The lamin CxxM motif promotes nuclear membrane growth

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Summary

We analyzed the influence of lamins on nuclear envelope growth in cultured *Xenopus* A6 cells by the overexpression of human lamin A, *Xenopus* and zebrafish lamins B2 and *Drosophila* lamins Dm0 and C as GFP fusion proteins. Lamins containing a CxxM motif in their primary sequence (lamins A, B2, Dm0) induced the formation of lobulated nuclei with multi-membrane-layered, highly folded nuclear membranes and intranuclear membrane assemblies, as observed by electron microscopy. Such morphological alterations were not observed with *Drosophila* lamin C, a lamin without this motif or with a lamin B2 mutant (B2-SxxM) where the cysteine of the CxxM motif is replaced by a serine. *Drosophila* lamin C mutants containing a CxxM motif behaved like B-type lamins thus confirming that this tetrapeptide is directly involved in the morphological changes we observed. Nuclear membrane proliferation could also be induced by lamin B2 in COS-7 cells and in zebrafish embryos but not by human lamin A in COS-7 cells. We speculate that the human lamin A is incompletely processed in *Xenopus* A6 cells and therefore behaves in this cell line like a B-type lamin. Our results indicate that the CxxM motif of B-type lamins has a dual function: it mediates lamin targeting to the inner nuclear membrane thereby promoting nuclear membrane growth.

Key words: Lamin, CxxM motif, Nuclear envelope, Intranuclear membranes, Nuclear membrane proliferation

Introduction

The nuclear envelope (NE) is composed of an outer nuclear membrane that is continuous with the endoplasmic reticulum (ER), the pore complexes and the structurally and functionally distinct inner nuclear membrane (Gruenbaum et al., 2003). Specific integral membrane proteins are found in the inner nuclear membrane that are responsible for a strong connection with the underlying lamina (reviewed by Cohen et al., 2001; Gruenbaum et al., 2003; Ye et al., 1998).

Lamins are type V intermediate filament (IF) proteins and major structural components of the nuclear lamina of metazoa but are absent from plants and yeast. They contain an α-helical rod domain centered between a non-helical N-terminal head domain and a globular C-terminal tail domain. The central 115 amino acids of the tail domain are organized into an immunoglobulin (Ig) fold (Krimm et al., 2002; Dhe-Pagano et al., 2002) that can be seen as spherical particle by electron microscopy (Aebi et al., 1986; for a review see Herrmann and Aebi, 2004).

The C-terminal tail contains two sequence elements that are not present in cytoplasmic IF-proteins. These are the nuclear localization signal and the CxxM motif (where C represents cysteine, x is any amino acid and M is methionine) at the direct C-terminus. The CxxM motif is posttranslationally processed in the cytoplasm by the attachment of a farnesyl moiety to the thiol group of the cysteine followed by the proteolytic removal of the last three amino acids. Finally the carboxyl group of the cysteine is methylated (for a review, see Maske et al., 2003). These modifications confer a higher hydrophobicity to the lamin C-terminus and are essential for the targeting to the inner nuclear membrane after lamin transport into the nucleus (Hofemeister et al., 2000; Maske et al., 2003) (reviewed by Nigg and Hennekes, 1995; Gruenbaum et al., 2003).

Two major lamin isotypes can be distinguished according to the presence or absence of the modified cysteine of the CxxM motif. In B-type lamins the farnesylated and carboxyl methylated cysteine of the CxxM motif remains permanently attached whereas in A-type lamins it is proteolytically removed during the final maturation of the C-terminus (reviewed by Nigg and Hennekes, 1995; Gruenbaum et al., 2003). No CxxM motif is present in the primary amino acid sequence of two splice variants of the mammalian lamin A gene (lamins C and C2) or in the lamin C of *Drosophila* (Gruenbaum et al., 2003; Krohne, 1998).

Lamins are essential proteins that have several functions in the interphase nucleus. First, they provide mechanical stability to the nuclear envelope. The reduction of the lamin content causes the aggregation of pore complexes in the nuclear envelope, nuclear fragility and premature death (Lenz-Böhme et al., 1997; Liu et al., 2000; Sullivan et al., 1999; Wagner et al., 2004). Second, lamins influence DNA replication (Spann et al., 1997; Newport et al., 1990; Meier et al., 1991) and organize the chromatin bordering the inner nuclear membrane by binding to chromatin (Höger et al., 1991a; Taniura et al., 1995) and to integral membrane proteins of the inner nuclear membrane (Foisner and Gerace, 1993; Lang and Krohne, 2003)

(for a review, see Dechat et al., 2000; Ye et al., 1998; Gruenbaum et al., 2003). Third, lamins are required for RNA polymerase II-dependent transcription (Spann et al., 2002) and are involved in the localization of transcription factors (reviewed by Gruenbaum et al., 2003). The direct and indirect interaction of lamins with different transcription factors is hypothesized from autosomal dominant inherited mutations of human lamins A and C that cause diseases as diverse as Emery-Dreifuss muscular dystrophy (EDMD) (Favreau et al., 2004), Dunningham-type familial partial lipodystrophy (FPLD) (Favreau et al., 2003) and progeria (Eriksson et al., 2003). The transcription factor SREBP1 has been shown to bind to the lamin A tail of the wild-type protein but not to a lamin A tail carrying a mutation characteristic for FPLD (Lloyd et al., 2002).

There are indications that lamins are involved in nuclear growth after the re-formation of the nuclear envelope at the end of mitosis. Experiments where the majority of lamins had been depleted in vivo (Benavente and Krohne, 1986) and in vitro (Newport et al., 1990) demonstrated that nuclei were enclosed by an intact nuclear envelope but the nuclei were not able to decondense the chromatin, to increase in size or to replicate their DNA.

To learn more about the involvement of lamins in nuclear growth we have selected cultured cells that were transiently overexpressing GFP-lamin fusion proteins. Here we present data demonstrating that the CxxM motif of lamins is directly involved in nuclear membrane growth in cultured cells.

Materials and Methods

Lamin constructs

All lamin constructs generated are listed in Fig. 1 and were controlled by DNA sequencing. All PCR fragments were subcloned into the 2.1-TOPO® vector (Invitrogen, Karlsruhe, Germany), excised and then cloned into the appropriate GFP expression vector.

Xenopus lamin B2 constructs

The various lamin mutants were constructed starting with the vector Bluescript KS (Stratagene, Amsterdam, Netherlands) containing the cDNA of the *Xenopus* lamin B2. All constructs were cloned in frame to the 3′ end of the GFP coding region of the vector pEGFP-C3 (Clontech Laboratories, Palo Alto, CA). The wild-type lamin B2 was cloned by using the *Sal*I/*Bam*HI fragment of the original cDNA. The encoded GFP fusion protein lacked amino acids 1-8 of lamin B2. Mutant lamin B2-ΔN2 was obtained by cloning the *Hin*dIII/*Bam*HI fragment. The lamin B2-ΔN1 construct was generated by PCR amplification (using primers: 5′-AACTCGACACTGCCCTAGAAGAATTAACTGAGCG-3′ and 5′-CACGCCTTCTTTGCTCCTAGAGG-3′) of a cDNA fragment coding for the C-terminal half of helix 2. The *Xho*I/*Hin*dIII fragment was cloned in-frame into the GFP-lamin B2-ΔN2-plasmid that had been restriction digested with *Xho*I and *Hin*dIII. Lamin B2 mutant B2-ΔN3 was generated by PCR using primers: 5′-AACTC-GAGCAAGCATTGGATGAACTCCGAAA-3′ and 5′-TTGGATCCT-TACATGACAGAGCAGCCTCTGGAT-3′. The *Xho*I/*Bam*HI fragment was cloned into vector pEGFP-C3. Lamin B2 mutant B2-SxxM was generated by PCR using primers: 5′-ACTACACCAAGCCGGTC-GACCCGGT-3′ and 5′-TTGGATCCTTACATGACAGAGGAGC-CTCTGGAT-3′. The fragment was digested with *Spe*I/*Bam*HI producing a fragment containing 498 bp from the 3′ end of the coding region. This fragment was cloned into the GFP-lamin B2 plasmid that had been digested with *Spe*I/*Bam*HI.

