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NUCLEAR MATRIX IN APOPTOTIC CELL DEATH AND CELL PROLIFERATION

The role of Nuclear Mitotic Apparatus (NuMA) Protein

by

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To my family

ABSTRACT

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Annales Universitatis Turkuensis, Painosalama Oy, 2011

The nucleus is a membrane enclosed organelle containing most of the genetic information of the cell in the form of chromatin. The nucleus, which can be divided into many sub-organelles such as the nucleoli, the Cajal bodies and the nuclear lamina, is the site for several essential cellular functions such as the DNA replication and its regulation and most of the RNA synthesis and processing. The nucleus is often affected in disease: the size and the shape of the nucleus, the chromatin distribution and the size of the nucleoli have remained the basis for the grading of several cancers.

The maintenance of the vertebrate body shape depends on the skeleton. Similarly, in a smaller context, the shape of the cell and the nucleus are mainly regulated by the cytoskeletal and nucleoskeletal elements. The nuclear matrix, which by definition is a detergent, DNase and salt resistant proteinaceous nuclear structure, has been suggested to form the nucleoskeleton responsible for the nuclear integrity. Nuclear mitotic apparatus protein, NuMA, a component of the nuclear matrix, is better known for its mitotic spindle organizing function. NuMA is one of the nuclear matrix proteins suggested to participate in the maintenance of the nuclear integrity during interphase but its interphase function has not been solved to date.

This thesis study concentrated on the role of NuMA and the nuclear matrix as structural and functional components of the interphase nucleus. The first two studies clarified the essential role of caspase-3 in the disintegration of the nuclear structures during apoptosis. The second study also showed NuMA and chromatin to co-elute from cells in significant amounts and the apoptotic cleavage of NuMA was clarified to have an important role in the dissociation of NuMA from the chromatin. The third study concentrated on the interphase function of NuMA showing NuMA depletion to result in cell cycle arrest and the cytoplasmic relocalization of NuMA interaction partner GAS41. We suggest that the relocalization of the transcription factor GAS41 may mediate the cell cycle arrest. Thus, this study has given new aspects in the interactions of NuMA, chromatin and the nuclear matrix.

Keywords: NuMA, nuclear matrix, interphase nucleus, apoptosis, caspase-3

TIIVISTELMÄ

Katri Kivinen

TUMAN PERUSAINE OHJELMOIDUN SOLUKUOLEMAN JA SOLUSYKLIN AIKANA - NuMA-proteiinin merkitys

Kliinis-teoreettinen laitos, Patologian oppiaine, MediCity-tutkimuslaboratorio ja Turun biolääketieteen tutkijakoulu (TuBS), Turun yliopisto, Turku
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Tuma on tärkeä monille solun toiminnoille: esimerkiksi solujakautumiselle välttämätön perintöaineksen kahdentuminen ja monet sen säätelymekanismit sekä proteiinisynteesin välivaiheet, RNA-synteesi ja -muokkaus, tapahtuvat tumassa. Tuma jakautuu erilaisiin toiminnallisiin alueisiin kuten tumajyväsiin, tumalevyyn ja kierteiskappaleisiin, joiden lisäksi tumassa sijaitsee pääosa solun toimintaa säätteleviä geenejä sisältävästä perintöaineksesta eli DNA:sta. Monissa sairauksissa havaitaan muutoksia tuman rakenteessa: syöpäsoluissa nähdään usein sekä tumakoon ja -muodon vaihtelua että tumajyväsrakenteiden ja perintöaineksen paikantumisen muutoksia. Nämä muutokset ovat tärkeitä arvioitaessa kasvaimen pahanlaatuisuuden astetta.

Soluissa ja solujen tumissa on osoitettu olevan erilaisia solun muodon ja rakenteen ylläpitoon osallistuvia proteiineja, jotka muodostavat niiden tukirangan. Tuman tukirangaksi on ehdotettu tuman perusainetta (nuclear matrix), joka määritelmän mukaan muodostuu detergentti-, DNaasi- ja suolakäsittelyn jälkeen liukenemattomasta proteiiniverkosta. NuMA (Nuclear Mitotic Apparatus) on tuman perusaineen proteiini, jonka tiedetään osallistuvan solujakautumisen aikana tumasukkulan rakentumiseen. NuMA-proteiinin on ehdotettu osallistuvan interfaasituman rakenteen ylläpitoon, mutta sen tehtävistä interfaasitumassa tiedetään vain vähän

Tässä väitöskirjassa selvitettiin NuMA-proteiinin ja tumanperusaineen merkitystä tuman rakenteen ja toiminnan ylläpitäjänä. Ensimmäisessä ja toisessa osatyössä osoitettiin kaspaasi-3:n olevan tärkeä sekä perintöaineksen että tuman perusaineen pilkkoutumiselle. Toisessa osatyössä todettiin NuMA-proteiinin sitoutuvan tuman perintöainekseen ja osoitettiin tämän vuorovaikutuksen häiriintyvän NuMA-proteiinin pilkkoutumisen myötä. Kolmannessa osatyössä osoitettiin siRNA-menetelmää käyttäen, että NuMA-proteiinin poistaminen tumasta aiheuttaa solusyklin pysähtymisen $G_{1/0}$-vaiheeseen. Tämän solusyklin pysähtymisen ehdotetaan tapahtuvan NuMA-proteiinin kanssa vuorovaikuttavan, geeniluentaa säätelevän GAS41-proteiinin kautta: NuMA-proteiinin poisto aiheutti GAS41-proteiinin siirtymisen tumasta solulimaan. Näin ollen väitöskirjatutkimus on antanut lisätietoa NuMA-proteiinin, perintöaineksen ja tumaperusaineen välisistä vuorovaikutuksista.

Avainsanat: NuMA-proteiini, tumaperusaine, apoptoosi, interfaasituma, kaspaasi-3

TABLE OF CONTENTS

ABBREVIATIONS

APL	Acute promyelocytic leukemia
Apaf-1	Apoptotic protease activating factor-1
ATCC	American type cell culture
APC	Anaphase promoting complex
Arp1α	Actin related protein 1α
BrdU	Bromo-deoxy-uridine
BSA	Bovine serum albumine
(k)Da	(kilo)Dalton
cDNA	Complementary DNA
Ced	CEll Death abnormality
DD	Death domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDMD	Emery-Dreyfuss muscular dystrophy
Emi1	Early mitotic inhibitor 1
END	Emi1-NuMA-Dynein complex
ER	Endoplasmic reticulum
FCS	Fetal calf serum
GAS41	Glioma amplified sequence 41
GFP	Green fluorescent protein
DNA	Deoxyribonucleic acid
FLICE	FADD-like ICE
H4K20m	Histone-4 methylated at lysine 20
HGPS	Hutchinson–Gilford progeria syndrome
hnRNP	Heterogeneous nuclear ribonucleoprotein
hnRNA	Heterogeneous nuclear RNA
IAP	Inhibitor of apoptosis
ICE	Interleukin converting enzyme
IF	Immunofluorescence
INM	Inner nuclear membrane
IP	Immunoprecipitation
LAP2α	Lamin associated polypeptide 2α
LGN	Leucine-Glycine-Asparagine
MAR	Matrix attachment region
MEF	Mouse embryonal fibroblast
MT	Microtubulus/es
NLS	Nuclear localization signal
NM	Nuclear matrix
NPC	Nuclear pore complex
NuMA	Nuclear Mitotic Apparatus protein

ONM	Outer nuclear membrane
p53ser15	p53 phosphorylated at serine 15
PAGE	Polyacrylamide gel electrophoresis
PARP-1	Poly(ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PCD	Programmed cell death
PKC	Protein kinase C
r/mRNA	Ribosomal/messenger RNA
RARα	Retinoic acid receptor α
RCC1	Regulation of chromosome condensation 1
ROD	Relative optical density
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SDS	Sodium Dodecyl Sulfate
SMC	Structural maintenance of chromosomes
siRNA	Small interfering RNA
snRNP	Small nuclear RNP
SPN	Spindle pole nucleus
SUN	Sad1 and UNC84 domain containing
STS	Staurosporine
TBS	Tris buffered saline
TNF(R)	Tumor necrosis factor (receptor)
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labeling
WB	Western blotting
z-DEVD-fmk	Bentsoylcarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone
z-VAD-fmk	Bentsoylcarbonyl-Val-Ala-Asp-fluoromethylketone

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, which are referred to in the text by the corresponding Roman numerals, I – III. In addition, some unpublished results are included.

I Kivinen K, Kallajoki M, Taimen P. (2005) Caspase-3 is required in the apoptotic disintegration of the nuclear matrix. *Exp Cell Res* 311(1):62-73

II Kivinen K, Taimen P, Kallajoki M. (2010). Silencing of Nuclear Mitotic Apparatus protein (NuMA) accelerates the apoptotic disintegration of the nucleus. *Apoptosis* 15(8): 936-45

III Kivinen K, Taimen P, Kallio M, Kallajoki M. Silencing of the nuclear mitotic apparatus protein (NuMA) results in proliferative arrest. *Submitted*.

1 INTRODUCTION

The skeleton forms the basis for the maintenance of the shape of the body of a vertebrate. Similarly, in a smaller context, the shape of the cell is mainly regulated by the cytoskeletal elements such as actin filaments, intermediate filaments or microtubules, and the nucleus of a cell also has structural elements involved in the maintenance of the nuclear structure. The best known structure to contribute to the nuclear integrity is the nuclear lamina but the inner non-chromatin, proteinaceous nuclear matrix has also been suggested to have a role in the maintenance of the nuclear structure. The nucleus has many roles in the cellular function. It harbors most of the genetic information enclosed in the chromatin and many important functions such as steroid hormone action, DNA replication, RNA transcription and RNA processing take place at specialized domains of the nucleus. During the cell division, the nucleus is degraded and the substructures and chromatin are divided to the reforming daughter nuclei.

Nuclear mitotic apparatus protein, NuMA, a component of the inner nuclear matrix, is better known for its mitotic function. During the early metaphase NuMA is sequestered to surround the spindle poles of the mitotic spindle where it functions in the maintenance of the spindle integrity. During early telophase NuMA is sequestered to the reforming nuclei where it resides during interphase. The role of NuMA during interphase is much debated. It has been suggested to function as a component of the inner nuclear matrix supporting the nuclear structure but it has also been linked to the differentiation process. The data on the interphase function of NuMA is controversial: some studies have shown that NuMA is essential for the maintenance of functional nuclei while others show that many highly differentiated cells lack NuMA and that functional nuclei may form in the absence of NuMA.

Apoptosis, a form of programmed cell death, constitutes a distinct set of morphological and biochemical changes in a cell. These include chromatin cleavage and condensation, shrinking of the cell, membrane blebbing and, finally, the formation of membrane-enclosed vesicles termed apoptotic bodies. The nuclear structure along with many functional domains of the cell is degraded during apoptosis and NuMA is a major target of apoptotic degradation: its cleavage has been suggested to be indispensable to the nuclear disintegration and precede the chromatin cleavage.

This study concentrates to study the interphase nuclear function of NuMA. Both the potential chromatin binding properties of the protein and the prospective structural role at the inner nuclear matrix are addressed. The study suggests that NuMA preferentially serves as a link between the chromatin structure and nuclear matrix but also suggests a role for NuMA in the cell cycle progression.

2 REVIEW OF THE LITERATURE

2.1 The nuclear structure

Most of the genetic information enclosed in an eukaryotic cell lies within the nucleus, a compartment of the cell comprising about 10% of the total cell volume (Alberts *et al.*, 2008). Nucleus is a cellular compartment enclosed by a double-membrane nuclear envelope. The interior of the nucleus can be divided into many functionally specialized domains which lack bounding membranes but can still be demarcated by a distinct protein profile they contain. The core structures of the nucleus described below are illustrated in a schematic drawing of a cell (Fig. 1A). Nucleus is often affected in different disease states such as tumor formation (Fig. 1B) and the diagnostic grading of a malignant tumor still often depends on the microscopic assessment of the tumor cell nuclei: although the knowledge encompassing the molecular changes behind cancer formation is growing, the nuclear shape and size, nucleolar number and size and chromatin contour remain the basis of grading in several cancers (Reviewed by Zink *et al.*, 2004).

The nuclear envelope is perforated by large multiprotein nuclear pore complexes (NPC) enabling the transport of large molecules in and out of the nucleus. The inner nuclear membrane (INM) is lined by the nuclear lamina, a filamentous protein layer consisting of the lamins, and the outer nuclear membrane (ONM) is continuous with the endoplasmic reticulum which is evidenced by the large number of ribosomes attached to it. The two membranes are continuous with each other at each NPC and the inner nuclear membrane differs from the outer membrane by a different composition of transmembrane proteins attached to it. Electron microscopy of the nuclear interior has revealed the granular nature of the interchromatin domains inside nuclei: nucleoli, Cajal Bodies (coiled bodies) and nuclear speckles are composed of electron-dense particles with diameters ranging from 25-50 nm. Cajal Bodies are present both free in the nucleoplasm and physically associated with the histones and small nuclear ribonucleoprotein (snRNP) loci associating mainly with nuclear RNA processing (Reviewed in Handwerger and Gall, 2006). Nuclear speckles are loci rich in splicing associated proteins such as splicing snRNPs, composition and localization of which responds to the changes in the transcriptional activity of the cell (Reviewed in Handwerger and Gall, 2006).

A typical nucleus encompasses 1-5 nucleoli which are the largest distinct structures inside the nucleus. The nucleolus is the site for ribosomal RNA (rRNA) transcription, pre-rRNA processing and the ribosome subunit assembly (Olson *et al.*, 2002). The chromosomes 13, 14 and 15 contain the ribosomal-DNA clusters (Henderson *et al.*, 1972). At telophase, the nucleolus assembles around these chromosomal areas containing the ribosomal gene repeats and disassembles again at the onset of mitosis. Nucleolus is not membrane-enclosed but its dense structure has enabled their isolation:

the mass-spectrometric analysis of isolated nucleoli has revealed over 4500 nucleolar proteins with differing functions which has given new insights into the nucleolar function (see nucleolar protein database at http://www.lamondlab.com/NOPdb3.0/). Most of the nucleolar proteome is involved in the ribosomal functions but the nucleolus also contains proteins involved in the cell cycle regulation, the DNA damage repair and the pre-mRNA processing (Lam *et al.*, 2005). An interesting finding is the cell-cycle dependent nucleolar association of telomerase: telomerase remains sequestered in the nucleoli until the early S phase of the cell cycle when its release enables the telomere replication and the entry into mitosis. This cell cycle dependent shuttling of the telomerase in and out of the nucleolus is lost in tumor and primarily transformed cells, and after the malignant transformation induced by the transfection of the simian virus 40 genome to primary cells (Wong *et al.*, 2002).

The chromatin inside the nucleus consists of chromosomes which are distributed in an orderly fashion. The classical differentiation can be made between transcriptionally active euchromatin in the nuclear interior and transcriptionally inactive heterochromatin localizing to the periphery of the nucleus and to the perinucleolar area. The chromosomes differ from each other not only by size but also in gene content. In an interphase nucleus, the chromosome localization varies in a radial manner depending on the gene content and the gene poor chromatin seem to localize to the peripheral nucleus at the heterochromatin areas (Reviewed by Zink *et al.*, 2004).

2.2. Nuclear matrix

2.2.1. Definition and conflict

The nucleus has been suggested to have a distinct nucleoskeleton similar to the cytoplasmic structures: the main element of this suggested structure is thought to be the nuclear matrix (henceforth NM), the proteinaceous, non-chromatin karyoskeleton of the nucleus, considered analogous to the cytoskeleton. The main components of the NM are the nuclear lamina and the interior fibrous network of ribonucleoproteins (RNPs); these structures have been implicated in numerous nuclear functions (Nickerson *et al.*, 1995). The protein composition of the NM is complex and may include almost 400 different proteins (Mika and Rost, 2005).

Russian scientists Zbarskii and Debov (1948) were the first to argue the existence of a NM protein fraction: by using high-salt solutions they were able to extract what they called the “residual nuclear protein fraction” (Reviewed by Pederson, 2000). Similar observations of a protein structure independent of the well-known DNA/histone-based chromatin were made later (Smetana *et al.*, 1963) but it was not until 1966 when Fawcett defined NM as the non-chromatin structures of the nucleus readily observed in unextracted cells under the electron microscope (Reviewed by Nickerson, 2001). Later Berezney and Coffey published the classical NM preparation method that justified the

use of the term nuclear matrix: by their definition, NM is the insoluble filamentous residue of the nucleus which remains after the treatment of the nuclei with a non-ionic detergent, DNase I and a high concentration of salt (Berezney and Coffey, 1974). Microscopically, NM is the fibrous network of protein filling the nucleus and the nuclear lamina underlying the nuclear membrane. Several different substructures have been identified in the NM: RNA transcription sites, DNA replication sites, speckled domains enriched in RNA splicing factors, Cajal bodies, chromosomal territories and interchromatin granule clusters can be identified as distinct features of the NM (Nickerson, 2001).

The harsh extraction method, difficulties in the *in vivo* imaging of the NM and controversial results have led to the questioning the existence of the whole proposed NM structure (Reviewed by Pederson, 2000). The proposed branching filament structure is inconsistent with the interchromatin space visualized in living cells: the interchromatin space seems to be a continuous dynamic space bounded by the changing chromatin contours (Politz *et al.*, 1999) and it hardly seems possible that this space would accommodate NM network consisting of straight filament structures described by Fey et al. (1986). Second, the ribonucleoprotein suggested to form the NM network (Fey *et al.*, 1986) was not supported by the electron-microscopy based studies which argued that the nuclear karyoskeleton was not a ribonucleoprotein network but rather structurally related to the cytoplasmic filament structures such as the intermediate-filaments (Hendzel *et al.*, 1999). Third, the main function of the NM has been suggested to be the organization the chromatin. Therefore, it seems reasonable to disfavor the NM structure characterization method based on procedures utilizing chromatin elution prior to analysis. At least certain RNPs tend to form filament structures when dislodged from their RNA binding site (Lothstein *et al.*, 1985). Similarly, a nuclear human immunodeficiency virus protein Rev, which normally binds to the viral pre-mRNA transcripts, undergoes spontaneous filament formation when released from its normal binding site (Heaphy *et al.*, 1991).

Although a large amount of criticism is directed against the theoretical structure consisting of protein and/or RNA, there is evidence that chromatin (Zink *et al.*, 1998) and non-chromatin structures such as nuclear speckles (Misteli *et al.*, 1997) are restrained from substantial Brownian motion, strongly supporting the view that there is a component or components of the cell nucleus that function to restrict the mobility of the macromolecular complexes. Newer techniques have been developed to address the criticism pointed against the use of the high salt concentration, isolation of the nuclei and the chromatin removal and these NM preparates contain nearly similar protein content as the one obtained by the classical method (Reviewed by Martelli *et al.*, 2002).

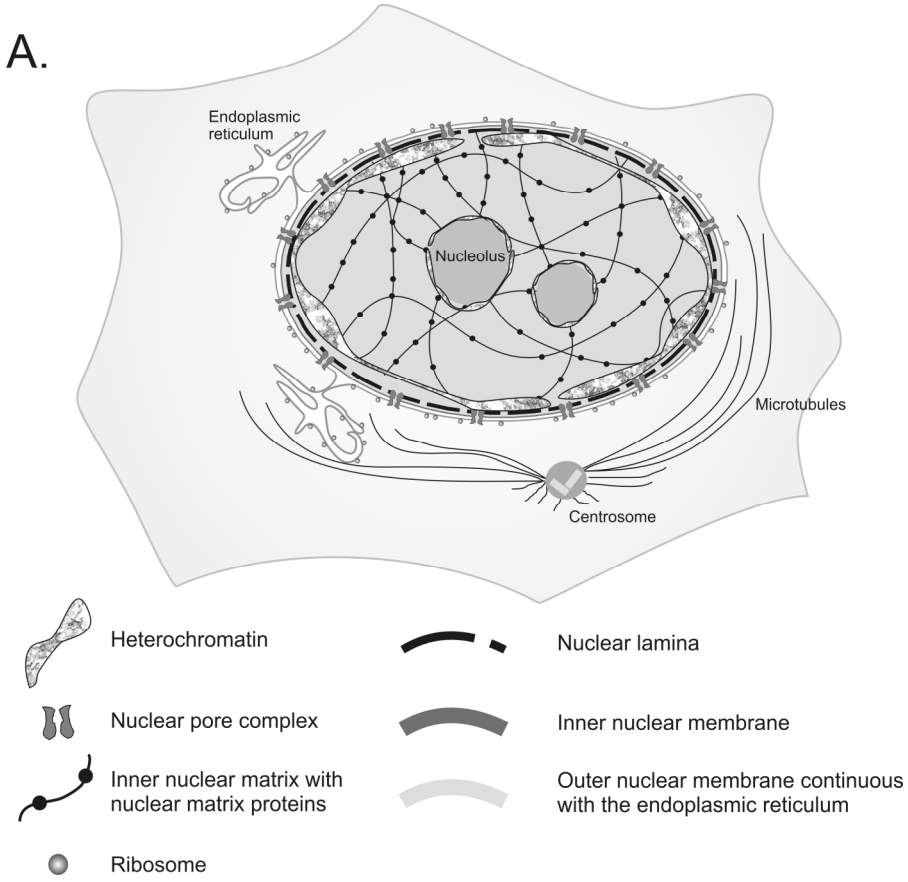

A.
Endoplasmic reticulum
Nucleolus
Microtubules
Centrosome
Heterochromatin
Nuclear pore complex
Inner nuclear matrix with nuclear matrix proteins
Ribosome
Nuclear lamina
Inner nuclear membrane
Outer nuclear membrane continuous with the endoplasmic reticulum
B.
I.
II.
III.
IV.

Figure 1. The nuclear structure. *A) The cytoplasm of a eukaryotic cell contains various complex structures (organelles, protrusions of the endoplasmic reticulum and cytoskeleton formed of different types of filaments, e.g. microtubules) and the nucleus comprising approximately 10% of the total cellular volume. The nucleus is surrounded by a double-membrane nuclear envelope. The outer nuclear membrane is continuous with the endoplasmic reticulum. Inside the inner nuclear membrane lies the nuclear lamina with various functions including the maintenance of the nuclear shape. The nuclear envelope is permeated by large nuclear pore complexes enabling the transport of substances in and out of the nucleus. Inside the nucleus lies the nuclear matrix: a proposed nucleoskeleton analogous to the cytoskeleton of the cytoplasm. Several substructures inside the nucleus, e.g. nucleoli, have been described. B) The nuclear size, shape and structure are affected in different tumors. I. The nuclei of a benign renal oncocytoma are small and round with small nucleoli. II. Cultured breast adenocarcinoma MCF-7 cells have variance in the nuclear shape and sixe. The nucleoli are abundant and large in size. III. Ovarian clear cell carcinoma shows marked variation in the nuclear size. IV. High grade osteosarcoma shows marked variation in the nuclear shape and size.*

2.2.2. The composition of the nuclear matrix

Two structural elements need to be distinguished when discussing the NM: the nuclear lamina underlying the inner nuclear membrane and the inner NM composed of a fibrous network of ribonucleoproteins (RNPs) filling the nucleus. These structures have been implicated in numerous nuclear functions, such as the chromatin compaction and organization, DNA replication, transcription and steroid hormone action (Reviewed by Nickerson *et al.*, 1995). The substructures inside the nucleus are generally considered a dynamic entity and the nuclear lamina is the only rigid nuclear structure which existence has been generally accepted. The protein content of nuclear matrices from different organisms have been mainly characterized by two-dimensional electrophoresis (Mika and Rost, 2005): a method separating proteins not only by their isoelectric points but also by their molecular weight similarly to the traditional SDS-PAGE (O'Farrell, 1975). The protein composition of the nuclear matrices analyzed by the two-dimensional gel electrophoresis is complex and may include almost 400 different proteins (Mika and Rost, 2005). This retrospective study used PubMed searches to filter papers concerning nuclear matrix/scaffold: over 3000 papers were identified and these papers described nearly 400 different proteins. While more than 50% of these are associated with the inner NM, approximately 130 are cell-type-specific or are related to a certain stage of the cell cycle and some are linked to a specific disease state such as some viral proteins produced by the viral genome. The inner NM is an entity of proteins, mainly formed by the lamins (Barboro *et al.*, 2002), NuMA (Lydersen and Pettijohn, 1980; Kallajoki *et al.*, 1991) and proteins bound to unprocessed pre-mRNA transcripts of the nucleus termed hnRNP (heterogeneous nuclear ribonucleoproteins, Barboro *et al.*, 2002).

