cleavage could be evaluated.

Cisplatin primarily forms intrastrand adducts, which are recognised by DNA repair proteins, by crosslinking the N7 positions of adjacent purines on the same strand; cisplatin-induced damage leads to the activation of the ATR checkpoint signalling pathway (Cliby et al., 1998). The conditions for cisplatin treatment were initially optimised to ensure that cells had not entered the apoptotic pathway under the chosen conditions. When HeLa cells were treated with 25 μM cisplatin over a period of 24 hours, it was observed that there was significant poly (ADP-ribose) polymerase (PARP-1) proteolysis, which is considered to be a sensitive marker for apoptosis, by 16 hours of treatment (Fig. 3A); by 24 hours, there was complete proteolytic degradation of PARP-1 and ~40% of the cells were apoptotic or dead. Lamin cleavage to form higher-mobility species and a small proteolytic fragment was also detectable by 16-24 hours; similar kinetics of proteolysis was observed with cells transfected with wild-type GFP-lamin A. By contrast, after 4 hours of cisplatin treatment, there was no degradation of PARP-1 or lamins, and ~98% of cells continued to display a normal nuclear morphology with intact peripheral lamina staining (Fig. 3B); emerin staining was also normal and there was no evidence of apoptotic markers such as cytochrome *c* release from mitochondria or labelling with annexin V in ~98% of cells (data not shown). Furthermore, wild-type GFP-lamin A gave a discrete nuclear rim pattern after 4 hours of treatment (shown in Fig. 4A), unlike the dispersal of GFP-lamin A observed at the onset of nuclear condensation (Broers et al., 2002). When untreated cells were stained with an antibody to γ-H2AX, <3% of cells displayed γ-H2AX foci (Fig. 3C). After 24 hours of cisplatin treatment, >95% of cells formed γ-H2AX foci, and nuclear blebbing and fragmentation were clearly discernible in ~40% of cells. After treatment with cisplatin for 4 hours, foci were detectable in ~75% of cells showing normal nuclear morphology, and only 2-3% of cells showed intense γ-H2AX staining and nuclear fragmentation and/or blebbing (similar to that shown in Fig. 3C for longer time points); these cells were not counted for the quantitative analysis as they

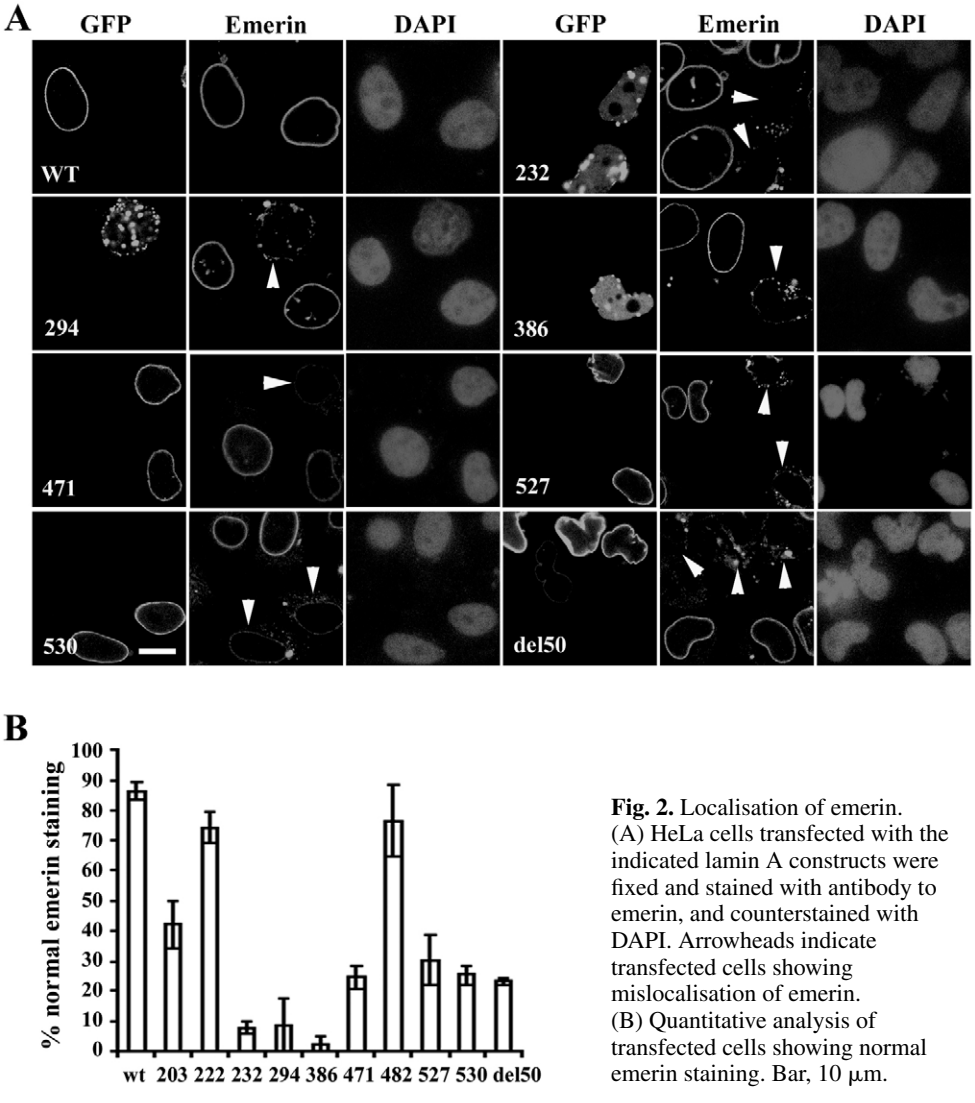


Fig. 2. Localisation of emerin. (A) HeLa cells transfected with the indicated lamin A constructs were fixed and stained with antibody to emerin, and counterstained with DAPI. Arrowheads indicate transfected cells showing mislocalisation of emerin. (B) Quantitative analysis of transfected cells showing normal emerin staining. Bar, 10 μm.