Drosophila lamin constructs

Bacterial expression vectors pET17 (Calbiochem-Novabiochem, Schwalbach, Germany) containing the coding sequence for the *Drosophila* lamins Dm0, C and mutant lamin C-CxxM have been described (Krohne et al., 1998). The lamin Dm0 cDNA was also cloned into the Bluescript KS vector. The complete coding regions were excised by restriction digestion with *Nde*I/*Eco*RI and cloned to the 3′ end of the GFP coding region of the vector pEGFP-C2 that had been digested with *Bgl*II and *Eco*RI. Cloning was facilitated by using adapter oligonucleotides that contained *Bgl*II and *Nde*I restriction sites. Lamin mutant C-CxxM-ΔN1 was obtained by digesting the pEGFP-C2 expression plasmid C-CxxM with *Sac*I/*Eco*RI and cloning the fragment in-frame to the 3′ end of the GFP coding region of vector pEGFP-C3.

Human lamin A

The pEGFP-C1 expression vector containing the coding region of the human wild-type lamin A cDNA (*Sst*II/*Bam*HI fragment) was kindly provided by J.L.V. Broers and a similar construct has been described (Broers et al., 1999).

Zebrafish lamin B2 mutant zB2-ΔN3

The complete coding region of zebrafish lamin B2 was amplified (nucleotides 202-1953 of GenBank accession number AJ005936) using total cDNA that had been synthesized by reverse transcription (Schoft et al., 2003) and was cloned into the 2.1-TOPO® vector. A DNA fragment encoding amino acids 238-583 of zebrafish lamin B2 was amplified by PCR using primers: 5′-TTGAATTCACAGGCGCT-GCAGGATCT-3′ and 5′-TATCTAGATCACATCACTGCACATTC-TCTGGC-3′. The *Eco*RI/*Xba*I fragment was cloned into pEGFP-C1. To allow the in vitro synthesis of mRNA coding for a GFP-zB2-ΔN3 fusion protein the required DNA fragment was amplified from the plasmid pEGFP-C1-zB2-ΔN3 using primers: 5′-AAAGGATCCAC-CATGGTGAGCAAGGG-3′ and 5′-TATCTAGATCACATCACTG-CACATTCTCTGGC-3′. The *Bam*HI/*Xba*I fragment was cloned into the pCS2P+ vector.

Antibodies

Monoclonal lamin antibodies used for immunofluorescence have been described (Höger et al., 1991b; Lourim and Krohne, 1993). Antibody X223 (specific for lamin LII/B2 of amphibian and higher vertebrates) was diluted 1:400 and antibody X94 (specific for amphibian lamin A) was diluted 1:300. Mouse monoclonal antibodies ADL 195, ADL 84 (against *Drosophila* Dm0) and LC28 (against Drosophila C) (Klapper et al., 1997), were diluted 1:10. Guinea pig antibodies against *Xenopus* LAP2β are described (Lang and Krohne, 2003). Secondary antibodies conjugated to Texas Red were purchased from Dianova (Hamburg, Germany). For immunoblotting, the mouse monoclonal antibody against GFP (Roche, Mannheim, Germany) was diluted 1:1000. Secondary antibodies coupled to peroxidase (anti-mouse) (Dianova, Hamburg, Germany) were diluted 1:10,000.

Cell culture, transfection and microinjection of cells

COS-7 (kidney cells of *Cercopithecus aethiops*) and A6 cells (kidney epithelial cells of *Xenopus laevis*) were cultured according to standard procedure (A6 cells: 27°C and 5% CO_2; COS-7 cells: 37°C and 5% CO_2). Cells were grown in petri dishes (3.5 cm diameter) for about 12 hours or plated on CELLocate (Eppendorf, Hamburg, Germany) coverslips and then transfected with DNA using Rotifect (Roth, Karlsruhe, Germany) according to the manufacturer's instructions. 24-30 hours after transfection the cells grown in petri dishes were used for protein analysis and coverslips were processed for microscopy (see below).

For microinjection experiments, *Xenopus* A6 cells and COS-7 cells were plated on CELLocate coverslips and grown for about 12 hours. Microinjection was performed using the Transjector S246, Mikromanipulator 5171 and Femtotips II (Eppendorf, Hamburg, Germany). The cells were injected with 40 ng/μl DNA for 0.2 seconds and an injection pressure of 130 hPa. 20-48 hours after injection the cells were analyzed microscopically (see below).

In vitro transcription and microinjection of zebrafish embryos

Capped mRNA was synthesized in vitro by transcription of *Not*I linearized pCS2P+ vector containing the zB2-ΔN3 DNA construct using the Sp6 mMessage Machine Kit (Ambion). Zebrafish embryos were microinjected with 50 pg capped mRNA. The injection was performed underneath the single blastomeres of 2-4 cell stages using a Microinjector 5242 (Eppendorf). The injected embryos were raised at 28°C.

Protein analysis and SDS-PAGE

Transfected COS-7 cells and *Xenopus* A6 cells grown in Petri dishes were washed with PBS (137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4), fixed in 100% ethanol, and collected by scraping with a rubber policeman. Cells were pelleted (1000 ***g*** for 5 minutes), washed once with acetone, air-dried, boiled in SDS-PAGE sample buffer and separated by SDS-PAGE (10% polyacrylamide) (Laemmli, 1970). Transfer to nitrocellulose filters (Stellar and School, Dassel, Germany) was performed using the semi-dry method of Kyhse-Anderson (1984). Antibodies were diluted in TBST buffer (149 mM NaCl, 0.3% Tween, 10 mM Tris-HCl, pH 8.0) containing 5% non-fat dry milk. Blocking of filters, dilution of antibodies, incubation with antibodies and washing the filters were performed as described (Schmidt et al., 1994). Bound secondary antibodies coupled to peroxidase were detected by exposure to X-ray film (X-Omat AR, Eastman Kodak, Rochester, NY) using an enhanced chemiluminescence system (Amersham Buchler, Braunschweig, Germany).

Immunofluorescence

Cells were fixed in methanol for 5 minutes at –20°C followed by fixation for 1 minute with acetone at –20°C and incubation for 15 minutes with PBS containing 0.5% bovine serum albumin (PBS/BSA). Antibody incubation was carried out in PBS/BSA for 30 minutes at room temperature. Bound primary antibodies were visualized with secondary antibodies conjugated to Texas Red (Dianova, Hamburg, Germany) diluted 1:100 in PBS/BSA. Photographs were taken with a confocal laser-scanning microscope (CLSM; TCS SP, Leica, Heidelberg, Germany). Digital images taken were recorded using standardized CLSM settings. The difference in the signal enhancement between cells exhibiting a weak and an intense GFP fluorescence was 70-80 V (weak fluorescence: 570-580 V; intense fluorescence: 490-500 V).

Electron microscopy

Xenopus A6 cells and COS-7 cells grown on CELLocate coverslips were fixed for 45 minutes with 2.5% glutaraldehyde (50 mM cacodylate pH 7.2, 50 mM KCl, 2.5 mM $MgCl_2$) at room temperature, then fixed for 2 hours at 4°C with 2% OsO_4 buffered with 50 mM cacodylate (pH 7.2), washed with H_2O and incubated overnight at 4°C with 0.5% uranyl acetate (in H_2O). The cells were dehydrated, embedded in Epon812 and ultrathin sectioned. Zebrafish embryos at 9, 24 and 36 hours post fertilization were fixed and processed for electron microscopy exactly as described (Schoft et al., 2003). Sections were analyzed with a Zeiss EM10 (Zeiss/LEO, Oberkochen, Germany).

Results

To investigate the influence on the nuclear morphology of ectopically expressed wild-type lamins of warm and cold-blooded vertebrates as well as of invertebrates, we selected *Xenopus* A6 cells for transfection and microinjection. It has previously been shown that the folding and assembly of some intermediate filament proteins of cold-blooded vertebrates is not optimal at temperatures above their body temperature (Herrmann et al., 1993; Cerda et al., 1998). Because *Drosophila* cells and *Xenopus* A6 cells are grown at the same temperature we expected that the targeting and assembly of insect lamins would not be negatively influenced in this amphibian cell line.