As Smetana had discovered in 1963, there was a nuclear network of RNA packed in proteins. This network is only detached from the nuclear remnant with harsh chemical or mechanical sheering (Smetana *et al.*, 1963). Only RNase but not DNase I treatment is sufficient to remove hnRNPs from the nucleus (Herman *et al.*, 1978). The release of hnRNPs results in the collapse of the chromatin and matrix fibers, suggesting the RNPs of the nucleus either to form a structure or alternatively to participate in the maintenance of another non-chromatin structure (Nickerson *et al.*, 1989). The ultrastructure of the inner NM is built on the core filaments with approximately 10 nm filament width and 23 nm axial repeats (Jackson and Cook, 1988; He *et al.*, 1990). The composition of these 10-nm core filaments has been the central problem in the NM research. Several different proteins such as lamins (Hozak *et al.*, 1995), NuMA (He *et al.*, 1995), structural protein 4.1 (Krauss *et al.*, 1997) and hnRNPs A2 and/or B1 (Tan *et al.*, 2000) have been suggested to form this structural base for the nuclear filaments.

2.2.3. The function of the nuclear matrix

The first insights into the NM function suggested the NM to form a nucleoskeletal structure analogous to the filamentous cytoskeleton of the cytoplasm (Berezney and Coffey, 1974, 1977). The nuclear lamina and its components participate in the maintenance of the nuclear shape (Reviewed by Stuurman *et al.*, 1998) and the integral proteins of the nuclear envelope establish a connection between the nucleus and the cytoskeletal structures which are thought to serve as a link for the signaling between nucleus and the cytoplasm (Stewart *et al.*, 2007). The role of the inner NM in the structural maintenance of the nucleus remains unclear to date but it is well known that the NM serves as an anchorage site for the chromatin. Genes contain repeating AT-rich 200 bp DNA sequences termed matrix attachment regions or scaffold attachment regions (MARs/SARs) which bind specific NM proteins, e.g. lamins and NuMA (Mirkovitch *et al.*, 1984; Luderus *et al.*, 1992; Luderus *et al.*, 1994). This results in the formation of DNA loop domains ranging in size from 5 to 200 kbp (Davie, 1995). The MAR sequences seem to be highly conserved as animal MARs have been shown bind plant NM and vice versa (Reviewed by Wang *et al.*, 2010). The function of the MAR sequences in DNA organization has been clarified in several papers. The bases of the DNA loop domains form the places where RNA and DNA come into close contact (Hozak *et al.*, 1993) and, therefore, are the suggested place for DNA replication, gene expression and regulation (Nickerson, 2001). The transcription is considered to occur at sites of DNA/NM interaction (Jackson and Cook, 1985) and although the binding of MAR usually enhances transcription, also repression of transcription has been described (Reviewed by Boulikas, 1995). Similarly to replication and transcription, hormone receptor binding (Simmen *et al.*, 1984; Stenoien *et al.*, 2001; Matsuda *et al.*, 2008) and hnRNA processing (Nickerson *et al.*, 1997) seem to take place at certain areas of the NM suggesting NM to act as a dynamic, coordinated base for a wide-range of functions of the nucleus.

2.3. Nuclear Mitotic Apparatus protein (NuMA)

A NM protein, Nuclear mitotic apparatus protein or NuMA, was first discovered by Lydersen and Pettijohn in 1980. They first analyzed non-histone chromosomal proteins from several human and hamster cell lines as well as human-hamster cell hybrids by gel electrophoresis and were able to find a predominantly nuclear ~ 300 kDa protein expressed by the studied human cell lines and one hybrid cell line (Lydersen *et al.*, 1980). Further studies revealed this protein to localize to the nucleus as a part of the NM during interphase and to the polar regions of the mitotic spindle during mitosis: hence the protein was named NuMA according to its mitotic localization (Lydersen and Pettijohn, 1980). Subsequently, several studies were to find a similar molecular weight protein with similar localization behavior: spindle pole nucleus (SPN)-antigen (Kallajoki *et al.*, 1991), SP-H (Maekawa *et al.*, 1991), centrophilin (Tousson *et al.*, 1991), 1F1/H1 (Compton *et al.*, 1991). These antigens were further proven to be one single protein NuMA (Compton *et al.*, 1992; Kallajoki *et al.*, 1993; Maekawa and Kuriyama, 1993).

2.3.1. From *NUMA1* gene to the NuMA protein

Two simultaneously published studies (Compton *et al.*, 1992; Yang *et al.*, 1992) used the cDNA expression libraries to solve the entire cDNA sequence and the primary structure of NuMA. The cDNA sequence published by Compton et al. contained a single open reading frame encoding for a 2101 amino acid polypeptide predicted to have a molecular mass of 236,278 Da. The cDNA sequence by Yang et al. contained a single open reading frame encoding for a 2115 amino acid polypeptide with a predicted molecular mass of 230,085 Da. An identical sequence was later analyzed by Maekawa and Kuriyama (1993): they suggested that the major difference between residues 1268 and 1299 is the result of a frame-shift and that the sequence lacking 1536-1549 in the sequence by Compton et al. may be due to alternative splicing. The human mRNA encoding the full-length protein is reported to be accompanied by two isoform of sizes 1763 and 1776 amino acids (p194 and p195 isoforms), respectively (Tang *et al.*, 1993). These isoforms had previously been suggested to be the result of proteolytic activity (Price and Pettijohn, 1986).

The decoded cDNA sequence was the key to localize the gene behind the protein. Sparks et al. (1993) used 2 kb cDNA clones for *in situ* hybridization and chromosome spread staining to detect a single copy, single locus gene *NUMA1* in the chromosome locus 11q3. *NUMA1* is a conserved gene in human, cow, chicken, domestic mouse, zebrafish and chimpanzee (HomoloGene, http://www.ncbi.nlm.nih.gov/homologene/). Genes encoding for NuMA protein have also been sequenced in dog, rabbit, horse, African clawed frog (*xenopus laevis*), rhesus monkey, platypus, grey short-tailed opossum, wild boar and Sumatran orangutan (Gene, http://www.ncbi.nlm.nih.gov/gene).

2.3.2. The structure of NuMA

The secondary structure prediction suggested NuMA to have a very large ~1500 amino acid long α-helical rod domain flanked by C- and N-terminal non-helical segments (See the schematic drawing in fig. 2 and Compton *et al.*, 1992; Yang *et al.*, 1992). The proposed central α-helical domain contained several hydrophobic heptad repeats interrupted by six proline rich regions. The structure is similar to a typical coiled-coil configuration seen in intermediate filament molecules (Fuchs and Weber, 1994). Later, Harborth et al. (1995) visualized the structure of the recombinant full-length NuMA using the rotatory shadowing technique in electron microscopy showing an unusually long 207 nm long α-helical rod domain with C- and N-terminal globular ends. The same study used chemical cross-linking technique to reveal the α-helices to arrange in parallel and register and suggested the dimerization of NuMA.

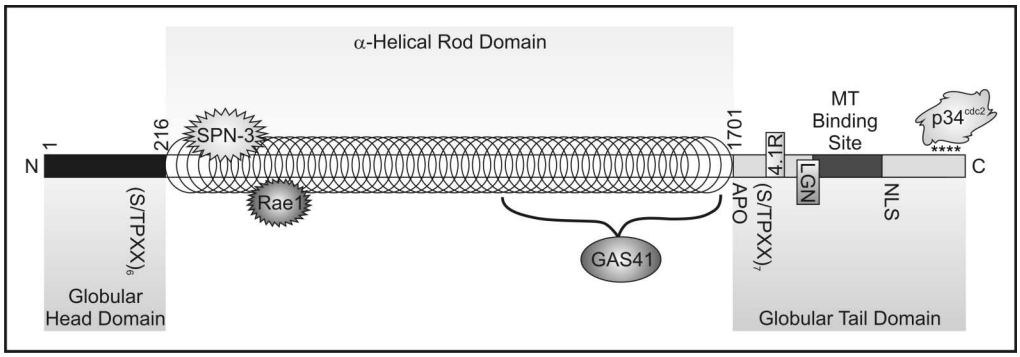


Figure 2. The schematic drawing of the structure and interactions of the NuMA protein, see text for details.

NuMA has not been shown to be a part of a particular protein family. Yang et al. (1992) suggested the α-helical rod domain of NuMA to contain sequence similarity to tropomyosins, myosin and intermediate filaments such as nuclear lamins or keratins. The sequence similarity (~20%) was the highest between NuMA α-helical domain and human cardiac myosin. Later, this finding was argued to be irrelevant since all coiled-coil α-helical fibrous proteins show this general level of sequence similarity (Parry, 1994). Parry used computational analysis to point out that the structure of α-helical rod domain favors parallel in-register double strand coiled-coil although NuMA is unlikely to form a filamentous structure of the type seen in intermediate or myosin thick filaments.

The targeting of NuMA to the nucleus requires the nuclear localization signal (NLS) which is bipartite consisting of the basic amino acids RKR and KK at positions 1987-1989 and 2004-2005, respectively (Fig. 2 and Gueth-Hallonet *et al.*, 1996). Mutation of

lysine residue on position 1988 results in exclusion of the protein to the cytoplasm (Tang *et al.*, 1994; Gueth-Hallonet *et al.*, 1996) whereas the mutation at position 1989 or both 2004+2005 seems only to impair the nuclear import of NuMA (Gueth-Hallonet *et al.*, 1996). This suggests that the lysine residue on position 1988 is indispensable for the nuclear import whereas the other residues of the NLS seem to promote this relocalization.

2.3.3. The role of NuMA in interphase

The role of NuMA in the interphase nucleus is largely unknown: it may serve as a structural component but several reports suggest NuMA to function in the genome organization and the regulation of the gene expression. According to Lydersen and Pettijohn (1980), NuMA is considered part of the NM which by definition is the residue of nuclei treated with high concentration salt, non-ionic detergent and nucleases (Berezney and Coffey, 1974). NuMA antibodies stain diffusely the whole nucleus excluding nucleoli and the secondary structure of NuMA is similar to some other known structural proteins: similarly to lamins and other intermediate filaments NuMA has a large coiled-coil rod domain and globular heads, (Compton *et al.*, 1992; Yang *et al.*, 1992). NuMA is an abundant (10^6 copies per cell) protein of the nucleus (Compton *et al.*, 1992) and although endogenous filament structures have not been visualized *in vivo*, NuMA is capable of forming dimers and multiarm oligomers *in vitro* (Harborth *et al.*, 1999), as well as ordered nucleoplasmic structures when over-expressed in HeLa cells (Gueth-Hallonet *et al.*, 1998). The over-expression of a NuMA mutant lacking the NLS results in large cytoplasmic NuMA aggregates (Saredi *et al.*, 1996). These aggregates consist of an extensive network of filaments with a diameter of ~5 nm. In the presence of dynamic microtubules, these filaments organize into solid ~23 nm cable structures. Immunoelectron microscopy of detergent extracted cells visualizes NuMA as a part of the intranuclear core filaments (Zeng *et al.*, 1994) and in association with the electron dense domains of the inner NM (Barboro *et al.*, 2002). This association is disrupted following RNase treatment suggesting NuMA interaction with the NM to be at least partially RNA dependent (Barboro *et al.*, 2002). The depletion of NuMA from the interphase cells has been considered a “mission impossible” due to the essential mitotic function of NuMA. Harborth et al. (2001) reported silencing of 21 different gene products in mammalian cultured cells. Among these, NuMA silencing induced apoptotic phenotype and growth arrest in HeLa cells, but the mechanism of the proposed cell death or the impact of the silencing on the nuclear structure were not further analyzed. Similarly, NuMA silenced human larynx carcinoma Hep-2 cells have been reported to exhibit apoptotic characteristics and growth arrest at 3 days post transfection (Yamauchi *et al.*, 2008). In this study, only 75% of NuMA silenced cells were still viable when compared to control siRNA transfected cells.

Although all the data above suggest NuMA to have an essential structural role in interphase nucleus, the assembly of postmitotic nuclei seems not to require NuMA:

NuMA immunodepleted *Xenopus* egg extracts treated with human sperm DNA are able to form functional nuclei in the absence of NuMA (Merdes and Cleveland, 1998). In addition to our own results, some siRNA experiments have reported NuMA depletion without significant degree of cell death. In a study describing the importance of NuMA for Kaposi's sarcoma-associated herpes virus infection, the authors cultured human embryonic carcinoma HEK293 cells stably transfected with plasmids encoding NuMA siRNAs for up to 9 days without evident signs of apoptotic cell death (Si *et al.*, 2008). Moreover, in two reports studying the role of NuMA in chromatin organization and mammary gland differentiation, NuMA silenced cells were viable 8 days after transfection and the amount of apoptotic cells was not increased when compared to controls (Abad *et al.*, 2007; Chandramouly *et al.*, 2007). There is also evidence that some differentiated and non-dividing cells lack NuMA, e.g. neurons and spermatozoa (Merdes and Cleveland, 1998; Taimen *et al.*, 2000; Taimen *et al.*, 2004). One statement suggests that NuMA is only sequestered from the cytosol during interphase to prevent its unwanted interaction with the microtubules (Radulescu and Cleveland, 2010).

The DNA content of the human genome is organized into linear chromosomes (initial length 1 meter) which are tightly packed into the nucleus (diameter approximately 10 μm) as chromatin (Radulescu and Cleveland, 2010). The chromosomes are organized inside the nucleus to so called chromosome territories (Reviewed by Cremer and Cremer, 2001) likely to be important to prevent random interaction of chromosomes resulting in unwanted fusions and translocations. The role of NuMA in the chromatin organization and gene expression control is supported by many studies. The first finding was notably the report of interaction of NuMA with matrix attachment region (MAR) sequences of DNA (Luderus *et al.*, 1994). Although the DNA binding S/TPXX motifs are located in both the N- and C-terminal ends of NuMA, the C-terminus of the protein seems to be more important for the nuclear DNA organization. The truncation of NuMA polypeptide at residues 2005 or 2030 causes a drastic reorganization of the nuclear components with relocation of the DNA, histone H1, and nucleoli to the nuclear rim (Gueth-Hallonet *et al.*, 1998). Similarly, Lelièvre et al. (1998) showed nuclear chromatin rearrangement as shown by changes in the acetylated histone H4 distribution when disrupting the function of NuMA with a C-terminus targeted antibody. Moreover, the expression of a C-terminal fragment of NuMA is sufficient to inhibit acinar differentiation and results in the redistribution of NuMA, chromatin markers acetyl-H4 and H4K20m, and regions of DNase I-sensitive chromatin. Only a small amount of NuMA may be sufficient to maintain the nuclear chromatin organization: a total NuMA silencing may be impossible to obtain due to the vital role of NuMA in mitosis and in a recent study NuMA siRNA treated HeLa cells maintained their interphase chromatin structure (Haren *et al.*, 2009). According to the information available on the interphase function of NuMA, it seems likely that NuMA preferably functions in the chromatin organization although it can not be concluded that some underlying structural function exists.

2.3.4. The role of NuMA during mitosis

Once every cell cycle, the nuclear envelope is broken down and the chromosomes are distributed evenly into the two newly forming daughter cells by the mitotic spindle consisting of a complex of two centrosomes nucleating the growth and the assembly of the mitotic microtubules (Fig. 3A). While the minus ends of the mitotic microtubules are anchored to spindle poles, the plus ends grow towards the equator of the cell to capture the kinetochores on the chromosomes (Fig. 3A). Immunoelectron microscopy reveals NuMA to reside in the area adjacent to the centrosomes but not directly interacting with them in mitotic cells (Tousson *et al.*, 1991; Dionne *et al.*, 1999). Figure 3 depicts the schematic structure of the mitotic metaphase cell and the function of NuMA during mitosis.

In the early paper NuMA was observed to localize to the spindle poles (Lydersen and Pettijohn, 1980) and to the reforming daughter nuclei in early telophase (Price and Pettijohn, 1986). Later papers described a more detailed localization pattern (Kallajoki *et al.*, 1991; Compton *et al.*, 1992; Yang *et al.*, 1992). The diffuse staining pattern of interphase nuclei is disrupted in early prophase as NuMA is excluded to the interchromosomal space coincident with the chromosomal condensation and during prometaphase it accumulates to the centrosomes (See fig. 3E and Yang *et al.*, 1992). During metaphase and early anaphase NuMA localizes to the pericentrosomal region, sometimes forming a ring-like staining pattern surrounding the centrosomes (See fig. 3E, box with dash line and Kallajoki *et al.*, 1991; Compton *et al.*, 1992). This staining pattern is dependent on the centrosomal integrity as spindle pole disruption with colcemid results in a dispersed staining pattern of NuMA (Lydersen and Pettijohn, 1980). Double staining of NuMA and tubulin show the bulk of NuMA to focus to the proximal minus-ends of the spindle microtubules (Compton *et al.*, 1992). In late anaphase and telophase, the staining intensity of NuMA at the polar region is decreased and NuMA accumulates in the periphery of the condensing chromosomes even before the nuclear lamina re-assembly (See fig. 3E and Yang *et al.*, 1992) but at least part of the import of NuMA into the reforming daughter nuclei requires nuclear pore function as the blocking of the nuclear pores at the end of mitosis leads to the exclusion of NuMA from the reforming daughter nuclei (Compton *et al.*, 1992).

The mitotic function of NuMA was originally addressed by microinjecting NuMA specific antibodies into mitotic cells (Yang and Snyder, 1992; Kallajoki *et al.*, 1993). The microinjection of monoclonal NuMA antibodies into mitotic HeLa and PtK2 cells induces the relocalization of NuMA into multiple foci from the pericentrosomal area, multipolar spindles and the formation of micronuclei (Kallajoki *et al.*, 1991; Kallajoki *et al.*, 1993). The microinjection of NuMA antibodies into African green monkey kidney CV-1 cells resulted in failure to form the mitotic spindle apparatus when microinjected into interphase and prophase cells and in a collapsed spindle apparatus and dissociated centrosomes when microinjected into metaphase cells (Yang and Snyder, 1992). Although it was initially speculated that NuMA is needed for the

mitotic re-assembly (Compton and Cleveland, 1994), the microinjection studies by Yang and Snyder also demonstrated that microinjection of NuMA antibodies into anaphase cells does not alter the mitotic progression suggesting that NuMA is needed in the mitotic spindle formation but not the later stages of the mitotic cell division. Similarly, functional nuclei can be formed in the absence of NuMA: NuMA immunodepleted *xenopus* egg extracts can form functional nuclei following the addition of human sperm DNA (Merdes and Cleveland, 1998).

The functional mitotic spindle consists of the microtubules and the centrosomes (fig. 3A) and NuMA is crucial for the stabilization of this core structure. The initial studies were based on the microtubule stabilizing properties of taxol (Kallajoki *et al.*, 1992) and microtubule destabilizing properties of nocodazole (Kallajoki *et al.*, 1991) and showed NuMA to disperse into multiple foci upon destabilization of the microtubules. Upon removal of nocodazole, NuMA foci act as microtubulus nucleating centers supporting the microtubulus bundling properties of NuMA. Similarly, NuMA depleted mitotic *Xenopus* egg extracts fail to assemble normal mitotic spindles when incubated with demembranated sperm DNA but form irregular, splayed microtubulus asters lacking characteristic spindle poles and the addition of purified NuMA to these extracts partially restores the spindle assembly (Merdes *et al.*, 1996). This paper was the first to describe the microtubulus binding and bundling properties of NuMA and the binding site on NuMA was later mapped to residues 1900-1971 in the C-terminus of the protein (Du *et al.*, 2002). The spindle pole assembly itself does not require the interaction between NuMA and the microtubules: according to a recent paper describing an inducible deletion of the exon 22 encoding for the microtubulus binding domain of NuMA, a normal bipolar spindle is formed in the presence of the mutant form, but the generation of the spindle forces induces detachment of the centrosome from the spindle and kinetochore fibers become defocused (Silk *et al.*, 2009). This suggests that instead of stabilizing the microtubulus bundling, NuMA is needed for the attachment of the microtubules and the centrosome at the pericentrosomal area.

2.3.5. The formation of the spindle

2.3.5.1. The recruitment of NuMA to the spindle pole

Early studies revealed NuMA to be a phosphoprotein (Price and Pettijohn, 1986). NuMA is phosphorylated at the onset of mitosis roughly at G_2/M transition simultaneously with the nuclear breakdown by $p34^{cdc2}$ (Compton and Luo, 1995; Sparks *et al.*, 1995) and four predicted $p34^{cdc2}$ phosphorylation sites (2000 threonine, 2040 threonine, 2072 serine and 2091 threonine) were predicted from the primary structure (Yang *et al.*, 1992). The paper Compton and Luo shows that the mutation of a $p34^{cdc2}$ phosphorylation target threonine residue at position 2040 is sufficient to abolish the ability of NuMA to associate typically with microtubules of the mitotic spindle and targets NuMA to the plasma membrane (Compton and Luo, 1995). The phosphorylation mediated by $p34^{cdc2}$ is thought to increase the solubility of NuMA

(Saredi *et al.*, 1997). A serine/threonine kinase Pim-1 is also thought to phosphorylate NuMA during mitosis (Bhattacharya *et al.*, 2002). Pim is proto-oncogene, the overexpression of which promotes lymphoma formation in lymphoid cells (van Lohuizen *et al.*, 1989). NuMA has two consensus sequences (residues 1986-1993 and 2022-2029) for phosphorylation by Pim-1 which colocalizes at the spindle poles and co-immunoprecipitates with NuMA-dynein-dynactin complex and phoshorylates NuMA *in vitro* (Bhattacharya *et al.*, 2002). The exact role of Pim-1 remains unclear but the researchers suggest it to function in a complex with NuMA and dynein-dynactin.

Without proper experimental evidence Compton et al. (1992) had speculated earlier that dynein may participate in the active transport of NuMA to the minus ends of spindle poles. The model has been clarified over the years by several reports. Following phosphorylation NuMA relocalizes to the spindle poles along the microtubules via movement by dynein/dynactin, a microtubule minus end directed motor protein complex (Merdes *et al.*, 1996). In addition to its motor protein function, dynein-dynactin increases the crossbundling of microtubules via the interaction with NuMA dimers (Fig. 3C). The subunit of dynactin, Arp1α, (actin related protein 1 α) is thought to act as the mediator of the binding NuMA to dynactin: the over-expression of Arp1α results in the ectopic cytoplasmic colocalization of Arp1α with NuMA, dynein and dynactin and, moreover, the over-expression of a mutant Arp1α results in the absence of NuMA in the ectopic Arp1α-dynactin-dynein complexes suggesting Arp1α is the subunit responsible for the NuMA-dynein-dynactin interaction (Clark and Meyer, 1999). The proper formation of a mitotic spindle, however, requires all the constituents of a complex formed by dynein, dynactin and NuMA as immunodepletion of any part resulted in disorganized microtubulus arrays. These results suggest NuMA, dynactin, and dynein to form an easily disassembling, low-affinity complex necessary for spindle pole organization (Merdes *et al.*, 2000). Although the precise dynein-dynactin binding sequence on NuMA has not been mapped, a computational model suggests the N-terminus of NuMA to have a CH (calponin homology) domain with affinity to actin-like proteins such as dynactin subunit Arp1 (Novatchkova and Eisenhaber, 2002).

During normal mitosis NuMA is retained at the spindle poles until anaphase. Anaphase begins with silencing of the spindle assembly checkpoint resulting in anaphase promoting complex (APC) mediated ubiquitination of cyclin B and securin and their subsequent proteasomal degradation leading to $p34^{cdc2}$ inactivation (Alberts *et al.*, 2008). The maintenance of $p34^{cdc2}$ activity by adding a mutant of nondegradable cyclin B to a mitotic *Xenopus* egg extract inhibits dephosphorylation of NuMA and its release from dynein-dynactin at the spindle poles (Gehmlich *et al.*, 2004). Prior to entering anaphase Emi1, an APC inhibitor, is sequestered to the spinde poles by an interaction with the END (Emi1, NuMA, Dynein-Dynactin) network (Ban *et al.*, 2007). The researchers state Emi1 not only to act in the spindle assembly but also to sequester inactive APC to the spindle poles along with $p34^{cdc2}$ and cyclin B. The high concentration of $p34^{cdc2}$ at the spindle poles promotes the maintenance of high level of

phosphorylation of NuMA essential for the normal mitotic function. If this is correct, this suggests a role for NuMA not only acting in the maintenance of the spindle structure but also in harvesting checkpoint regulatory components from action. Taken together, the data described above creates a model where following phosphorylation NuMA is recruited to the MT minus ends via dynein/dynactin motor protein activity. At the spindle NuMA bundles microtubules together at the pericentrosomal area via interaction with various proteins and also possibly controls some cell cycle regulatory proteins.