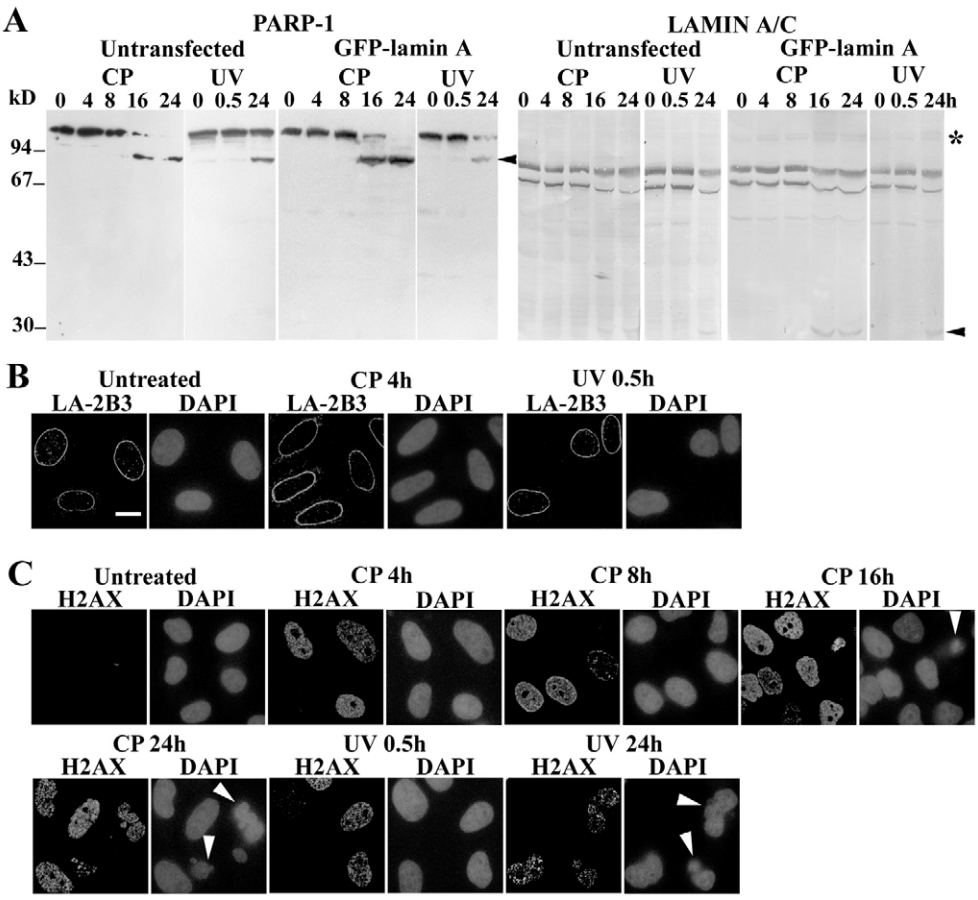


Fig. 3. Optimisation of treatment with DNA-damaging agents. (A) Western blot analysis of untransfected HeLa cells and cells transfected with wild-type GFP-lamin A, treated with cisplatin (CP) for 0-24 hours, or UV irradiation with a recovery period of 0.5 or 24 hours, and probed with antibodies to PARP-1 and lamin A/C. Arrowheads indicate cleavage products as a result of apoptosis. Wild-type GFP-lamin A is indicated by an asterisk. Molecular mass markers indicated on the left are: phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa. (B) HeLa cells treated with cisplatin for 4 hours or UV irradiation with a recovery time of 0.5 hours were stained with antibody to lamin A/C (LA-2B3). (C) HeLa cells treated with cisplatin for 0-24 hours were stained with antibody to γ-H2AX, and counterstained with DAPI. Arrowheads indicate fragmented nuclei. Percentages of apoptotic cells after cisplatin treatment were <3% (4h), 5% (8h), 15% (16h) and 40% (24h); and after UV irradiation, <3% (0.5h) and 50% (24h). Bar, 10 μm.

were likely to be apoptotic. The localisation of γ-H2AX foci and the incorporation of bromodeoxyuridine into DNA was coincident in >90% of nuclei after DNA damage (data not shown), consistent with formation of foci during S phase described in earlier studies (Cliby et al., 1998; Ward and Chen, 2001).

