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# Functional diversity of LAP2α and LAP2β in postmitotic chromosome association is caused by an α-specific nuclear targeting domain

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**Lamina-associated polypeptide 2α (LAP2α) is a non-membrane-bound isoform of the LAP2 family implicated in nuclear structure organization. We show that during postmitotic nuclear assembly LAP2α associates with chromosomes prior to accumulation of the membrane-bound isoform LAP2β, although both proteins contain the same putative chromatin interaction domains located in their common N-terminal regions. By transient and stable expression of various N- and C-terminal LAP2α deletion mutants in HeLa cells, we identified an ~350-amino-acid-long region in the C-terminal α-specific domain of the protein that is required for retention of LAP2α in interphase nuclei and for association with mitotic chromosomes, while the N-terminal domain seemed to be dispensable for these interactions. *In vitro* chromosome binding studies using recombinant LAP2α mutants revealed that this LAP2α-specific 'nuclear targeting domain' was essential and sufficient for association with chromosomes. These data suggested a functional diversity of chromosome binding properties of LAP2 isoforms.**

*Keywords*: chromosome/lamina-associated polypeptide/mitosis/nuclear lamina/nuclear matrix

## Introduction

Mammalian nuclei are complex organelles that carry out a number of important functions, such as DNA replication, transcription and RNA processing. These functions largely depend on an integrated, highly ordered spatial organization of the nuclear compartment (Lamond and Earnshaw, 1998). A key to understanding the relationship between nuclear architecture and function is the identification and investigation of components that establish nuclear organization by linking chromatin to the nuclear envelope and the nucleoskeleton. Potential candidates for such structural molecules are the lamins, nuclear-specific intermediate filament-type proteins, and lamin-associated polypeptides. They form the major structural framework of the nuclear envelope, the nuclear lamina, and provide mechanical stability to the nucleus (for a review, see Moir *et al*., 1995). In addition, lamins have also been found in nucleoplasmic foci and DNA replication centers (Bridger *et al*., 1993; Moir *et al*., 1994; Spann *et al*., 1997), and have been identified as components of an internal nucleoskeleton (Hozak *et al*., 1995; Neri *et al*., 1999), but the functional significance of these findings is controversial. The observed interactions of lamins with DNA *in vitro* (Luderus *et al.*, 1992; Baricheva *et al.*, 1996; Zhao *et al.*, 1996) and *in vivo* (Rzepecki *et al*., 1998), and their *in vitro* association with chromosomes and chromatin (Glass and Gerace, 1990; Hoger *et al*., 1991; Yuan *et al*., 1991; Glass *et al*., 1993; Taniura *et al*., 1995) suggested a role for the lamins in chromatin organization, but the molecular mechanisms and their biological relevance remain unclear.

In addition, several proteins interacting with the lamins have been implicated in the structural organization of chromatin: p58, also called lamin B receptor (LBR) (Worman *et al*., 1990), is an integral protein of the inner nuclear membrane and has been found to interact with B-type lamins (Simos and Georgatos, 1992; Meier and Georgatos, 1994; Ye and Worman, 1994), DNA (Ye and Worman, 1994) and human chromodomain proteins homologous to *Drosophila* HP1 (Ye and Worman, 1996; Ye *et al*., 1997). Based on its interactions with chromosomes, p58 was proposed to serve as a chromatin docking site at the nuclear envelope (Pyrpasopoulou *et al*., 1996). Members of the lamina-associated polypeptide 2 (LAP2) family are also candidate proteins involved in the organization of chromatin. The originally described rat LAP2, now called LAP2β, has been identified as an inner nuclear membrane protein that binds to lamin B and chromosomes (Foisner and Gerace, 1993; Furukawa *et al*., 1995, 1998). Microinjection of recombinant mutant LAP2β into mammalian cells inhibited nuclear volume increase and progression into S phase (Yang *et al*., 1997b), and addition of human recombinant LAP2β truncation mutants to cell-free *Xenopus laevis* nuclear assembly reactions severely affected nuclear envelope and lamina assembly (Gant *et al*., 1999). These experiments clearly indicated a role of the membrane protein LAP2β in lamin assembly and nuclear organization. Analyses of the human and mouse LAP2 genes and transcripts revealed that LAP2β is one of 3–6 LAP2 isoforms derived from a single gene by alternative splicing (Harris *et al*., 1994, 1995; Berger *et al*., 1996). While most of these isoforms are closely related structurally, the largest isoform LAP2α was found to contain only a 187-amino-acid N-terminal domain identical to the N-termini of the other isoforms and a unique α-specific, 506-amino-acid-long C-terminal part lacking a trans-membrane domain. Accordingly, LAP2α was recently characterized as a nuclear, non-membrane protein, which based on its resistance to detergent/high-salt extraction may be a component of the nucleoskeleton (Dechat *et al*., 1998). LAP2α was also identified as the only member of the LAP2 family constitutively expressed during spermiogenesis, suggesting that it may be involved in chromatin remodeling during this process (Alsheimer *et al*., 1998).

In addition to their structural role in defining nuclear architecture in interphase, the LAP2 isoforms have also been suggested to serve important functions in the dynamic disassembly/reassembly of the nuclear envelope and the reorganization of chromatin during mitosis. Both LAP2α and β have been found to dissociate from chromosomes in a phosphorylation-dependent manner during metaphase and to accumulate around or between decondensing chromosomes during early stages of nuclear reassembly prior to the formation of the nuclear membrane and the lamina (Foisner and Gerace, 1993; Yang *et al*., 1997a; Dechat *et al*., 1998). The potential role of these proteins in chromatin reorganization upon exit from metaphase was also supported by their interaction with chromosomes *in vitro* and its regulation by mitosis-specific phosphorylation (Foisner and Gerace, 1993; Dechat *et al*., 1998; Furukawa *et al*., 1998).

While the lamin-binding region of the membrane protein LAP2β was found to reside in its C-terminal domain, which is not present in the LAP2α isoform, its chromatin interaction domain (Furukawa *et al*., 1998) as well as an interaction domain with the chromosomal protein BAF (barrier to autointegration factor; Furukawa, 1999) were localized within the N-terminal LAP2 common domain. Although these findings suggested that targeting of LAP2α and β to chromosomes is mediated by the same regions at the proteins' N-termini, *in vitro* binding studies with bacterially expressed LAP2α recombinant proteins indicated the presence of a chromosome binding domain in the α-specific region of LAP2α (Dechat *et al*., 1998).

Here, we present data suggesting that the chromosome binding properties of LAP2α differ significantly from those of LAP2β. By transient and stable expression of various LAP2α domains in HeLa cells, and by two independent *in vitro* chromosome binding assays, we show that an α-specific region, which is absent in LAP2β, is essential for chromosome binding of LAP2α and for nuclear retention in interphase. This difference in the chromosome binding properties was also reflected by different assembly kinetics of LAP2α and LAP2β around chromosomes after mitosis, suggesting a functional diversity of LAP2 isoforms due to different chromosome binding domains.

## Results

### *LAP2α-specific region(s) are important for nuclear localization of the protein*

In order to identify functional domains of LAP2α, we generated a series of deletion mutants missing various parts of the protein's N- and C-termini, including LAP2 common and α-specific regions (Figure 1). LAP2α deletion mutants containing a C-terminal Myc-tag were transiently expressed in HeLa cells using the tetracycline-inducible expression system (Gossen *et al*., 1995), and their subcellular localization was analyzed by confocal immunofluorescence microscopy using antibodies to the Myc-epitope tag. Ectopically expressed full-length LAP2α (Figure 2, LAP2α 1–693, fl) and a truncation mutant lacking 78 amino acids at its C-terminus (LAP2α 1–615, ΔC78) were localized exclusively in the nucleus, like the endogenous protein (Dechat *et al*., 1998). In contrast, LAP2α mutants missing 279 C-terminal amino acids, which comprise about half of the protein's α-specific domain (LAP2α 1–414, ΔC279), and mutants lacking about three-quarters (LAP2α 1–254, ΔC439) or all (LAP2α 1–187, ΔC506) of the α-specific domain, were localized in both the nucleus and the cytoplasm. Also, the smallest C-terminal truncation mutant analyzed (LAP2α 1–85, ΔC608), covering the N-terminal 85 amino acids of the LAP2 common domain, which has previously been described to contain the chromatin interaction domain of LAP2β (Furukawa *et al.*, 1998), was found to distribute equally between cytoplasm and nucleus.

Conversely, N-terminal deletion mutants lacking the chromatin interaction domain (LAP2α 81–693, ΔN80) or lacking the entire LAP2 common domain (LAP2α 188–693, ΔN187) were located exclusively in the nucleus, like the endogenous protein (Figure 3A). Taken together, the observed cellular distribution of the LAP2α truncation mutants showed that the α-specific domain of the protein is sufficient to target the protein into the nucleus effectively, leading to an exclusive nuclear localization, while the potential chromatin interaction domain in its N-terminal LAP2 common domain is dispensable.

To test whether the potential nuclear localization signal (NLS) at the beginning of the α-specific region (Figure 1) is essential for the nuclear translocation of the LAP2α mutant ΔN187, which consists of the α-specific region, we deleted an additional eight amino acids from the N-terminus of this polypeptide, giving rise to the LAP2α mutant protein ΔN195 (LAP2α 196–693), which contained most of the α-specific region but lacked the potential NLS. As shown in Figure 3A, the removal of the potential NLS had no effect on the subcellular distribution of the transiently expressed mutant. Both polypeptides covering the α-specific region with and without the NLS were detected exclusively in the nucleus (Figure 3A, ΔN187 and ΔN195). Thus, the nuclear accumulation of the LAP2α-specific region was apparently independent of active nuclear transport mediated by its NLS. In contrast, further deletion of the N-terminal half of the α-specific region (amino acids 188–409) significantly affected the ability of the mutant protein LAP2α 410–693 (ΔN409) to accumulate exclusively in the nucleus. A considerable amount of the expressed polypeptide was also found in the cytoplasm in addition to its nuclear localization (Figure 3A). Thus, deletion of the N-terminal half of the α-specific domain had similar effects on the localization of the respective LAP2α mutants as deletion of its C-terminal half (compare Figure 2, ΔC279). Therefore, we concluded that domains located in both N- and C-terminal α-specific regions collaborate in the efficient targeting of LAP2α into the nucleus. In support of this hypothesis, a construct covering the central part of the α-specific region (amino acids 270–615) of LAP2α was detected exclusively in the nucleus upon transient transfection (Figure 3B). Similar results were obtained upon transient expression of these LAP2α mutants in normal rat kidney (NRK) cells (data not shown).