For this project the complete coding regions and mutants of human lamin A, *Xenopus* lamin B2, zebrafish lamin B2, *Drosophila* lamins Dm0, and C had been cloned in frame to the 3′ end of the GFP coding region contained in the pEGFP expression vector (Fig. 1). To verify that each expression vector encoded the correct protein, total proteins of transfected COS-7 cells (Fig. 2, lanes 1-12) or *Xenopus* A6 cells (Fig. 2, lanes 13,14) were analyzed by SDS-PAGE and immunoblotting with GFP antibodies (Fig. 2). Total proteins of 24-hour-old zebrafish embryos that had been microinjected at the 2-4 cell stage with the mRNA coding for the zebrafish lamin B2 mutant zB2-ΔN3 were analyzed in the same way (Fig. 2, lane 15). The immunoblots demonstrated that the GFP fusion proteins had the expected size. COS-7 cells have been used for most experiments because they can be transfected with a much higher efficiency than *Xenopus* A6 cells. We noted the presence of degradation products (Fig. 2, lanes 2,3,13,14) indicating that a subpopulation of these GFP fusion proteins lacked part of the lamin C-terminus including the CxxM motif.

Light microscopy of cells expressing wild-type lamins

When the expression plasmids encoding a wild-type lamin were introduced by transfection or microinjection into *Xenopus* A6 cells we noted no alteration of the nuclear morphology by immunofluorescence microscopy when cells exhibited a weak GFP fluorescence. However, in cells showing an intense GFP fluorescence we observed that wild-type lamins B2 (*Xenopus*), A (human) and Dm0 (*Drosophila*) (Fig. 3A-C) could induce two different alterations in the nuclear structure. One such alteration we noted was a change in the nuclear shape. The nuclear envelope was more folded than in control cells (Fig. 3A,B) and some nuclei became heavily lobulated (Fig. 3A). In addition, in a number of cells we have seen dot-like structures in the nuclear periphery that were also stained by antibodies against endogenous lamins (Fig. 3C,C′). A6 cells expressing higher levels of *Drosophila* lamin C (Fig. 3D,D′) always possessed in their nuclei small dot-like or streak-like aggregates of the GFP fusion protein. Several of these aggregates also contained endogenous lamins (Fig. 3D,D′).

When the transfected cells were stained with antibodies specific for the lamin contained in the GFP fusion protein we have always observed a colocalization of the GFP fluorescence and the lamin staining (Fig. 3E,E′; shown for lamin Dm0) demonstrating that GFP stained structures seen did contain the specific lamin. These results indicate that the population of GFP fusion proteins lacking a C-terminal

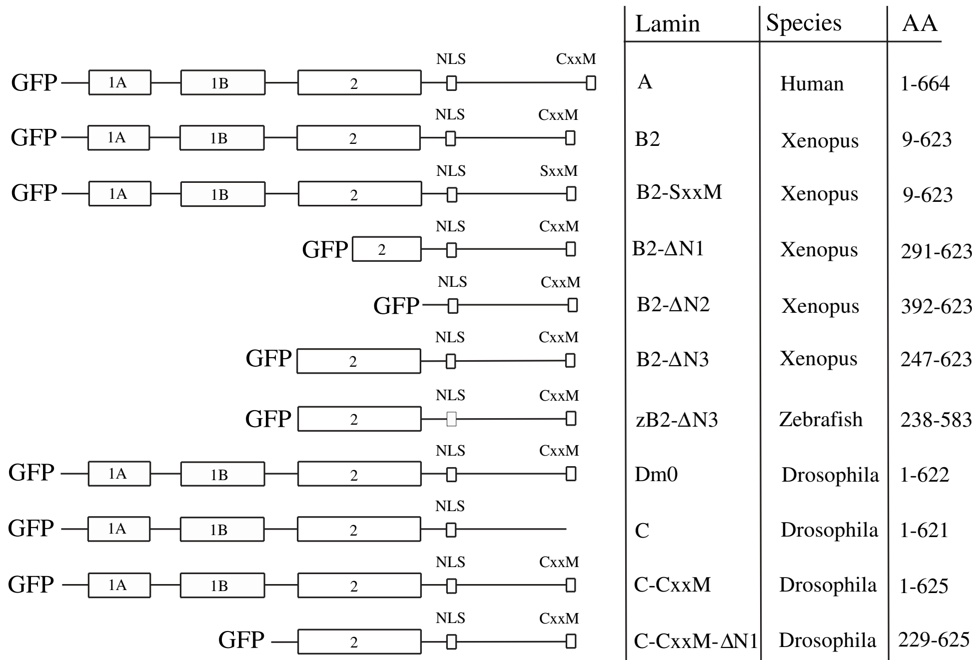

Fig. 1. Schematic of GFP-lamin fusion proteins. These are wild-type human lamin A, *Xenopus* lamin B2, *Drosophila* lamins Dm0 and C, mutants derived from these lamins and an N-terminal deletion mutant of zebrafish lamin B2 (zB2-ΔN3). The position of the GFP, the α helical region (1A, 1A, 2), the nuclear localization signal (NLS) and the CxxM-motif are indicated. Three N-terminal deletion mutants (B2-ΔN1, B2-ΔN2, B2-ΔN3) and a point mutation (B2-SxxM) where the cysteine in the CxxM-motif had been replaced by a serine have been generated from the *Xenopus* lamin B2. C-CxxM is a *Drosophila* lamin C mutant containing the CxxM motif of lamin Dm0 and C-CxxM-ΔN1, a *Drosophila* lamin C mutant with the CxxM motif lacking the N-terminal 228 amino acids. The numbers of amino acids (AA) of each lamin contained in the GFP fusion protein are listed.

segment of the specific lamin (see Fig. 2) is under the limit of detection.

Electron microscopy of cells expressing wild-type lamins

Inspection of ultrathin sections at low magnification of A6 cells overexpressing the wild type lamins A, B2 and Dm0 (Fig. 4A-C) demonstrated a drastic increase in nuclear membranes causing a shape change from spherical into a highly folded structure with lobulated nuclei. In these nuclei we observed additional membranes that were always located in close vicinity to the nucleoplasmic surface of the nuclear envelope (Fig. 4B,C, arrows). In some of the nuclei we saw no or very few additional membranes (Fig. 4A) but they were more frequent in other nuclei (Fig. 4C,D). The electron microscopic analysis of dot-like structures seen by light microscopy in a number of cells overexpressing lamins A, B2 or Dm0 revealed that they consist of parallel aligned membranes that are arranged into spherical structures (Fig. 4D, arrow and inset). These spherical multi-membrane layers were always in contact with the nuclear lamina (Fig. 4D).

The GFP fluorescence suggested that dot-like structures regularly seen in cells overexpressing *Drosophila* lamin C and less frequently observed in cells expressing one of the other three lamins have a similar ultrastructure. The electron microscopic inspection, however, demonstrated that *Drosophila* lamin C formed longitudinal electron-dense aggregates that were found in association with the nuclear envelope and protruded into the nuclear interior (Fig. 4E). These aggregates never contained membranes and exhibited a banded morphology in several areas (Fig. 4E, inset) very similar to that of the lamin C paracrystals assembled in vivo in baculovirus-infected cultured insect cells (Klapper et al., 1997; Krohne et al., 1998). The paracrystalline ultrastructure was only visible in polymers that were sectioned parallel to their longitudinal axis and no regular substructures could be seen in cross-sectioned or oblique-sectioned polymers (see Klapper et al., 1997; Krohne et al., 1998).