When cells transfected with wild-type GFP-lamin A were treated with cisplatin for 4 hours, the number of transfected cells displaying γ-H2AX foci was similar to that of untransfected cells, as shown in Fig. 4A. (Fewer than 3% of transfected cells displayed foci prior to cisplatin treatment.) Importantly, cells transfected with E203G, G232E, Q294P, R386K, R471C, R527C, L530P or lamin A del50 showed a substantially reduced ability to form γ-H2AX foci and 70-80% of transfected cells did not exhibit foci. In cells expressing other mutants such as H222P and R482L, the extent of foci formation was nearly similar to that of wild-type GFP-lamin A or untransfected cells. The similar response with untransfected cells and cells transfected with wild-type GFP-lamin A provides evidence that the transfected cells were not under stressful conditions that might affect their response to DNA damage. Representative images are displayed in Fig. 4A and a quantitative analysis of the data is shown in Fig. 4C. There were no obvious alterations in localisation of GFP lamins after treatment, although minor changes could not be ruled out.

UV irradiation of cells results in the formation of pyrimidine dimers that lead to stalled replication forks and activation of the ATR signalling pathway (Guo et al., 2000; Ward and Chen, 2001). To check whether formation of γ-H2AX foci upon UV irradiation was altered in cells expressing lamin constructs, transfected HeLa cells were irradiated with UV at 50 J/m^2 and allowed to recover for 30 minutes in fresh medium. Under these conditions, no significant effects were observed on nuclear morphology or staining of the peripheral lamina (Fig. 3B) and <3% of cells were apoptotic (Fig. 3C). Furthermore, there was no detectable cleavage of lamin A or PARP-1 after 30 minutes of recovery (Fig. 3A). Cells transfected with E203G, G232E, Q294P, R386K, R471C, R527C, L530P or lamin A del50 showed reduced ability to form γ-H2AX foci and 60-75% of transfected cells did not exhibit foci. In cells expressing other mutants such as H222P and R482L, the extent of foci formation was nearly similar to that of wild-type GFP-lamin A or untransfected cells. Representative images are displayed in Fig. 4B and a quantitative analysis of the data is shown in Fig. 4D. Interestingly, the individual mutants showed similar trends for foci formation in response to cisplatin or UV.

As an additional marker for repair sites, we analysed the localisation of 53BP1, a p53-binding protein that rapidly redistributes from a diffuse nuclear location to nuclear foci that co-localise with γ-H2AX foci in response to various DNA-damaging agents (Rappold et al., 2001). When HeLa cells were treated with cisplatin for 4 hours, 53BP1 was predominantly localised in nuclear foci in ~80% of cells (Fig. 5A). In cells expressing E203G, G232E, Q294P, R386K, R471C, R527C, L530P or lamin A del50 mutants, 53BP1 remained diffusely localised in the majority of cells after cisplatin treatment; by

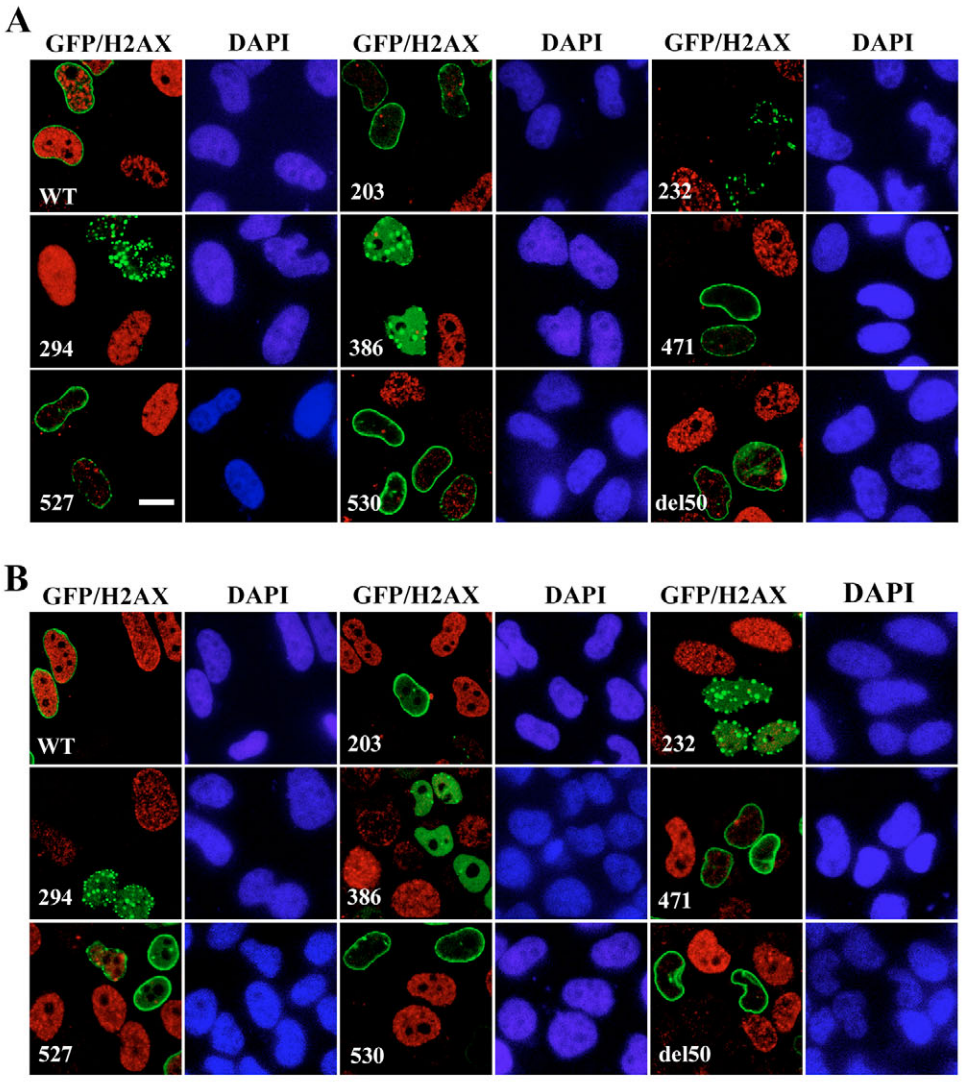
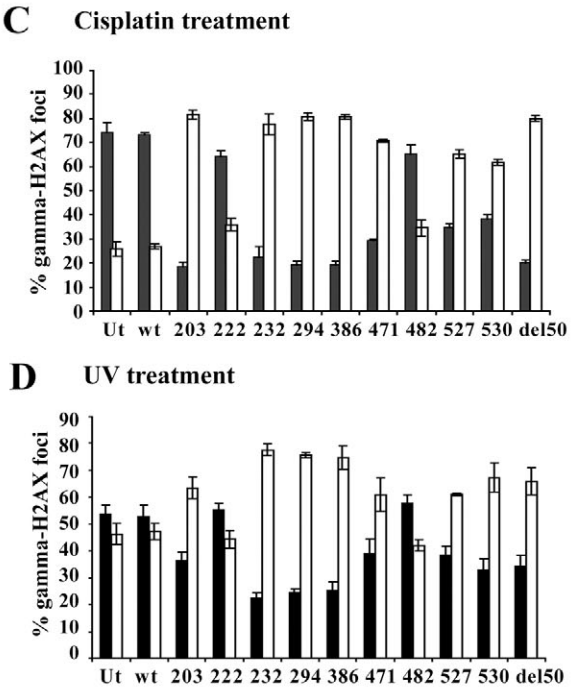