### *LAP2α mutants missing the nuclear targeting domain are not integrated into the nuclear structure*

Our expression studies showed that the α-specific region of LAP2α contains a 'nuclear targeting domain' (amino

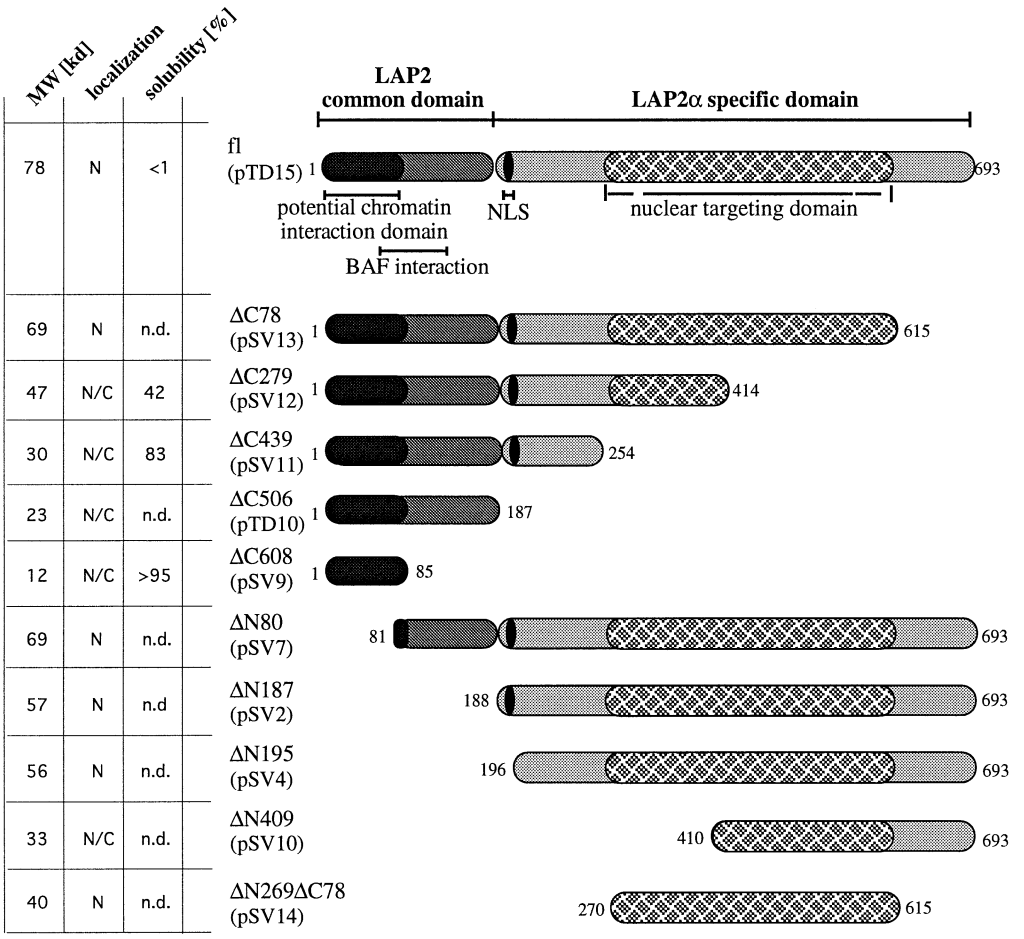


**Fig. 1.** Molecular organization of LAP2α deletion mutants. The LAP2 common domain, containing a potential chromatin interaction domain (Furukawa *et al*., 1998) and a region mediating BAF interaction (Furukawa, 1999), and the LAP2α-specific domain containing a NLS, and the nuclear targeting domain are indicated by bars and/or different filling patterns. Names of expression plasmids coding for respective LAP2α mutants and deleted C- and N-terminal amino acids are indicated on the left. Numbers next to bars denote the position of amino acids in the human LAP2α sequence; fl, full length. The table on the left shows calculated molecular weights (MW) of Myc-tagged mutants, cellular localization upon transient transfection (N, nuclear; N/C, nuclear plus cytoplasmic) and solubility of mutants upon lysis of stably transfected cells; relative amounts of mutants in cytoplasmic fractions were determined by densitometric scanning of bands in immunoblots shown in Figure 4B.

acids 270–615), which is essential and sufficient for exclusive accumulation of LAP2α in the nucleus. As all LAP2α mutants missing parts of the nuclear targeting domain were sufficiently small to enter the nucleus by passive diffusion (see Figure 1) and could also be found in the nucleus, albeit they did not accumulate there, we speculated that the nuclear targeting domain might function in a transport-independent mechanism. Considering that LAP2α has previously been described to be part of a Triton X-100/salt-resistant nuclear structure (Dechat *et al*., 1998), the nuclear targeting domain might be involved in anchoring LAP2α firmly to structural components within the nucleus.

To test this hypothesis and to analyze the association of LAP2α deletion mutants with nuclear structures at the biochemical level, we generated HeLa cell clones that stably express recombinant full-length (fl) LAP2α (clone 15-1) or LAP2α mutants ΔC279 (clone 12-2), ΔC439 (clone 11-21) and ΔC608 (clone 9-43). Immunoblot analyses of total cell lysates using anti-Myc antibody showed that all clones expressed the respective mutant protein in a tightly regulated, tetracycline-dependent manner and at comparable levels (Figure 4A), which were similar to the expression level of endogenous LAP2α (for ΔC279 see Figure 4B and C; others not shown). Upon lysis of cells in hypotonic, detergent-free buffer and separation of cytoplasmic supernatant and insoluble nuclei-enriched fractions, endogenous LAP2α and ectopically expressed recombinant full-length LAP2α (fl) were detected exclusively in the nucleus fraction by immunoblot analyses using LAP2α-specific (Figure 4B, clone 15-1) and anti-Myc antibodies (data not shown). In contrast, LAP2α deletion mutants missing parts of the protein's α-specific domain were also found in the cytoplasmic fractions to different extents (Figure 4B), while the distribution of endogenous LAP2α was unchanged (Figure 4B, clone 12-2; data not shown). LAP2α mutant ΔC279 (Figure 4B, clone 12-2), missing half of the α-specific domain, was almost equally distributed between cytoplasmic and nuclear fractions, whereas LAP2α mutants ΔC439 (clone 11-21) and ΔC608 (clone 9-43), lacking almost all or all of the LAP2α-specific domain, were predominantly detected in the soluble fraction (Figure 1). Thus, the more of the LAP2α-specific domain that was deleted, the greater the level of the protein found in the cytoplasmic fraction. As nuclei in these samples still contained a nuclear

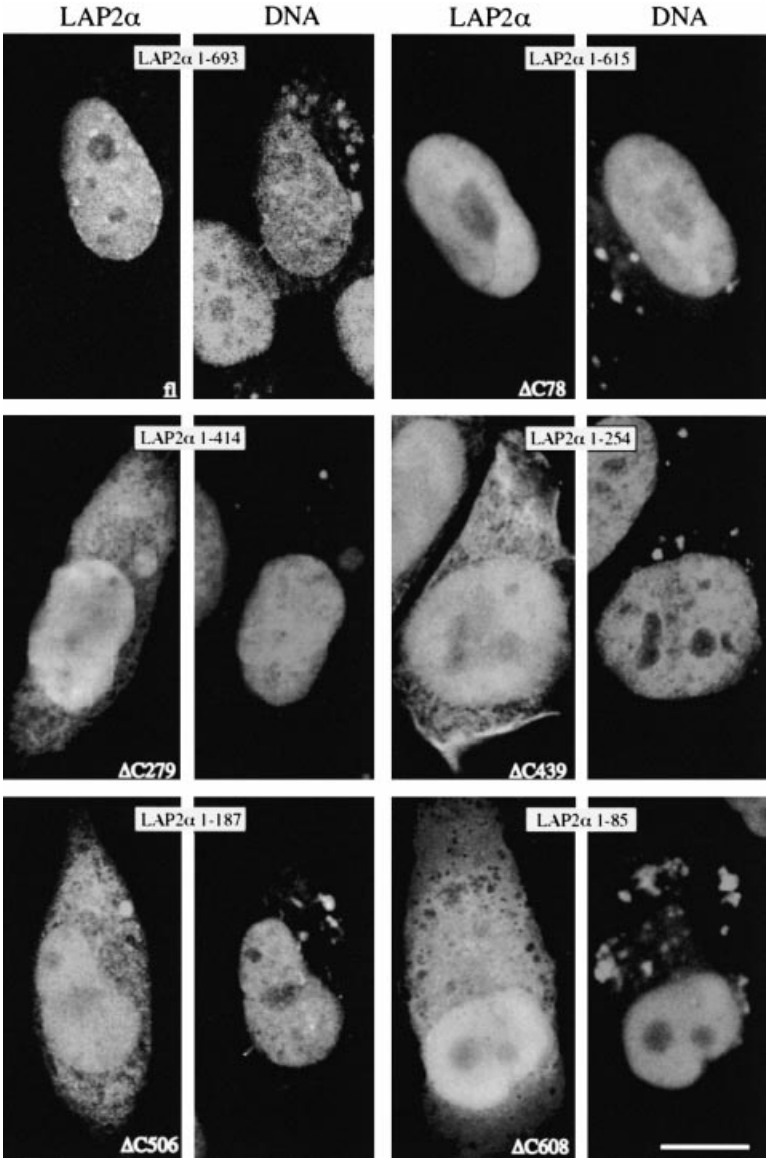


**Fig. 2.** Subcellular localization of transiently expressed Myc-tagged C-terminal LAP2α deletion mutants. HeLa Tet-On cells were transfected with plasmids encoding different N-terminal regions of LAP2α (respective amino acids of LAP2α sequences and deletions are indicated), incubated with doxycycline for 12–24 h and processed for immunofluorescence microscopy using monoclonal anti-Myc antibody for detection of recombinant proteins (LAP2α) and propidium iodide for detection of DNA. Confocal images are shown. Bar, 10 μm.

membrane, one has to envisage that the membrane was ruptured during mechanical cell homogenization and soluble nuclear proteins not bound to nuclear structures diffused out of the nuclei into the supernatant during fractionation. Therefore, it can be concluded that, unlike endogenous and exogenously expressed full-length protein, LAP2α deletion mutants are not firmly anchored in the nucleus. Considering the different extents of release of mutant proteins into the cytoplasmic fraction, one might speculate that LAP2α mutant ΔC279 missing the C-terminal half of the LAP2α-specific region (amino acids 415–693) maintained a partial capability to interact with nuclear structures. Only deletion of additional α-specific residues (amino acids 255–414) abolished the protein's capability to associate with nuclear structures completely. These data correlate nicely with the different subcellular localization of LAP2α mutants upon transient expression, and suggest that the nuclear targeting region, which contains stretches upstream and downstream of amino acids 410–414 (Figure 1), is involved in linking LAP2α to nuclear structures.