2.3.5.2. The control of NuMA at the spindle

At the onset of mitosis the microtubulus dynamics increases and interphase microtubulus arrays are dismantled (Hyman and Karsenti, 1996). One model suggests that the microtubulus arrangements during mitosis are organized by the function of two molecules, Ran and Importin-β involved in the nuclear import during interphase. During interphase, importins bind the NLSs of proteins to be imported through the nuclear pore complexes to the nucleus and the binding of Ran-GTP to the importin-cargo complex mediates the dissociation of the cargo from the importins in the nucleus (Nachury *et al.*, 2001). The initial idea for Ran acting in the mitotic spindle formation rose from a study showing that excess Ran-GTP in mitotic extracts induces the polymerization of microtubules and formation of spindle- or aster-like structures independent of centrosomes or chromatin, and that this phenomenon is also seen when excess RCC1 (Regulator of chromosome condensation 1) is added to the extract (Carazo-Salas *et al.*, 1999). RCC1 is a chromatin binding nuclear guanosine nucleotide exchange factor (GEF) that mediates the change of Ran-GDP to its GTP bound form. RCC1 is bound to the chromatin throughout the cell cycle and its activity at the chromosomes creates a Ran-GTP gradient extending away from the chromatin. According to the model, importins would sequester NuMA early in mitosis to the area near the chromatin where the concentration of RanGTP is the highest resulting in the release of NuMA from the NuMA-importin complex, the microtubulus stabilization and, thus, aster formation (Nachury *et al.*, 2001; Wiese *et al.*, 2001). This model was, however, later criticized by another group based on the finding that an unrelated stable microtubulus (MT) producing agent Tau could also generate asters in the presence of functional NuMA (Du *et al.*, 2002).

The study by Du et al. (Du *et al.*, 2001; Du *et al.*, 2002) suggested that LGN (leu-gly-asn), the mammalian analog for the drosophila Pins (partner of inscuteables) involved in the asymmetrical cell division, controls NuMA in the spindle pole assembly. The overexpression of LGN and its depletion by siRNA results in defective spindle-pole organization (Du *et al.*, 2001). The same paper described LGN to localize to the cytoplasm in interphase cells and to interact with NuMA during mitosis regulating the mitotic microtubules: the addition of anti-LGN antibodies or the LGN binding domain of NuMA to a mitotic extract induces spontaneous aster formation whereas the addition of NuMA binding domain of LGN to the mitotic extract blocks the aster formation. Later paper by the same group (Du *et al.*, 2002) described LGN binding site of NuMA

(residues 1878-1910) to overlap with the MT binding site of NuMA (residues 1900-1910). The LGN binds NuMA with higher affinity than MTs and due to the overlapping binding sites NuMA can not bind MTs and LGN simultaneously.

Importantly, the role of NuMA is not to function only in the spindle assembly at the spindle pole and stabilization but also in the anchoring the spindle to the cell cortex. Endogenous NuMA has been detected on the cell cortex in mitotic cells (Du and Macara, 2004). This finding resulted in a new model of NuMA regulated spindle assembly. In addition to the inhibitory effect of LGN on the NuMA-MT interaction, LGN binds NuMA to the cell cortex where NuMA-LGN-complex binds membrane bound GDP-Gαi (Du and Macara, 2004). The writers speculate that this interaction could modulate the pulling forces mediated by the LGN-NuMA-dynein complex attached to the cell cortex moving towards the minus ends of the astral microtubules. LGN has higher affinity for GDP-Gαi than for the GTP bound form and the formation of the LGN-NuMA- GDP-Gαi complex is regulated by Ric (Resistance to inhibitors of cholinesterase) 8A acting as a guanine exchange factor (Tall and Gilman, 2005). Ric 8A also stimulates the release of NuMA from LGN and may therefore act as a regulator of NuMA-LGN interaction not only at the cell cortex but also at the spindle poles.

Another binding partner of NuMA, an mRNA transporting protein Rae1, is also suggested to mediate the indirect binding of the N-terminus of NuMA to the microtubules forming a “tetravalent” binding of a NuMA dimer to microtubules (See fig. 3C, Wong *et al.*, 2006). The role of this interaction is suggested to be important for the formation of a bipolar spindle but the role needs to be clarified by further experimentation. Human cohesin is a multi-protein complex mediating the interaction between sister chromatids in S-phase prior to mitosis. In addition to the chromatid cohesion, cohesin is shown to interact with NuMA and the NM during interphase. During mitosis only a small amount of cohesin is retained at the chromosome pairing domain at the centromeres but the majority is relocalized to the spindle pole to interact with NuMA and function in the mitotic spindle aster assembly (Gregson *et al.*, 2001; Kong *et al.*, 2009). The role of the cohesins during mitosis is not characterized in detail but they are involved in the aster assembly as the depletion of cohesin proteins SMC1, SMC3 or SA1N results in aberrant aster formation (Gregson *et al.*, 2001).

2.3.6. Interaction partners of NuMA

NuMA is a large protein with several distinct functional domains and multiple interaction partners (Table 1). The mitotic interactions between NuMA and several proteins were described earlier. Only a few factors have been described to interact with NuMA during interphase: the DNA and importin interactions were described earlier in the text.

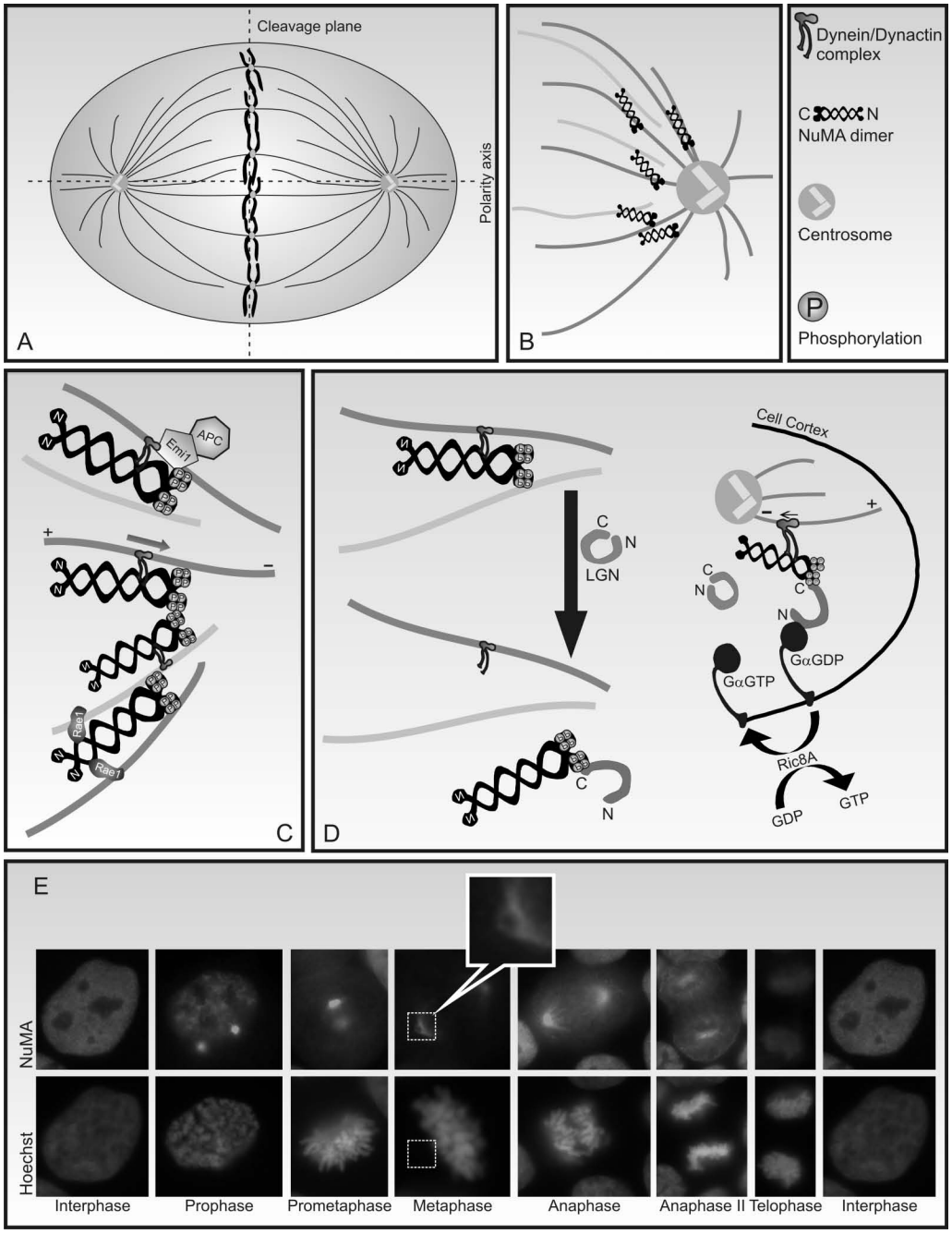

Cleavage plane
Polarity axis
A
B
Dynein/Dynactin complex
C N
NuMA dimer
Centrosome
P
Phosphorylation
APC
Emi1
Rae1
Rae1
C
LGN
C
N
D
Cell Cortex
GαGDP
GαTP
Ric8A
GDP
GTP
E
NuMA
Hoechst
Interphase
Prophase
Prometaphase
Metaphase
Anaphase
Anaphase II
Telophase
Interphase

Figure 3. NuMA in mitosis. *A) A schematic drawing of a metaphase cell where the chromosomes are aligned along the cleavage plane and the centrosomes are localized at the polarity axis with minus-ends of the microtubules attached to the kinetochores on the chromosomes. B) NuMA dimers may crosslink the microtubules in different ways. C) NuMA forms several interactions at the microtubules. Interaction with dynein mediates the minus-end directed movement along the microtubules. The Emi/NUMA/Dynein (END) complex may regulate cell cycle progression by binding APC. Protein Rae1 may cause the cross-bundling of parallel microtubules. D) LGN has a higher affinity to NuMA than to microtubules and, thus, regulates NuMA binding to the microtubules. It may also regulate the pulling forces on astral microtubules by attaching them via NuMA/LGN/GαGDP complex to the cell cortex. E) The localization of NuMA at different mitotic stages. See text for details.*

GAS41 (glioma amplified sequence 41), originally described as a gene amplified in glioblastoma multiforme and astrocytomas (Fischer *et al.*, 1997), was identified as a binding partner of NuMA using Yeast two-hybrid system (Harborth *et al.*, 2000). The paper identified GAS41 to bind the C-terminal part of NuMA α-helical rod domain (residues 1048-1700). GAS41 is a nuclear transcription factor involved in the suppression of the p53 pathway (Llanos *et al.*, 2006). The silencing of GAS41 in wild type p53-expressing U2OS cells causes G_2/M phase arrest and p53 pathway activation including the transcriptional of p21 activation and the phosphorylation of p53ser15 (Park and Roeder, 2006). Targeted disruption of both GAS41 alleles results in a loss of viability of chicken DT40 cells and the cell death is preceded by a significant decrease in RNA synthesis suggesting that GAS41 is required for RNA transcription (Zimmermann *et al.*, 2002). The functional significance of the interaction between NuMA and GAS41 has not been clarified in detail.

Protein 4.1R has been identified as a binding partner of NuMA not only during interphase but also during mitosis (Mattagajasingh *et al.*, 1999; Mattagajasingh *et al.*, 2009). Protein 4.1R was first described in erythrocytes as a 80 kDa structural protein stabilizing the membrane structure (Ungewickell *et al.*, 1979). The family of 4.1 proteins consists of several proteins. One protein of family 4.1R (or EPB41) is encoded by a single gene but expressed as multiple different isoforms in a tissue-specific and developmental-stage dependent manner (Hoover and Bryant, 2000): the 135 kDa isoform binds NuMA i*n vitro* and *in vivo* and residues 1788-1810 of NuMA are crucial for the interaction (Mattagajasingh *et al.*, 1999). Protein 4.1R colocalizes partially with NuMA in interphase nuclei and redistributes to the spindle poles to interact with the NuMA-Dynein-Dynactin complex through its C-terminal motif (Mattagajasingh *et al.*, 1999). 4.1R directly binds microtubules (Huang *et al.*, 2004). Phosphorylation of 4.1R by $p34^{cdc2}$ enhances its binding to NuMA and tubulin (Huang *et al.*, 2005). The depletion of 4.1R from a mitotic extract results in disorganized mitotic spindle and mislocalization of NuMA from the spindle poles and the addition of 4.1R to the immunodepleted extract rescued the spindle formation (Krauss *et al.*, 2004). Although the data here strongly suggests 4.1R to act in the spindle formation, 4.1R is a nuclear protein and mutations disrupting the interaction between NuMA and 4.1R result in

cytoplasmic mislocalization of 4.1R (Mattagajasingh *et al.*, 2009). A Yeast two-hybrid screen showed 4.1N, member of the 4.1 family, to interact with NuMA (residues 1440-1913) via its C-terminus (Ye *et al.*, 1999). The treatment of PC12 cells with nerve growth factor induces 4.1N translocation from the cytoplasm to the nucleus, where it interacts with NuMA, induces a cell cycle arrest in G_1 and causes nuclear structure changes (Ye *et al.*, 1999). Another member of the 4.1 family, 4.1B, has a similar NuMA binding motif but its interaction has not been described (Parra *et al.*, 2000).

2.3.7. NuMA in diseases

The first reference of NuMA to human diseases was the discovery of circulating autoantibodies in patients with autoimmune diseases. Patients with various connective tissue diseases (Sjögren´s syndrome, CREST, polyarthritis, SLE) reacting with NuMA (McCarty *et al.*, 1981; Price *et al.*, 1984). NuMA-antibodies have been described in patients with Sjögren´s syndrome, non-spesific connective tissue diseases, systemic lupus erythematosus, vitiligo and autoimmune thyroiditis, scleroderma, a single psoriasis patient (Auer-Grumbach and Stangl, 1993; Herrera-Esparza *et al.*, 1999; Mozo *et al.*, 2008; Tampoia *et al.*, 2009; Szalat *et al.*, 2010). The wide variety of diseases connecting with NuMA autoantibodies and the small number of patients presenting with NuMA antibodies, has made the authors to suggest that only in a case of very high NuMA titer it could lead to a more thorough screening for a connective tissue disease (Mozo *et al.*, 2008).

Malignant transformation turns normal cells into over-actively dividing cancer cells showing aneuploidy, partial immortality and chromosomal instability. The locus of *NUMA1* gene, 11q13, is a commonly mutated gene locus in several cancer types, e.g. almost 15% of breast cancer show an amplification of the locus and several oncogenes are located at this chromosomal area (Dickson *et al.*, 1995). Some cancer cell lines show an overexpression of NuMA leading to multipolar spindles (Quintyne et al., 2005) and it was suggested that mutation of *NUMA1* gene resulting in an amino acid shift Ala794Gly is related to a higher risk of breast cancer (Kammerer et al., 2005). This conception was, however, overruled by a Finnish research group who found no relation between this mutation of NuMA and breast cancer risk, neither did this mutation result in a particular tissue phenotype although it did, however, slightly increase the risk of lymph node involvement (Kilpivaara *et al.*, 2008).

Acute promyelocytic leukemia (APL), a disease characterized by a block in the myeloid cell differentiation at the promyelocyte stage, associates with NuMA. The hallmark for the APL generation is the chromosomal translocation t(15;17)(q22;q21) detected in the majority of APL patients and not in patients with other forms of acute lymphatic leukemia. The translocation results in the generation of an oncogenic fusion protein containing PML (promyelocytic leukemia) and RARα (Retinoic acid receptor α). As rarities, four other APL associated fusion proteins, PLZF (promyeolocytic leukemia zinc finger) protein, NPM (nucleophosmin), NuMA or STAT5B (Signal

transducer and activator of transcription 5B) protein fused to RARα, have been identified and characterized (Zelent *et al.*, 2001). *NuMA-RARα* fusion gene was first described by Wells et al. (1997) in a patient case of a 6-month-old boy who represented with typical APL without PML fusion protein involvement. The genotyping revealed t(11;17)(q13;q21) translocation and the generated fusion protein consisting of the 1883 N-terminal amino acid residues of NuMA fused to the ligand binding domain of RARα -identical portion of the protein seen in other fusion proteins identified (Wells *et al.*, 1997). The fusion protein did not alter the function of PML and the writers suggested that the APL is generated via alterations in RARα function. NuMA-RARα fusion protein binds endogenous NuMA and localizes similarly to endogenous NuMA. The α-helical domain of NuMA in the fusion protein is critical for the interaction with retinoic acid response elements and the inhibition of the endogenous RARα transcriptional activity (Dong *et al.*, 2003).

Cell cycle stage	**Interaction partner**	**Interaction domain on NuMA**	**Ref.**
Interphase	GAS41	Residues 1048-1700 interact with the C-terminus of GAS41	Harborth et al. (2000)
	MAR	Six S/TPXX motifs on the N-terminus and seven on the C-terminus	Luderus et al. (1994)
Interphase and mitosis	Importin	Residues 1971-1991 coding for the NLS in the C-terminus	Nachury et al. (2001)
	4.1R	Residues 1788-1810	Mattagajasingh et al. (2001 and 2009)
	4.1N	Unknown	Ye et al. (1999)
	Cohesin	Unknown	Gregson et al. (2001)
Mitosis	Dynein/dynactin complex	Unknown	Merdes et al. (1996 and 2000) Clark and Meyer (1999)
	Microtubules	Residues 1900-1971	Merdes et al. (1996) Du et al. (2002)
	Rae1	Residues 325-829	Wong et al. (2006)
	Emi1	Unknown	Ban et al. (2007)
	LGN	Residues 1878-1910	Du et al. (2001 and 2002)
	Pim-1	Unknown	Bhattacharya et al. (2002)

Table 1. Interaction partners of NuMA

2.4. Nuclear envelope and nuclear lamina

The inner and the outer nuclear membranes form the nuclear envelope which defines the barrier between the cytoplasm and the nuclear compartment. The inner and outer nuclear membranes are continuous at the sites of the nuclear pore complexes (NPCs) which are large protein complexes that form channels in the nuclear envelope enabling the transport from the cytoplasm to the nucleus and vice versa. The outer membrane is continuous with the rough endoplasmic reticulum and houses attached ribosomes on the surface in addition to the linker proteins mediating the connection between the cytoskeleton and the inner nucleus. The inner nuclear membrane contains several transmembrane proteins which e.g. anchor the nuclear lamina but also serve as a link between the cytoskeleton and the inner nuclear scaffold. The nuclear lamina consisting of the intermediate filaments termed lamins underlies the inner nuclear membrane. It is the only nuclear structure proven to function in the maintenance of the nuclear shape but has also been shown to take part in many crucial functions of the nucleus. Figure 4 depicts the schematic structure of the nuclear envelope and the nuclear lamina.

2.4.1. The seven lamin subtypes are encoded by three genes

The major constituent of the nuclear lamina are the lamins (approximately 60-70 kDa) which are type V intermediate filament proteins. Like other intermediate filament proteins, lamins consist of an N-terminal non-helical head domain, a long α-helical rod domain and a C-terminal non-helical tail-domain. Lamins form filaments consisting of dimers and polymers: the α-helical rod domain mediates the homodimerization and the dimers assemble in a head-to-tail manner to form filaments (Heitlinger *et al.*, 1991). Lamins are classified as A- and B-type lamins according to their biochemical properties. Two types of B-type lamins, lamin B1 and B2, exist and they are encoded by distinct genes *LMNB1* and *LMNB2* (Peter *et al.*, 1989; Vorburger *et al.*, 1989). The latter gene also encodes for a less abundant variant lamin B3 (Furukawa and Hotta, 1993). Lamins A and C are regarded as the A-type lamins and are splice variants of a single mRNA of the *LMNA* gene (Lin and Worman, 1993). Two less abundant isoforms, lamin AΔ10 and C2, are also produced in some vertebrates from *LMNA* gene (Nakajima and Abe, 1995; Machiels *et al.*, 1996).

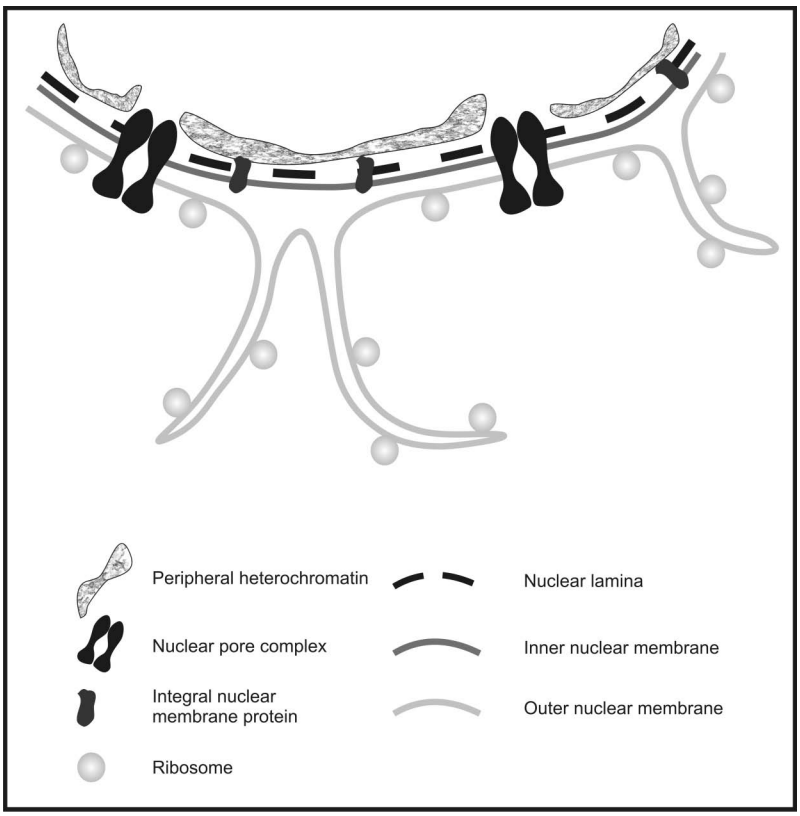


Figure 4. A schematic drawing of the coarse structure of the nuclear envelope and the nuclear lamina. *The nuclear envelope is a double-membrane structure. The outer nuclear membrane is continuous with the endoplasmic reticulum and has ribosomes attached on the surface and the lamina underlies the inner nuclear membrane. The inner nuclear membrane is permeated by several different integral proteins which act in tethering of the nuclear lamina to the membrane. Some of the integral proteins also form a link between the inner nuclear matrix and the cytoskeleton.*

Although it has been suggested that other intermediate filament proteins would have evolved from lamins through the loss of both the nuclear localization signal and the CaaX box (where C is cysteine, A is an aliphatic amino acid and X is any amino acid) of the lamin gene (Doring and Stick, 1990), there are some major differences (Fig. 5). The globular head domain of lamins is significantly smaller and lamins contain an additional six-heptad repeat extension in one (1B) of the four central coiled-coil domains (Reviewed in Dechat *et al.*, 2010). In addition, the membrane targeting CaaX-box and the nuclear localization signal (NLS) characteristic of lamins are absent in the tail domain of other intermediate filament proteins (Reviewed in Dechat *et al.*, 2010). Also, an important similarity between A-type and B-type lamins, but absent in other intermediate filament proteins, is the IgG-fold, a 105 amino acid globular domain

between the rod and the tail domains (Dhe-Paganon *et al.*, 2002). The IgG-fold seems to be associated with interactions with DNA and other proteins.

The B-type lamins have been suggested to be the ancestral lamins. The A-type lamins are thought to have risen from a gene duplication of lamin B and an addition of an extra exon resulting in the insertion of the approximately 90 amino acid sequence preceding the CaaX box at the C-terminus of lamin A (Stick, 1992). The rod domain important for the homodimerization is similar in length in A- and B-type lamins but there is variation in the amino acid sequence. All vertebrates express at least one B-type lamin whereas the expression of A-type lamins is developmentally regulated (Reviewed in Dechat *et al.*, 2010). In mouse embryos, A-type lamins are not expressed until birth excluding muscle cells where the expression starts on the day 10-12 of the embryogenesis (Rober *et al.*, 1989). Similar patterns of expression are seen in other vertebrates and *Drosophila*. The minor lamin isoforms B3 and C2 are only expressed in germ cells whereas lamin AΔ10 is expressed in a variety of cell types.

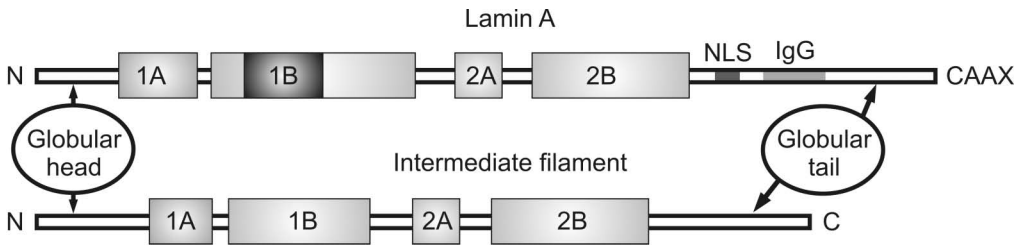


Figure 5. Lamins and other intermediate filaments differ in their structure. *In lamins the globular head domain is much shorter than the corresponding domain in a cytoplasmic IF proteins. The 1B central coiled-coil domain contains six-heptad repeat repeat extension absent in cytoplasmic IF. In lamins, the C-terminal globular tail contains the nuclear localization signal (NLS), CaaX box susceptible for posttranslational isoprenylation and the IgG-fold thought to function in DNA and protein interactions. (Image modified from an image by Stuurman et al., 1998)*

Lamins A, B1 and B2 share a common type of post-translational modification termed isoprenylation at their C-terminal CaaX box to become mature proteins. Lamin C does not contain the CaaX box and, thus, is not isoprenylated. The isoprenylation begins by farnesylation of the pre-lamins at the cysteine residue of the CaaX box (Farnsworth *et al.*, 1990; Lutz *et al.*, 1992). The farnesylation is followed by the cleavage of the C-terminal three amino acids (aaX) (Maske *et al.*, 2003; Corrigan *et al.*, 2005) and the carboxymethylation (Winter-Vann and Casey, 2005). Pre-lamin A is finally cleaved to remove the last 15 C-terminal amino acids including the isoprene residue prior to the release of the mature lamin A (Corrigan *et al.*, 2005). The B-type lamins are permanently associated with the inner nuclear membrane probably through the isoprenylation of the C-terminal CaaX box: the isoprenylation increases the affinity of

proteins to membranes and the impairment of the isoprenylation results in decreased accumulation of the B-type lamins to the nuclear envelope (Kitten and Nigg, 1991; Mical and Monteiro, 1998).