Fig. 4. Formation of γ-H2AX foci after DNA damage. HeLa cells transfected with the indicated GFP-tagged lamin A constructs (green) were (A) treated with cisplatin for 4 hours or (B) irradiated with UV and allowed to recover for 0.5 hours, fixed and stained with antibody to γ-H2AX (red) and counterstained with DAPI. Bar, 10 μm. (C,D) Quantitative analysis of γ-H2AX staining with cisplatin-treated and UV-treated HeLa cells, respectively. Percentage of untransfected (Ut) or transfected cells positive (solid bars) or negative (open bars) for γ-H2AX foci are plotted.



contrast, with H222P and R482L mutants or wild-type GFP-lamin A, recruitment to nuclear foci was observed in most of the transfected cells (Fig. 5B). A quantitative analysis of the data is shown in Fig. 5C. Expression of lamin mutants did not affect the diffuse nuclear staining of 53BP1 in untreated cells (data not shown). Upon 30 minutes of recovery after UV irradiation, 53BP1 was localised in very small 53BP1 foci with a diffuse background (Fig. 5A), so we did not quantitate the effects of lamin mutants after UV treatment. Importantly, the lamin mutants that affect 53BP1 redistribution correspond to those that are impaired in γ-H2AX production, which is consistent with the reported requirement of γ-H2AX for binding to and recruitment of 53BP1 to repair sites (Ward et al., 2003).

Aberrant localisation of ATR

To determine whether lamin mutants could affect localisation of specific signalling components and thus cause an impaired response to DNA damage, we checked the localisation of ATR and ATM kinases in cells expressing lamin mutants in cotransfection studies performed in cells not subjected to DNA-damaging conditions. Cells expressing FLAG-ATR or