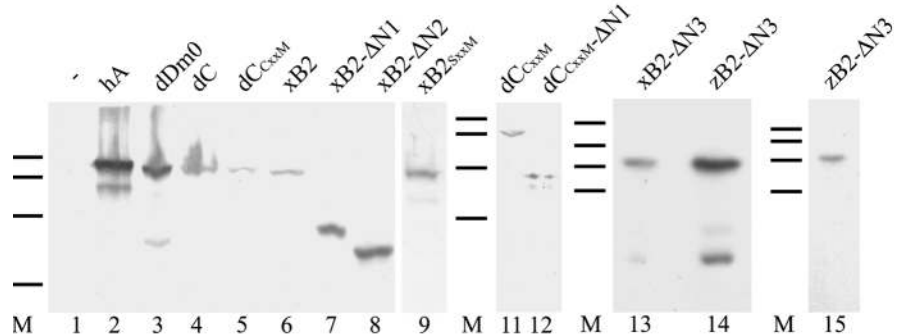

Fig. 2. Characterization of GFP-lamin fusion proteins. Total protein of COS-7 cells (lanes 1-12), *Xenopus* A6 cells (lanes 13, 14) and zebrafish embryos (lane 15) expressing the GFP-lamin fusion proteins listed in Fig. 1 were separated by SDS-PAGE and immunoblotted with GFP antibodies. COS-7 and A6 cells were transfected and zebrafish embryos were microinjected with in vitro synthesized lamin mRNA at the 2-4 cell stage. Embryos were analyzed 24 hours post fertilization. Lane 1, non transfected cells; lane 2, human lamin A (hA); lane 3, *Drosophila* lamin Dm0 (dDm0); lane 4, *Drosophila* lamin C (dC); lanes 5 and 11, *Drosophila* lamin C-CxxM (dC_{CxxM}); lane 6, *Xenopus* lamin B2 (xB2); lane 7, *Xenopus* lamin B2-ΔN1 (xB2-ΔN1); lane 8, *Xenopus* lamin B2-ΔN2 (xB2-ΔN2); lane 9, *Xenopus* lamin B2 mutant B2-SxxM ($xB2_{SxxM}$); lane 12, *Drosophila* lamin C_{CxxM} –ΔN1 (dC_{CxxM} –ΔN1); lane 13, *Xenopus* lamin B2-ΔN3 (xB2-ΔN3); lanes 14 and 15, zebrafish lamin B2-ΔN3 (zB2-ΔN3). Molecular masses of reference proteins (lanes M) are marked and are from top to bottom: 116, 97, 66 and 45 kDa.

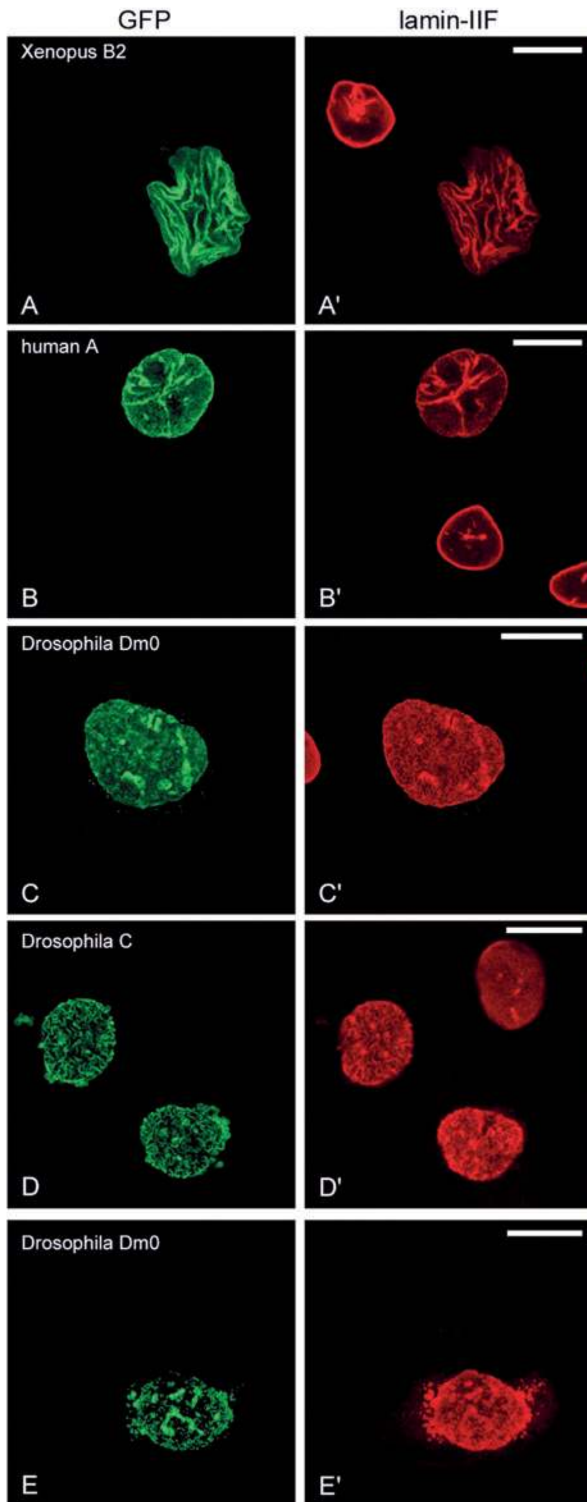

Fig. 3. Light microscopy of *Xenopus* A6 cells overexpressing GFP fusion proteins of wild-type lamins. (A-E′) Cells overexpressing wild-type *Xenopus* lamin B2 (A,A′) human lamin A (B,B′), *Drosophila* lamin Dm0 (C,C′,E,E′) and *Drosophila* lamin C (D,D′) were examined for GFP fluorescence (left panels) and lamin immunofluorescence (right panels). Lamin antibodies specific for lamins A (antibody X94; A′,C′), B2 (antibody X223; B′,D′) and *Drosophila* lamin Dm0 (mixture of antibodies ADL195 and ADL84; E′) were used. Digital images taken by confocal laser-scanning microscopy are shown. Bars, 10 μm.

When we inspected the cross-sectioned nuclear envelopes of the highly lobulated nuclei shown in Fig. 4A-C and of other nuclei overexpressing lamins A, B2 or Dm0 at higher magnification we found areas where flat membrane cisternae of variable size were bordering the inner nuclear membrane (Fig. 5A-C). These membrane cisternae were not continuous with the inner nuclear membrane but separated from it by an electron-dense layer of approximately 15-25 nm thickness that had the same electron density as the lamina subjacent to the inner nuclear membrane (Fig. 5B, arrow). The detailed inspection of several nuclei indicates that the flat membrane cisternae can be formed independently from each other and at several sites (Fig. 5A-C) eventually growing into an extended cisterna thus forming in some areas of the nuclear periphery a nuclear envelope consisting of four membranes (Fig. 5C). As extra membrane cisternae grow, they form a second and third layer (Fig. 5D, arrowheads 1-3). In these membrane stacks each cisterna is separated from the neighboring cisterna by a layer similar in electron density to the lamina (compare Fig. 5B with Fig. 5D). The electron-dense layers localized between the stacked intranuclear membranes also had a thickness of approximately 15-25 nm**.** When two multi-membrane-layered nuclear envelopes came into such a close contact in highly lobulated nuclei that most of the nucleoplasm was excluded, then eventually a nuclear envelope with a ‘zebra-stripe’ pattern was generated that was bordered on both sides by cytoplasm (Fig. 5E).

The existence of extra membrane layers also induced membrane loops or lobes protruding into the nucleoplasm (Fig. 5F) that later eventually could grow into multi-membrane-layered spherical structures of variable size (Fig. 5G,H). The larger of these spherical membrane assemblies (Fig. 5G,H; see also Fig. 4D) corresponded to the dot-like structures seen by light microscopy (see Fig. 3C,E). In addition, we noticed nuclear envelope areas where the extra membranes formed irregular vesicle-like structures (Fig. 5I). No pore complexes were found in areas where the nuclear envelope consisted of several membrane layers (Fig. 5D,E).

The influence of the CxxM motif

Our results indicate that the overexpression of wild-type lamins A, B2 and Dm0 in *Xenopus* A6 cells induces the production of extra nuclear membranes, whereas *Drosophila* lamin C did not have this effect. The major structural difference between the four tested lamins is the absence of the C-terminal CxxM motif in the *Drosophila* lamin C. It is known from previous studies that this tetrapeptide is involved in the targeting of lamins to the inner nuclear membrane (Krohne et al., 1989; Holtz et al., 1989; for review see Krohne, 1998; Gruenbaum et al., 2003). To investigate the influence of this motif on the lobulation of nuclei and the production of intranuclear membranes we performed two sets of experiments.