In order to compare in more detail the potential interaction of LAP2α mutant ΔC279 with nuclear structures with that of the endogenous protein, we extracted nuclei in buffers containing low or high concentrations of salt and/or Triton X-100 and analyzed the distribution of the proteins between low- and high-speed pellet and supernatant fractions. While endogenous LAP2α was completely retained in the nuclei-enriched low-speed pellet fraction ($P_L$) at low ionic strength in the absence and presence of detergent, the ΔC279 mutant was distributed almost equally between soluble and insoluble fractions in the absence of Triton X-100 and was predominantly soluble in the presence of detergent (Figure 4C). At high-salt condition (200 mM), the mutant protein was predominantly soluble in both cases, whereas a

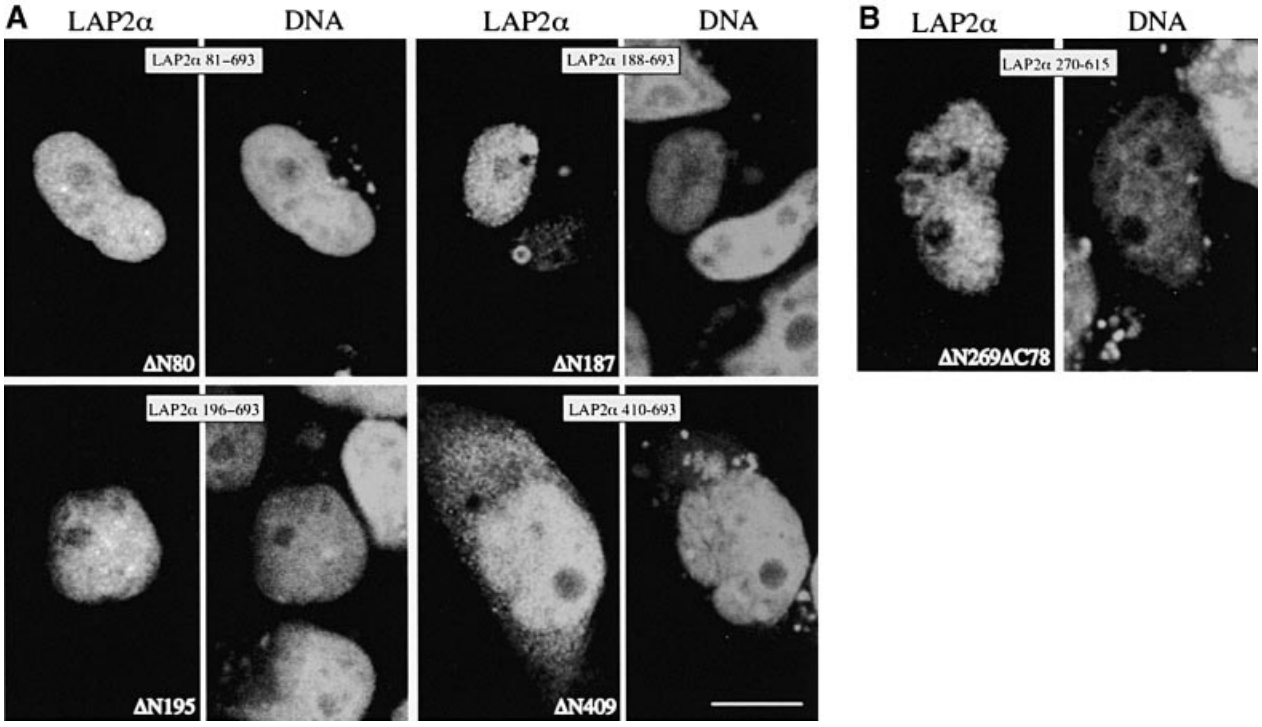


**Fig. 3.** Subcellular localization of transiently expressed Myc-tagged N-terminal LAP2α deletion mutants (**A**) and of a mutant comprising the nuclear targeting domain (**B**). HeLa Tet-On cells were transfected with plasmids encoding different LAP2α mutants (respective amino acids of LAP2α sequences and deletions are indicated), incubated with doxycycline for 12–24 h and processed for immunofluorescence microscopy with monoclonal anti-Myc antibody for detection of recombinant proteins (LAP2α) and propidium iodide for detection of DNA. Confocal images are shown. Bar, 10 μm.

considerable amount of endogenous LAP2α was still found in the insoluble fractions. Together, these data showed that the binding of LAP2α mutant protein to intranuclear structures was significantly weaker than that of the endogenous protein and confirmed the importance of regions downstream of amino acid 414 for stable association of LAP2α with nuclear structures.

### *LAP2α mutants missing the nuclear targeting domain do not associate with chromosomes during postmitotic nuclear reassembly*

As LAP2α has previously been shown to associate with chromosomes early during nuclear reassembly and to bind to mitotic chromosomes *in vitro* (Dechat *et al.*, 1998), it was intriguing to test whether the predicted nuclear targeting domain in the α-specific region, which seems to be responsible for the integration of LAP2α into the nuclear structure during interphase, is also involved in the protein's interaction with chromosomes during mitosis. Therefore, we followed the cellular localization of full-length and C-terminally truncated LAP2α mutants in stably transfected cells at various cell cycle stages by confocal immunofluorescence microscopy. In one set of experiments we analyzed the distribution of mutant proteins in relation to chromosomes using a monoclonal antibody to the Myc-tag and the DNA stain propidium iodide (Figure 5a–d); in another series we compared the distribution of mutant proteins with that of endogenous LAP2α using affinity-purified polyclonal anti-Myc antibodies and a monoclonal antibody to LAP2α that did not detect the truncation mutants (Figure 5a′–d′). Like endogenous protein, LAP2α mutant ΔC279 was not associated with chromosomes during metaphase and appeared diffusely distributed throughout the cells (Figure 5a and a′). At very early stages of nuclear reassembly during late anaphase, when endogenous LAP2α accumulated in distinct structures around and between chromosomes (Figure 5b, b′, c and c′; see also Dechat *et al.*, 1998), LAP2α mutant protein ΔC279 still remained mostly cytoplasmic in the majority of cells (Figure 5c and c′). Only in ~30% of the cells at this mitotic stage was a translocation of the mutant protein to chromosomes observed similar to the wild-type protein (Figure 5b and b′). The LAP2α truncation mutant, however, was uniformly distributed in the cytoplasm and failed to associate with chromosomes in all cells analyzed during telophase/$G_1$, when endogenous LAP2α accumulated at the periphery of and between decondensing chromosomes (Figure 5d and d′) and within the newly formed nuclei. Exogenously expressed full-length LAP2α, on the contrary, behaved exactly like the endogenous protein at all cell cycle stages tested (data not shown). Thus, it can be concluded that the deletion of LAP2α's C-terminal 279 amino acids clearly affected the ability of the protein to associate with chromosomes during early stages of nuclear reassembly and to accumulate in the newly formed nuclei.

This conclusion was further supported at the biochemical level by *in vitro* nuclear assembly studies. Cell lysates from mitotically arrested cells expressing LAP2α mutant ΔC279 were incubated at 30°C for various time periods, as reported previously (Burke and Gerace, 1986; Dechat *et al.*, 1998), adjusted to 0.5% Triton X-100, and separated into low- and high-speed pellet and supernatant fractions. Immunoblot analysis of fractions revealed that both endogenous LAP2α and LAP2α mutant ΔC279, as well as

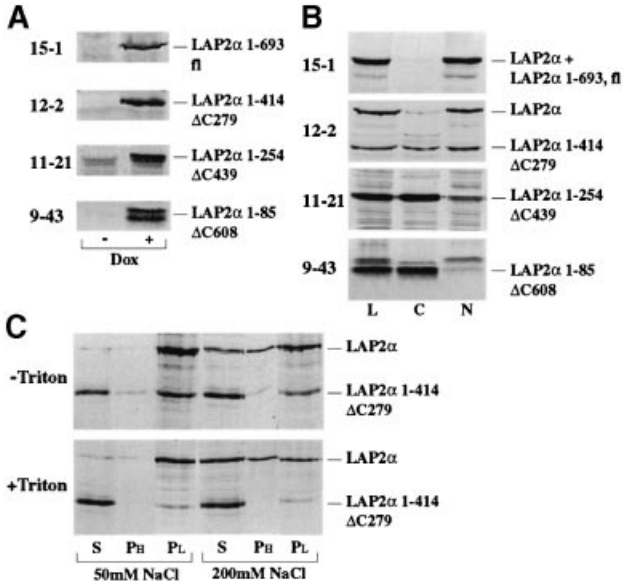


**Fig. 4.** Subcellular distribution and Triton X-100/salt resistance of stably expressed exogenous full-length (fl) LAP2α (1–693), LAP2α mutants (1–414, 1–254, 1–85) and endogenous LAP2α. (**A**) Total cell lysates of stably transfected HeLa Tet-On clones 15-1, 12-2, 11-21 and 9-43, grown in the presence (+) or absence (–) of doxycycline (Dox), were analyzed by immunoblotting using monoclonal anti-Myc antibodies. (**B**) Interphase cultures of HeLa Tet-On clones 15-1, 12-2, 11-21 and 9-43 expressing the constructs indicated were lysed in hypotonic buffer and total cell lysates (L), soluble cytoplasmic (C) and insoluble nuclear (N) fractions were separated by centrifugation and analyzed by immunoblotting using LAP2α- (panel 15-1) or LAP2-specific (all others) antibodies. (**C**) Nuclei isolated from clone 12-2 were extracted with Triton X-100 and salt as indicated, and insoluble low-speed pellet fractions ($P_L$) were collected by centrifugation at 2000 *g*. The supernatant fraction was centrifuged at 100 000 *g* to yield high-speed pellet ($P_H$) and soluble fraction (S). Immunoblots of fractions using anti-LAP2 antibodies are shown. Positions of endogenous LAP2α and/or ectopically expressed mutants are indicated on the right.

LAP2β, lamin B and lamins A/C, were predominantly soluble in the mitotic lysate without prior incubation (Figure 6). Upon 30 min incubation of the mitotic cell lysate, the majority of endogenous LAP2α shifted into the low-speed, chromosome-containing pellet fraction, similar to LAP2β and lamin B, while the mutant protein stayed mostly soluble. Lamins A/C, which have been shown to accumulate at chromosomes considerably later than LAP2α (Dechat *et al*., 1998), also did not shift to the pellet fraction significantly. Thus, although LAP2α mutant ΔC279 showed accumulation in the chromosomal area in a subset of postmitotic cells at very early stages of nuclear reassembly (Figure 5b and b′), the mutant was unable to assemble into chromosome-associated structures. These data indicated that LAP2α mutant ΔC279 might still contain regions that directly or indirectly mediate weak association with chromosomes during early stages of nuclear assembly, but clearly misses domains that mediate the stable association with chromosomes and/or nucleoskeletal structures.