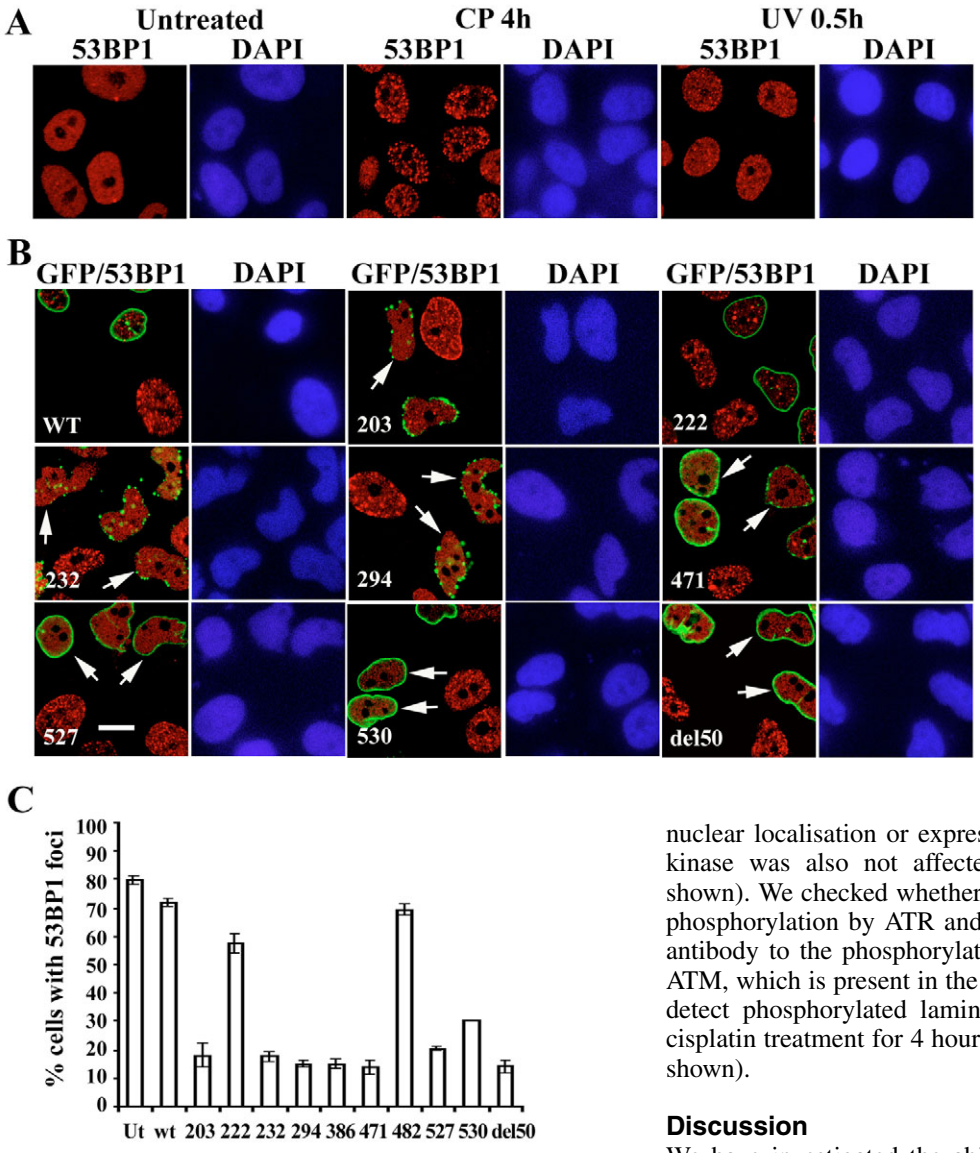


Fig. 5. Localisation of 53BP1 after DNA damage. (A) Untreated HeLa cells or cells treated with cisplatin for 4 hours or UV irradiation with a recovery time of 0.5 hours were stained with antibody to 53BP1 and counterstained with DAPI. (B) HeLa cells transfected with the indicated GFP-tagged lamin A constructs (green) were treated with cisplatin for 4 hours, fixed and stained with antibody to 53BP1 (red) and counterstained with DAPI. Arrows indicate transfected cells showing diffuse 53BP1 staining. Bar, 10 μm. (C) Quantitative analysis of cells showing 53BP1 staining in nuclear foci after cisplatin treatment. Ut, untransfected.

FLAG-ATM showed a diffuse nuclear staining as described earlier for both the endogenously and exogenously expressed kinases; the properties of the exogenous kinases have been shown to be similar to the endogenous enzymes and their overexpression does not have deleterious effects (Cliby et al., 1998; Bakkenist and Kastan, 2003). This normal localisation of ATR kinase was not affected by expression of wild-type lamin A, or the H222P and R482L mutants. Interestingly, we observed a distinct mislocalisation of ATR to the cytoplasm on expression of G232E, Q294P or R386K, with the majority of cells showing exclusive cytoplasmic location (shown in Fig. 6A). Although ATR kinase was detected mostly in the nucleus in cells expressing the E203G, R471C, R527C, L530P or lamin A del50 mutants, with occasional cytoplasmic localisation, the number of cells expressing ATR was substantially lower. This loss of ATR expression was also observed with the G232E, Q294P and R386K mutants, and a quantitative analysis of the data is given in Fig. 6C. By contrast, we did not detect significant changes in the localisation or expression of ATM in the presence of any of the above mutants (Fig. 6B,C). The nuclear localisation or expression of DNA-dependent protein kinase was also not affected by lamin mutants (data not shown). We checked whether lamin A is a direct substrate for phosphorylation by ATR and ATM in a western blot with an antibody to the phosphorylated target sequence for ATR and ATM, which is present in the lamin A sequence. We could not detect phosphorylated lamins after mild DNA damage, i.e. cisplatin treatment for 4 hours or UV for 30 minutes (data not shown).

Discussion

We have investigated the ability of cells expressing disease-causing lamin A mutant proteins to respond to DNA damage. Our data indicate that HeLa cells expressing several highly deleterious lamin mutations are impaired in their ability to form DNA repair foci containing γ-H2AX in response to cisplatin or UV irradiation. These mutant lamins displayed altered localisation of emerin and caused mislocalisation of ATR kinase in untreated cells. The implications of our results are discussed with respect to the molecular phenotypes of the studied mutants and the role of phosphoinositide 3-kinase-related protein kinases in the repair of DNA damage.

Molecular phenotypes of lamin mutants

The lamin mutants investigated in this study have been linked to autosomal dominant forms of disease, and could be expected to exhibit specific phenotypes in the presence of wild-type endogenous lamin A and C. Our results shows that the AD-EDMD mutants G232E, Q294P and R386K form aggregates and are strongly disruptive of lamin assembly. The observed assembly properties and localisation of ectopically expressed mutants that have also been studied by other laboratories (E203G, R386K, R482L, L530P and lamin A del50) are