First, we generated a *Xenopus* lamin B2 mutant where the cysteine of the CxxM motif was replaced by a serine (mutant B2-SxxM). The light microscopic inspection of cells overexpressing mutant B2-SxxM as a GFP fusion protein revealed a diffuse nuclear staining by GFP and the presence of dot-like aggregates in the nuclear periphery that were also stained by GFP (data not shown). Electron microscopic analysis revealed that nuclei of cells overexpressing lamin B2-

SxxM contained spherical proteinaceous aggregates subjacent to the inner nuclear membrane (Fig. 6A,B; asterisks). In these cells the nuclear membrane was indistinguishable from those of control cells (Fig. 6A,B) and we did not detect lobulated nuclei or membranes inside the nucleus.

Second, we constructed a *Drosophila* lamin C that contained the CxxM motif of lamin Dm0 (sequence: CAIM) at its C-terminus (Krohne et al., 1998). The overexpression of this lamin C mutant as a GFP fusion protein resulted in the formation of several nuclear envelope protrusions (Fig. 6C,D).

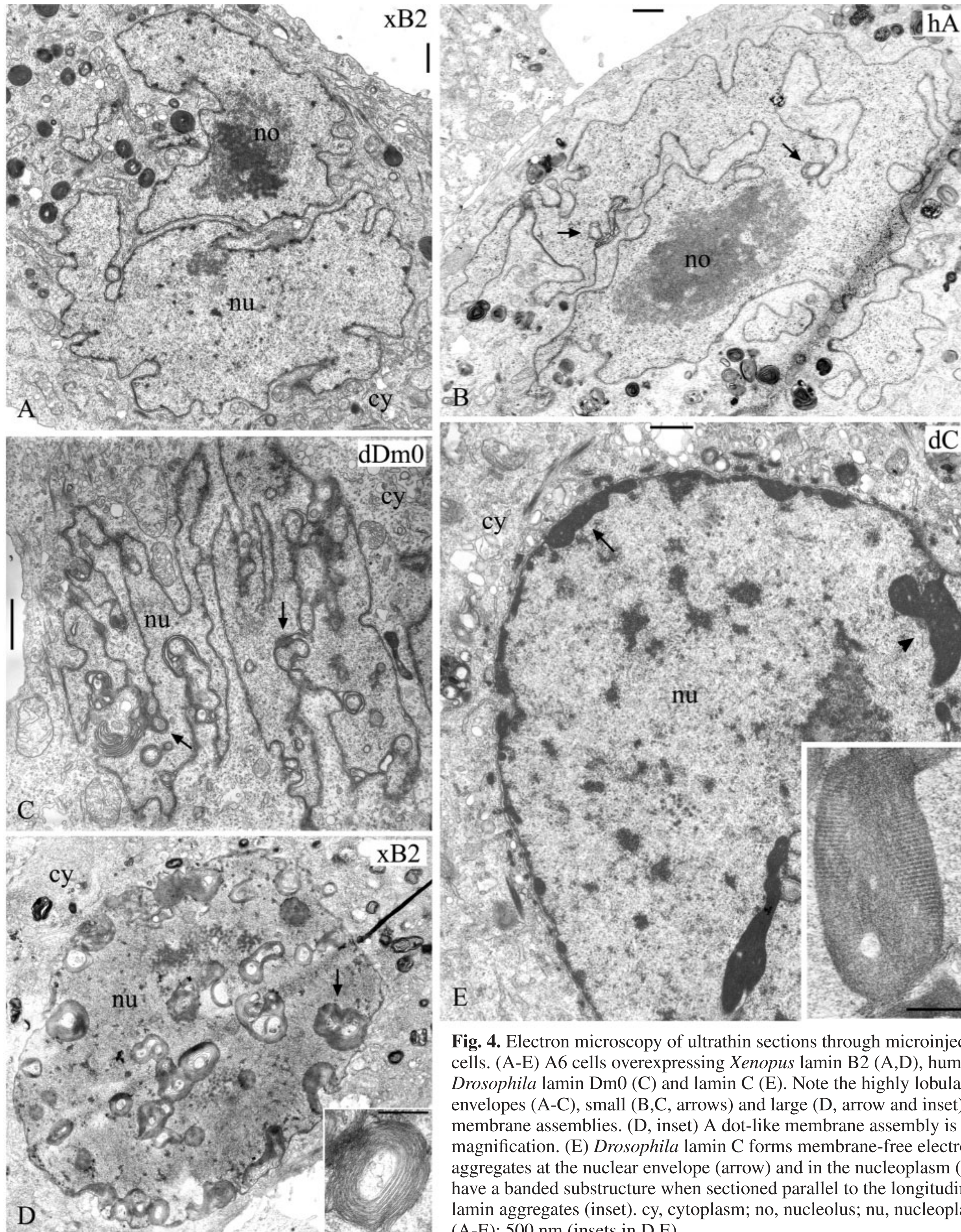

Fig. 4. Electron microscopy of ultrathin sections through microinjected *Xenopus* A6 cells. (A-E) A6 cells overexpressing *Xenopus* lamin B2 (A,D), human lamin A (B), *Drosophila* lamin Dm0 (C) and lamin C (E). Note the highly lobulated nuclear envelopes (A-C), small (B,C, arrows) and large (D, arrow and inset) intranuclear membrane assemblies. (D, inset) A dot-like membrane assembly is shown at higher magnification. (E) *Drosophila* lamin C forms membrane-free electron-dense aggregates at the nuclear envelope (arrow) and in the nucleoplasm (arrowhead) that have a banded substructure when sectioned parallel to the longitudinal axis of the lamin aggregates (inset). cy, cytoplasm; no, nucleolus; nu, nucleoplasm. Bars 1 µm (A-E); 500 nm (insets in D,E).