Further deletion of 160 amino acids from LAP2α mutant ΔC279 at its C-terminus, which yielded mutant ΔC439 missing about three-quarters of the protein's α-specific domain, completely abolished the translocation of the mutant to chromosomal areas and the association of the protein with interchromosomal structures during all stages of nuclear reassembly in stably transfected cells (Figure 7). Double immunofluorescence confocal microscopy revealed that endogenous LAP2α structures at or between the chromosomes in early telophase (Figure 7b) or LAP2α in the nucleus during late telophase/$G_1$ (Figure 7c) did not co-localize significantly with LAP2α mutant protein ΔC439. The mutant remained uniformly distributed throughout the cytoplasm and was apparently excluded from the newly formed nucleus in late telophase/$G_1$. Similarly, the smallest construct covering LAP2α's N-terminal 85 amino acids (ΔC608), which is common to all LAP2 isoforms and has been shown to associate with chromosomes *in vitro* (Furukawa *et al*., 1998), did not accumulate in the chromosomal area and in the newly formed nuclei after mitosis in stably transfected cells (Figure 7a′–c′). The redistribution of the endogenous protein in these cells, however, remained unchanged.

These data suggested that the 'nuclear targeting domain' within amino acids 254–615, which was involved in the efficient nuclear localization of LAP2α in interphase, was also essential for the protein's stable association with chromosomal structures early during postmitotic nuclear assembly. In stably transfected cells, mutant proteins missing this domain stayed mostly cytoplasmic during telophase/$G_1$.

### *The nuclear targeting domain is essential and sufficient for association of LAP2α with chromosomes in vitro*

Our experiments using stably transfected HeLa cells clearly demonstrated that LAP2α mutants missing the nuclear targeting domain in the α-specific region failed to accumulate at chromosomal areas during postmitotic nuclear assembly. Since N-terminal deletion mutants could not be stably expressed in cells (see Discussion) we were unable to test the chromosome binding activity of the nuclear targeting domain directly *in vivo*. Alternatively, we tested the ability of the different LAP2α mutant proteins to interact with chromosomes *in vitro.* Mitotic chromosomes were mixed with recombinant, bacterially expressed LAP2α mutant proteins and sedimented through a sucrose cushion. Immunoblot analyses of the chromosome-enriched pellet fractions revealed that 50–60% of full-length LAP2α and of C-terminal deletion mutant ΔC78 (Figure 8B, 1–615), as well as of the N-terminal LAP2α mutants ΔN80 and ΔN187 (Figure 8A and B), co-sedimented with chromosomes, while they did not sediment on their own in the absence of chromosomes (Figure 8). Immunofluorescence microscopy of chromosomal pellets using various LAP2α-specific antibodies revealed association of these recombinant proteins with chromosomes along their entire surface (Figure 9, and not shown for ΔC78). Control blots and immunofluorescence analyses of chromosomal fractions using the same anti-LAP2 antibodies did not reveal endogenous chromosomal LAP2α staining. Thus, it can be concluded that the recombinant full-length LAP2α and mutants ΔC78, ΔN80, ΔN187, which all contained the nuclear targeting domain (amino acid 270–615), associated with chromosomes *in vitro.* In contrast, C- and N-terminal truncation mutants missing significant parts of the nuclear targeting domain (LAP2α 1–414, Figures 8A, B and 9; and LAP2α 410–693, Figure 8A and B) or the entire domain (LAP2α 1–

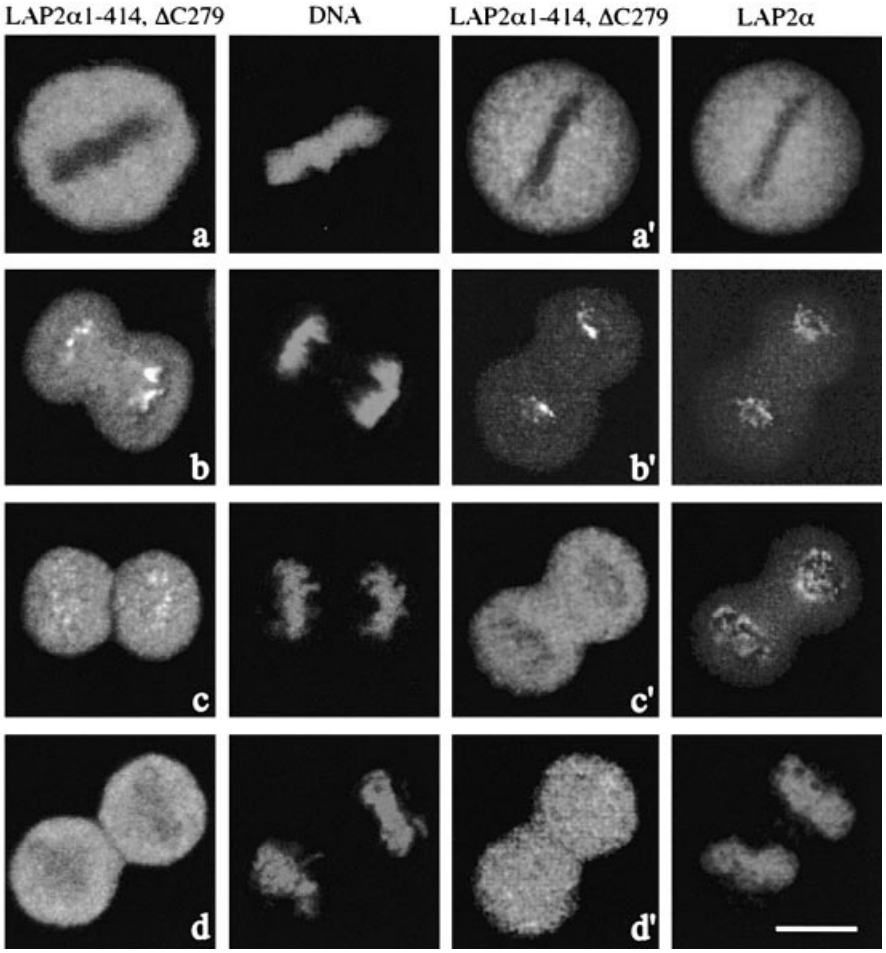


**Fig. 5.** Redistribution of LAP2α mutant (1–414) and endogenous LAP2α during different stages of mitosis. Mitotic cells of HeLa clone 12–2 were processed for immunofluorescence microscopy using monoclonal (**a**–**d**) or affinity-purified polyclonal anti-Myc antibodies (**a′**–**d′**) for detection of LAP2α mutant 1–414, LAP2α-specific antibody for detection of endogenous protein (LAP2α) and propidium iodide for detection of DNA. Confocal double immunofluorescence images are shown. Bar, 10 μm.

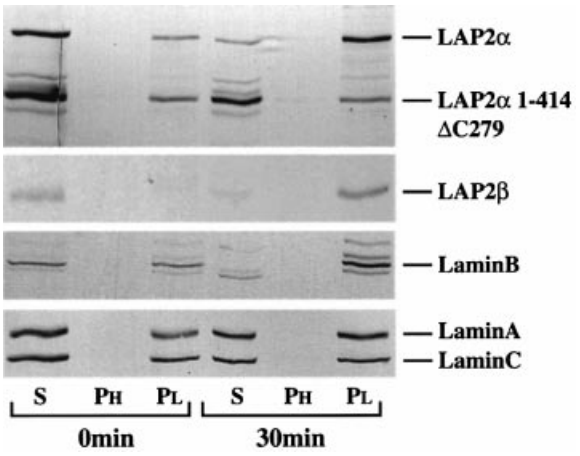


**Fig. 6.** Distribution of LAP2α mutant 1–414 and endogenous LAP2α during *in vitro* nuclear assembly. Cell lysates of mitotic 12-2 cells were incubated for 0 and 30 min at 30°C, supplemented with 0.5% Triton X-100, and insoluble low-speed pellet fractions were collected by centrifugation at 2000 *g* ($P_L$). The supernatant fraction was centrifuged at 100 000 *g* to yield high-speed pellet ($P_H$) and soluble fractions (S). Immunoblots of fractions using antibodies to the proteins indicated are shown.

85, Figures 8A, B and 9; LAP2α 1–187, Figure 8A and B; and LAP2α 1–254, Figure 8B) were neither found to co-sediment significantly with chromosomes (Figure 8) nor did they bind to chromosomes in immunofluorescence microscopic studies (Figure 9; data not shown). The nuclear targeting domain alone (LAP2α 270–615), however, was sufficient to associate with chromosomes (Figures 8 and 9). Thus, the *in vitro* binding studies confirmed the important role of the nuclear targeting domain in mediating the chromosomal association of LAP2α.