In addition to isoprenylation, lamins are widely modified post-translationally. Phosphorylation of lamins was first described by Ottaviano and Gerace (1985) and is known to regulate the nuclear import of lamins (Hennekes *et al.*, 1993; Leukel and Jost, 1995) and the mitotic disassembly of the nuclear lamina (Collas *et al.*, 1997). Similarly, reassembly of the nuclear lamina at the onset of telophase/G_1 transition requires dephosphorylation (Thompson *et al.*, 1997). Lamins are also phosphorylation targets during the apoptotic disintegration of the nucleus (Weaver *et al.*, 1996). The exact implication of the sumoylation of lamin A remains to be solved but it is known to be crucial: decreased sumoylation of lamin A due to mutations increases cell death and associates with familial type cardiomyopathy (Zhang and Sarge, 2008). Similarly, the functional role of lamin ADP-ribosylation (Adolph, 1987) and glycosylation (Ferraro *et al.*, 1989) remains unsolved.

2.4.2. The networks of lamins

The nuclear lamina is a dense fibrillar structure underlying the inner nuclear membrane consisting of the nuclear lamin filaments and transmembrane proteins tethering the nuclear lamina to the nuclear membrane. According to the traditional model, two sets of parallel 10-nm-diameter intermediate filament –like structures are arranged at right angles to each other to form an orthogonal meshwork attached to the inner nuclear membrane with 50 nm repeat distance (Aebi *et al.*, 1986). This model is based on transmission electron microscopic images of detergent extracted metal shadowed nuclear envelopes. Field electron scanning electron microscopy (feSEM) has enabled the visualization of the nuclear lamina without detergent extraction and a recent paper describes a new model in *Xenopus* oocytes where the lamina formed by the B-type lamin LIII consists of a single set of tightly packed 8-10 nm thick filaments running parallel to each other with a 15 nm repeat distance (Goldberg *et al.*, 2008b). The orthogonality of the structures in the new model is due to the cross-connecting thin filaments with a diameter of 5 nm and 15 nm repeat similar to the thicker filaments (Goldberg *et al.*, 2008a). The A-type lamins are not as tightly associated with the nuclear membranes as the B-type lamins due to the lack of the isoprenylated residue at the C-terminus and it seems A- and B-type lamins form separate filamentous networks in the nuclear lamina. Ectopically expressed A-type lamins seem to top the endogenous B-type lamin network excluding areas of NPCs (Goldberg *et al.*, 2008a). Although being separate networks, there is evidence that these structures comprising of either A-type or B-type lamins are interacting since the removal/absence of A-type lamins disturbs B-type lamin distribution and vice versa (Shimi *et al.*, 2008).

The majority of the nuclear lamins are associated with the nuclear lamina. There is, however, a long established model also for an intranuclear network of lamins. These

intranuclear lamins are suggested to function in e.g. DNA replication and transcription and some of these lamins may also represent lamin intermediates. The intranuclear network of B-type lamins is rather static whereas intranuclear A-type lamins are highly mobile (Shimi *et al.*, 2008). The B-type lamins have been speculated to form an intranuclear structure or attach to an underlying immobile structural component of the nucleus. This speculation is also supported by the fact that the silencing of lamin B1 results in the reorganization of other nuclear lamins and increased mobility of lamin A whereas the silencing of other lamins does not affect lamin B1 distribution (Shimi *et al.*, 2008).

2.4.3. The function of lamins

The role of lamins in the regulation of the size, the shape and the stability of the nucleus is a well established conception (Reviewed by Dechat *et al.*, 2010). This is supported by several studies: e.g. lamin A deficient mouse embryonal fibroblasts (MEFs) display increased deformability of the nuclei and decreased viability under mechanical constraint when compared to control MEFs (Houben *et al.*, 2009) and *Xenopus* oocyte germinal vesicles display increased stiffness following ectopic lamin A expression (Houben *et al.*, 2009).

Lamins act in the organization and function of the chromatin. Light microscopic observations suggest that there is a close association between the peripheral heterochromatin and the nuclear lamina (Fawcett, 1966). The α-helical rod domain of the lamins has been suggested to mediate this interaction either directly or indirectly (Glass *et al.*, 1993) and the binding may be mediated through the lamins associating with the histones (Goldberg *et al.*, 1999) or the DNA-binding matrix attachment regions (MARs) of the lamins (Luderus *et al.*, 1994). The origin of replication sites are stated to colocalize with the AT-rich MAR-binding sequences of DNA and lamins in a specialized manner: lamins A and C are present at the replicational foci in early replicating fibroblasts (Kennedy *et al.*, 2000) whereas these foci and lamin B colocalize in late S phase in mouse 3T3 cells (Moir *et al.*, 1994). Lamins interact with proliferation factors such as the proliferating nuclear antigen (PCNA) present at the replication foci suggesting a role for lamin in the regulation of the replication (Shumaker *et al.*, 2008). DNA repair function is also suggested as the disease causing mutations of lamins have been shown to have defective telomere function and DNA repair (Gonzalez-Suarez *et al.*, 2009a; Gonzalez-Suarez *et al.*, 2009b).

The lamins are frequently found in association not only with the gene-poor heterochromatin but also with the inactive genes. The artificial relocalization of chromosomes from the nuclear interior to the periphery of the nucleus by attaching them to the integral INM proteins inactivates some genes but not all (Finlan *et al.*, 2008). On the other hand, the artificial attachment of a genetic locus to lamin B1 does not affect its transcriptional activity (Kumaran and Spector, 2008). Lamins interact with several transcription factors either directly, e.g. c-Fos and oct-1, or indirectly via

interaction with several lamin-binding proteins e.g. LAP2α and pRb (Reviewed in Dechat *et al.*, 2010). Although lamin disruption has been shown to interfere with RNA polymerase pol II activity, the exact role of lamins in the transcriptional regulation remains unclear (Shimi *et al.*, 2008).

Nuclear lamina is considered analogous to the cytoskeleton involved in the regulation of many cellular functions e.g. the cell shape and motility. Lamins are connected to the cytoskeleton through the interaction with a protein complex LINC (Linker of nucleoskeleton and cytoskeleton) that forms when the integral SUN (Sad1p, UNC-84) domain containing proteins (e.g. SUN1, SUN2) of the INM interact in the intermembrane space with ONM transmembrane proteins nesprins (also known as Klarsicht, ANC-1 Syne homology or therefore KASH) (Crisp *et al.*, 2006). Similarly, a lamin interacting INM integral protein emerin has been shown to interact with nesprins in the NE lumen creating a second link between lamins and the cytoskeleton (Wheeler *et al.*, 2007; Zhang *et al.*, 2007). The nesprins bind directly to the cytoplasmic actin, intermediate filaments, microtubules and centrosomes constituting a link between the nuclear structure and the cytoplasmic structures (Wilson and Foisner, 2010).

2.4.4. Laminopathies

Diseases caused by mutations of *LMNA* gene are classically referred to as the laminopathies. Earlier it was considered that a single gene has one function and that the mutation of the gene can lead to one kind of disease: it is well known that for *LMNA* this is not a true conception. An autosomal dominant form of Emery-Dreifuss muscular dystrophy (EDMD) was the first discovered syndrome associated with *LMNA* mutation (Bonne *et al.*, 1999). Since 1999, a wide variety of diseases connected to lamins and lamin associated proteins have been described and over 400 different mutations have been described for *LMNA* alone (See LMNA mutation database at http://www.umd.be/LMNA/ and Worman *et al.*, 2010). Laminopathies can be divided into diseases affecting mainly striated muscle, e.g. EDMD and limb girdle muscular dystrophy, adipose tissue, e.g. partial lipodystrophy, peripheral nerve, e.g. Charcot-Marie-Tooth disease type 2B1, or multiple tissues resulting in progeroid phenotypes such as Hutchinson–Gilford progeria syndrome (HGPS) (Worman *et al.*, 2010). Hundreds of different mutations and several different tissue phenotypes of laminopathies have made it hard to understand the pathogenesis behind these diseases.

The mutation of *LMNA* most commonly associating with HGPS is a point mutation at the exon 11 resulting in the accumulation progerin, a farnesylated lamin A with deletion of 50 amino acids in the C-terminus of the protein (Eriksson *et al.*, 2003). This paper also noted the appearance of deforming nuclei in HGPS patients showing irregularity of the nuclear contour caused by nuclear blebs. Similarly, cells from HGPS patients carrying a mutation causing E145K amino acid substitution show accumulating changes in lamin B distribution, nuclear blebbing, changes in the heterochromatin distribution, centromere and telomere localization and premature

senescence (Taimen *et al.*, 2009). Also, the expression of progerin in human mesenchymal stem cells alters their differentiation pathways (Scaffidi and Misteli, 2008), which would explain the preferential affision of the mesenchymal tissues in this syndrome.

2.5. Programmed cell death

Programmed cell death (PCD, Lockshin and Williams, 1965) is originally referred to instances where sequential, characteristic events lead to the death of a cell. PCD can be classified according to morphological (apoptotic, necrotic, autophagic or linked to mitosis), enzymological (such as activation of certain nucleases or proteases) or immunological characteristics. Several different mechanisms have been described and probably the best classification divides the phenomenon into apoptotic and non-apoptotic cell death (Okada and Mak, 2004). The non-apoptotic cell death can be divided into several sub-groups such as anoikis, autophagy, cornification, mitotic catastrophe, necrosis, senescence but newer forms have been characterized later (Kroemer *et al.*, 2009).

Anoikis is the form of cell death where an anchorage dependent cell detaches from surrounding tissues and, in the absence of extracellular matrix signaling or cell-to-cell interactions, is destined to die (Frisch and Francis, 1994). Metastasizing tumor cells are a good example of cells evading anoikis as their removal from the original site of growth does not constitute a trigger for cell death.

Autophagy, a form of cell death often described as “self-cannibalism”, is a way of cells to eradicate unwanted proteins and organelle parts into membrane enclosed autophagosomes which are degraded by the lysosomal pathway (Kim and Klionsky, 2000). As a differentiation to apoptosis, cells dying by autophagy do not show chromatin condensation but rather a characteristic massive cytoplasmic vacuolization and have no association with phagocytes (Kroemer *et al.*, 2009).

Cornification is a specialized form of cell death occurring within the maturating squamous epithelium. The cell proliferation of the epidermis takes place at the basal layer. During maturation, the keratinocytes move up in the epithelium and lose their viability via cytoskeletal rearrangements and DNA degradation in a process termed cornification (Lippens *et al.*, 2005). The dead cell on the skin surface provide protection from the outside prior to desquamation.

The term mitotic catastrophe is used to explain a mechanism of a delayed mitotic-linked cell death resulting in typical morphological features such as the formation of multiple micronuclei. Mitotic catastrophe is usually the consequence of preterm or inappropriate entry into mitosis caused by chemical or physical stresses and is sometimes referred to as “reproductive death” as the cells usually remain viable for some time after cell death but are unable to generate viable progeny (Vakifahmetoglu

et al., 2008). There is however controversies whether mitotic catastrophe should be considered a separate entity because late stages of this form of cell death may resemble apoptosis or necrosis (Kroemer *et al.*, 2009).

Necrosis is morphologically characterized by cellular swelling, organelle swelling, plasma membrane rupture and loss of cellular contents to the surrounding tissues. Necrosis was long considered as unorganized, accidental form of cell death but evidence is accumulating that the cell death through the necrotic pathway is tightly regulated through biochemical signaling pathways (Golstein and Kroemer, 2007).

One feature of living cells is considered to be the replicative potential and benign cells have a limited potential for cell proliferation. This phenomenon was first discovered by Hayflick (1965): after a certain amount of cell divisions benign cells reach a state when they are no longer able to divide. This state of being a "living dead" is considered as the form of cell death termed senescence. In addition to limited replication potential, other causes for a cell to enter senescence are thought to connect to the risk of malignant transformation: cell encountering e.g. oxidative stress, DNA damage or telomere shortening may enter senescence (Campisi and d'Adda di Fagagna, 2007). Senescent cells adopt a flattened, enlarged morphology and exhibit specific molecular markers like senescence-associated β-galactosidase (Dimri *et al.*, 1995). The senescent cell death is thought to be mainly mediated via p53-pathway but the pRb-pathway is also affected (Vaziri and Benchimol, 1999; Helmbold *et al.*, 2006): upon stress, such as DNA damage, p53 is no longer targeted for degradation by MDM2 mediated ubiquitination and the p53 is able to activate cyclin dependent kinase inhibitor p21. Similarly, increased cyclin dependent kinase inhibitor $p16^{INK4a}$ levels lead to pRb hypophosphorylation. The hypophosphorylated pRb binds E2F leading to the repression of the cell cycle progression. The degree of DNA damage seems to be important as major DNA damage is known also to activate the intrinsic apoptotic pathway (described later in the text). The senescent cells have exited cell cycle permanently but are still able to communicate with surrounding cells: accumulating senescent cells release various degradative proteases, growth factors and inflammatory cytokines that compromise the function of non-senescent neighboring cells.

PCD is a physiological phenomenon necessary for functions, such as the renewal of tissues and the removal of unwanted cells during organogenesis. Cell death, independent on the type of it, is also needed to remove of unnecessary or non-functional cells from an organism. This constitutes a threat as the errors in the mechanisms mediating PCD may enable increased cell death resulting in the removal of cells needed for the normal function such as the beta cell apoptosis inducing diabetes (Thomas *et al.*, 2009) and, on the other hand, decreased cell death is considered a common hallmark of cancer formation as harmful cancer cell are not eliminated and continue to grow (Hanahan and Weinberg, 2000).

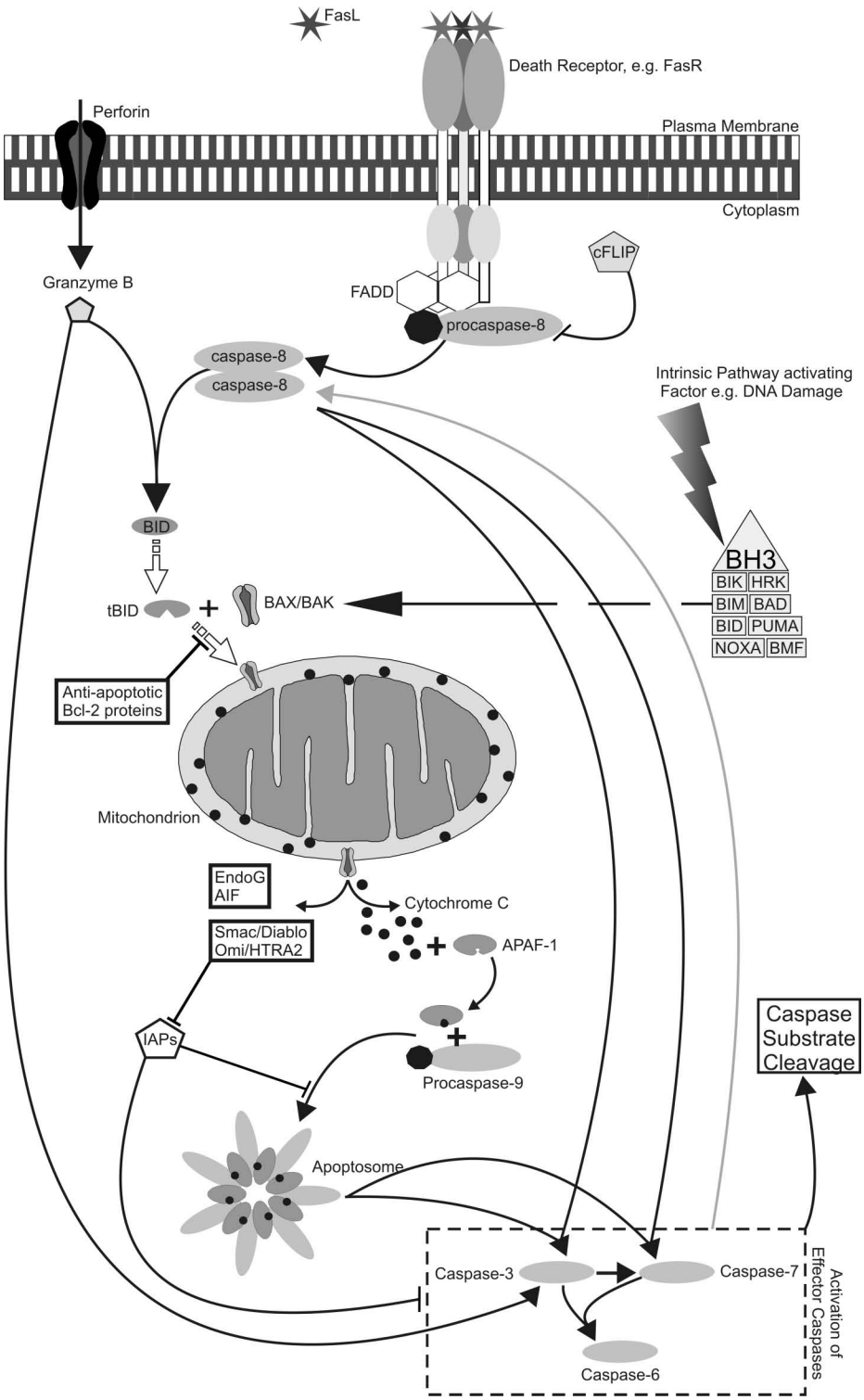


Figure 6. Apoptotic pathways. *See text for details.*

2.5.1. Apoptosis

Apoptosis is characterized by chromatin condensation, shrinking of the cell, membrane blebbing and, finally, the formation of membrane-enclosed vesicles termed apoptotic bodies (Kerr *et al.*, 1972). The paper by Kerr et al (1972) was the first to introduce the concept of apoptosis. However, a German biologist Walther Flemming had described similar morphological changes in cells almost a century earlier in 1885 (Reviewed by Lockshin and Zakeri, 2001). Characteristic of apoptosis, the apoptotic bodies are recognized and eaten by the phagocytes without an inflammatory response of the surrounding tissues. During embryonic development apoptosis is activated in order to remove unwanted cells to achieve successful organogenesis. This phenomenon was first described in the development of the nematode *Caenorhabtidis elegans* which undergoes apoptotic execution of the defined 131 cells out of its 1090 cells during its development to a mature 959 cell nematode. Sydney Brenner envisioned *C. elegans* to be an ideal organism to study this developmental condemnation of cells (Brenner, 1974), John Sulston noted the invariability in the particular cells that were doomed to die during *C. elegans* development (Sulston, 1976) and Robert Horvitz mutated genes of the nematode to identify apoptosis regulating genes (Ellis and Horvitz, 1986) later turning out to be homologues for the proteases regulating the mammalian apoptosis machinery. The three men received the 2002 Nobel Prize in Physiology or Medicine for their achievements in the cell death field.

2.5.2. The apoptotic pathways

In addition to its activation in developmental processes such as organogenesis, apoptosis can be triggered by several stimuli in the need to eliminate harmful cells. Three overlapping apoptosis activating pathways can be distinguished.

The death receptors are integral proteins of the plasma membrane that mediate the signals of the death ligands when the extrinsic pathway is activated (Fig. 6). Death receptors, e.g. FasR/CD95, TNFR (tumor necrosis factor receptor) and TRAILR (TNF related apoptosis inducing ligand receptor), are part of the TNF receptor superfamily containing a death domain (DD) in their intracellular part. The binding of the death ligand to the receptor induces trimerization of the receptor and the binding of the adaptor molecule FADD (Fas-associated protein with a death domain) to the DD and recruitment of procaspase-8 to the receptors via its interaction with FADD. This complex termed DISC (Death-inducing signaling complex) of the trimerized receptor, FADD and procaspase-8 is needed for the initiator caspase-8 activation. Active caspase-8 cleaves bcl-2 homology domain 3 (BH3)-only protein BID (BH3 interacting domain death agonist) to its active counterpart tBID (truncated BID) which enables the recruitment of BAX/BAK (Bcl-2-associated X protein/Bcl-2 homologous antagonist killer) to the mitochondrion outer membrane and the release cytochrome c and other factors, e.g. endonuclease G, apoptosis inducing factor (AIF), Smac/Diablo (Second

mitochondria-derived activator of caspase/direct IAP binding protein with low pI) and Omi/HTRA2 (High-temperature requirement protein A2) from the intermembrane space of the mitochondrion. The human homologue for Ced-4 protein needed for the apoptosis in *C. Elegans* turned out to be Apaf-1 (apoptotic protease activating factor). Apaf-1 binds cytochrome c via its WD40 domain to form a complex, which is able to recruit caspase-9 in the presence of dATP/ATP. The interaction is mediated via caspase recruitment (CARD) domains present in both procaspase-9 and Apaf-1. Apaf-1-cytochrome c-procaspase-9 complex termed the apoptosome contains approximately ~7 Apaf-1-cytochrome c complexes and similar amount of procaspase-9 molecules enabling the autocatalytic activation of caspase-9. Active caspase-9 cleaves and activates the effector caspases, caspases 7 and 3, responsible for the cleavage of apoptotic substrate proteins.

The cells can also activate the apoptotic machinery from within the cell. Harmful events, such as DNA damage or toxic chemicals may contribute to the activation of the intrinsic pathway. The activation is mediated via bcl-2 family proapoptotic BH3-only proteins (BIK, HRK, BAD, BIM, BID, PUMA, NOXA, BMF). Similarly to BID, the other BH3-only proteins mediate either directly or indirectly the cytochrome c release by facilitating the BAX/BAK oligomerization at the mitochondrial outer membrane and, thereafter, the apoptosome formation. BAD phosphorylation and dephosphorylation regulates its activity in response to trophic factors to promote cell death or survival (Danial, 2008) whereas the expression of both PUMA and NOXA are under the transcriptional regulation of p53 when the cell faces DNA damage (Oda *et al.*, 2000; Nakano and Vousden, 2001).

Additional apoptotic pathway is activated by cytotoxic granzyme B granules released by the cytotoxic T lymphocytes and natural killer cells. In addition to granzyme B the granules contain perforin needed to the release of granzyme B into the cells where it directly cleaves BID activating the mitochondrial apoptotic pathway and also directly activates the effector caspase, caspase-3 and apoptotic target proteins such as lamin B and PARP-1.

2.5.3. Caspases

Ellis and Horvitz (1986) initially identified *ced-4* and *ced-3* as the genes responsible for mediating the death pathways in *C. elegans*. Later, *ced-9*, a gene preventing cell death, was identified as a regulator and suppressor of cell death in *C. elegans*. The cloning and characterization of *ced-3* led to the discovery of the mammalian protease homologues for *ced*s: the mammalian interleukin 1β converting enzyme (ICE) was identified as the first mammalian homologue for the protein encoded by *ced-3* (Yuan et al., 1993).

In fact, ICE (or caspase-1) was the first identified member of the mammalian apoptotic proteases termed caspases, a family of proteases dependent on the cysteine nucleophile

to cleave motifs possessing aspartate (Thornberry and Lazebnik, 1998). Caspases are expressed as inactive zymogens possessing a large and a small subunit preceded by an N-terminal prodomain. They are activated by cleavage via upstream proteases or by auto- and transactivation (Hengartner, 2000): the active caspase derived from two zymogens is a heterotetramer composed of two large and two small subunits possessing two active cleaving sites. Based on the length of the prodomain at the N-terminus, caspases are divided into two groups. A long prodomain usually contains several protein-interaction sites important for the caspase activation and the long prodomain containing initiator caspases, e.g. caspase-9 and caspase-8 (also termed FLICE for FADD-like ICE) are capable of autocatalytic activation when recruited to the death domains where their activation initiates the cascade of caspase activation. The effector caspases (caspases 3, 6 and 7) possessing a short prodomain are activated upstream via cleavage by initiator caspases. Nearly 400 caspase substrate proteins have been identified by 2007 (Lüthi and Martin, 2007) and the number is growing. The cleavage of substrate proteins such as structural proteins is believed to result in the characteristic morphological features of apoptosis.