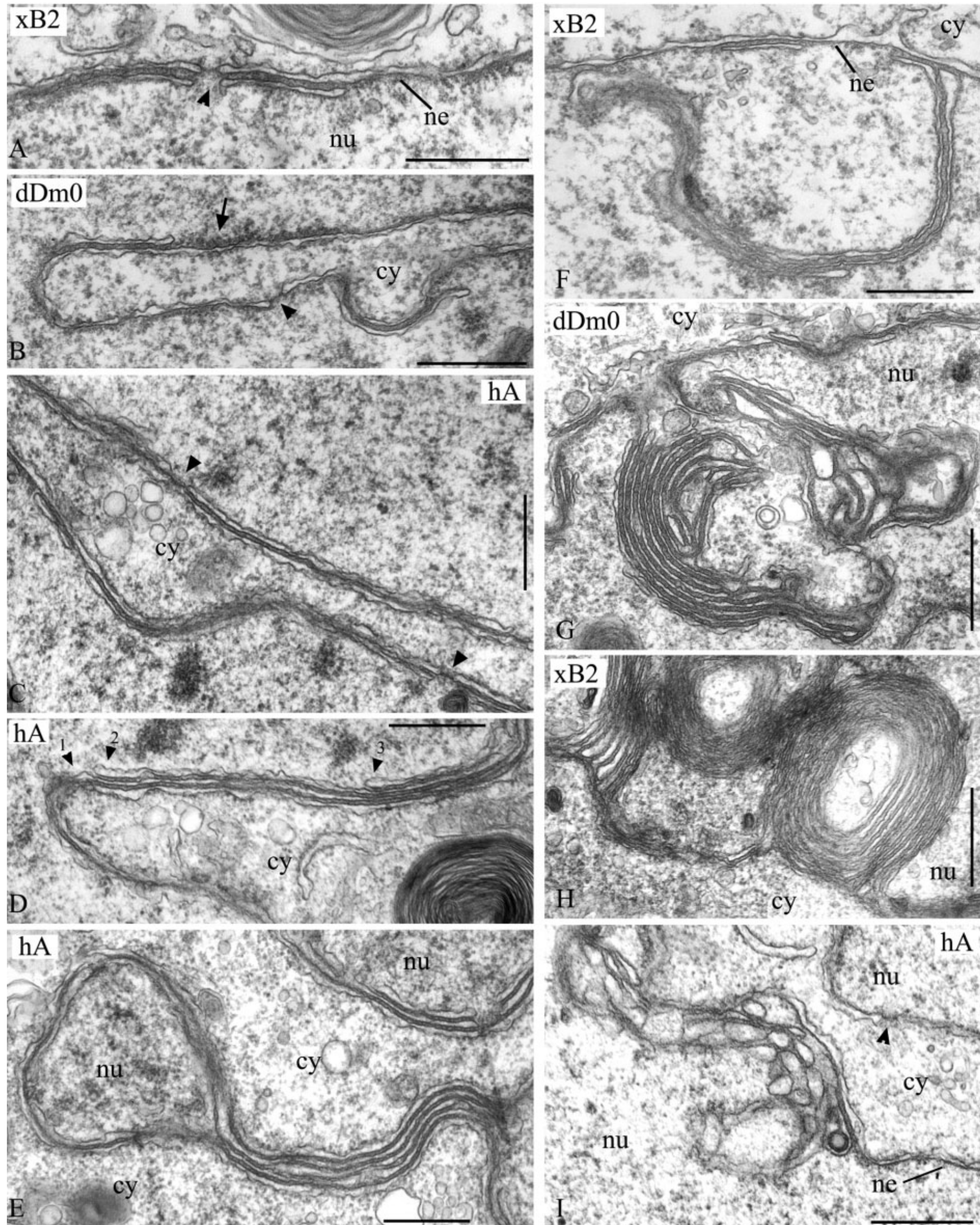

Fig. 5. Intranuclear membranes are formed in cells overexpressing wild-type lamins. (A-I) High magnification electron micrographs of ultrathin sections through microinjected *Xenopus* A6 cells expressing *Xenopus* lamin B2 (A,F,H), human lamin A (C-E,I) and *Drosophila* lamin Dm0 (B,G). Intranuclear membranes form flat cisternae that are separated from the inner nuclear membrane by a 15-25 nm-thick layer similar in electron density to the lamina (B, arrow). Examples where only a few locally restricted intranuclear membranes were found are shown in A and B, and a nuclear envelope nearly completely bordered by an intranuclear membrane cisterna in C. (D) Nuclear envelope bordered in neighboring areas by one (arrowhead 1), two (arrowhead 2) and three (arrowhead 3) membrane cisternae. (E) Part of a highly lobulated nucleus showing an area where the nucleoplasm is nearly excluded between the multi-membrane-layered nuclear membranes. (F-H) Lobulated intranuclear membrane cisternae of increasing complexity. (I) Irregular vesicle-like intranuclear membranes. Nuclear pore complexes are indicated by arrowheads in A-C, I. cy, cytoplasm; ne, areas of the nuclear envelope with an unaltered morphology; nu, nucleoplasm. Bars, 500 nm.

In these areas of the nuclear envelope proteinaceous aggregates were found in association with the inner nuclear membrane that were similar in electron density to polymers formed by the wild-type *Drosophila* lamin C (compare Fig. 6C,D with Fig. 4D).

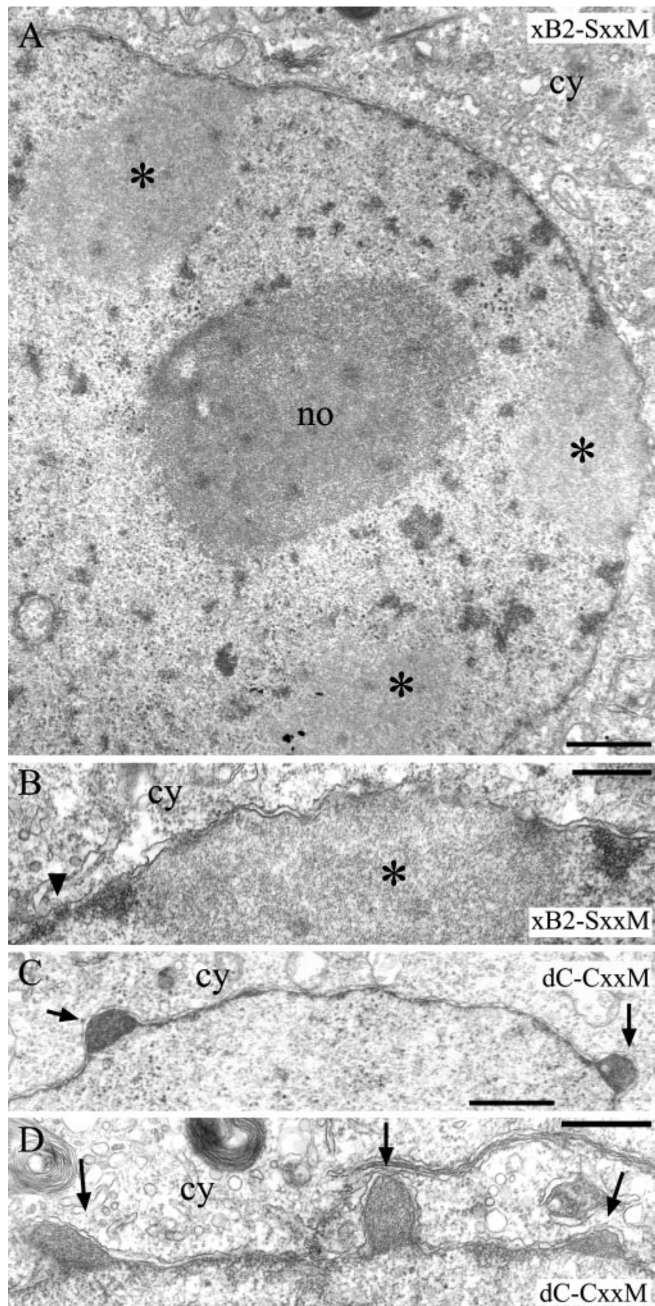

Fig. 6. The influence of the CxxM motif on the formation of intranuclear membranes. (A-D) Electron micrographs of ultrathin sections through microinjected *Xenopus* A6 cells expressing *Xenopus* lamin B2 mutant B2-SxxM (A,B) and *Drosophila* lamin mutant C-CxxM (C,D). (A,B) Nuclei of cells expressing mutant lamin B2-SxxM contain proteinaceous aggregates that are associated with the nuclear envelope (asterisks) but no intranuclear membranes. (C,D) Protrusions in the nuclear envelope (arrows) are seen in cells expressing the lamin C-CxxM mutant. The protrusions are filled with protein aggregates similar in electron density to those formed by *Drosophila* lamin C (see Fig. 4E). A pore complex is indicated with an arrowhead in B. cy, cytoplasm; no, nucleolus. Bars, 1 µm (A,C); 500 nm (B,D).

The influence of other lamin domains

Our results indicate that the wild-type lamins containing a CxxM motif are involved in nuclear membrane growth and the generation of intranuclear membranes in *Xenopus* A6 cells. To verify whether other lamin domains modulate this function of the CxxM motif we analyzed five lamin mutants as GFP fusion proteins that contained N-terminal deletions of different lengths (Fig. 1). These were three mutants of *Xenopus* lamin B2 (B2-ΔN1, B2-ΔN2, B2-ΔN3), one of the zebrafish lamin B2 (zB2-ΔN3) and one mutant of the *Drosophila* lamin C (C-CxxM-ΔN1). All mutants contained the complete C-terminal tail with a CxxM motif. One mutant also contained linker sequences and the complete helix 2 (C-CxxM-ΔN1), two the complete helix 2 (B2-ΔN3, zB2-ΔN3), and one the C-terminal half of helix 2 (B2-ΔN1). The expression of each of the lamin mutants in *Xenopus* A6 cells resulted in the formation of extra membranes in the nuclei (Fig. 7A-C). The intranuclear membrane cisternae were predominantly found in close association with the inner nuclear membrane when lamin mutants were expressed that possessed at least part of helix 2 (Fig. 7A,B; data not shown for mutants B2-ΔN3, zB2-ΔN3) thus resulting in the formation of a multi-membrane-layered nuclear envelope. The overexpression of the lamin mutant lacking the complete helical domain (mutant B2-ΔN2; Fig. 7C) caused no alteration of the nuclear envelope but resulted in the formation of spherical multi-membrane layers in the nuclear interior that only had contact with the nuclear lamina at few sites (Fig. 7C). The data presented indicate that in our experimental system, membrane proliferation is primarily controlled by the CxxM motif.