Interestingly, the N-terminal LAP2 common region of LAP2α (1–187), which has been reported to contain a chromatin binding domain in its first 85 amino acids using a different assay based on permeabilized mitotic cells (Furukawa *et al*., 1998), and which was recently shown to contain a binding domain for the chromosomal protein BAF within amino acids 67–137 (Furukawa, 1999), failed to interact in our assay. These discrepancies might be due to the different chromosome binding assays applied or due to the use of different LAP2-fusion proteins [His-tagged proteins in this study versus glutathione *S*-transferase (GST)-fusion proteins in the previous study]. To address these possibilities, we tested the His-tagged LAP2α mutant proteins for their ability to bind to chromo-

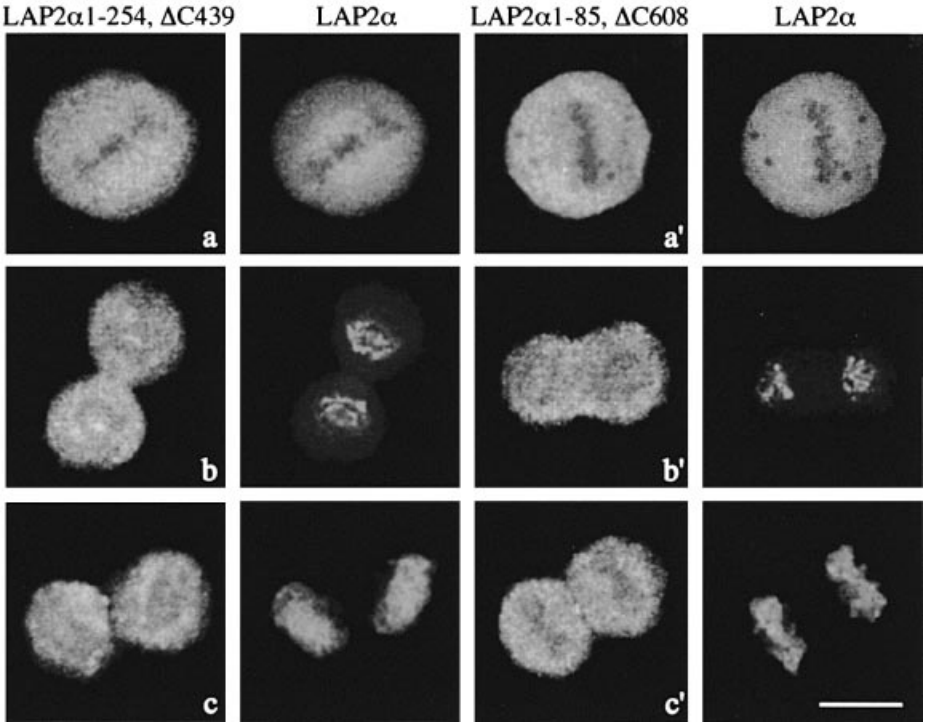


**Fig. 7.** Subcellular distribution of LAP2α mutants (1–254 and 1–85) and endogenous LAP2α during different stages of mitosis. Mitotic cells of HeLa clones 11-21 and 9-43 stably expressing LAP2α 1–254 and LAP2α 1–85 were processed for immunofluorescence microscopy using affinity-purified polyclonal anti-Myc antibody for detection of mutant proteins and anti-LAP2α antibodies for detection of endogenous LAP2α. Confocal double immunofluorescence images are shown. Bar, 10 μm.

somes in the permeabilized cell assay described by Furukawa *et al*. (1998). Mitotic NRK cells on glass coverslips were permeabilized in digitonin-containing buffer and incubated with soluble bacterial cell lysates containing His-tagged LAP2α mutant proteins, and bound proteins were detected by immunofluorescence microscopy using antibodies specifically reacting with the recombinant human proteins. As in the co-sedimentation assay, full-length LAP2α and mutants ΔN80 and ΔN187 were able to bind to chromosomes in permeabilized NRK cells in metaphase and anaphase (Figure 10A), whereas the N-terminal fragments (LAP2α 1–85, not shown; and LAP2α 1–187, Figure 10A) still did not interact, although all constructs were applied at comparable concentrations (data not shown).

In contrast, a fusion protein containing GST fused to the N-terminal LAP2α fragment 1–187 (Figure 10B, GST-fusion) was able to interact with metaphase and anaphase chromosomes, while control proteins containing GST alone (Figure 10B, GST control) did not bind. Since GST-fusion proteins may form dimers via their GST domain and might adopt a specific three-dimensional structure, our data indicate that the putative chromosome interaction domain(s) in the common region of LAP2 isoforms may only be functional in a specific three-dimensional conformation obtained upon dimerization and/or complex formation of the protein.

Altogether, our data indicate that LAP2α contains at least two different chromatin binding sites: one located in the LAP2α-specific nuclear targeting domain, which was found to be essential and sufficient for chromosome interaction *in vitro* (Figures 8–10) and essential for chromosome association *in vivo* during early stages of nuclear assembly (Figures 5 and 7); and a second domain in the LAP2 common region, which seems to be dispensable for chromosome interaction of LAP2α *in vitro* (Figures 8–10) and was not sufficient to mediate chromosomal interaction during nuclear reassembly *in vivo* (Figure 7). As the latter domain apparently needs oligomerization and/or complex formation for efficient binding, this interaction might be functional at different stages of the cell cycle.

### *LAP2α accumulated at chromosomes prior to association of LAP2β*

Our data demonstrated that the chromosome binding properties of LAP2α differ significantly from those of LAP2β, involving a unique LAP2α-specific interaction domain. These data also implied that the two proteins behave differently during postmitotic nuclear assembly when they associate with chromosomes. To test this hypothesis, we simultaneously analyzed the cellular distribution of endogenous LAP2α and LAP2β at various cell cycle stages following metaphase by double immunofluorescence microscopy. While both proteins were distributed throughout the cytoplasm in metaphase cells (Figure 11a), the majority of LAP2β was still found in distinct cytoplasmic structures during telophase, when LAP2α accumulated in interchromosomal structures (Figure 11b). At stages when LAP2α was localized exclusively around decondensing chromosomes, LAP2β started to accumulate at the nuclear periphery, but numerous LAP2β-containing structures still extended into the cytoplasm (Figure 11c). At late stages of nuclear reassembly LAP2α was found diffusely distributed in the newly formed nuclei and LAP2β became concentrated at the nuclear periphery (Figure 11d). Taken together, these

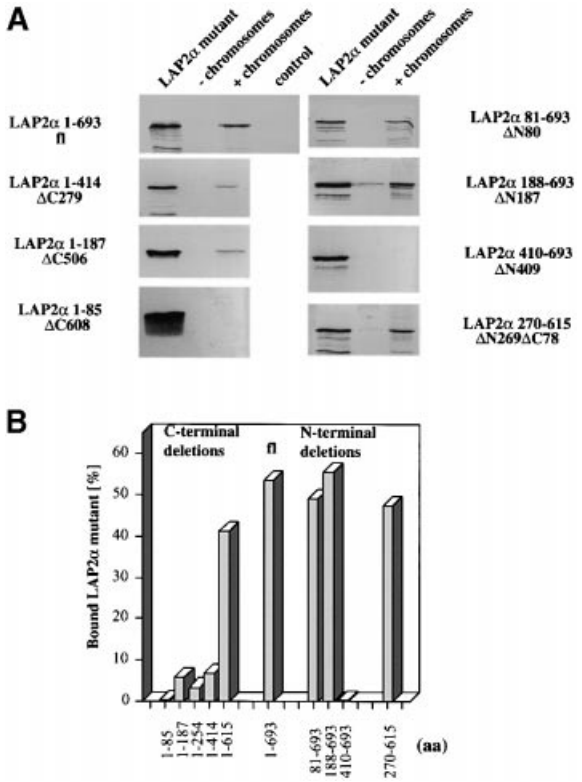


**Fig. 8.** Co-sedimentation of LAP2α mutants with mitotic chromosomes. (**A**) Bacterial cell lysates containing the indicated recombinant LAP2α mutant proteins (lanes LAP2α mutant) were mixed with buffer (– chromosomes) or mitotic chromosomes (+ chromosomes), sedimented through a sucrose cushion, and pellet fractions were analyzed by immunoblotting using anti-LAP2α antibodies. The lane control shows immunoblot analysis of the chromosomal fraction. (**B**) The relative amount of co-sedimented LAP2α mutant protein was analyzed by densitometric scanning of bands in immunoblots shown in (A).

data showed that LAP2α associated with chromosomes prior to accumulation of LAP2β, supporting the proposed role of LAP2α in very early stages of nuclear assembly and suggesting a functional diversity of LAP2 isoforms during nuclear assembly mediated at least in part by different chromosome binding properties.

## Discussion

In the present study, which was aimed at the identification of functional domains in the non-membrane-bound isoform of the LAP2 protein family, we identified a domain in the LAP2α-specific region of the molecule, termed the nuclear targeting domain, which was essential and sufficient for mediating nuclear retention of LAP2α in the interphase nucleus and essential for association with chromosomes during postmitotic nuclear reassembly. As other LAP2 isoforms, such as LAP2β, which has also been shown to bind to chromosomes (Foisner and Gerace, 1993; Furukawa *et al.*, 1997), miss the LAP2α-specific nuclear targeting domain, our studies clearly indicated a functional diversity of LAP2 isoforms in chromosome binding properties. This conclusion was supported by the observed different assembly kinetics of LAP2α and LAP2β around chromosomes during nuclear assembly.

### *Potential mechanisms of nuclear accumulation of LAP2α in interphase*

In the transient expression studies we found that all LAP2α mutant proteins that contained the nuclear targeting domain (amino acids 270–615) localized exclusively in the nuclear compartment, whereas all the mutants missing parts or all of this domain were found equally distributed in the nucleus and the cytoplasm. These findings suggested that the nuclear targeting domain is essential for exclusive nuclear localization of LAP2α. The partial nuclear localization of LAP2α mutants missing the nuclear targeting domain can be explained by their low molecular weights (<47 kDa; see Figure 1) allowing passive diffusion through pore complexes (Nigg, 1997; Ohno *et al.*, 1998). Among various potential molecular mechanism(s) for how the nuclear targeting domain may mediate exclusive nuclear localization of LAP2α, nuclear retention of the molecule due to specific interactions of the nuclear targeting domain with nuclear components seems to be the most likely one for several reasons. First, the nuclear targeting domain, which is still exclusively targeted into the nucleus, does not contain a putative NLS but is small enough to diffuse freely through the pore complexes. Secondly, the previously reported resistance of LAP2α against Triton X-100/salt extraction suggested its incorporation in a structural nuclear scaffold (Dechat *et al.*, 1998). Thirdly, the subcellular fractionation studies presented here support the role of the nuclear targeting domain in the tight association of LAP2α with the nucleoskeleton. We show that endogenous and ectopically expressed full-length LAP2α distributed exclusively in nuclear fractions, while mutants missing parts or all of the nuclear targeting domain were increasingly found in the supernatant fraction the more of the targeting domain that was missing. As the nuclear membrane was likely to be ruptured during cell homogenization and centrifugation, only proteins tightly associated with structural nuclear components were retained in the nuclear pellet fractions in these experiments. The nature of LAP2α-interacting components in the nucleus remains unknown. *In vitro* binding studies, using trypsin-, salt- or DNase-treated chromosomes and extraction of nuclear fractions in various buffer conditions (Dechat *et al.*, 1998; data not shown), suggested that chromosomal proteins rather than DNA itself comprise direct interaction partners for LAP2α.

Specific binding to nuclear structures has also been implicated in the efficient nuclear targeting of related proteins, including LAP2β (Furakawa *et al.*, 1995, 1998), p58 (Soullam and Worman, 1993) and otefin (Ashery-Padan *et al.*, 1997).

Aside from the nuclear targeting domain, a short NLS-like region of basic amino acids (amino acids 189–195) located outside of the nuclear targeting domain (Harris *et al.*, 1994) in LAP2α might contribute to the nuclear localization of newly synthesized LAP2α in interphase cells by mediating its active transport through the pore complexes. It is unclear, however, whether the potential internal NLS is functional as LAP2α mutants, which lacked the C-terminal half or the entire nuclear targeting domain but contained the potential NLS, were not targeted into the nucleus effectively.