2.5.4. Other players of apoptosis

In order to prevent unnecessary apoptosis, faulty caspase activation is tightly controlled by different mechanisms. The inhibitor of apoptosis proteins (IAPs) act in different ways in the inhibition. E.g. the X-chromosome related IAP (XIAP) inhibits directly the cleavage action of caspases 3 and 7 (Deveraux *et al.*, 1997). Cellular FLICE inhibitory protein (cFLIP) is a protein homologous to caspase-8/FLICE that interacts with both the caspase-8 and the adaptor protein FADD inhibiting the Fas-mediated activation of caspase-8 (Irmler *et al.*, 1997). The binding of IAPs to their targets is overcome by the IAP regulatory proteins Smac/Diablo and Omi/HtrA2 which are released from the mitochondrial intermembrane space during apoptosis (Du *et al.*, 2000; Verhagen *et al.*, 2000; Hegde *et al.*, 2002).

The *Ced-9* encoded protein was noticed to act as an inhibitor of apoptotic cascades and this protein is homologous to the mammalian bcl-2 (B-cell lymphoma 2) protein first identified as a proto-oncogene in follicular B-cell lymphoma. Similarly to ced-9 protein, bcl-2 proved not to promote cell proliferation but instead to block the apoptotic cell death. A whole family of bcl-2 proteins has been characterized: the members may possess pro-apoptotic or anti-apoptotic properties and can be divided into three subclasses defined by the homology shared by the four bcl-2 homology domains (BH)-domains. The antiapoptotic bcl-2 proteins (Bcl-2 and bcl-X_L) are characterized by the presence of BH4-domain in their structure. Multidomain proapoptotic members (BAK and BAX) contain only BH1-3 domain whereas the group of BH3-only proteins are always proapoptotic. The antiapoptotic properties of the BH1-4 containing proteins may rely on the binding of BH-3 proteins: The X-ray crystallographic studies reveal that the BH1-3 domains of Bcl-X_L form a hydrophobic pocket which is able to bind BH3-only proteins (Muchmore *et al.*, 1996; Petros *et al.*, 2000). The binding of the

BH3-only proteins to Bcl-2/Bcl-X_L is mainly thought to act as a way to sequester unwanted proapoptotic stimuli away from the cytosol (Danial and Korsmeyer, 2004; Alberts *et al.*, 2008) but it has also been suggested that, upon activation, the binding of excess BH3-only proteins may neutralize the antiapoptotic properties of Bcl-2 and Bcl-X_L (Cory *et al.*, 2003). The BH3-only protein activation either directly or indirectly leads to the BAX/BAK multimerization at the mitochondrion outer membrane leading to release of cytochrome c and the activation of the intrinsic apoptotic pathway.

2.5.5. The apoptotic degradation of the nucleus

The condensation and the fragmentation of the nucleus are major hallmarks of apoptosis. Upon caspase activation, the degradation of DNA occurs at the A/T rich MAR regions producing a typical ladder of fragments seen in the gel electrophoretic analysis of the apoptotic DNA. The major DNase responsible for the DNA degradation, CAD/DFF40 (caspase activated DNase or DNA fragmentation factor 40), is normally controlled by its binding partner ICAD/DFF45 (Inhibitor of CAD/DNA fragmentation factor 45) but the cleavage of ICAD/DFF45 by caspase-3 results in the liberation of CAD and subsequent DNA cleavage (Enari *et al.*, 1998; Sakahira *et al.*, 1998). Supporting this, MCF-7 breast cancer cell line defective in caspase-3 due to a point mutation of *CASP-3* gene do undergo apoptosis but lack some common features of apoptosis including the formation of apoptotic bodies and the oligonucleosomal DNA fragmentation (Jänicke *et al.*, 1998). ICAD/DFF45 is needed for the proper function of the DFF40 as it is suggested to function as a molecular chaperone enabling the proper folding of CAD/DFF40 but ICAD/DFF45 deficient mice still show some residual nuclease activity (Zhang and Xu, 2000). Some caspase-independent apoptotic degradation of the DNA has been attributed to mitochondrial proteins, apoptosis inducing factor (AIF) and endonuclease G, released from the mitochondrion simultaneously with cytochrome c (Susin *et al.*, 1999; Li *et al.*, 2001).

DNA, NuMA, lamins and several other nuclear factors are targeted for apoptotic degradation following apoptosis induction. Some of them are known structural proteins such as lamins, some are involved in the apoptotic execution such as the apoptotic nucleases. The exact role for NuMA cleavage is not clarified thoroughly but it may be involved in the structural degradation of the nucleus or in the disruption of the interaction between NuMA and DNA.

2.5.5.1. The apoptotic cleavage of NuMA

The nuclear matrix and its components are major targets for the apoptotic disintegration of the nucleus and the cleavage of NuMA is needed for the proper apoptotic nuclear disintegration. Initial finding suggested NuMA to be cleaved into approximately 200 kDa and 48 kDa fragments (Weaver *et al.*, 1996) in dexamethasone treated thymocytes. The same study showed that NuMA is phosphorylated in apoptotic

cells and that the cleavage of NuMA precedes the apoptotic DNA ladder formation. The apoptotic phosphorylation was speculated to trigger a conformational change within the protein targeting it to proteolytic cleavage. This was speculated thereafter to alter the interactions of NuMA with other nuclear proteins. Similarly, Hsu et al. (1996) also showed the appearance of a ~ 180 kDa truncated form in 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole treated HeLa cells and in camptothecin, staurosporine, cycloheximide, and calcimycin treated HL60 cells where the extent of NuMA cleavage product amount was comparable with the degree of oligonucleosomal laddering. Fas-antibody treated apoptotic Jurkat T cells first produced both 190 kDa and 180 kDa NuMA fragments which were further processed to 180 and 160 kDa fragments and the cleavage coincided the nuclear degradation (Casiano *et al.*, 1996; Taimen and Kallajoki, 2003). Hirata et al. (1998) used recombinant caspases to show that NuMA is cleaved by caspases 3, 4, 6 and 7 *in vitro* suggesting that caspases could be responsible for the apoptotic cleavage of NuMA. Similarly, Taimen and Kallajoki (2003) also showed that the specific inhibition of caspases 3, 6 or 8 led to a decrease in cleavage of NuMA in FasL+PD98059 treated HeLa cells whereas caspase-3 deficient cells MCF-7 were unable to cleave NuMA following staurosporine treatment. The induction of apoptosis in the presence of Ac-DEVD-CHO (caspase-3 inhibitor), z-VAD-fmk (pancaspase inhibitor), Ac-VEID-CHO (caspase-6 inhibitor), Ac-DMQD-CHO (caspase-3 inhibitor), TPCK (chymotrypsin inhibitor) inhibits NuMA cleavage whereas Ac-YVAD-CHO (Caspase-1 inhibitor), TLCK (inhibitor of trypsin and other serine proteases) and E-64 (inhibitor of cystein proteases) had little or no effect on the cleavage of NuMA (Greidinger *et al.*, 1996; Gueth-Hallonet *et al.*, 1997; Hirata *et al.*, 1998).

Gueth-Hallonet at al. (1997) generated different mutated constructs of NuMA and BHK cells transfected with these constructs were treated with staurosporine or hydroxyurea to induce apoptosis. They were able to map the apoptotic cleavage site of NuMA to 1701-1725 amino acid residues at the border of the α-helical rod domain and the C-terminal globular tail. A single amino acid mutation at the suggested site at the residue 1705 did not, however, prevent the apoptotic cleavage of NuMA. The junction between the central coiled-coil domain and the C-terminal globular head contains four potential apoptotic cleavage sites (Lin *et al.*, 2007). Importantly, the deletion of the region containing the proposed apoptotic cleavage sites (residues 1701–1828) in NuMA protects cells from the apoptotic disruption of the nucleus. The latter study also showed that the transfection of a tail-less NuMA, a mutant mimicking the N-terminal cleavage fragment, induces chromatin condensation and activates the death machinery in highly apoptosis-resistant 293T cells carrying the SV40 large T antigen. This may be due to various reasons: this NuMA construct lacks several functionally important sites, such as the MT binding domain (Fig. 2) and a potential region for NuMA-DNA interaction (See fig. 2 and Radulescu and Cleveland, 2010). The authors suggest that the apoptotic phenotype caused by this mutant protein resembling the cleaved form of NuMA may be due to the potential tendency of this mutant protein to compete with the full length native protein, leading to the collapse of the nuclear architecture and eventually to the apoptotic phenotype. On the other hand, the lack of the MT binding

domain may result in mitotic defects causing the apoptotic phenotype either directly through mitotic catastrophe or through inappropriate completion of mitosis leading to abnormalities in the chromosome content and defective consequent mitosis. The immunofluorescense analysis of apoptotic HeLa cells showed NuMA to be excluded from the apoptotic chromatin containing apoptotic bodies (Gueth-Hallonet *et al.*, 1997) and this dissociation is prevented in caspase-3 deficient MCF-7 cells (Taimen and Kallajoki, 2003). Later, the analysis of activated peripheral blood mononuclear cells treated with UV-B irradiation or staurosporine showed the absence of NuMA in the isolated apoptotic bodies (Schiller *et al.*, 2008). All the three studies (Gueth-Hallonet *et al.*, 1997; Taimen and Kallajoki, 2003; Schiller *et al.*, 2008) showing the exclusion of NuMA from the apoptotic bodies were made using an antibody recognizing the N-terminal part of NuMA whereas the fate of the C-terminal cleavage fragment remains unsolved.

2.5.5.2. The apoptotic cleavage of lamins

The disassembly of the nuclear lamina is a key feature during the apoptotic nuclear disintegration. Caspase-6 has been shown to be responsible for the apoptotic cleavage of A-type lamins (Orth *et al.*, 1996; Takahashi *et al.*, 1996; Slee *et al.*, 2001) which has been proposed as the main mechanism for lamina disassembly. The A-type lamins have a conserved caspase-6 target sequence VEID located at residues 227-230. This region is known to contain the chromatin-binding sites of lamin A (Glass et al., 1993), and is also involved in the formation of the nuclear lamina structure (McKeon, 1991). Lamin B can also be degraded by caspase-6 at residues 227-230 but a study using either caspase-3 or caspase-6 immunodepleted cell-free extracts showed that the proteolysis of lamin B was unaffected by the removal of caspase-6 whereas caspase-3 cleaves efficiently lamin B during the execution phase of apoptosis (Slee *et al.*, 2001).

Lamins are phosphorylated both in mitosis and in apoptosis. In mitotic cells, lamins are dispersed throughout the cytoplasm following phosphorylation by $p34^{cdc2}$ kinase (Peter *et al.*, 1990). Phosphorylation of lamins has been speculated to interfere with the head-to-tail interactions responsible for the fibrous architecture of the lamina and, thus, result in depolymerisation. There are also reports on different protein kinase C (PKC) isoforms phosphorylating lamins during cell cycle as well as in apoptosis. In ara-C-treated apoptotic HL-60 cells PKC-δ is activated by caspase-3 which leads to the translocation of the catalytically active fragment of PKC-δ to the lamina and the phosphorylation of lamin B (Cross *et al.*, 2000). This phosphorylation is delayed by a target specific inhibition of PKC-δ. Another study (Shimizu *et al.*, 1998) implies PKC-α to be the kinase responsible for lamin B phosphorylation: in camptotecin treated HL-60 cells the phosphorylation of lamin B preceding the lamina disintegration was inhibited by immunodepletion of PKC-α and the phosphorylation was restored by the addition of recombinant active PKC-α. The exact role of the apoptotic phosphorylation of lamins is currently unknown. It has been speculated that the phosphorylation may

loosen the tight lamina to ensure proper cleavage of lamins by caspases (Cross *et al.*, 2000).

3 AIMS OF THE STUDY

The focus in this thesis work is in the role of NM proteins in the apoptotic disruption of an interphase nucleus, and, on the other hand, in the overall maintenance of the nuclear structure. The main emphasis is on the NuMA protein but other NM proteins are used for the comparison of different nuclear domains, e.g. lamins for the nuclear lamina in the nuclear periphery and fibrillarin for the nucleoli.

There is contradictory evidence whether NuMA functions as a structural component of the controversial “nuclear matrix”. Some studies suggest that NuMA functions in the chromatin organization and gene regulation and others that it is retained in the nucleus to prevent its harmful interaction with cytoplasmic factors such as microtubules.

The specific aims of this doctoral thesis are:

1. To characterize the apoptotic cleavage of NuMA and the role of the cleavage of NuMA in the process of the apoptotic disintegration of the nuclear structure.

2. To study the effect of NuMA silencing on the structure and function of the cell.

3. To clarify the role of NuMA in the chromatin organization.

4 MATERIALS AND METHODS

4.1. Cell lines, constructs and antibodies

Table 2. Cell lines

Cell line	Origin	Modification	Donator	ATCC	Ref.
MCF-7	Mammary adenocarcinoma cell line		Prof. Pirkko Härkönen, University of Turku, Finland	HTB-22	
MCF-7c3	Mammary adenocarcinoma cell line*	pBabepuro/ caspase 3 plasmid*	C.J. Froelich, North Western University Medical School	*	Yang et al., 2001
MCFv	Mammary adenocarcinoma cell line*	Empty pBabepuro plasmid*	C.J. Froelich	*	Yang et al., 2001
MCF-10A	Benign mammary gland epithelial cell line		Docent Marko Kallio, University of Turku	CRL-10317	
HeLa	Cervical adenocarcinoma cell line		Prof. John Eriksson, Åbo Akademi, Finland	CCL-2	
HaCaT	Human immortalized non-tumorigenic keratinocyte cell line		Prof. Stina Syrjänen, University of Turku		
ROL-SED	Benign skin fibroblast		Prof. Risto Penttinen, University of Turku		

*A cell line derived from stably transfected MCF-7 cell line.

Table 3. Constructs

Construct	Vector	Donator	Reference
NuMA-N1-GFP	pEGFP-N1 (BD Biosciences Clontech, CA, USA).	Prof. Duane Compton, Darthmouth Medical School, NH, USA	Kisurina-Evgenieva et al., 2004
NuMA-C3-GFP	pEGFP-C3 (BD Biosciences).	Prof. Duane Compton	Kisurina-Evgenieva et al., 2004
Lamin B Receptor	pEGFP-N1 (BD Biosciences)	Prof. Eija Jokitalo, University of Helsinki, Finland	Ellenberg et al., 1997
pssGFP-KDEL	pEGFP-N1 (BD Biosciences)	Prof. Eija Jokitalo	Kuokkanen et al., 2007

Table 4. SiRNA oligonucleotides

siRNA target	siRNA sequence	Corresponding bases of the *NuMA1* gene	Producer
NuMA	CUAGCUGAGCUCCAUGCCA*	2792-2810	Dharmacon, Chicago, IL, USA
NuMA1	GGGAACAGUUUGAAUAUAA*	519-537	Dharmacon
Non-targeting 1	-	-	Dharmacon
Non-targeting 2	-	-	Dharmacon

* Target specific siRNA duplex sequences were of type $AA(N_{19})dTdT$ (N is any nucleotide) in order to obtain 21 nucleotide sense and anti-sense strands and 2-nt overhangs.

4.2. Antibodies

Mouse monoclonal NuMA antibody (used in I-III; epitope: amino acids 256-267; clone: SPN-3) was used for immunofluorescence (IF) and western blotting (WB) (Kallajoki *et al.*, 1991; Kallajoki *et al.*, 1993). Rabbit polyclonal NuMA antibody (used

in II-III, epitope: full length recombinant protein) used for immunofluorescence microscopy (IF) and western blotting (WB) was obtained from Abcam, UK.

Goat polyclonal lamin B antibody (used in I-III and fig. 7; epitope: C-terminus of the protein; clone: c-20) used for IF and WB was obtained from Santa Cruz Biotechnology, CA, USA. Mouse monoclonal Lamin A/C antibody (used in I-III; clone: 636) used for IF and WB was obtained from Novocastra Laboratories ltd, UK. Rabbit polyclonal fibrillarin antibody (used in II-III) epitope: amino acids 1-100) used for IF and WB was obtained from Abcam. Goat polyclonal GAS41 antibody (used in III; epitope: N-terminus of GAS41; clone: N14) used in IF was obtained from Santa Cruz Biotechnology.

Rabbit polyclonal pericentrin antibody (used in fig. 11; epitope: amino acids 100-600) used for IF was obtained from Abcam. Rabbit polyclonal tubulin antibody (used in fig. 11; epitope: pig brain tubulin) used for IF was obtained from Mary Osborn (Max-Planck-Institute for Biochemical Chemistry, Germany) (Osborn *et al.*, 1978). Mouse monoclonal BrdU antibody (used in III, clone: BU-1) was obtained from Amersham Biosciences, UK.

Rabbit polyclonal PARP-1 antibody recognizing the p85 apoptotic cleavage fragment (used in I; clone: G7341; epitope: p85 apoptotic cleavage fragment of PARP-1) used for IF was obtained from Promega, WI, USA. Mouse monoclonal PARP-1 antibody (used in I-III; epitope: amino acids 216-375; clone: c-2-10) used for WB was obtained from Sigma-Aldrich, MO, USA.

Rabbit polyclonal cleaved caspase-3 antibody (used in I; epitope: aminoterminal to Asp175 of caspase-3) used for IF was obtained from Cell Signaling Technology, MA, USA. Rabbit polyclonal caspase-8 (Neomarkers, CA, USA), rabbit polyclonal caspase-9 (Neomarkers), rabbit polyclonal caspase-6 (Neomarkers), rabbit polyclonal caspase-3 (BD Biosciences, CA, USA), mouse monoclonal caspase-7 (BD Biosciences) antibodies were used for western blotting in I.

Mouse monoclonal p53 (used in III; clone: DO-7) used for WB was obtained from BD Pharmingen, CA, USA. Mouse monoclonal pRb antibody (used in III; clone: G3-245) used for western blotting was obtained from BD Pharmingen. Mouse monoclonal antibody recognizing p53 phosphorylated at serine 15 (used in III; clone: 16G8) used in IF was obtained from Cell Signaling Technology.

4.3. Cell culture and cell synchronization (I-III)

MCF-7, HaCaT, HeLa and ROL-SED cells were cultured in DMEM (Gibco, Invitrogen, CA, USA) supplemented with 10% fetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) except if otherwise stated. In addition, MCF-7 cells were supplemented with 10 nM β-estradiol

(Sigma Chemical Co., MO, USA). MCF-7c3 and MCF-7v cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) and 1 mg/ml G418 (Sigma). MCF10A cells were grown in 1:1 DMEM:Ham´s F12 basal medium supplemented with 5% horse serum, 2mM L-glutamine, 10 μg/ml insulin, 5 μg/ml hydrocortisone, 20 ng/ml EGF and 100 ng/ml cholera toxin except if otherwise stated.

Cells were cultured in 50 mm Petri dishes or 6-well plates and split twice a week to a concentration of approximately $2x10^5$ cells/dish. In serum starvation experiments, normal growth medium was replaced with starvation media (growth medium supplemented with 0.1% FCS and 2 mM L-glutamine) 12 hours after plating and transfection was performed 24 hours after the beginning of starvation. For nocodazole block, cells were treated with growth medium containing 1 μg/ml nocodazole (Sigma) for 20-24 hours.

4.4. Transfections

4.4.1. SiRNA (II and III)

Target specific siRNA duplex sequences were of type $AA(N_{19})dTdT$ (N is any nucleotide) in order to obtain 21 nucleotide sense and anti-sense strands and 2-nt overhangs. The duplex targeted against NuMA, a ready-made siGenome NuMA1 oligonucleotide, a ready-made non-mRNA-targeting siRNA oligonucleotide numbers 1 and 2 or plain transfection reagent Oligofectamine (Invitrogen, CA, USA) were used. See 1.1.3 for sequence details. The final concentration for different oligos used was 100 nM. 24 hours prior to transfection, cells were divided into 6-well plates or 50 mm Petri dishes to a concentration of 70 000 to 300 000 cells per well/dish (confluence of 30-50%) with fresh growth medium excluding antibiotics. Transient transfection of siRNAs was performed using Oligofectamine. Optimem 1 medium (Gibco) was used as a diluent in the liposome complex formation.

4.4.2. GFP-transfection (II)

The full-length-NuMA constructs with either N-terminal or C-terminal GFP-label were used in this study (See table 3 for construct details). HeLa cells were plated to a confluence of 80% 24 hours prior to transfection. Fugene HD transfection reagent (Roche Applied Science) was used according to instructions by the manufacturer. DNA (μg) to transfection reagent (μl) ratio used was 1:2. Optimem 1 medium (Gibco) was used as a diluent in the liposome complex formation.

4.5. Induction and inhibition of apoptosis (I, II)

To induce apoptosis, cells were treated with 2 μM staurosporine (Sigma-Aldrich) or with 100 ng/ml of human Fas receptor IgM antibody (CH-11, MBL Medical & Biological Laboratories, Nagoya, Japan) in the presence of 30 μM MAPK kinase inhibitor PD 98059 (Calbiochem, La Jolla, CA) as described previously (Holmstrom *et al.*, 1999). To inhibit apoptosis, cells were treated in the presence of pancaspase inhibitor 100 μM bentsoylcarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk; R&D Systems, UK) or 100 μM caspase-3 inhibitor bentsoylcarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (z-DEVD-fmk; R&D Systems). All preceding reagents, apart from Fas receptor IgM, were dissolved in DMSO and added into cell culture coincidently with other reagents. Control samples were treated with an equal amount of DMSO.

4.6. Protein extraction (II)

The growth media were collected and the cells were trypsinized. The trypsin suspension was combined with the media and centrifuged at 1000 g for 5 minutes. The cells were subsequently washed with ice cold PBS and centrifuged for 5 minutes at 1000 g. Part of the PBS suspension was collected to produce a whole cell lysate by centrifugation at 12,000 g for 5 minutes. The cell pellet was prepared for electrophoresis as described later.

For soluble protein extracts, the cell pellets were resuspended into extraction buffer (0.5% Triton X-100 in PBS supplemented with Complete protease inhibitor cocktail; Roche Applied Science, Indianapolis, IN), vortexed briefly and incubated 5 minutes on ice. Cell suspension was then centrifuged for 5 minutes at +4ºC at 1000 g and supernatant was used as a soluble protein fraction. The insoluble cell pellet was directly resuspended into 100ºC SDS-PAGE electrophoresis sample buffer at a concentration of $2 \cdot 10^7$ cells/ml and sonicated for 5 seconds.

To further extract DNA binding proteins and NM fractions, the pellet produced as described above was first incubated in a buffer containing 1 μg/ml DNase I (Sigma), 50 μg/ml RNase A (Sigma) in PBS supplemented with Complete protease inhibitor cocktail for 20 minutes at room temperature. Next, NaCl in PBS was added to a final concentration of 500 mM and the suspension was incubated at room temperature for 20 minutes. The suspension was then centrifuged at 12000 g for 5 minutes and the supernatant and pellet were separated. The insoluble nuclear matrix proteins containing pellet was directly resuspended into 100ºC SDS-PAGE electrophoresis sample buffer at a concentration of $8 \cdot 10^7$ cells/ml and sonicated for 5 seconds.

Soluble proteins were precipitated from the supernatants using the methanol chloroform method described by Wessel and Flügge (1984). Protein pellet was resuspended into 1xSDS-PAGE electrophoresis sample buffer and heated for 5 minutes at 100°C. Both insoluble and soluble protein fractions were subjected to electrophoresis and immunoblotting.

The quantitation of the relative optical intensity (ROD) was made using the MCID 5+ image analysis software (InterFocus Imaging, Cambridge, UK). A horizontal analysis was made to quantitate similar molecular weight bands in different lanes. The ROD values were divided by the number of cells loaded on to the gel to obtain a ROD/cell value. The relative amounts of ROD/cell values for a distinct sample were combined and the percentage of protein in different fractions is shown in the table (II, fig. 3b).

4.7. Electrophoresis and immunoblotting (I-III)

Cells grown on Petri dishes were first scraped into the medium. Cell numbers were counted with a haemocytometer. Cell suspensions were centrifuged at 1000g for 5 minutes, washed with PBS and finally pelleted at 12000g for 10 minutes. Cell pellets were directly resuspended into 100^{o}C SDS-PAGE electrophoresis sample buffer at a concentration of 10^7 cells/ml and sonicated for 5 seconds. Samples were loaded on 5% gels for NuMA and PARP-1, on 10% gels for lamins and on 12% gels for caspases. Two parallel gels were run: one for Coomassie blue staining to determine equal loading and another for immunoblotting. Proteins were transferred electrophoretically to Optitran BA-S 83 reinforced nitrocellulose (Schleicher & Schuell, Dassel, Germany) in a buffer containing 25mM Tris, 192mM glysine, 0.05%SDS, and 10% methanol at 300mA constant current for 1.5 h. The transfer was controlled by Ponceau red staining. The filter was preincubated with 4% BSA in 0.2% Tween 20 in TBS (Tris-buffered saline; 20mM Tris-HCl, pH 7.4, 0.15mM NaCl) overnight and in 5% powdered milk in 0.2% Tween 20 in TBS for 1h and incubated with primary antibody diluted in 1% BSA in 0,2% Tween 20 in TBS overnight at 4^{o}C. The filters were washed three times with 0.2% Tween 20 in TBS and incubated for 1h at room temperature with peroxidase labeled secondary antibody. The filters were washed three times with 0.2% Tween 20 in TBS and the immunoreactivity was detected by using enhanced chemiluminescence reaction (ECL Western blotting detection system, Amersham). When reacted with another primary antibody filters were first washed twice with 0.2% Tween 20 in TBS, then incubated at 50^{o}C with stripping buffer (2% SDS, 100mM β-mercaptoethanol, 63mM Tris-HCl, pH 6.8) for 1h and washed three times with 0.2% Tween 20 in TBS.