Expression of GFP-lamin fusion proteins in mammalian cells and zebrafish embryos

To verify whether the nuclear membrane proliferation seen in *Xenopus* A6 cells is specific for this cell line or is also relevant for other vertebrate cells we expressed human lamin A and two lamin B2 mutants (B2-ΔN3, zB2-ΔN3) in COS-7 cells (human lamin A, Xenopus mutant B2-ΔN3) and in zebrafish embryos (zebrafish mutant zB2-ΔN3). The *Xenopus* lamin B2 mutant (B2-ΔN3) was tested in COS-7 cells (Fig. 8A) and the equivalent zebrafish lamin B2 mutant (zB2-ΔN3) in zebrafish embryos (Fig. 8B,C). Zebrafish embryos had been microinjected with the capped mRNA encoding lamin zB2-ΔN3 at the 2-4 cell stage. In successfully microinjected embryos a few hundred cells expressed the GFP fusion protein. The inspection of these cells under the light microscope revealed that several nuclei possessed bleb-like protrusions at their surface (data not shown; see Fig. 8B). Electron microscopy revealed that the expression of the two lamin B2 mutants caused the formation of multi-membrane-layered nuclear envelopes in cultured mammalian cells (Fig. 8A) and in tissues of embryos at 9 (Fig. 8B), 24 (data not shown) and 36 hours (Fig. 8C) post fertilization. The bleb-like protrusions seen by light microscopy correspond to areas where two nuclear membranes came in such close contact that most of the nucleoplasm was excluded (Fig. 8B, arrow). Our results

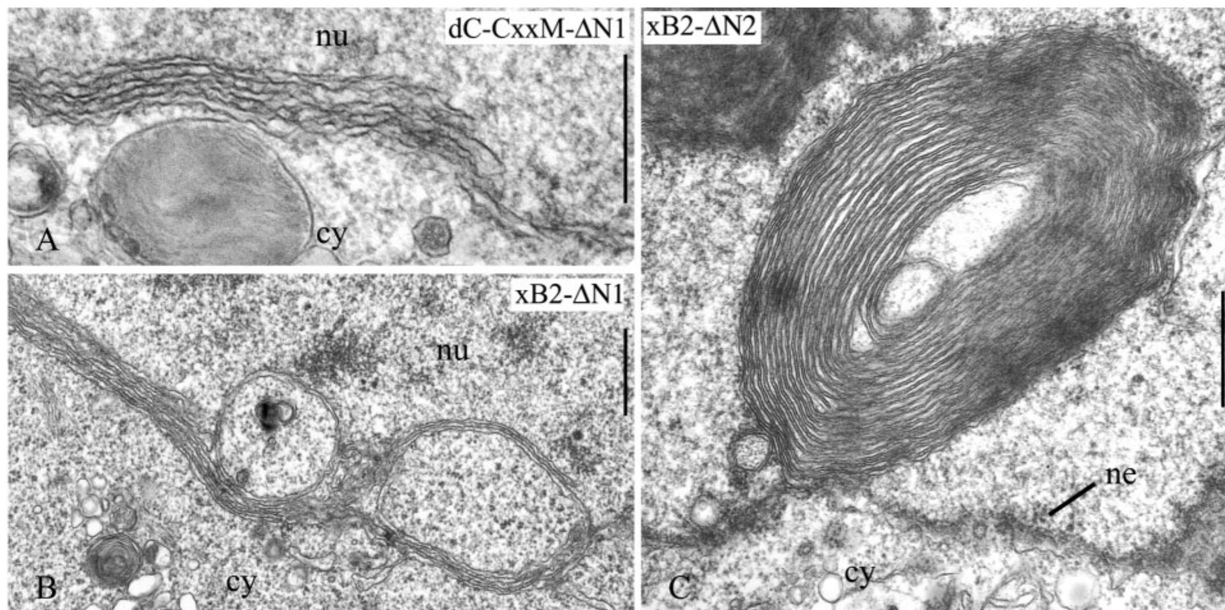

Fig. 7. Intranuclear membranes formed by lamin mutants containing a CxxM motif and N-terminal deletions. (A-C) Electron micrographs of ultrathin sections through microinjected (A,C) or transfected (B) *Xenopus* A6 cells expressing *Drosophila* lamin C-CxxM-ΔN1 (A), *Xenopus* lamin B2-ΔN1 (B) and *Xenopus* Lamin B2-ΔN2 (C). Lamin mutants containing the complete helix 2 (A) or the C-terminal half of helix 2 (B) can induce the formation of multi-layered nuclear envelopes. (C) Mutant B2-ΔN2 induced the formation of intranuclear spherical assemblies of multiple membrane cisternae that were in contact with the nuclear envelope. Note that the layers between adjacent cisternae (A,B) are less electron dense than in cells expressing wild-type lamins. cy, cytoplasm; ne, nuclear envelope; nu, nucleoplasm. Bars, 500 nm.

therefore demonstrate that the data obtained with respect to B-type lamins in *Xenopus* A6 cells can be generalized.

As a further control we expressed the human lamin A in COS-7 cells by the microinjection of the corresponding plasmid. At the light microscopy level cells exhibited a rim-like nuclear staining, and in several cells we also noticed small dot-like structures in the nuclear periphery. On ultrathin sections through cells overexpressing human lamin A we observed an electron-dense layer of irregular thickness underneath the inner nuclear membrane (Fig. 8D). In addition, we have seen spherical electron-dense aggregates of variable size attached to the inner nuclear membrane (Fig. 8E) but found no indication for the proliferation of the nuclear membrane as seen in *Xenopus* A6 cells. Intranuclear membranes were found in association with the surface of some spherical aggregates (Fig. 8E, arrow). Our results obtained on the expression of the human lamin A in mammalian cells are in agreement with published data (Bechert et al., 2003; Izumi et al., 2000) indicating that the ‘B-type-like’ behavior of human lamin A observed in *Xenopus* A6 cells seems to be specific for this cell line.

Discussion

By the overexpression of lamins in mammalian, amphibian and fish cells we have detected that the CxxM motif of B-type lamins is involved in nuclear membrane growth. We are convinced that the nuclear lobulation and the extra membranes seen inside the nuclei can be attributed to the function of the CxxM motif of B-type lamins because the overexpression of lamins without a CxxM (*Drosophila* lamin C) or a mutated CxxM motif (lamin B2-SxxM) did not result in a changed nuclear shape or the formation of intranuclear membranes. The ‘B-type-like’ behavior of human lamin A in *Xenopus* A6 cells suggests that it is not processed in this amphibian cell line.

In *Xenopus* A6 cells with stacked intranuclear membrane cisternae we noticed that neighboring cisternae were separated by a 15-25 nm-thick electron-dense layer in cells expressing lamins A, B2 or Dm0 (see Fig. 5A-H). The electron-dense layer only had a thickness of 8-10 nm in A6 cells expressing a lamin B2 mutant lacking the head domain and the complete α-helical rod domain (Fig. 7C) suggesting that these two domains contribute to its thickness. It is likely that antiparallel interactions of the rod domains in lamin polymers (Fig. 5; Fig. 7A,B) contribute to the arrangement of the intranuclear membranes (for reviews, see Stuurman et al., 1998; Herrmann and Aebi, 2004). Presently we cannot exclude the fact that the stacking of intranuclear membranes may be enhanced by the low affinity antiparallel dimerization of the GFP especially in fusion proteins lacking the complete α-helical domain (Fig. 7C) (Snapp et al., 2003).

How is nuclear membrane proliferation controlled?