Another alternative mechanism by which the nuclear targeting domain of LAP2α might mediate nuclear

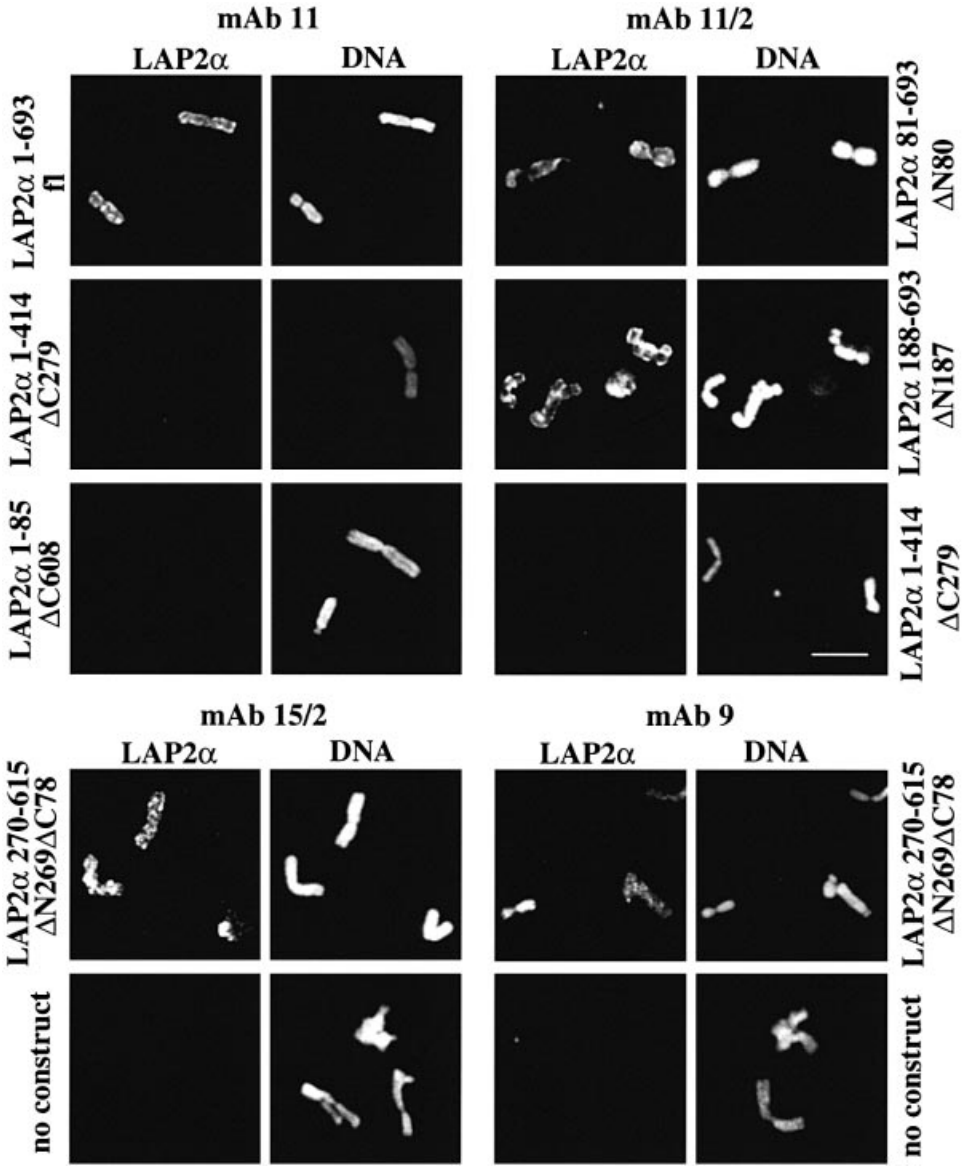


**Fig. 9.** Association of LAP2α mutants with chromosomes. Soluble bacterial cell lysates containing recombinant LAP2α mutants as indicated were mixed with chromosomes and processed for immunofluorescence microscopy using various monoclonal antibodies (mAb) to LAP2α as indicated. Bar, 2 μm.

accumulation of LAP2α may be the specific interaction of this domain with NLS-containing, import-competent proteins in the cytoplasm, which transport LAP2α into the nucleus in a supramolecular complex.

### *The nuclear targeting domain is essential for association of LAP2α with chromosomes after mitosis*

LAP2α, which is not associated with chromosomes at metaphase, has been reported to accumulate in structures at and between chromosomes at very early stages of post-mitotic nuclear reassembly, before an intact nuclear membrane is formed (Dechat *et al*., 1998). Our studies here clearly showed that LAP2α associated with chromosomes prior to the accumulation of LAP2β-containing membrane vesicles around the chromosomal surface. Therefore, it is very likely that the majority of LAP2α does not have to be actively transported into the nucleus through newly formed pore complexes after mitosis but may access chromosomal binding sites before decondensing chromosomes are fully enclosed by the nuclear membrane. Following the cell-cycle-dependent distribution of LAP2α mutant proteins in stably transfected HeLa cells, we found that the nuclear targeting domain is also essential for efficient association with chromosomes during mitosis. This was also confirmed by *in vitro* chromosome binding studies in two different independent assays. Thus, it may be concluded that the same interactions of LAP2α are essential for both the correct redistribution of the protein after mitosis and the stable integration in the nuclear scaffold.

### *Functional significance of the nuclear targeting domain*

Interestingly, we did not succeed in generating cell lines that stably expressed the N-terminal deletion mutants LAP2α 81–693 (ΔN80), LAP2α 188–693 (ΔN187), LAP2α 196–693 (ΔN195) and LAP2α 270–615 (ΔN269ΔC78). The reason for this is presently unknown,

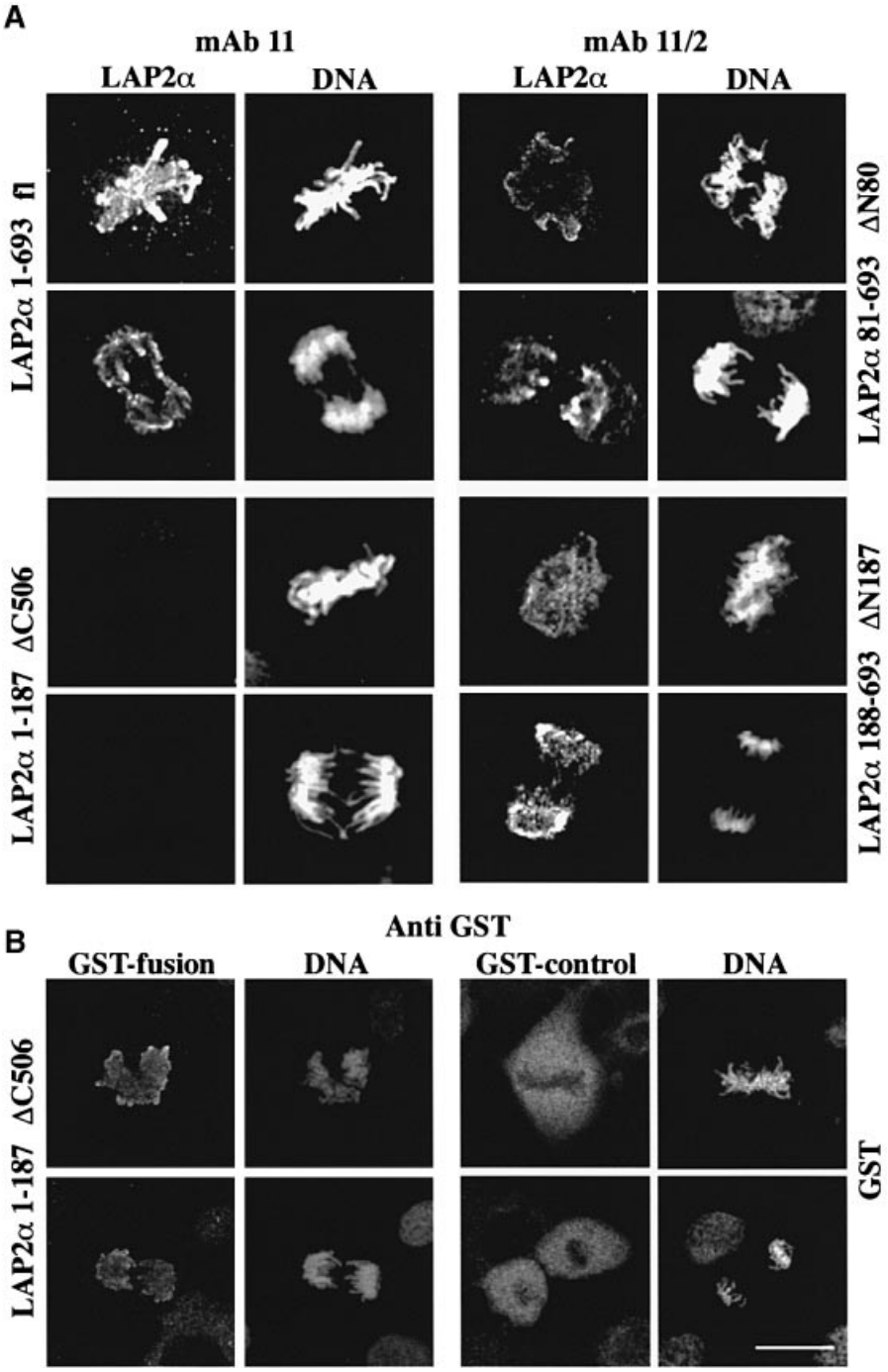


**Fig. 10.** Binding of LAP2α mutant proteins to chromosomes in permeabilized NRK cells. NRK cells at metaphase (upper panels) and anaphase (lower panels) were permeabilized in digitonin-containing buffer, incubated with soluble bacterial cell lysates containing the indicated His-tagged LAP2α mutant proteins (**A**) or GST-fusions (**B**) at comparable concentrations and processed for immunofluorescence microscopy using monoclonal antibodies (mAb) to human LAP2α or to GST for detection of recombinant proteins and propidium iodide for detection of DNA. Confocal double immunofluorescence images are shown. Bar, 10 μm.

but it might have been caused by a greatly decreased stability of mutants missing N-terminal sequences. This seems unlikely, however, as a mutant protein consisting of the N-terminal 85 amino acids fused to the nuclear targeting domain also failed to become stably expressed in cells (our unpublished data). Alternatively, these N-terminal deletion mutants might exhibit a dominant negative effect on the function of the endogenous protein. A leaky repression of the tetracycline-dependent expression system in the absence of tetracycline would cause cell growth arrest and eventually kill the cells if the function(s) of LAP2α were essential for cell cycle progression and/or viability. In line with this hypothesis, we also found a very low transfection efficiency of the constructs coding for these mutants in transient transfections.