4.8. Immunofluorescence microscopy (I-III)

For immunofluorescence microscopy cells were grown on 12 mm glass coverslips. At the time points indicated, coverslips were collected, fixed in 3,7% formaldehyde in PBS

(145 mM NaCl, 7,5 mM Na_2HPO_4, 2,8 mM NaH_2PO_4) for 15 minutes at room temperature, permeabilized with 0,1% Triton X-100 in PBS for 15 minutes and washed with PBS. Coverslips were incubated with primary antibodies diluted in 1% BSA in PBS for 2 h at room temperature, washed three times with PBS and incubated for 1 h with secondary antibody/antibodies. After being washed three times with PBS, cells were stained for DNA with Hoechst 33258 (1μg/ml in 25% ethanol/ 75% PBS) for 5-10 minutes and embedded in Mowiol 4.88 (Hoechst AG, Frankfurt, Germany). Samples were analyzed using Olympus BX60 fluorescence microscope (Olympus Optical Co. LTD, Tokyo, Japan) and AnalySIS software (Soft Imaging System) or Zeiss LSM510 confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany). To determine the amount of apoptotic morphological changes in chromatin structure, p85 fragment of PARP-1 and cleaved caspase-3 or lamin B granules containing cells, or TUNEL-positive cells, 400-600 cells were counted from at least five randomly selected areas of each sample.

4.9. DNA flow cytometry (III)

For DNA flow cytometric analysis, the media supernatants were collected and attached cells were harvested by trypsinization. Media supernatant and trypsin suspension were combined and the suspensions were centrifuged at 1000 g for 5 minutes and the supernatants were discarded. For fixation, 1 ml of -20°C 70% ethanol was added to the cell pellets dropwise while vortexing at medium speed. Cells were fixed over night in ethanol at - 20°C. The following day, cells were centrifuged at 300 g for 5 minutes and the pellet was resuspended in staining solution (0.3% Triton X-100, 4 mM citrate, 0.15 mM propidium iodide, 20 μg/ml RNase A) and stained for 30-60 minutes on ice. Cells were analyzed using FACSCalibur (BD Biosciences) and the cell cycle data analysis was performed with Cyflogic software (CyFlo Ltd, Turku, Finland) on a FL2-A histogram from gated single cells.

4.10. Senescence associated β-galactosidase staining (III)

Senescent cells were visualized by histochemical staining for senescence associated β-galactosidase at pH 6 (Dimri et al., 1995) using a senescence detection kit as described by the manufacturer (Sigma). All solutions for the kit were diluted or prepared in ultrapure filtered water. Cells grown on coverslips were first washed with PBS and, thereafter, fixed in 1x fixation buffer for 6-7 minutes. Following fixation, cells were washed three times with PBS and incubated in the staining solution at 37°C for 8 hours. After staining the staining mixture was replaced with 70% glycerol for visualization and the samples were analyzed with Olympus BX60 microscope.

4.11. BrdU staining (III)

For BrdU staining, cells synchronized with serum starvation were labeled with 10nM BrdU in 10% FCS containing growth medium 12 hours prior to fixation and fixed as described above. An antibody detection based kit for BrdU (RPN202, Amersham) was used. Following fixation, cells were treated with nuclease to produce single stranded DNA accessible to the antibody and stained with anti-BrdU antibody and secondary antibody to detect labeling. The samples were analyzed with Olympus BX60 microscope.

4.12. TUNEL staining (I)

For Terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-12-dUTP Nick-End Labeling (TUNEL) assay (DeadEnd™ Fluorometric TUNEL System, Promega) the cells grown on coverslips were fixed and permeabilized as described above. Samples were pre-equilibrated using equilibrating buffer for 10 minutes at room temperature and then incubated with buffer containing equilibrating buffer, nucleotide mix and TdT enzyme for 1 hour at 37^{o}C protected from daylight. The reaction was terminated by immersing the samples in 2xSSC (1xSSC; 0,15M NaCl, 0,015M trisodiumcitrate) for 15 minutes at room temperature. Samples were then washed three times in PBS for 5 minutes and then further stained with desired antibodies and Hoechst 33258 to detect DNA. The samples were analyzed with Olympus BX60 microscope.

4.13. Statistical analysis (II)

SAS/STAT software (SAS Institute Inc., NC, USA) was used to analyze the data for figure 6 in II. For the statistical analysis, the data from three experiments were pooled and Mantel-Haenszel khi-squared test was used. Distribution of the frequencies of normal morphology, the early and late apoptotic cells differed statistically significantly in the groups of NuMA siRNA and non-targeting siRNA treated cells after 6 and 12 hours of staurosporine treatment ($p<0.001$).

5 RESULTS

5.1. The role of caspase-3 in the apoptotic disintegration of the nucleus (I and II)

5.1.1. The apoptotic disruption of the nuclear chromatin structure depends on caspase-3 (I)

According to the previous knowledge, caspase-3 is responsible for the activation of caspase activated DNase (CAD) and, thus, the apoptotic fragmentation of the chromatin associated with the apoptotic nuclear disintegration (Enari *et al.*, 1998). MCF-7 cell line is an interesting model for studying apoptosis as it has lost *CASP-3* gene encoding the procaspase-3 due to a point mutation resulting in a shift in the reading frame (Jänicke *et al.*, 1998). MCF-7 cells undergo apoptosis but lack the typical apoptotic DNA laddering and the formation of the apoptotic bodies. To elucidate the role of caspase-3 in the degradation of the nuclear chromatin structure following chemically induced apoptosis, MCF-7 cells transfected with a plasmid containing *CASP-3* (MCF-7c3) or an empty vector (MCF-7v) were grown on coverslips, collected after 0, 4, 8, or 12 hours of staurosporine (STS) treatment, fixed, permeabilized and stained for DNA with Hoechst 33258. The chromatin changes following STS treatment were quantified in both cell lines: slightly clumpy chromatin and the disappearance of the nucleoli were regarded as the early apoptotic changes, chromatin marginalization as a late change and the formation of apoptotic bodies as the final stage (I, see fig.1 for representative images). As an early event, both cell lines showed typical cytoplasmic shrinkage (I, fig. 4) which was not prevented when the cells were treated with STS in the presence of a pancaspase inhibitor z-VAD-fmk (I, fig.5). The DNA staining differed significantly between the two cell lines. After 4 hours of STS treatment, 85% of the MCF-7v cells showed early apoptotic changes as the nucleoli disappeared and the chromatin became a clumpy and fibrous meshwork. About 10% of the MCF-7v cells showed unaffected chromatin following 12 hours of STS treatment (I, Fig.1). The number of the late apoptotic MCF-7v cells with condensed, occasionally marginalized chromatin grew in a time dependent manner but no apoptotic bodies were detected, which is consistent with an earlier study (Johnson *et al.*, 2000). The transfection of *CASP-3* gene into MCF-7 cells restored their ability to form apoptotic bodies. After 4 hours of STS treatment ~ 15% of MCF-7c3 cells showed late apoptotic changes and the number of cells presenting with apoptotic bodies grew in a time dependent manner being about 30% after 12 hours. The treatment with STS in the presence of a pancaspase inhibitor z-VAD-fmk drastically inhibited late apoptotic changes in both cell lines: only single MCF-7c3 cells showing apoptotic bodies were observed. Pancaspase inhibitor does not inhibit the early changes or the cytoplasmic shrinkage as in both cell lines ~ 80% of cells presented with early

chromatin changes at all the time points. The chromatin did not condense similarly to cells treated with STS alone but the nucleoli were difficult to identify and the chromatin appeared atypically organized which suggests that the early observed chromatin changes are due to some caspase-independent activity or the caspase inhibition is not complete.

To assess the degree of chromatin fragmentation following the caspase-3 reconstitution, both cell lines were processed for TUNEL (Terminal deoxynuclotidyl transferase- mediated dUTP Nick-End Labeling) staining following STS treatment. Following 12 hours of STS treatment 70% MCF-7v cells showed weak staining whereas approx. 60% of the MCF-7c3 cells were strongly positive for TUNEL (I, fig.3). The TUNEL staining of both cell lines was totally abolished when the cells were treated with STS in the presence of z-VAD-fmk. This supports, the previous knowledge that caspase-3 is needed for the proper DNA cleavage during apoptosis but the weak positivity in the MCF-7v cells suggests that some fragmentation also occurs in the absence of caspase-3. Taken together, these results confirm the previous observation that caspase-3 is important in the late stages of apoptotic chromatin changes and indispensable for the formation of the apoptotic bodies.

5.1.2. Staurosporine activates both initiator caspases 8 and 9 (I)

Staurosporine is a broad-spectrum kinase inhibitor and its function seems to be mediated via the intrinsic apoptotic pathway (See section Review of Literature, fig. 6). We tested the activation and cleavage of different caspases in STS treated MCF-7v and MCF-7c3 cells. Immunoblotting showed strong expression of procaspase-3 in MCF-7c3 cells, the amount of which decreased as the amount of active p17 and p12 sub-units accumulated in a time-dependent manner following STS treatment (I, fig. 6A). Both initiator caspases, caspase-8 and caspase-9, were processed to the active forms in MCF-7c3 cells as were also the effector caspases, caspase-6 and caspase-7 (I, fig. 6B). Only the amount of 35 kDa procaspase-7 was diminished in MCF-7v cells but the processed ~ 30 kDa or the active p20 form were not detected (I, fig. 6B). No processing of other caspases was detected in MCF-7v cells, suggesting that only minimal caspase activation takes place in the absence of caspase-3. Nearly 80% of the MCF-7c3 cells stained positive for the active caspase-3 after 12 hours of STS treatment but surprisingly a small number of MCF-7 cells (~ 4%) also stained positive for the active caspase-3 (I, fig. 2). To exclude the possibility of MCF-7c3 contamination in the MCF-7v cell cultures, three subclones were cultured from single cells and, similarly to the original MCF-7v cells, the subclonal cells showed weak staining for the active caspase-3 staining following STS treatment (data not shown). Active caspase-3 staining was mainly cytoplasmic in the early apoptotic cells but gathered to the nucleus and to surround the apoptotic bodies in late stage apoptotic MCF-7c3 cells (I, fig. 2). Poly(ADP)-ribose polymerase-1 (PARP-1), 116 kDa nuclear enzyme participating indirectly in the DNA repair machinery, is a widely used marker for caspase-3 activation (Tewari *et al.*, 1995). Approximately 68% of MCF-7v and 80% of MCF-7c3 cells stained positive for the cleaved form of PARP-1 after 12 hours of STS treatment

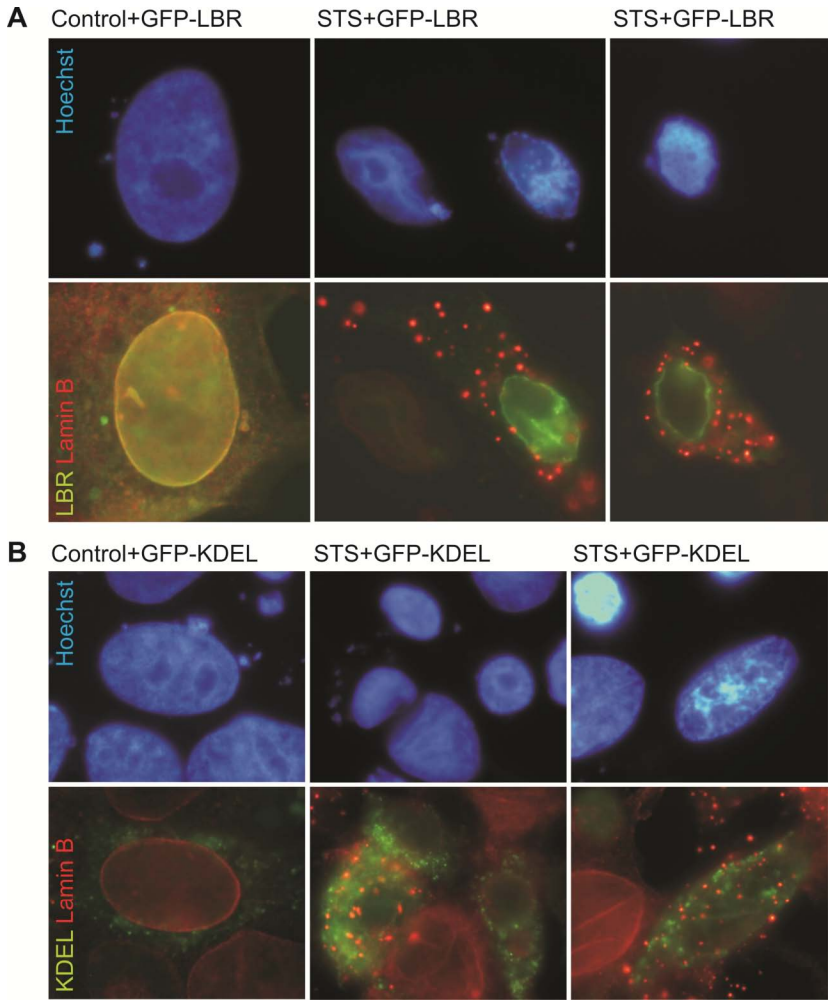
(I, fig. 2) suggesting that some other caspase than caspase-3 is responsible for PARP-1 cleavage. Taken together, there is a significant caspase-3 activation in MCF-7c3 cells which explains the activation of the extrinsic pathway initiator caspase caspase-8 in a downstream activation manner. Also importantly, some activation of caspases remains in the absence of caspase-3 in MCF-7v cells.

5.1.3. The cleavage of the nuclear lamina (I, II)

Nuclear lamina is an insoluble part of the nuclear matrix which is targeted to apoptotic degradation by caspases. The degradation of the nuclear lamina precedes the apoptotic fragmentation of DNA (Weaver *et al.*, 1996). Our group has earlier described the lack of degradation of both lamins A/C and B in STS treated MCF-7 cells (Taimen and Kallajoki, 2003). We tested the effect of caspase-3 reconstitution on the lamin cleavage: both MCF-7v and MCF-7c3 cells cleaved lamin B into a typical 45 kDa fragment but a more pronounced effect was detected in MCF-7c3 cells (I, fig. 6A). The lack of caspase-3 lead to defective lamin A/C cleavage: no apoptotic lamin A/C fragments were observed in MCF-7v cells whereas MCF-7c3 cells showed diminished amount of the intact 67 kDa protein (I, Fig. 6A). Similarly, neither MCF-7 nor HeLa cells showed the cleavage product of lamin A/C but lamin B was cleaved in both cell lines to the 45 kDa apoptotic fragment, and the cleavage is attenuated when the cells were treated in the presence of both STS and the pancaspase-inhibitor z-VAD-fmk (II, fig.1c).

Both lamins, lamin B and lamin A/C, localized mainly to the nuclear periphery in control MCF-7, MCF-7v, MCF-7c3 and HeLa cells (I, fig.4 and II, fig. 1 and data not shown). In apoptotic MCF-7v and MCF-7 cells, lamin B staining first appeared wrinkled in the early apoptotic cells. Interestingly, lamin B gathered thereafter to large cytoplasmic granules distinct from the nuclear structures (I, figs. 4A and 4B, II, fig. 5a). Typically, the nucleus lost approximately half of the lamin B staining when these granules were seen and 44% of the MCF-7v cells presented with this feature following 12-hour STS treatment (I, fig. 4C). In MCF-7c3 cells, lamin B gave similar wrinkled staining pattern in the early apoptotic cells but later mainly scattered around the cytoplasm or condensed to surround the apoptotic bodies. Only ~ 22% of MCF-7c3 cells contained cytoplasmic lamin B granules following STS treatment and the granules were significantly smaller than in MCF-7v or MCF-7 cells. The treatment of MCF-7v cells with STS in the presence of z-VAD-fmk blocked the formation of these lamin B granules (I, figs. 4C and 5A-B) whereas the treatment of MCF-7c3 cells with both STS and a specific caspase-3 inhibitor z-DEVD-fmk restored the formation of these granules (I, fig. 5C). To test whether other nuclear lamina components also were associated with these granules, we double stained the MCF-7v and MCF-7c3 cells with lamin A/C and lamin B antibody (I, fig. 4B). Similarly to lamin B, lamin A/C showed a wrinkled staining pattern in the beginning of apoptosis but later condensed around the apoptotic bodies and did not relocate to the cytoplasm.

During mitosis some of the nuclear envelope components are localized to the endoplasmic reticulum (ER) as shown by lamin B receptor (LBR) relocalization (Puhka *et al.*, 2007). We questioned whether LBR, an integral protein of the inner nuclear membrane, would relocalize with lamin B to the ER in the apoptotic MCF-7 cells. MCF-7 cells were transfected with GFP-fusion proteins encoding for either LBR or an ER-targeting peptide sequence KDEL. The transfected cells were treated with STS for 12 hours, fixed, permeabilized and stained for lamin B. In control cells, GFP-LBR localized to the nuclear periphery where it also remained in cells treated with STS for 12 hours (Fig. 7A). In both control and STS treated MCF-7 cells, GFP-KDEL localized in a punctated pattern to the cytoplasm but lamin B granules were distinct of the GFP-KDEL staining (Fig. 7B). These results suggest that lamin B distinct from other nuclear lamina structures is released from the nuclear lamina to the cytoplasmic granules in the absence of caspase-3 activity.



5.1.4. The caspase-3 dependent cleavage of NuMA during apoptosis results in relocalization of the N-terminal fragment whereas the C-terminus remains associated with the chromatin (I, II)

Several studies have shown that NuMA is specifically cleaved in different models of PCD (Casiano *et al.*, 1996; Weaver *et al.*, 1996; Gueth-Hallonet *et al.*, 1997; Hirata *et al.*, 1998; Bortul *et al.*, 2001; Taimen and Kallajoki, 2003; Lin *et al.*, 2007). Following apoptotic stimulus, NuMA is cleaved to an approximately 180-200 kDa fragment at the junctional zone between the central coiled-coil region and the C-terminal globular tail-region (Gueth-Hallonet *et al.*, 1997; Lin *et al.*, 2007). Earlier results suggested that MCF-7 cell line is unable to cleave NuMA following STS treatment (Taimen and Kallajoki, 2003). We therefore tested whether caspase-3 reconstitution restores the ability for NuMA cleavage. STS treated MCF-7c3 and MCF-7v cell lines were treated in the absence or presence of 100 μM z-VAD-fmk and prepared for immunoblotting. Both cell lines expressed the full-length 238 kDa NuMA polypeptide and additional 190 and 200 kDa polypeptides which correspond to the isoforms described by Tang et al. (1993). No cleavage of NuMA was detected in MCF-7v cells (I, fig. 6A) which is consistent with earlier results (Taimen and Kallajoki, 2003). MCF-7c3 cell, on the contrary, showed cleavage of NuMA to the ~ 180 kDa fragment and further smaller cleavage products (Fig. 8 and I, fig. 6A). Similarly, HeLa cells but not MCF-7 cells cleave NuMA into an approximately 180-200 kDa fragment and this cleavage is prevented in the presence of z-VAD-fmk (II, fig.1c).

In the untreated control cells, NuMA was dispersed throughout the nucleus excluding the nucleoli in interphase cells and gathered to the spindle poles during mitosis (I, fig. 4A and II, fig. 1a, upper panel, arrowheads). MCF-7v cells gradually lost NuMA staining in the early apoptotic cells with partially condensed chromatin. In MCF-7c3, a clear separation from the condensed chromatin was visible and the intensity of NuMA staining decreased significantly (I, fig. 4A). HeLa cells showed similar NuMA staining pattern as MCF-7c3 cells and at the end of apoptosis NuMA staining gathered to surround the apoptotic bodies (I, fig.4A and II, fig. 1a). As caspase inhibition did not completely abolish the apoptotic chromatin changes, cells treated with STS in the presence of 100 μM z-VAD-fmk were stained for NuMA and chromatin. NuMA staining changed to a more diffuse pattern when the nucleoli disappeared (I, fig. 5A).

Figure 7. The cytoplasmic Lamin B relocalization in apoptotic MCF-7 cells. *MCF-7 cells transfected with either GFP-tagged lamin B receptor (LBR) or GFP-tagged endoplasmic reticulum marker (KDEL) were treated 12 hours with STS to induce apoptosis. The cells were fixed and stained for lamin B and DNA (Hoechst 33258). A) The localization of lamin B (red) in MCF-7 cells transfected with GFP-tagged lamin B receptor (LBR, green). B) The localization lamin B (red) in MCF-7 cells transfected with GFP-tagged endoplasmic reticulum marker KDEL (green).*

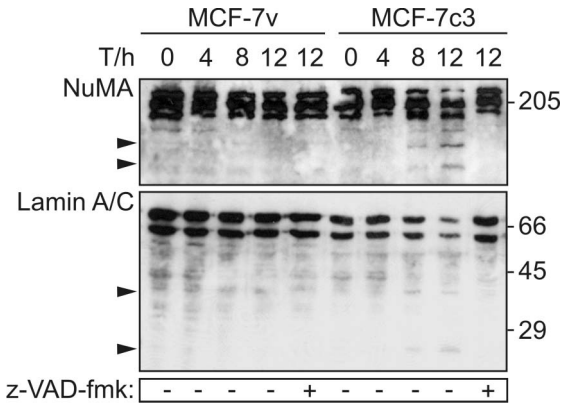


Figure 8. NuMA cleavage during apoptosis. *During STS induced apoptosis, NuMA is processed to a ~ 180 kDa fragment and further to smaller fragments in caspase-3 transfected MCF-7c3 cells. Lamin A/C is cleaved in minor proportions. The cleavage of both proteins is prevented when the cells are treated with STS in the presence of pancaspase inhibitor z-VAD-fmk. MCF-7v cells do not cleave NuMA.*

The NuMA/SPN-3 antibody recognizes the amino acid residues 256-267 near the transition of the N-terminal globular head and the coiled-coil domain of NuMA (Harborth *et al.*, 1995) suggesting that the observed 180 kDa fragment is the N-terminal cleavage fragment. To further assess the faith of the C-terminal NuMA during apoptosis, we transfected C- and N-terminally GFP-tagged NuMA-fusion protein encoding plasmids (Kisurina-Evgenieva *et al.*, 2004) to HeLa cells and treated the transfected cells with STS to induce apoptosis. Similar to SPN-3 staining both N- and C-terminally tagged GFP-NuMA constructs were nucleoplasmic during interphase cells (II, fig. 4a) and localized to the spindle poles during mitosis (II, fig. 4a, arrows). Interestingly, the C-terminally tagged GFP-NuMA co-aligned within the apoptotic bodies (II, fig. 4b, upper panel, arrowhead), while the N-terminal GFP construct was excluded from the highly condensed chromatin similarly to SPN-3 (II, fig. 4b, lower panel, arrowhead).

5.1.5. The apoptotic solubilization of the nuclear matrix: cleavage of NuMA results in the solubilization of the N-terminal cleavage product while the C-terminal fragment remains bound to chromatin (II)

NuMA is highly insoluble in interphase nuclei but its phosphorylation at G2/M transition leads to its mitotic solubilization (Kallajoki *et al.*, 1991; Compton and Luo, 1995). Interestingly, NuMA is also known to be phosphorylated during apoptosis

(Weaver *et al.*, 1996). The relocalization of the N-terminal apoptotic NuMA suggested that its mobility and/or solubility might have altered due to the cleavage. This hypothesis was addressed by serially extracting soluble proteins from apoptotic cells, from untreated cells and cells arrested in mitosis with 1 μM nocodazole. Only a minor fraction of NuMA and lamins was extractable from untreated control cells, whereas they were considerably more soluble in mitotic cells (II, fig. 2a). In both Fas-antibody and STS-treated apoptotic HeLa cells, the N-terminal cleavage product of NuMA was solubilized almost completely, while the full-length NuMA remained in the insoluble fraction (II, fig. 2a). The cleavage and subsequent solubilization was prevented by the pancaspase inhibitor z-VAD-fmk (II, fig. 2a). Lamins remained insoluble following the induction of apoptosis (II, fig. 2a, asterisks). Similarly to HeLa cells, all the studied proteins were partially solubilized following nocodazole treatment but remained uncleaved and insoluble in the apoptotic MCF-7 cells (II, fig. 2b).