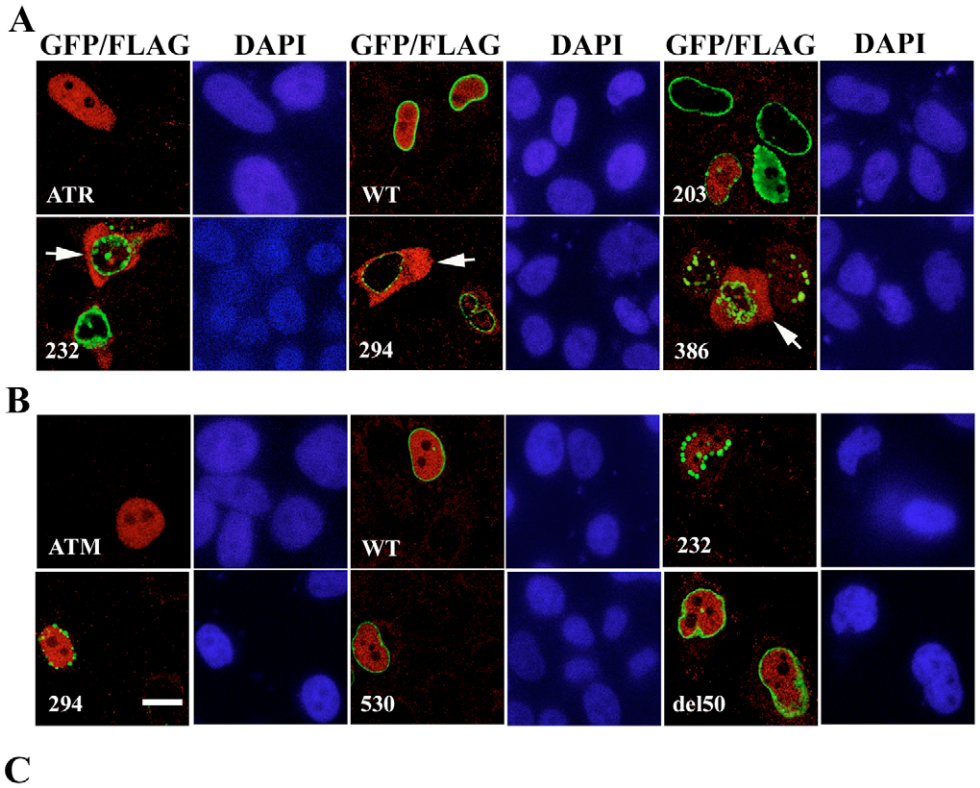


Fig. 6. Localisation of ATR and ATM kinases in untreated cells. (A) HeLa cells transfected with a FLAG-ATR construct (ATR) or cotransfected with the indicated GFP-tagged lamin A constructs (green) were fixed and stained with anti-FLAG antibody (red), and counterstained with DAPI. Arrows indicate cells showing exclusive cytoplasmic staining of ATR. (B) As in A, except that a FLAG-ATM construct (ATM) was used. Bar, 10 μm. (C) Quantitative analysis of transfected cells expressing ATR, including nuclear and/or cytoplasmic staining (open bars), or ATM (solid bars), both normalised to wild type as 100%.

consistent with previous reports (Ostlund et al., 2001; Bechert et al., 2003; Goldman et al., 2004; Scaffidi and Misteli, 2005). Lamin A del50 has been shown to have strongly deleterious effects on nuclear morphology and chromatin organisation (Goldman et al., 2004), which have been attributed to retention of the farnesylated C-terminus. In our study, the expression of lamin A del50 also gave rise to highly convoluted nuclei with abnormal morphology, whereas the other progeroid constructs – R471C, R527C and L530P – were predominantly localised with smooth staining at the nuclear rim and displayed low levels of lobulated nuclei. Although L530P has been linked to AD-EDMD in humans, it gives rise to HGPS-like symptoms in mice in a knockin experiment; this might be a result of aberrantly spliced forms of lamin A observed in the mouse model (Mounkes et al., 2003). However, we did not detect any products from differentially spliced forms of L530P in western blots of transfected cell lysates. Constructs such as H222P predominantly showed a typical nuclear rim localisation and normal localisation of emerin, consistent with the milder properties observed in a recently reported knockin mouse model of AD-EDMD with the H222P mutation (Arimura et al., 2005). Similar localisation patterns of the mutants were observed in HeLa and C2C12 cells, although variations in expression and localisation of lamin mutants in other cell types cannot be ruled out. There is considerable evidence for the involvement of lamins in the spatial organisation of RNA pol II transcription (Spann et al., 2002; Kumaran et al., 2002), and specific genes are misregulated in HGPS, FPLD and DCM cells (Csoka et al., 2004; Capanni et al., 2005; Mounkes et al., 2005). When we analysed RNA pol II transcription in cells expressing lamin mutants, we did not observe significant alterations in staining of active pol II (data not shown), although we cannot rule out effects on transcription of specific genes.