A dominant negative effect of LAP2β truncation mutants, which lacked the transmembrane domain but contained the lamin-binding region, on cell cycle progression and nuclear growth has recently been demonstrated upon microinjection into mammalian cells (Yang *et al.*,

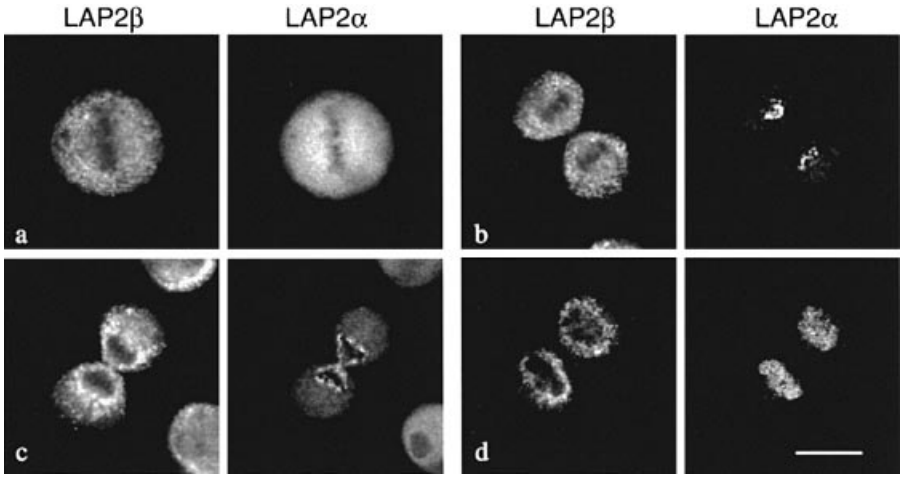


**Fig. 11.** Redistribution of endogenous LAP2α and endogenous LAP2β during different stages of mitosis. Mitotic HeLa cells were processed for immunofluorescence microscopy using monoclonal antibody to LAP2α and antiserum 1688 to LAP2β. Confocal double immunofluorescence images are shown. Bar, 10 μm.

1997b). Similarly, the addition of recombinant human LAP2β mutant proteins containing either the N-terminal 187-residue-long LAP2 common domain or the full nucleoplasmic domain of LAP2β to cell-free *X.laevis* nuclear assembly reactions negatively affected nuclear envelope assembly and nuclear growth (Gant *et al*., 1999).

### *Chromosome binding activity of the nuclear targeting domain versus the N-terminal potential chromatin interaction domain*

The N-terminal 85 amino acids in the first 187 residues of LAP2β, which are also present in LAP2α, have been reported to contain a chromatin interaction domain (Furukawa *et al*., 1998), and a region between amino acids 67 and 137 was recently shown by yeast two-hybrid assays to interact with the chromosomal protein BAF (Furukawa, 1999). Therefore, we reckoned that these domains might also be involved in the interaction of LAP2α with chromosomes. However, LAP2α mutants that contained these N-terminal potential chromosome binding domains, but lacked the nuclear targeting domain, were not found to associate with chromosomes after mitosis in stably transfected HeLa cells. The lack of chromosome binding of these mutants might be for different reasons. First, the number of interaction sites at the chromosomal surface at this stage of the cell cycle might be limited and mutant proteins might not be able to compete efficiently with endogenous protein for binding sites. Secondly, the simultaneous interaction of different binding domains in the molecule might be important for stable association of LAP2α with chromosomal structures. Thirdly, the interaction of the LAP2 common region with the chromosomal LAP2 binding protein BAF, which is not observed in metaphase cells (Furukawa, 1999), might be activated by post-translational modifications of either BAF or LAP2α at later stages of assembly. However, based on the observations that transiently expressed N-terminal fragments of LAP2β (Furukawa *et al*., 1995, 1997) and of LAP2α were not efficiently targeted into the nucleus even in interphase cells, it seems likely that both post-translational modifications and simultaneous interactions of several binding domains in the molecule account for stable integration of the proteins into the nuclear structure.

More surprisingly, His-tagged proteins containing one (LAP2 1–85) (Furukawa *et al*., 1998) or two (LAP2 1–187) (Furukawa, 1999) putative chromosome interaction domains in the LAP2 common domain, but that lacked the LAP2α-specific nuclear targeting domain, did not bind to chromosomes *in vitro* using two different assays. However, unlike the His-tagged LAP2α mutant 1–187, a GST-fusion protein containing LAP2α 1–187 was found to interact with chromosomes specifically. As GST-fusion proteins can dimerize through their GST domain, they might differ in their three-dimensional structure and conformation and exhibit different binding properties as compared with monomeric proteins. Unfortunately, it was impossible to test directly the chromosome binding properties of GST-fusions of the LAP2α-specific regions due to insolubility and aggregation of this construct under the conditions of the assay.

Although these data indicate that LAP2 proteins might have a common region that interacts with chromatin under certain circumstances, our data clearly demonstrate the existence of an alternative mechanism for chromosome association that is unique for LAP2α and is mediated by the LAP2α-specific nuclear targeting domain. Furthermore, our *in vitro* binding studies and transient and stable expression of various LAP2α mutants in mammalian cells showed that the LAP2α-specific region is essential for interaction of LAP2α with chromosomes at early stages of nuclear assembly. On the other hand, the chromosome interaction domain(s) in the N-terminal LAP2 common region are apparently dispensable for the chromosome binding of LAP2α at this cell cycle stage as ectopically expressed mutant proteins covering the LAP2 common domain failed to associate with chromosomes, and mutants lacking the common domain were still able to interact with chromosomes *in vitro*. Nevertheless, these domains might be important for tight association of all LAP2 proteins with chromatin during later stages of nuclear assembly or during interphase.

This has important implications for the potential functions of various LAP2 isoforms during nuclear assembly and suggests a functional diversity of LAP2 isoforms in their chromosome binding properties. Double immunofluorescence microscopy showed that there is indeed a

temporal difference in the association of LAP2α and LAP2β with mitotic chromosomes during nuclear assembly *in vivo*.

Furthermore, other LAP2 isoforms may also contain additional isoform-specific domains involved in chromosome interaction. In line with this notion, LAP2β-specific domains, distinct from the potential chromosome binding domain, have been implicated in DNA binding and nuclear targeting of the protein (Furukawa *et al*., 1997). Therefore, it seems likely that several LAP2 isoform-specific and LAP2 common domains may cooperate to target these proteins to chromosomes and nuclear structures efficiently. The identification of specific binding partners for the various LAP2 isoforms will be an important goal for the future to elucidate specific functions for the diverse molecular domains of LAP2 isoforms.

## Materials and methods

### Construction of expression plasmids encoding LAP2α deletion mutants

The Tet-On response plasmid pCM108, a derivative of pUHD10-3 (Gossen and Bujard, 1995; Gossen *et al*., 1995) that contains a Myc-tag cDNA downstream of the multicloning sequence, was kindly provided by C.Maercker. pTD6 was generated from pCM108 by deletion of the *Xho*I site through insertion of an oligonucleotide (5′-TCGATGGCCA-3′) into the *Xho*I site, and by introducing *Nde*I, *Nhe*I and *Xho*I restriction sites into the multicloning sequence through insertion of oligonucleotides (5′-GGCATATGGCTAGCGCTGAGCTCGAGGG-3′, 5′-AATTCCTCGAGCTCAGCGCTAGCCATATGCCGC-3′) via *Sac*II and *Eco*RI. pTD10 was generated by subcloning LAP2α cDNA (bp 1–561) from pET 23a-LAP2 1–187 (Dechat *et al*., 1998) into pTD6 via *Nde*I and *Xho*I. cDNA encoding full-length LAP2α (bp 1–2079; Harris *et al*., 1994) was subcloned from pET 17b-LAP2α (Dechat *et al*., 1998) into pTD6 via *Nhe*I and *Xho*I. The stop codon (TAG), 5′ of the Myc-tag sequence, was mutated into a leucine codon (TTG) by exchanging the *Bst*XI–*Xho*I fragment with the respective PCR product containing the mutation (primers: 5′-CACTAGGAGGTATTCAAGCAG-3′; 5′-GAATTCCTCGAGCaAGTGTTT-3′), giving rise to plasmid pTD15. pTD15 was used to generate response plasmids encoding various LAP2α deletion mutants (Figure 1). For construction of pSV2 and pSV4, LAP2α cDNA fragments covering bp 562–916 and 586–916, respectively, were amplified by PCR using oligonucleotides 5′-CCATCGATGCTAGCGGAAAGAAGAAAGAACACAAGAAAGTG-3′ and 5′-CCATCGATGCTAGCGTGAAGTCCACTAGGGATATTGTTCC-3′, respectively, as forward primers and 5′-CCATCGATTAGAGACCAACATGGCACTGTGTTC-3′ as the reverse primer. The primers were designed to code for external *Cla*I and *Nhe*I sites at the 5′ end. The PCR fragments were subcloned into pBluescript II KS(–) (DDBJ/EMBL/Genbank accession No. X52329) via *Cla*I, checked by sequencing, and cloned into pTD15 via *Nhe*I and *Eco*NI. To obtain pSV7 and pSV9, LAP2α cDNA was subcloned into the bacterial expression vector pET-23a(+) (Novagen, Madison, WI) via *Nhe*I–*Xho*I. The generated plasmid pSV5 was digested with *Nhe*I–*Sac*II and *Sac*II–*Xho*I, respectively, ligated to oligonucleotides (5′-CTAGCGCCGCCGC-3′,5′-GGCGGCG-3′) and (5′-GGGCCGGC-3′, 5′-TCGAGCCGGCCCGC-3′) (pSV6 and pSV8), and cloned into pTD15 via *Nhe*I and *Xho*I. pSV10 was generated by digestion of pTD15 with *Nhe*I and *Bcl*I and ligation to oligonucleotides 5′-CTAGCAGAATT-3′ and 5′-GATCAATTCTG-3′. For construction of pSV11, pSV12 and pSV13, pTD15 was digested with *Avr*II–*Xho*I, *Bcl*I–*Xho*I and *Bst*XI–*Xho*I, respectively, and ligated to oligonucleotides (5′-CTAGGAGCCTC-3′, 5′-TCGAGAGGCTCC-3′), (5′-GATCAGTCTC-3′, 5′-TCGAGAGACT-3′) and (5′-TTGGGC-3′, 5′-TCGAGCCCAAGCGC-3′), respectively. pSV13 was digested with *Nhe*I–*Eco*NI and ligated to oligonucleotides (5′-CTAGCATGGCCTCT-3′, 5′-CAGAGGCCATG-3′) to obtain pSV14. For bacterial expression of His-tagged proteins, truncated LAP2α cDNAs from pSV2, 4, 10, 11, 12, 13 and 14 were subcloned into pET23a(+) via *Nhe*I and *Xho*I.