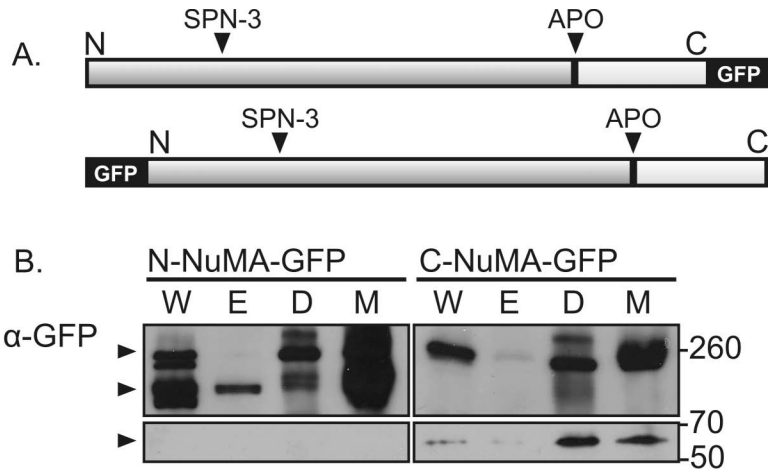


Figure 9. The N-terminal GFP-NuMA is solubilized following apoptotic cleavage. *HeLa cells were transfected with either C- or N-terminally tagged NuMA and treated with STS for 12 hours to induce apoptosis. A) The schematic drawing of the apoptotic cleavage of the NuMA polypeptide. The GFP-tag is only at either end of the polypeptide. B) The cleaved N-terminal GFP NuMA is found in the soluble fraction (E) whereas the C-terminal GFP-tagged cleavage product is found mainly in the DNA binding (D) and NM binding fractions (M).*

To further study the association and release of the cleaved NuMA protein from the chromatin fraction, the soluble, the DNA binding and the nuclear matrix (NM) associated proteins were separated from apoptotic and untreated HeLa cells by using a method modified from the classical NM preparation method used by Berezney and Coffey (1974). In untreated cells, NuMA was found in both the DNA-binding and the NM fractions (II, fig. 3a). Lamins were found in the DNA binding and the NM fraction

following apoptosis induction. In apoptotic cells, the DNA-binding fraction contained mainly full length NuMA, while the NM fraction contained both the cleaved and the full length protein. Quantification of the extracted proteins showed that the presence of all the proteins studied was diminished in the DNA binding fraction during apoptosis and approximately 80% of the N-terminal cleavage product of NuMA was in the soluble form (II, fig. 3b).

Our antibody, SPN-3, does not recognize the C-terminal part of NuMA associating with chromatin. We therefore used this extraction method to study the staurosporine-treated HeLa cells transfected with either the C- or N-terminally GFP-tagged NuMA fusion protein encoding plasmids to search for the C-terminal cleavage fragment. Extracted proteins probed with a GFP-antibody revealed an ~ 60 kDa protein band in both the DNA binding fraction and NM fraction but not in the soluble fraction (Fig. 9 and II, fig. 4c). This corresponds to the size of the predicted apoptotic C-terminal GFP-NuMA fragment. Taken together, the apoptotic cleavage of NuMA results in the release and solubilization of the N-terminal globular head and rod domains from the chromatin, whereas the C-terminal globular tail remains associated with the chromatin fraction.

5.2. NuMA silencing has no major effect on the interphase nuclear structure (III)

NuMA depletion has drastic effects on the dividing cell and the silencing of NuMA in HeLa cells has been shown to result in apoptosis (Harborth *et al.*, 2001). SiRNA method was also used in order to study the effect of NuMA silencing on the cell function and structure and to further characterize the apoptotic phenotype of the NuMA negative cells. An epithelial adenocarcinoma cell line MCF-7 with intact p53 and a keratinocyte cell line HaCaT known to be p53 negative due to a mutation in the *TP53* gene (Lehman *et al.*, 1993) were transfected with siRNA oligonucleotide targeted against NuMA, a non-targeting (NT) siRNA oligonucleotide or the transfection reagent Oligofectamine alone (OF), and followed for 3 days. The cells were stained for NuMA and chromatin to observe any structural changes but the chromatin structure remained unchanged in NuMA silenced cells although NuMA was successfully silenced in approximately 50% of the cells in both cell lines (III, fig. 1). The unchanged chromatin structure was further confirmed by staining the cells for chromatin markers H4K20m and Acetyl-H3, the distribution of which did not change (data not shown). To detect any structural changes of the nucleus, cells were stained for a marker for the nuclear lamina, lamin B, and a nucleolar marker fibrillarin. Both the nuclear lamina and structure of the nucleoli, however, remained unaffected (III, supplemental fig. 1). Stainings for cytokeratins (pan-cytokeratin antibody clone AE1/AE3 and Cam5.2) and tubulin were unable to show changes in these cytoplasmic filament networks in NuMA silenced cells (data not shown).

The silencing was further confirmed by western blotting. In MCF-7 cells, the level of NuMA expression was decreased while the amount other nuclear marker proteins,

lamin B and lamin A/C, remained unaffected (III, fig. 1C). Neither PARP-1 nor lamins, both known to be apoptotic target proteins, were cleaved in MCF-7 cells (III, fig. 1C). A faster migrating prominent pRb band appeared in NuMA silenced MCF-7 cells whereas control MCF-7 cells showed a cluster of several closely migrating pRb bands presumably representing different phosphorylated forms of pRb. In HaCaT cells, the level of NuMA expression was decreased while the amount of other nuclear marker proteins, lamin B and lamin A/C, remained unaffected. Lamins as well as NuMA itself, remained uncleaved whereas PARP-1 was cleaved to a minor degree in NuMA silenced and control samples after 72 hours presumably due to extended culture (III, fig. 1D). No hypophosphorylated form of pRb was detected in NuMA silenced HaCaT cells. Taken together, NuMA silencing has no major effect on the nuclear chromatin structure or other nuclear markers as the distribution and protein level of these markers remains unchanged.

5.3. NuMA depletion results in cell cycle arrest and mitotic defects (III)

5.3.1. Cell cycle arrest following NuMA depletion (III)

Observed hypophosphorylated form of pRb in MCF-7 cells suggested changes in the cell cycle progression as pRb is known to be phosphorylated at the G_1/S transition (Delston and Harbour, 2006). We quantified the amount of mitotic cells in siRNA treated MCF-7 and HaCaT cells. NuMA was successfully silenced in both cell lines (III, fig. 1C-D) The number of mitotic MCF-7 cells decreased following NuMA silencing cells (mitotic index 0.5 ± 0.5% at 72 hours, III, fig. 2A) when compared with NT siRNA treated (mitotic index 5.2 ± 0.6% at 72 hours). The amount of mitotic HaCaT cells was only slightly reduced in NuMA depleted cells: the difference being at its largest after 48 hours of silencing (mitotic index 3.8 ± 0.5% for NuMA siRNA and 6.9 ± 0.9% for NT siRNA) and balancing out thereafter (4.5 ± 1.7% for NuMA siRNA and 5.0 ± 1.7% for NT siRNA at 72 hours). Ki-67 is a proliferation marker present in G_1, S and G_2 cells as well as in mitotic cells, but absent in G_0 and senescent cells (Scholzen and Gerdes, 2000). Ki-67 staining was used to further assess the proliferation rate. The Ki-67 index of MCF-7 cells decreased following NuMA silencing (III, fig. 2B-C; NuMA siRNA 62.4 ± 7.4% v.s. NT siRNA 88.3 ± 3.7% at 72 hours) whereas nearly all HaCaT cells were Ki-67 positive following NuMA siRNA treatment (III, fig. 2D). Taken together, NuMA silencing decreases the mitotic activity in both MCF-7 and HaCaT cells but only MCF-7 cells exit cell cycle as shown by Ki-67 staining.

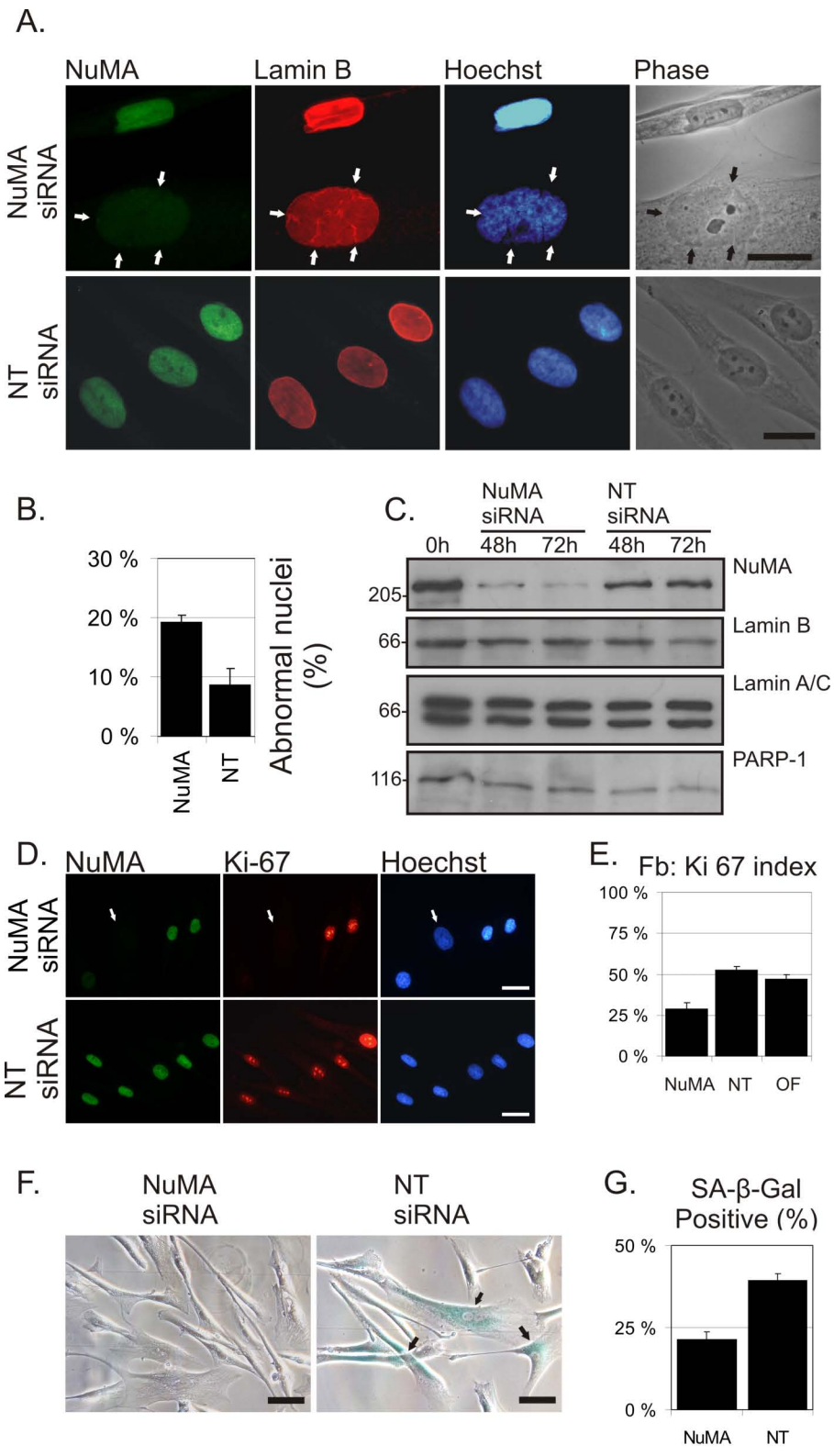
Flow-cytometric DNA content analysis was performed to further analyze the cell cycle progression of NuMA silenced MCF-7 and HaCaT cells. Flow cytometry confirmed a proliferative arrest in $G_{1/0}$ of NuMA silenced MCF-7 cells: 77.8 ± 9.4% of NuMA silenced MCF-7 cells were in $G_{1/0}$ while 57.9 ± 3.8% and 57.3 ± 3.8% of NT siRNA treated and untreated control cells were in $G_{1/0}$, respectively (III, fig. 3A-B).

Correspondingly, there were ~ 50% less S phase (8.0 ± 5.3%) and G_2/M phase (12.7 ± 4.9%) cells among NuMA silenced MCF-7 cells when compared to NT siRNA treated (16.8 ± 3.0 % in S and 24.3 ± 1.6% in G_2/M) and untreated controls (18.0 ± 0.8% in S and 23.5 ± 2.3% in G_2/M). There was no increase in the amount of apoptotic sub-G_1 population following NuMA silencing (III, fig. 3A-B). HaCaT cells seemed to progress in the cell cycle irrespective of the NuMA expression level and no increase in the amount of apoptotic sub-G_1 cells was detected (III, fig. 3B). Similarly, for apoptosis detection neither TUNEL assay, staining for cleaved active caspase-3 nor cleaved PARP-1 (p85) showed difference between NuMA silenced, NT treated and control cells (data not shown).

5.3.2. Benign fibroblasts undergo decreased proliferation following NuMA silencing

Cancer cells have to be always considered as abnormally acting cell types. We therefore further studied benign human skin fibroblasts (passages 9-12) transfected with NuMA siRNA oligonucleotides, non-targeting (NT) siRNA oligonucleotides or oligofectamine alone (OF), and followed for 3 days. NuMA negative cells were morphologically different from untransfected cells: they were typically dilated and had larger nucleus, invaginations of the nuclear lamina and a loss of peripheral condensed heterochromatin in Hoechst stain (Fig. 10A). The amount of cells with atypical nuclear morphology (including abnormally large nuclei, nuclei with blebs or uneven nuclear envelope, and nuclei with the loss of peripheral condensed heterochromatin) increased

Figure 10. Silencing of NuMA in benign fibroblasts results in aberrations in the nuclear morphology and decreased proliferative activity but restrains replicative senescence. *Human skin fibroblasts treated with NuMA siRNA, non-targeting siRNA (NT) or oligofectamine alone (OF) for 72h were stained for NuMA, lamin B, Ki-67 and DNA. A: Atypically large NuMA negative cell showing uneven nuclear rim, pleated lamina (arrows) and loss of peripheral condensed heterochromatin. Scale bars 10 μm. B: The amount of the cells with atypical nuclei was quantified. 200-300 randomly selected cells were counted per sample. Table represents the mean and standard deviations from three separate experiments. C: Western blot analysis of fibroblasts. NuMA is efficiently silenced while no apoptotic cleavage of the studied proteins is detected. D: NuMA negative cells showing nuclear deformity are devoid of Ki-67 antigen (arrow). Scale bars 10 μm. E: The proportion of Ki-67 positive cells. 200-300 randomly selected cells were counted per sample. Table shows the mean and standard deviations from three separate experiments. F: Senescence associated β-galactosidase staining for fibroblasts treated with NuMA siRNA or non-targeting siRNA for 72 hours. Scale bars 20 μm G: 200-300 randomly selected cells were analyzed per sample and the number of positively stained cells was determined. The table represents the mean and standard deviations from four separate experiments.*


A.
NuMA
Lamin B
Hoechst
Phase
NuMA siRNA
NT siRNA
B.
30 %
20 %
10 %
0 %
NuMA
NT
Abnormal nuclei (%)
C.
NuMA siRNA
NT siRNA
0h
48h
72h
48h
72h
205
66
66
116
NuMA
Lamin B
Lamin A/C
PARP-1
D.
NuMA
Ki-67
Hoechst
NuMA siRNA
NT siRNA
E.
Fb: Ki 67 index
100 %
75 %
50 %
25 %
0 %
NuMA
NT
OF
F.
NuMA siRNA
NT siRNA
G.
SA-β-Gal Positive (%)
50 %
25 %
0 %
NuMA
NT

up to 19.3 ± 1.1% at 72 hours post-transfection in NuMA silenced fibroblasts while 8.7 ± 2.7% of NT siRNA treated cells had similar changes (Fig. 10B). Immunoblotting showed efficient silencing of NuMA after 48 and 72 hours but no apoptotic cleavage of the proteins studied (Fig. 10C). Interestingly, NuMA silenced fibroblasts were less frequently positive for Ki-67 antigen (28.5 ± 3.9% at 72 hours post-transfection, Fig. 10D and E) than control cells (52.4 ± 2.5% for NT and 47.1 ± 2.5% for OF) suggesting that silencing of NuMA decreases proliferative activity. Fibroblasts are rather slowly proliferating cells and we did not detect a significant difference in the flow cytometric DNA content analysis (data not shown).

5.3.3. NuMA silencing results in centrosome dissociation and mitotic defects but the cell cycle exit is not only the result of these mitotic defects (III)

Haren et al. (2009) showed recently that NuMA silencing in HeLa cells results in spindle abnormalities including centrosome spindle dissociation, spindle assembly check point activation and lagging chromosomes altogether leading to reduced growth. All the observed mitotic NuMA silenced MCF-7 cells stained for NuMA in minor amounts and totally NuMA negative mitotic cells were not present. Consistent with earlier studies, tubulin staining revealed NuMA silencing to cause misshaping of the spindle apparatus and a parting of the centrosome from the mitotic apparatus was often present (Fig. 11A, arrowheads). In NT siRNA treated MCF-7 cells, NuMA distributed to the pericentrosomal area to surround the centrosome (Fig. 11B, upper panel). The small amount of NuMA present in NuMA silenced cells gathered near the spindle pole but this diminished amount of NuMA did not form the normal mitotic distribution and localize to surround the centrosome but was either scattered near the spindle pole or did not extend to the proximal part of the mitotic spindle (Fig. 11B, lower panel). NuMA silenced cells seemed not to form a symmetrical metaphase plate: NuMA silencing increased the number of mitotic MCF-7 cells with defective chromosome alignment and a growing number of mitotic cells with misaligned chromosomes was seen (Fig. 11C, NuMA 42.6 ± 3.2% v.s. NT siRNA 30.0 ± 2.8%). The defects in the chromosome alignment were accompanied with misshapen mitotic spindle (Fig. 11D, NuMA siRNA 34.2 ± 2.0% v.s. NT siRNA 22.1 ± 3.1%) as judged by the tubulin staining: the spindle was often widespread and asymmetrical. It seems that the formation of the metaphase spindle was prolonged as a growing number of mitotic cells stayed in the prometaphase-like situation following NuMA depletion (Fig. 11E, NuMA siRNA 55.3 ± 5.6 % v.s. NT siRNA 38.9 ± 3.3%) and did not enter metaphase as efficiently as controls (Fig. 11F, NuMA siRNA 15.5 ± 2.8% v.s. NT siRNA 22.1 ± 1.1%).

To study whether the accumulating mitotic defects cause the cell cycle arrest, we used serum starvation (0.1% FCS) to synchronize cells into quiescence 24 hours prior to and during NuMA siRNA treatment. After 48 hours of siRNA treatment cells were released to a medium containing normal serum and

nocodazole for 24 hours. 25.4% of NuMA silenced MCF-7 cells remained in $G_{1/0}$ whereas congruent number for NT siRNA treated MCF-7 cells was 8.8%. The percentage of cells entering mitosis decreased ~30% when cells were treated with NuMA siRNA (III, fig. 4A-B, NuMA siRNA 50% and NT siRNA 75%). The polyploid population present in MCF-7 cell line is most probably due to a defective mitotic check point following prolonged nocodazole treatment. This polyploid population was not present in HaCaT cells negative in p53 (Lehman *et al.*, 1993) which progressed through the cell cycle disregarding the NuMA silencing (III, fig. 4B). We further compared two other cell lines, MCF-10A known to have a functional p53, and HeLa having a defective p53, in a similar experiment to confirm the role of p53 in the cell cycle exit. Similar results of a MCF-10A population arresting in $G_{1/0}$ were seen whereas the cell cycle progression of HeLa cells did not alter (III, supplemental fig. 2). The BrdU incorporation assay showed that 49 % of the NuMA siRNA treated but NuMA positive MCF-7 cells incorporated BrdU similarly to control NT siRNA treated cells (53 %) whereas only 13 % of the NuMA negative NuMA siRNA treated cells progressed to S phase to incorporate BrdU (III, fig. 4C, arrowheads).

5.3.4. NuMA interaction partner GAS41 becomes cytoplasmic following NuMA depletion (III)

GAS41, an interaction partner of NuMA, is a transcription factor involved in the regulation of the p53 pathway (Llanos *et al.*, 2006). Cytoplasmic relocalization of GAS41 following NuMA silencing was evident in both in 10% serum and serum starved MCF-7 cells (III, fig. 5A). In serum starved cells, GAS41 seemed to gather into cytoplasmic granules (III, fig. 5A, arrowhead). The depletion of GAS41 has been shown to activate p53 tumor suppressor pathway by increase in p53 and p21 expression and p53 serine 15 phosphorylation (Park and Roeder, 2006). We stained the MCF-7 cells with an antibody recognizing the p53 phosphorylated at serine 15 (p53ser15). There was no evident p53ser15 positive nuclear staining following NuMA silencing. In both in 10% serum and serum starved NuMA silenced MCF-7 cells, we did, however, discover p53ser15 positive cytoplasmic aggregates (III, fig. 5B) which did not colocalize with GAS41 aggregates (III, fig. 5C). Although the level of p53 expression remained unchanged in MCF-7 cells following NuMA silencing (III, fig. IC), the amount of a p53 down-stream tumor suppressor protein p21 was seen to increase in NuMA silenced MCF-7 cells (III, fig. 5D-E). To conclude, NuMA silencing seems to cause p53 mediated $G_{1/0}$ arrest combined with the cytoplasmic relocalization of transcription factor GAS41, phosphorylation of p53 at serine 15 and an increased level of p21.