We have observed that emerin is distinctly mislocalised in cells expressing mutants like R386K, Q294P and G232E that form aggregates and are defective in assembly at the nuclear periphery. Furthermore, emerin levels at the nuclear envelope were depleted on expression of constructs with mutations at the C-terminus (R471C, R527C, L530P and lamin A del50), which might be unable to bind to emerin and localise it to the nuclear envelope, since emerin has been shown to associate with the C-terminus of lamin A (Vaughan et al., 2001). Expression of the DCM mutant E203G, which forms small aggregates at the periphery in ~40% of cells, also resulted in mislocalisation of emerin. By contrast, expression of the FPLD mutant R482L did not alter the nuclear rim localisation of emerin in the majority of cells. However, co-localisation of lamin and emerin might not always imply correct intermolecular interactions. In a previous study with fibroblasts from FPLD cells expressing this mutant, about 80% of cells showed normal rim localisation of emerin, whereas 15-20% cells displaying lamin A/C aggregates showed reduced staining of emerin; binding of emerin to lamin A but not to lamin C was affected in the FPLD

fibroblasts (Capanni et al., 2003). Emerin is an important binding partner for lamin A, and also binds to barrier-to-autointegration factor (BAF), a DNA-binding chromatin protein (Lee et al., 2001). Emerin and BAF, together with other lamin-binding proteins such as lamina-associated polypeptide 2α (Dechat et al., 2000), have been proposed to cooperate with lamins in the spatial organisation of chromatin. An important correlation we have observed is that the lamin mutants that show altered localisation of emerin are hindered in their response to DNA damage and show mislocalisation of ATR kinase, as discussed in the next section.

Aberrant chromatin organisation has been observed in various conditions of lamin mis-expression. Expression of lamin A del50 leads to the depletion of heterochromatin and abnormal nuclear morphology, which have been attributed to the accumulation of prelamin A (Goldman et al., 2004). Also, cardiomyocytes from lamin A/C-deficient mice display relocalisation of heterochromatin from the periphery to the interior of the nucleus (Nikolova et al., 2004). Analysis of the dynamics of various GFP-tagged lamin mutants in live cells has given further insights into the disparate effects of different mutations in lamin A and C on lamin organisation and chromatin structure. Wild-type lamin A and the R482W mutant, which causes FPLD, showed slow kinetics of movement at the nuclear periphery, consistent with their incorporation into a stable polymer (Gilchrist et al., 2004). By contrast, AD-EDMD mutants in the rod domain as well as L530P displayed increased dynamics; the rod domain mutants are likely to be impaired in filament assembly whereas L530P, being buried in the C-terminal core domain, might destabilise protein folding. The R482W mutant is surface exposed at the C-terminus and thus might not affect the structure of the protein (Dhe-Paganon et al., 2002). In another study, significant decrease in integration into intranuclear sites was observed for the R386K mutant, in addition to increased mobility at the nuclear rim, which was proposed to be a result of loss of interaction with chromatin (Broers et al., 2005). With reference to the other mutants we have studied, the structure of the globular tail domain predicts that substitutions at R527 are likely to perturb structure as a result of disruption of a salt bridge at the domain surface, whereas those at R471, which is buried in the domain core, are likely to disrupt protein folding and stability (Dhe-Paganon et al., 2002). Of the rod domain mutations we have analysed, G232E and Q294P markedly affect filament assembly and the mutants form large nuclear aggregates. E203G also affects filament assembly as small aggregates are formed in 40% of transfected cells. However, H222P assembles normally into filaments at the periphery.

Implications for repair of DNA damage

When DNA is damaged by agents such as UV irradiation or crosslinking agents, this leads to stalled replication forks in S phase and activation of the ATR pathway (Zhou and Elledge, 2000; Abraham, 2001; Bartek et al., 2004). Treatment with ionising radiation or certain radiomimetic drugs results in random double-strand breaks that lead primarily to the activation of the ATM pathway by autophosphorylation of ATM, with a requirement for ATR kinase also in this response (Shiloh, 2003; Bakkenist and Kastan, 2003). Furthermore, ATR appears to carry out genome surveillance functions in normal cell cycles (Ward and Chen, 2001). Defects in ATR or ATM signalling pathways are highly deleterious. ATR-knockout mice are embryonic lethal (Brown and Baltimore, 2003) and a hypomorphic mutation in ATR has been linked to the human disease Seckel syndrome in which there is severe growth retardation as well as skeletal and brain abnormalities (O'Driscoll et al., 2003). In humans, lack of ATM results in ataxia telangiectasia, which is characterised by neuronal degeneration, immunodeficiency, premature ageing and a predisposition to cancer; similar abnormalities are seen in ATM-null mice (Shiloh, 2003). In the absence of the gene encoding H2AX in mice, cells show increased genomic instability and more sensitivity to DNA damage, and are impaired in the formation of DNA repair foci. Prevention of H2AX phosphorylation by specific kinase inhibitors also blocks the formation of DNA repair foci, and recruitment of other repair proteins such as BRCA1, Nbs1, 53BP1, RAD50 and RAD51 to sites of DNA damage (Redon et al., 2002). DNA repair pathways are also defective in cells accumulating prelamin A as a result of lack of functional Zmpste24 or in HGPS cells subjected to DNA damage (Liu et al., 2005; Varela et al., 2005).

Our present findings provide a mechanistic basis for the defects in DNA repair caused by mutations in lamin A. We show that ATR kinase is mislocalised and/or mis-expressed in cells expressing lamin mutants that are impaired in the DNA damage response to UV and cisplatin. ATR normally resides in the nucleus bound to chromatin and is directly complexed with ATR-interacting protein, but details of its mechanism of nuclear transport have not been reported. We suggest that the mislocalisation of ATR might be attributed to loss of specific binding sites on chromatin, leading to altered nuclear transport properties, retention in the cytoplasm and subsequent degradation. In response to DNA damage, replication protein A (a single-stranded-DNA-binding protein) bound at the DNA lesion is able to recruit the complex of ATR-interacting protein and ATR, thus facilitating the phosphorylation of crucial substrates such as γ-H2AX by ATR; this might involve additional proteins such as claspin, minichromosome maintenance proteins and RAD proteins (Bartek et al., 2004). Mislocalisation of ATR by lamin mutants may thus lead to impaired formation of γ-H2AX foci, which in turn reduces recruitment of 53BP1, as seen with the same set of mutants. Although lamin mutants do not directly affect ATM localisation, we suggest that effects on ATR might be sufficient to impair DNA repair pathways requiring both ATM and ATR functions. As the mutants showing mislocalisation of ATR also display altered localisation of emerin, we propose that normal lamin-chromatin interactions are required for the correct nuclear localisation of ATR and subsequent activation of the ATR signalling pathway.