For bacterial expression of GST-fusion proteins, oligonucleotides 5′-AATTCTCGAGCCCGCTAGC-3′, 5′GATCGCTAGCGGGCTCGAG-3′ containing *Nhe*I and *Xho*I sites were introduced into the multiple cloning site of pGEX-3X (Pharmacia Biotech, Vienna, Austria) via *Bam*HI and *Eco*RI to generate pTD56, coding for GST. pTD58 and pTD60 coding for fusions of GST and LAP2α 188–693 and LAP2 1–187, respectively, were generated by subcloning the respective cDNAs from pSV2 and pTD10 into pTD56 via *Nhe*I and *Xho*I.

### Cell culture and synchronization

HeLa, NRK and Chinese hamster ovary (CHO) cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose, 10% fetal calf serum, 20 mM HEPES pH 7.0, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Life Technologies, Paisley, UK) at 37°C in a humidified atmosphere with 5% $CO_2$. Media were supplemented with 100 μg/ml G418 (Life Technologies) for culture of HeLa Tet-On cells (Clontech Laboratories, Palo Alto, CA) and an additional 200 μg/ml hygromycin B (Boehringer Mannheim, Germany) for stably transfected HeLa Tet-On clones 15-1, 12-2, 11-21 and 9-43. To induce expression of exogenous proteins, 2 μg/ml doxycycline (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were added to the medium for at least 15 h. For synchronization of cell growth, cells were arrested in $G_1$/S phase by an overnight incubation in complete medium containing 2 mM thymidine (Sigma-Aldrich Chemie GmbH), followed by an 8 h release from the block in medium without thymidine. Weakly attached mitotic cells were collected by mechanical shake-off, incubated on adhesion slides (Bio-Rad, Hercules, CA) for 30–45 min at 37°C and processed for immunofluorescence microscopy. For *in vitro* nuclear assembly experiments, cells were incubated in medium containing 0.2 μg/ml nocodazole (Sigma-Aldrich Chemie GmbH) overnight and harvested by mechanical shake-off.

### Transfection

All transfections were performed according to the procedures of the manufacturer using lipofectamine reagent, Opti-MEM (Life Technologies) and DNA prepared with a Jet Star plasmid kit (Genomed, Bad Oeynhausen, Germany). In brief, for transient transfections HeLa Tet-On cells were seeded on Falcon 4 chamber tissue culture glass slides (Becton Dickinson, Franklin Lakes, NJ), grown overnight and incubated for 4 h in transfection mixture using 0.6 μg DNA per chamber. Cells were then incubated overnight in complete medium plus 2 μg/ml doxycycline and processed for immunofluorescence microscopy. For stable transfections $5 \times 10^5$ HeLa Tet-On cells were plated on 6-cm culture dishes, grown overnight and co-transfected with 0.4 μg pTK-Hyg selection vector (Clontech Laboratories) and 6 μg of response plasmid. After 4 h cells were grown in complete medium without hygromycin for 48 h, split into five 10-cm culture dishes and cultured in medium containing 200 μg/ml hygromycin B for 14 days. Single clones were grown in 24-well plates in the presence and absence of doxycycline, and expression of mutant proteins was analyzed by immunoblotting and immunofluorescence microscopy.

### Subcellular fractionation and in vitro assembly

Stably transfected HeLa cell clones 15-1, 12-2, 11-21 and 9-43 were incubated in ~5 vols of ice-cold hypotonic buffer [10 mM HEPES pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)] containing 20 μM cytochalasin B and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, 10 μg/ml aprotinin, leupeptin and pepstatin (all from Sigma-Aldrich Chemie GmbH)] for 10 min and lysed in a glass–glass homogenizer. After addition of 8% sucrose, the soluble cytoplasmic and the insoluble nuclear fractions were separated by centrifugation at 2000 *g* for 15 min at 4°C. The nuclei-containing pellets were extracted in the same buffer with 50 or 200 mM NaCl either with or without 1% Triton X-100 and centrifuged at 2000 *g* for 10 min to obtain low-speed pellet and at 100 000 *g* for 30 min to yield high-speed pellet and supernatant fractions. For *in vitro* nuclear assembly, mitotic 12-2 cells were homogenized in KHM buffer as described previously (Burke and Gerace, 1986) supplemented with 20 μM cytochalasin B and incubated at 30°C. Phosphatase inhibitors (0.1 μM calyculin A and okadaic acid; Life Technologies), kinase inhibitors (1 μM staurosporin; Sigma Chemical Co., St Louis, MO; and 80 μM olomoucine; Calbiochem-Novabiochem GmbH, Nottingham, UK) and 0.5% Triton X-100 were added and samples were centrifuged at 2000 *g* for 10 min and at 100 000 *g* for 30 min to yield low-speed pellet, high-speed pellet and supernatant fractions.

### Isolation of metaphase chromosomes

Nocodazole-arrested CHO cells were incubated in medium containing 20 μM cytochalasin B and 0.2 μM nocodazole for 30 min, pre-swollen in 5 mM HEPES pH 7.4, 5 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and protease inhibitors for 10 min on ice and homogenized

in chromosome buffer (10 mM HEPES pH 7.4, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.1% Triton X-100 and protease inhibitors) in a glass–glass homogenizer. The cell lysates were layered on top of a discontinuous sucrose gradient (3 ml of 20, 30, 40, 50 and 60% sucrose in chromosome buffer), centrifuged at 500 *g* for 10 min at 4°C, and chromosomes enriched in a diffuse band in the middle of the gradient were collected, frozen in liquid nitrogen and stored at –80°C.

### *Expression and isolation of recombinant LAP2α proteins*

His-tagged recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) using the inducible T7 RNA polymerase-dependent pET vector system (Novagen). Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2–4 h. Bacteria were frozen in 1/10 of the original culture volume of Tris buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl, 2 mM EGTA, 1 mM DTT, protease inhibitors), thawed on ice, lysed by addition of 0.1 mg/ml lysozyme, 0.1% Triton X-100, 10 mM $MgCl_2$, 50 µg/ml DNase and 20 µg/ml RNase, and incubated for 30 min at 30°C. Following the addition of 7 M urea and homogenization in a glass–glass homogenizer, cell lysates were spun at 100 000 *g* for 30 min at 4°C, and supernatants were frozen at –20°C.

To prepare extracts of GST-fusion proteins, IPTG-induced bacteria containing the respective pGEX-derived plasmids were frozen in 1/10 of the original culture volume of binding buffer [20 mM HEPES pH 7.4, 115 mM $Na(CH_3COO)$, 2 mM $Mg(CH_3COO)_2$, 0.5 mM EGTA, 1 mM DTT and 1 mM PMSF], thawed on ice, sonicated five times for 20 s, and cleared by centrifugation at 16 000 *g* for 30 min.

### *Chromosome binding assays*

For co-sedimentation analyses, bacterial cell lysates containing recombinant His-tagged proteins were dialyzed in 20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.2% Triton X-100, 1 mM DTT, 1 mM PMSF, and 25 µl of these fractions were mixed either with 50 µl of chromosomes or with 50 µl of chromosome buffer, incubated for 30 min at 30°C and centrifuged through 75 µl of a 35% sucrose cushion in chromosome buffer at 3000 *g* for 10 min. Pellets were analyzed by SDS–PAGE and immunoblotting. For microscopic analyses the mixture was applied to adhesion slides, and chromosomes were allowed to sediment for 30 min before they were processed for immunofluorescence microscopy.

For the *in situ* chromosome binding assay using permeabilized cells, mitotic NRK cells enriched by a 7 h release from a thymidine block were permeabilized in binding buffer containing 10 µg/ml digitonin and washed in binding buffer containing 2% bovine serum albumin and 0.02% Triton X-100. Soluble bacterial cell lysates in binding buffer or dialyzed against the same buffer were applied to the permeabilized cells on coverslips for 20 min at room temperature. Cells were immediately fixed with 3.7% formaldehyde and processed for immunofluorescence microscopy.

### *Immunofluorescence microscopy*

Cells grown on Falcon 4 chamber tissue culture glass slides or mitotic cells or chromosomes attached to adhesion slides were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, incubated with 50 mM $NH_4Cl$ in PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 min each. After incubation in PBS/0.2% gelatin for 30 min, primary and secondary antibodies were applied in the same buffer for 1 h each. Primary antibodies used were undiluted hybridoma supernatants of antibodies to LAP2α (Dechat *et al*., 1998) and monoclonal Myc 1-9E10.2 antibody (American Type Culture Collection CRL-1729, Rockville, MD), or affinity-purified polyclonal Myc antibody diluted 1:50 (a generous gift from M.Toegel and F.Propst), or antiserum 1688 to LAP2β diluted 1:100 (kindly provided by Larry Gerace), or monoclonal antibody to GST (Serotec, UK) diluted 1:10. Secondary antibodies used were affinity-purified goat anti-mouse IgG and goat anti-rabbit IgG conjugated to Bodipy FL (Molecular Probes, Leiden, The Netherlands) and goat anti-mouse IgG conjugated to Texas Red (Jackson Immuno-Research, West Grove, PA). RNA was digested with 1 mg/ml DNase-free RNase for 20 min and DNA was stained with 0.1 µg/ml propidium iodide (Sigma Aldrich Chemie, GmbH) in PBS for 10 min. Samples were mounted in Mowiol and viewed in a Zeiss Axiophot microscope and a MRC-600 confocal microscope (Bio-Rad).

### *Other methods*

SDS–PAGE was performed according to Laemmli (1970). For immunoblotting, proteins were electrophoretically transferred onto nitrocellulose (0.2 µm; Schleicher and Schuell, Dassel, Germany) in 48 mM Tris–HCl pH 9.4, 39 mM glycine using the Mini Transblot system (Bio-Rad). Proteins were immunologically detected with undiluted hybridoma supernatants of anti-LAP2 antibodies (Dechat *et al*., 1998), monoclonal Myc 1-9E10.2 antibody, and monoclonal antibodies to lamin B (Ab-1; Calbiochem) and to lamins A/C (Loewinger and McKeon, 1988), using the Protoblot Immunoscreening System (Promega, Madison, WI).

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