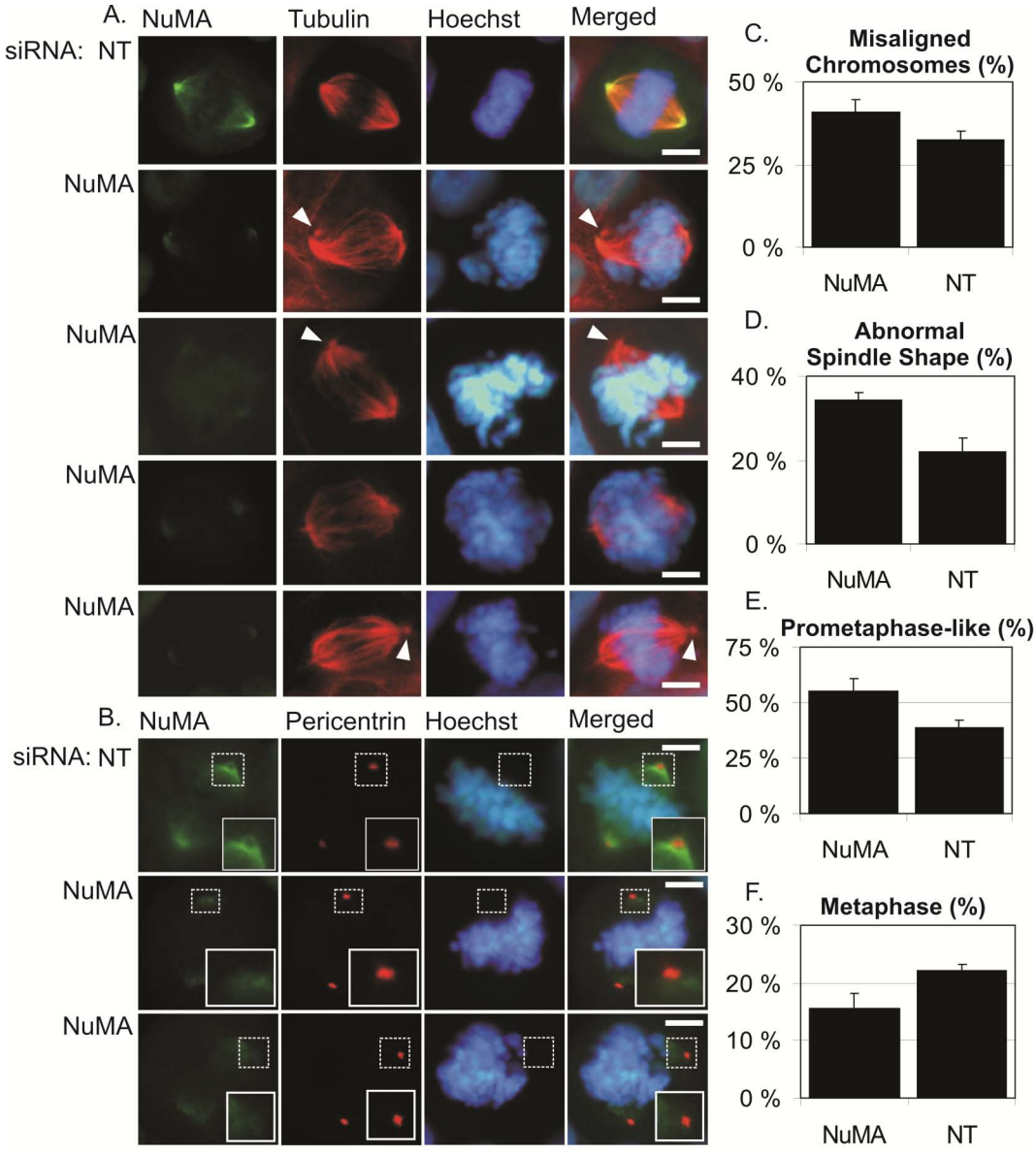

A.
NuMA
Tubulin
Hoechst
Merged
siRNA: NT
NuMA
NuMA
NuMA
NuMA
B.
NuMA
Pericentrin
Hoechst
Merged
siRNA: NT
NuMA
NuMA
C.
Misaligned Chromosomes (%)
50 %
25 %
0 %
NuMA
NT
D.
Abnormal Spindle Shape (%)
40 %
20 %
0 %
NuMA
NT
E.
Prometaphase-like (%)
75 %
50 %
25 %
0 %
NuMA
NT
F.
Metaphase (%)
30 %
20 %
10 %
0 %
NuMA
NT

Figure 11. Mitotic chromosome alignment, spindle formation and centrosome localization are disturbed in NuMA depleted cells. *MCF-7 breast cancer cells treated with NuMA siRNA or non-targeting siRNA (NT) for 72 hours were fixed, permeabilized and stained for NuMA (green), tubulin or pericentrin (red) and DNA (blue). All mitotic NuMA siRNA treated cells typically show a minor amount of NuMA staining. 80-120 mitotic cells were counted to quantify changes in tables C-F. Tables represent the mean and standard deviations from three separate experiments. A) The structure of the mitotic spindle is altered in NuMA silenced cells. Upper panel shows a metaphase cell with a normal spindle localization of NuMA. NuMA depleted cells typically showed more misaligned chromosomes and slightly disconnected centrosomes (arrowheads). Scale bars 5 µm. B) Centrosomal misalignment following NuMA silencing. Upper panel shows the typical ring-like staining pattern of NuMA around the centrosome. Pericentrin is slightly mislocalized from the end of the spindle in NuMA depleted cells. Scale bars 5 µm. C) NuMA silencing causes increase in the number of mitotic cells with misaligned chromosomes. D) NuMA silencing results in increased number of cells with abnormal spindle shape. E) The percentage of mitotic cells with prometaphase-like chromosome distribution. F) The percentage of metaphase cells in the mitotic cell population.*

5.4. NuMA siRNA accelerates the nuclear changes following the induction of apoptosis (II)

MCF-7 cells deficient in caspase-3 do not cleave NuMA as shown by earlier results (Taimen and Kallajoki, 2003). Although caspase-3 activated CAD is needed for the chromatin cleavage into apoptotic bodies we wanted to examine the effect of NuMA silencing on the apoptotic chromatin changes. NuMA silencing using siRNA was used prior to STS treatment and similar quantification of normal, early or late apoptotic chromatin changes were made. Prior to 12-hour STS treatment, MCF-7 cells were plated and treated with 100 nM NuMA siRNA or a non-targeting control (NT) siRNA for 60 hours. The changes were quantified at 0, 6 and 12 hours post STS-treatment. NuMA is a DNA binding protein (Luderus *et al.*, 1994) but no major changes were detected in the chromatin distribution prior to STS treatment (II, fig. 5a). Following

STS treatment we observed increasing numbers of cells with early and late changes in NuMA-silenced compared to NT siRNA treated cells (II, fig 6). The percentage of cells with late chromatin changes was almost tripled in NuMA siRNA treated cells compared to NT siRNA treated cells after 12 h (II, fig. 6c, 19.2 ± 5.8% vs. 7.0 ± 1.1%, $p<0.001$). Although NuMA and NT siRNA treated cells showed similar cytoplasmic shrinkage in phase contrast microscopy following STS treatment (Fig. 5a), there was a significantly higher number of cells without any morphological nuclear changes among NT siRNA treated cells compared to NuMA-siRNA-treated cells (Fig. 6a, 24.5 ± 3.1% vs. 7.4 ± 5.2%, $p<0.001$). The MCF-7 cell line used in this study (II) was obtained from a different source than the ones used in the first thesis study (I) which may explain the differences: the number of NT siRNA treated showing late apoptotic changes is only half of the MCF-7v cells showing late apoptotic changes in the first study (I).

6 DISCUSSION

6.1. NuMA – maintaining the chromatin structure (II and III)

NuMA is mainly known for its function in the maintenance of the spindle during mitosis. Due to its essential role, it has been a challenge to study the interphase function of NuMA. According to the predominant view, NuMA would function as a nuclear matrix protein and take part in the maintenance of the nuclear structural integrity. Lydersen and Pettijohn (1980) were the first to show the presence of NuMA in the nuclear matrix preparations and several studies have shown results supporting this (Kallajoki *et al.*, 1991; Harborth *et al.*, 1995; Barboro *et al.*, 2002). The results in this thesis show that NuMA is present in the NM preparations but mainly associates with the chromatin binding fraction (II, fig. 3).

Although the predicted structure of the NuMA polypeptide suggested a structural role for NuMA (Compton *et al.*, 1992; Yang *et al.*, 1992), endogenous NuMA filament structures have not been visualized in living cells. The experimental data also, however, favors the structural role: NuMA is capable of forming dimers and multiarm oligomers *in vitro* (Harborth *et al.*, 1999), as well as ordered nucleoplasmic structures when overexpressed in HeLa cells (Gueth-Hallonet *et al.*, 1998). Also supporting this, is the study by Saredi et al. (1996): overexpressed deletion mutant of NuMA lacking the NLS organizes into an extensive cytoplasmic network of filaments with a diameter of ~5 nm which organizes into solid ~23 nm cable structures in the presence of microtubules. Immunoelectron microscopic studies have visualized NuMA as a part of the intranuclear core filaments (Zeng *et al.*, 1994) and in association with the electron dense domains of the inner NM (Barboro *et al.*, 2002). We extracted ~ 90% of NuMA in the chromatin fraction of HeLa cells (II, fig. 3b) and similarly ~ 75% of NuMA was present in the DNase I sensitive and 2 M NaCl extractable fraction of human mammary epithelial cells (HMECs, Abad *et al.*, 2007). Our extraction method also included the treatment with RNase A in combination with DNase I, which may in part explain the larger amount of extracted NuMA. The interaction of NuMA and the electron dense domains of the NM was disrupted following RNase treatment suggesting that the interaction of NuMA with the nuclear matrix is at least partially RNA dependent (Barboro *et al.*, 2002). Also the differences in the nuclear organization of benign HMECs and malignant HeLa cells may contribute to the differences in the NuMA extraction.

The association of NuMA with the chromatin is evident but exact site for the interaction is only a suggestion. NuMA polypeptide has DNA binding MAR sequences in both the N- and the C-terminal globular domains (Luderus *et al.*, 1994; Radulescu and Cleveland, 2010). This thesis shows that the C-terminal cleavage fragment of NuMA remains associated with the condensed chromatin of the apoptotic bodies

indicating that the chromatin interaction with the C-terminus is tight and apoptosis resistant whereas we received no evidence for a chromatin interaction of the N-terminal cleavage fragment. The C-terminal cleavage fragment contains several important functional interaction sites which may also contribute to the chromatin interaction indirectly. According to the predicted structure, the carboxy-terminal domain of NuMA contains two particularly basic regions: one is located at residues 1830-1908 and other at 2029-2116 (Yang *et al.*, 1992). The basic nature of these regions makes them perfect candidates for binding the acidic DNA. The area C-terminal to the NLS seems to be responsible for the chromatin organization as the truncation of NuMA polypeptide at residues 2005 or 2030 causes a drastic reorganization of nuclear components with relocalization of the DNA, histone H1, and nucleoli to the nuclear rim (Gueth-Hallonet *et al.*, 1998). The blockade of the C-terminal function by the introduction of a C-terminus targeted antibody induces also nuclear chromatin rearrangements as shown by changes in the acetylated histone H4 distribution (Lelievre *et al.*, 1998). Similarly, Abad (2007) showed that the overexpression of the C-terminal part of NuMA (residues 1965-2101) results in the redistribution of endogenous NuMA, chromatin markers acetyl-H4 and H4K20m, and regions of DNase I-sensitive chromatin of HMECs. In our siRNA studies (II and III), we did not detect any changes in the chromatin structure or the heterochromatin marker H4K20m and Acetyl-H3 distribution (data not shown). We assume that only a small amount of NuMA may be sufficient to maintain the nuclear chromatin organization but more research should be aimed at understanding the exact site of chromatin binding of NuMA.

According to the information available on the interphase function of NuMA, it seems likely that NuMA preferably functions in the chromatin organization and gene expression although it can not be concluded that some underlying NM related structural function exists. In summary, the association between the C-terminal NuMA and chromatin seems to be rather tight overcoming the apoptotic degradation of the chromatin and the interaction between the C-terminus of NuMA and chromatin seems be a prerequisite for the function of NuMA during interphase, as shown in the previous studies with differentiating mammary epithelial cells (Lelievre *et al.*, 1998; Abad *et al.*, 2007).

6.2. NuMA – from chromatin to gene expression (III)

This thesis study shows that NuMA depletion results in cell cycle arrest in $G_{1/0.}$ The cell cycle exit can be partially explained by the accumulating mitotic defects which were also shown Haren, et al. (2009). We synchronized the cells in interphase during silencing to prevent the silencing-induced mitotic defects but, still, similar cell cycle exit and decreased BrdU incorporation was detected following the release from the interphase blockade (III, fig. 4A-B) suggesting that the cell cycle exit has some other underlying mechanism. Controversially, the silencing of NuMA in a 10 nM or 50 nM siRNA concentration results in increased Ki-67 index of differentiating HMECs in a three dimensional culture (Chandramouly *et al.*, 2007). This phenomenon was further

abolished when the cells were treated with 100 nM siRNA. The authors claim this change to be the result of off-target effects on the cell cycle progression which present at higher concentrations of siRNA. We and Haren (*personal communication*) used 100 nM concentration of siRNAs in our studies: this concentration was the smallest sufficient to obtain silencing in approximately 50% of the cells and 200 nM was seen to be somewhat toxic to the cells (data not shown). The studies by Abad et al. (2007) and Chandramouly et al. (2007) do not show a western blot to see the true efficiency of the silencing for comparison but they do show clear silencing effect. The silencing seems to inhibit differentiation and change the chromatin organization: the antibody recognizing the histone H4K20 marginalizes to the periphery of the nuclei following NuMA silencing (Abad *et al.*, 2007).

GAS41, a conserved transcription factor interacting with NuMA (Harborth *et al.*, 2000), normally inhibits the activity of p53(Llanos *et al.*, 2006). Similar report also speculated that GAS41 participated in the destabilization of p53. The silencing of GAS41 in U2OS cells with intact p53 causes G_2/M phase arrest and p53 pathway activation including p21 transcriptional activation and p53ser15 phosphorylation (Park and Roeder, 2006). Interestingly, we found NuMA silencing to result in the translocation of GAS41 into the cytoplasm in conjunction with upregulation of p21 expression and the appearance of cytoplasmic p53ser15. The p53 phosphorylated at serine 15 normally has a nuclear staining pattern and the role of this phosphorylation seen in our experiments needs to be further studied. The cytoplasmic relocalization GAS41 might impair the destabilization of p53 but we did not detect changes in the p53 level (III, fig. 1C). Also, the silencing of the multifunctional structural protein 4.1R, another interaction partner of NuMA in interphase cells (Mattagajasingh *et al.*, 1999) induces G_1 arrest in non-transformed cells with intact p53. There are very few functional antibodies available for 4.1R and we were not able to study the localization of the protein in NuMA silenced cells. We tested two antibodies in different staining conditions and were unable to gain any staining of the protein.

This thesis study suggests NuMA to regulate the cycle progression regulation through some mechanism which seems be connected to the change in the GAS41 distribution. It would be interesting to study the expression profiles of NuMA silenced cells with gene expression chips: as NuMA silencing led to the cell cycle arrest, this approach could provide general information about the overall mechanism and pathways behind the phenomenon. The traditional silencing using siRNAs or other silencing vectors is rather slow and the mitotic defects accumulate during silencing. A possible model for efficient NuMA depletion could be the targeting of NuMA to rapid degradation by inserting a destabilizing domain to the DNA sequence to precede *NUMA1* sequence. The expression of fusion proteins containing destabilizing domain in their sequence may be regulated by a small molecule Shld1, the removal of which results in rapid degradation of the destabilizing domain containing fusion protein (Russell and Hirata, 1998). The problem with this approach is the targeted incorporation of the destabilizing domain encoding sequence to precede *NUMA1* in the genome. Adeno Associated Virus (AAV) targeted homologous recombination may be one option to solve this problem:

AAVs seem to use homologous recombination when inserting its genetic information to the host genome (Nagata *et al.*, 2003). AAV-mediated transfection of a fusion sequence of the destabilizing domain sequence and the *NUMA1*-terminal sequence may be sufficient to produce a cell line expressing a NuMA fused to the destabilizing domain. In the presence of Shld1 these transgenic cells express the fusion protein but upon removal of Shld1 the fusion NuMA is targeted to rapid degradation. Such a cell line does not exist yet but its construction would enable the study of direct effects of NuMA depletion on an interphase cell.

6.3. The effect of caspase-3 on the nuclear morphology and the DNA fragmentation (I, II)

Apoptosis is typically characterized by the apoptotic degradation of the nucleus and the eradication of the chromatin into 50-300 kb high molecular weight (HMW) fragments which are further cleaved into oligonucleosomal 180-200bp pieces (Nagata et al., 2003). The latter requires caspase-3 mediated activation of the Caspase activated DNase (Enari et al., 1998), whereas the endonuclease(s) responsible for HMW fragmentation are less well understood. By comparing caspase-3-deficient MCF-7v cells and MCF-7c3 cells transfected with functional caspase-3 gene, we found both cell lines to exhibit early chromatin condensation and chromatin marginalization, whereas caspase-3 was found to be required for the apoptotic body formation (I, fig. 1) consistent with earlier studies (Johnson et al., 2000). As expected, apoptotic MCF-7c3 cells with highly condensed chromatin showed intense TUNEL staining, but still some TUNEL staining was also present in caspase-3-deficient MCF-7v cells. In addition to CAD, at least two apoptotic nucleases have been identified: Apoptosis Inducing Factor (AIF) (Li et al., 2001) and endonuclease G (Susin et al., 1999) are both released from the mitochondria during apoptosis in conjunction with cytochrome c (Fig. 6) and mediate the cleavage of DNA. We assume that the weak TUNEL staining of apoptotic MCF-7v cells may be due to AIF or endonuclease G mediated fragmentation of the DNA.

In the presence of caspase blockade both cell lines still showed cytoplasmic shrinkage and minimal alterations in the chromatin distribution (I, fig. 5). The role of caspase-3 in the apoptotic cleavage of the chromatin is inevitable. Although minor changes such as blurring of the chromatin structure and disappearance of the nucleoli was present during apoptosis induced in the presence of pancaspase inhibition with z-VAD-fmk, both MCF-7v and MCF-7c3 cell lines were negative for TUNEL assay (I, fig. 3) suggesting that the DNA was still intact. The morphological changes present may be due to protease activity other than caspases. For example, cathepsins and calpains have been shown to mediate programmed cell death during caspase inhibiton (Leist and Jaattela, 2001). As 100 μM z-VAD-fmk may inhibit several other proteinases in addition to caspases, it is also possible that the morphological changes are not due to protease activity but represent changes in cellular ion concentration or in protein kinase activity. The cleavage of NuMA and lamins, both of which bind to DNA at MARs

(Luderus et al., 1994), is completely prevented in the cells (I, fig. 6) suggesting that their proteolysis is not responsible for the changes. However, the removal of NuMA from the nucleus accelerates the apoptotic chromatin changes although no TUNEL staining was detected in these cells (II, fig. 6 and data not shown). The evidence on the connection between NM degradation and the apoptotic degradation of the chromatin is inevitable but the exact mechanism still can not be described in detail according to recent knowledge. If NuMA is needed for the maintenance of the chromatin-NM structure, a transient deletion or mutation of the chromatin binding site of NuMA could give new information on whether the chromatin-NuMA interaction truly is necessary for the nuclear organization. Prior to this experiment, however, the exact site for this interaction needs to be clarified.

6.4. The role of caspases in the apoptotic cleavage of lamins and PARP-1 (I)

Our results show caspase-3 to be crucial in the apoptotic cleavage of NuMA and lamin A/C in STS treated MCF-7 cells. Some cleavage of lamin B may occur in the absence of caspase-3, whereas PARP-1, another nuclear protein, seems to be cleaved regardless of caspase-3 (I, fig. 2 and 6, and II, fig. 1C). PARP-1 was originally identified as an apoptotic substrate for caspase-3 (Tewari *et al.*, 1995) but later studies identified caspase-7 involvement in the cleavage process (Germain *et al.*, 1999). Since caspase-3 and -7 share similar substrate specificity (DEVD at the cleavage site), it has been suggested that, in the absence of capsase-3, caspase-7 could replace the activity of caspase-3 (Korfali *et al.*, 2004). Seeing that caspase-3 is important for the cleavage of lamin B (Peter *et al.*, 1990), it is possible that caspase-7 is responsible for minimal cleavage of lamin B seen in caspase-3-deficient MCF-7v cells (I, fig. 6A).

In this thesis study, lamin B, cleaved in the presence of broad spectrum kinase inhibitor STS, relocated into cytoplasmic granules in caspase-3-deficient MCF-7v cells whereas the presence of caspase-3 attenuated the granule formation or the granules were fine in appearance. Furthermore, caspase-3 specific inhibitor, z-DEVD-fmk, restored granule formation in MCF-7c3 cells. The cells with granules showed meshwork-like chromatin morphology indicating that granule formation precedes late apoptotic chromatin condensation. The abnormal relocalization of lamin B into cytoplasmic foci may be due to lack of PKC activation or to incomplete cleavage of lamin B in caspase-3-deficient cells. These mechanisms must be clarified in future experiments. Live cell imaging of GFP-lamin B transfected apoptotic MCF-7 cells could give information on the mobility of lamin B and the segregation of different cellular fractions such as cytosolic and nuclear proteins may give new insights into the relocalization of lamin B in apoptotic cells.

6.5. The effect of NuMA cleavage on the nuclear integrity (I, II)

Several studies have shown that NuMA is specifically cleaved in different models of apoptotic cell death (Weaver *et al.*, 1996; Gueth-Hallonet *et al.*, 1997; Hirata *et al.*, 1998; Taimen and Kallajoki, 2003) as well as in necrotic cells (Bortul et al., 2001). The apoptotic degradation of the nucleus coincides with the cleavage of NuMA (Taimen and Kallajoki, 2003) and, when the cleavage of NuMA is impaired by mutating the cleavage site, the nucleus does not collapse into apoptotic bodies (Lin et al., 2007). Several proteases have been suggested to cleave NuMA but caspase-3 is the major candidate for the protease responsible: this thesis study showed NuMA cleavage to be impaired in caspase-3-deficient MCF-7v cells whereas a nearly complete cleavage into ~180 kDa was observed in MCF-7c3 cells (Fig. 8 and I, fig. 6). Some further degradation into ~160 and ~150 kDa fragments was also observed in MCF-7c3 cells after 12 hours (Fig. 8). Other candidates for the cleaving protease include caspase-6 and –7, both of which are able to cleave NuMA *in vitro* (Hirata et al., 1998). As procaspase-7 was processed and its target PARP-1 was cleaved also in MCF-7v cells (I, fig. 6B), it seems unlikely that caspase-7 would be responsible for the cleavage of NuMA. In the present study we could not detect caspase-6 activation in the absence of caspase-3. Therefore, we cannot exclude the possibility that caspase-3 is only a mediator for the cleavage of NuMA and activates caspase-6 or some other protease to execute the cleavage.

The junction between the central coiled-coil domain and the C-terminal globular head contains four potential apoptotic cleavage sites (Lin et al., 2007). Importantly, the deletion of the region containing the proposed apoptotic cleavage sites (residues 1701–1828) in NuMA protects the cells from the apoptotic disruption of the nucleus. The N-terminal apoptotic fragment containing the coiled-coil domain important for the dimer formation is solubilized following cleavage (II, fig. 2-3). Thus, the apoptotic degradation and following solubilization would abolish the cross-linking properties of the dimer partly explaining the need for NuMA cleavage during apoptotic nuclear disintegration: the C-terminal globular tail may remain associated with collapsing chromatin whereas the cleavage results in the elution of the cross-linking N-terminal NuMA. A mutant form NuMA unable to form homodimers would therefore alter the chromatin structure as well. Indeed, a mutant NuMA lacking the N-terminal globular head domain and much of the coiled-coil region (deletion of residues 7-1502) has been shown to result in dotted pattern of NuMA staining and the chromatin staining relocalizing into foci associating with C-terminal NuMA dots (Gueth-Hallonet et al., 1998). According to the literature and our observations of the apoptotic fragments, NuMA may act as a linker protein between the chromatin structure and the underlying nuclear matrix, to which it partly attaches itself and, the cleavage of which is needed for these structures to collapse.

7 SUMMARY AND CONCLUSIONS

My aim was to investigate the role of NuMA and other NM proteins in the maintenance of the nuclear structure. It was previously known that NuMA is a vital NM protein, the cleavage of which is needed for the apoptotic degradation of the nucleus. I also wanted to study the role NuMA plays in the interphase nucleus function, especially the chromatin organization and function.

The nuclear matrix undergoes active degradation during the apoptotic nuclear disintegration. In this study, caspase-3 was further clarified to be necessary for the cleavage and redistribution of the nuclear matrix proteins NuMA and nuclear lamins, as well as for the typical DNA cleavage and formation of the apoptotic bodies. Some degradation of lamin B, PARP-1 and chromatin as judged by the TUNEL staining was evident in the absence of capsase-3. We suggest that caspase-7 or some other protease may participate in the cleavage of these structures mainly degraded by caspase-3.

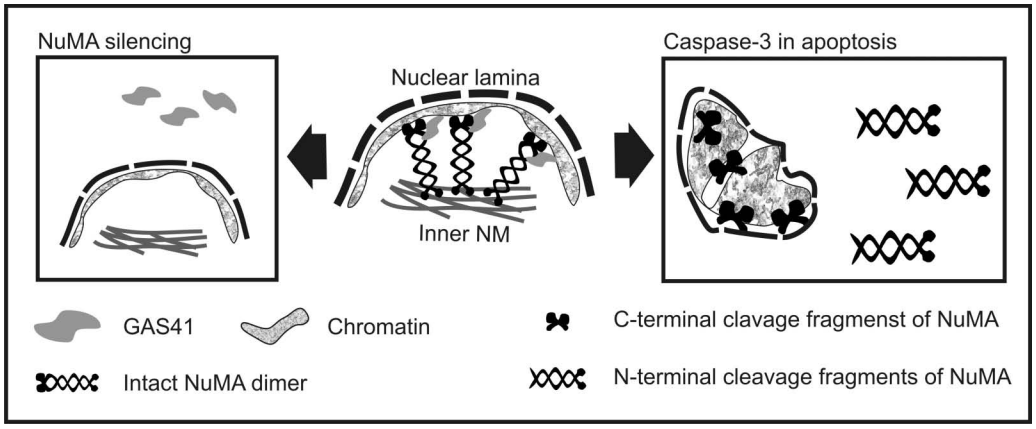


Figure 12. Functions of NuMA in the interphase nucleus

According to a broad spectrum of literature NuMA is considered a NM protein. My study showed that NuMA is mainly found in the DNA binding fraction of the nuclei and that its depletion accelerates the apoptotic chromatin changes. The same study also pointed out that the C-terminal cleavage product of NuMA is retained colocalized with the apoptotic chromatin fragments while the N-terminal NuMA is solubilized following cleavage (Fig. 12). These results suggest NuMA to function in the maintenance of the chromatin structure.

The depletion of NuMA from an interphase nucleus does not, however, affect the chromatin structure but, instead, causes a cell cycle arrest, which we suggest to be mediated via NuMA interaction partner and a known transcription factor GAS41. The

study showed that NuMA depletion results in relocalization of GAS41 to the cytoplasm (Fig.12) and p53 mediated cell cycle arrest to $G_{1/0}$. The results clarify the role of the interaction between NuMA and GAS41 suggesting this to serve as a link between the nuclear integrity and cell cycle progression.

My results suggest that rather than being just a protein retained from the cytosol functioning as a NM protein, NuMA may be important in the maintenance of the nuclear chromatin serving as a link between the chromatin structure and the cell cycle progression. The possible role of NuMA in the regulation of the gene expression remains to be elucidated.

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Internet resources:

Nucleolar protein database	http://www.lamondlab.com/NOPdb3.0/
HomoloGene	http://www.ncbi.nlm.nih.gov/homologene/
Gene	http://www.ncbi.nlm.nih.gov/gene/
LMNA mutation database	http://www.umd.be/LMNA/