Our study has demonstrated that several lamin mutants causing progerias and muscle-specific disorders induce mislocalisation of emerin and aberrant localisation of ATR prior to damage, resulting in defects in ATR signalling pathways such as reduced phosphorylation of H2AX and inadequate recruitment of 53BP1 to repair sites in response to DNA damage in cell culture. With regard to disease mechanisms, ectopic expression studies in an immortal cell line such as HeLa do have potential limitations in interpretation for the disease in the whole animal. However, in the context of our findings, it may be noted that wastage of tissues is a

hallmark of several laminopathies. Hence, impairment of DNA repair processes ultimately leading to cell death might be a more general mechanism that could contribute to pathogenesis in some laminopathies, in addition to the well-documented effects on tissue differentiation.

Materials and Methods

Plasmid constructs

Lamin A constructs were made as fusions with green fluorescent protein (GFP). The wild-type GFP-lamin A construct has been described earlier (Mariappan and Parnaik, 2005). Point mutations were introduced into lamin A cDNA by PCR-based mutagenesis of the 1.4 kb *Hin*dIII fragment (502-1886 bp) in a two-step PCR using the appropriate mutant primers and flanking normal primers. The mutated segments were ligated to lamin A cDNA fragments to give full-length constructs. All constructs were verified by automated DNA sequence analysis. To enable ectopic expression of lamin A del50, appropriate fragments of lamin A cDNA were amplified by PCR and religated so as to delete the 1819-1968 bp segment (amino acids 607-656). All lamin constructs were expressed as GFP fusions from the cytomegalovirus (CMV) promoter in the pEGFP-C vector. Mammalian expression vectors for FLAG-tagged ATR and ATM kinases were generous gifts from P. Ngheim (Massachusetts General Hospital, Charlestown, MA) and M. Kastan (St Jude's Children's Research Hospital, Memphis, TN), respectively.

Cell culture, DNA transfection and treatment with DNA-damaging agents

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. DNA transfections were carried out with Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Transfection efficiencies were ~25%. At 24 hours after transfection, cells were treated with 25 μM cisplatin for 4 hours (or longer as indicated). For treatment with UV irradiation, transfected cells were subjected to UV irradiation at 50 J/m^2 and then allowed to recover in fresh medium for 30 minutes.

Immunofluorescence microscopy

HeLa cells were washed with phosphate-buffered saline (PBS) and then routinely fixed by treatment with 3.5% formaldehyde for 10 minutes followed by 0.5% (v/v) Triton X-100 for 6 minutes at room temperature, or with methanol at –20°C for 10 minutes for LA-2B3 antibody. Cells were then incubated with 0.5% gelatin in PBS for 1 hour followed by incubation with first antibody for 1 hour and then biotinylated second antibody and avidin-Cy3 for 1 hour each at room temperature. Samples were mounted in Vectashield (Vector Laboratories) containing 1 μg/ml DAPI. The primary antibodies used were mouse monoclonal antibodies (mAbs) to lamin A/C (LA-2B3), which stains the nuclear periphery (Jagatheesan et al., 1999), γ-H2AX from Upstate Biotechnology, emerin (4G5) from Novocastra Laboratories, 53BP1 (H-300) from Santa Cruz Biotechnology, and FLAG (M2) from Sigma-Aldrich. Secondary antibody conjugates were from Jackson ImmunoResearch Laboratories, Molecular Probes or Vector Laboratories. There was no crossreactivity of the fluorescent second antibodies in control experiments in which primary antibodies were omitted. Confocal laser-scanning immunofluorescence microscopy (CLSM) was carried out with a Zeiss LSM510 META confocal microscope. Image analysis was done using LSM510 META Software (Carl Zeiss), and images were assembled using Adobe Photoshop 6.0. Quantitative analysis was carried out by inspection of n=100 cells per sample in three separate experiments and values expressed as mean ± s.d.

Western blot analysis

HeLa cells were harvested, lysed in Laemmli's sample buffer, boiled and electrophoresed through SDS-10% polyacrylamide gels. Gels were electroblotted onto PVDF membrane filters and blocked overnight in 5% BLOTTO in Tris-buffered saline containing 0.1% Tween-20. Filters were incubated with primary antibody for 2 hours, followed by secondary antibody for 1 hour. The primary antibodies used were polyclonal antibodies to lamin A from Santa Cruz Biotechnology, poly ADP-ribose polymerase-1 (PARP-1) from Roche Applied Science and GFP from Clontech. Bound antibody was visualised using a chemiluminescence kit (Roche Applied Science) for peroxidase-conjugated secondary antibody (for PARP-1) or by colour reaction using nitroblue tetrozolium and 5-bromo-4-chloro-indolyl phosphate for alkaline phosphatase-conjugated secondary antibody (for lamin A and GFP).

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