Studies of ER-resident membrane proteins show that their overproduction can induce a marked proliferation of ER membranes in which the overproduced proteins accumulate, and these studies reveal that membrane proliferation is coupled with lipid synthesis (Ohkuma et al., 1995; Chin et al., 1982; Ravid et al., 1999; Snapp et al., 2003). It is known that the synthesis of new lipids is regulated via a feedback inhibition. The depletion of sterols from the cytosol induces the proteolytic activation of ER-localized inactive transcription factors named sterol regulatory element binding proteins

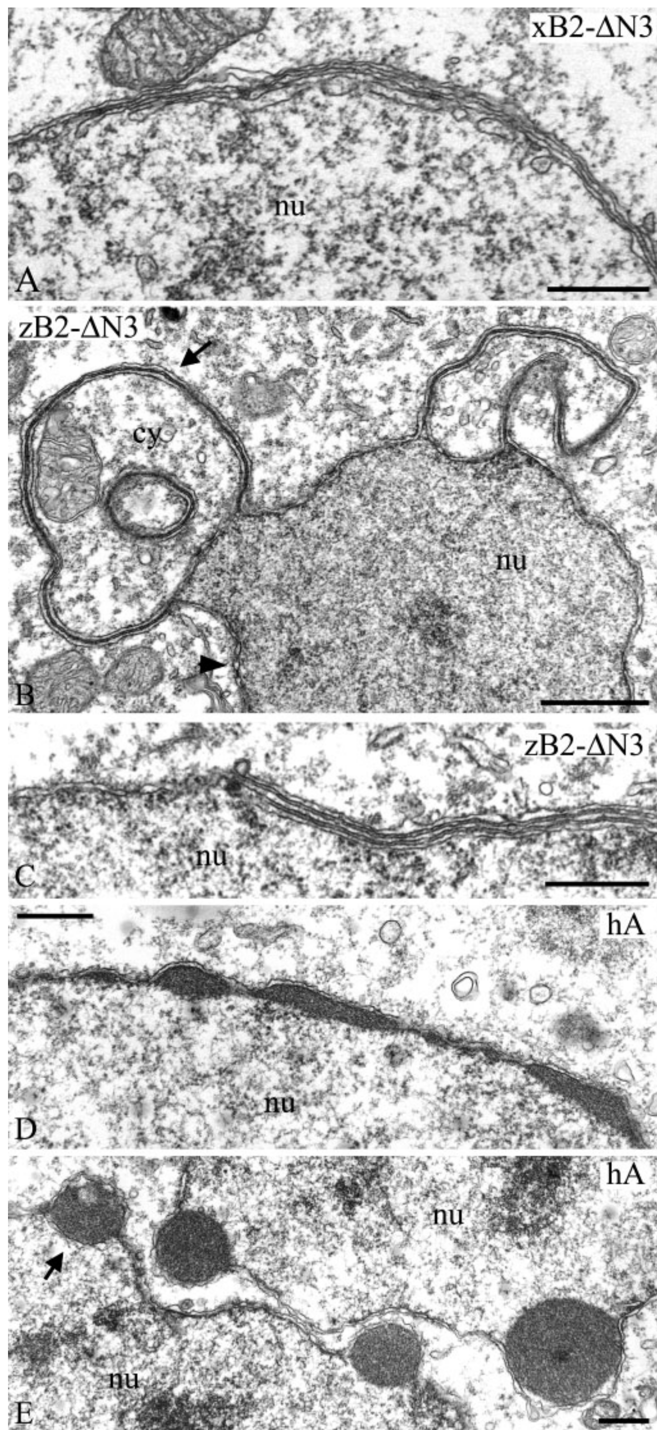

Fig. 8. Expression of GFP-lamin fusion proteins in COS-7 cells and zebrafish embryos. (A-E) Electron micrographs of ultrathin sections through transfected (A) and microinjected COS-7 cells (D,E) and zebrafish embryos at 9 (B) and 36 (C) hours post fertilization. Cells express *Xenopus* lamin B2-ΔN3 (A) and human lamin A (D,E). Zebrafish embryos were microinjected at the 2-4 cell stage with mRNA encoding zebrafish lamin B2 mutant zB2-ΔN3 (B,C). Multi-layered nuclear membranes (A,C) and nuclear lobulations (B, arrow) were seen in cells overexpressing this lamin B2 mutant. The arrowhead in B indicates an unaltered area of the nuclear envelope containing pore complexes. (D,E) COS-7 cells expressing human lamin A possess an electron-dense layer of irregular thickness underneath the inner nuclear membrane (D) or spherical aggregates attached to the inner nuclear membrane (E). Intranuclear membranes are attached to some spherical aggregates (E, arrow). cy, cytoplasm; nu, nucleoplasm. Bars, 500 nm (A,C-E); 1 μm (B).

(SREBPs). After their detachment from the ER, SREBPs are translocated into the nucleus, and as a further consequence, genes required for the synthesis of sterols and fatty acids are transcribed (Horton et al., 2003) (reviewed by Rawson, 2003).

Because the nuclear envelope is continuous with the ER it is worth speculating that nuclear membrane growth is controlled by the same mechanism. Our data suggest that in B-type lamins the farnesylated cysteine of the posttranslationally processed CxxM motif is one trigger for nuclear membrane growth. Following the attachment of newly synthesized lamins at the inner nuclear membrane, lipids would be incorporated in a coordinated manner into the nuclear membrane resulting in nuclear envelope growth.

Electron microscopy studies have demonstrated that the surface area of the nuclear envelope and the pore complex number are continuously increasing during interphase with the highest surface increase during S-phase (Maul et al., 1972). Biochemical experiments revealed that lamins are synthesized throughout the interphase of cultured CHO cells (Gerace et al., 1984), and that in vitro translatable lamin mRNAs are predominantly detectable in Ehrlich ascites cells at S-phase (Bludau et al., 1986). These data suggest that nuclear envelope growth and lamin synthesis are coordinated during the cell cycle.

The formation of highly lobulated nuclear envelopes in cells overexpressing lamins at a moderate level does reflect nuclear envelope growth in our experimental system. These nuclei possessed no or very few intranuclear membranes (Fig. 4A), and the distribution of endogenous lamins and proteins of the inner nuclear membrane was indistinguishable from that in control cells (data not shown). In contrast, the distribution of endogenous nuclear envelope proteins is altered in lobulated nuclei formed by the expression of lamins with a shortened α-helical domain (Schirmer et al., 2001) and in cells expressing N-terminally truncated lamins (mutants B2-ΔN1, B2-ΔN3, C-CxxM-ΔN1; data not shown). In yeast, highly lobulated nuclei are formed when the genes encoding the integral membrane proteins Nem1p or Spo7p have been mutated (Siniossoglou et al., 1998).

When higher concentrations of GFP-lamin fusion proteins were synthesized we did not see a coordinated surface increase of the outer and the inner nuclear membrane but rather the formation of numerous intranuclear membranes (Fig. 4C,D; Fig. 5G,H). This observation suggests that the coordinated nuclear envelope growth is not controlled exclusively by lamins.

We saw no continuities of the inner nuclear membrane and intranuclear membranes by electron microscopy (Fig. 5). However, we cannot exclude the fact that temporary contacts between a subpopulation of these membranes may exist. Light microscopy observations support this idea. Few but not all of the dot-like GFP-lamin containing structures seen in nuclei could be stained by an antibody specific for an integral membrane protein of the inner nuclear membrane (antibodies against LAP2β; data not shown) (Lang and Krohne, 2003). It

is also possible that some of the intranuclear membranes have been formed de novo using lipids present in the nucleoplasm (reviewed by Irvine, 2003) or have been generated from vesicles that budded from the inner nuclear membrane.

Intranuclear membranes are not uncommon. They are characteristic of insect cells that have been infected with the baculovirus (Braunagel et al., 1998), for cells of the human endometrium (Isaac et al., 2001) and have been observed in cells overexpressing the pore complex proteins Nup53p (Marelli et al., 2001), Nup153 (Bastos et al., 1996) and a mutant of the fibroblast growth factor receptor 4 (Søerensen et al., 2004). In contrast to lamins, these proteins did not affect the nuclear shape of cells when overexpressed.

Our demonstration that lamins containing a CxxM motif are involved in nuclear membrane growth are in agreement with previously described experiments where lamin depletion has prevented nuclear envelope growth in vivo and in vitro (Benavente and Krohne, 1986; Newport et al., 1990). Future experiments should help to gain insight into the mechanism by which the CxxM motif might influence nuclear membrane proliferation via lipid metabolism.

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