

Charles University in Prague
Faculty of Natural Sciences
Department of Genetics and Microbiology

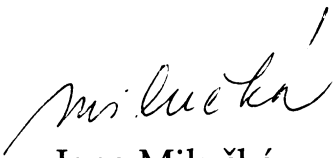
**Analysis of molecular determinants responsible for
subcellular localisation of nuclear DNA helicase II**

Jana Milučká

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Tutor of the diploma work: MUDr. Zdeněk Hodný, CSc.
Consultant of the diploma work: Mgr. Zora Nováková

I hereby declare that I elaborated this diploma work independently,
headed by my tutor, and all utilised literature was properly cited.


Jana Milučká

I thank on my tutor, Zdeněk Hodný, for daring to give me freedom in my work and for introducing me to the world of science.

I would like to express my hearty thanks to our working team, for making me look forward to each single day in the laboratory and creating me such light-hearted conditions for my work.

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Analysis of molecular mechanisms responsible for the subcellular localisation of nuclear DNA helicase II (NDH II)

NDH II belongs to the family of nuclear RNA helicases. Besides nucleoplasmic localisation, presence of NDH II in specific nuclear compartments such as PML nuclear bodies and nucleolus was reported. Its function in these domains is still unknown. To investigate the molecular determinants responsible for nuclear compartmentalization of NDH II, we analyzed *in vitro* behaviour of NDHII and its deletion mutants fused with green fluorescent protein.

In addition to nucleoplasmic staining identical to localisation of endogenous NDHII, EGFP-NDH II localised into distinct nuclear bodies adjacent to nucleoli, which we termed NDH II perinucleolar bodies. Intriguingly, these bodies were not identical with any of the nuclear bodies or compartments tested (i.e PML nuclear bodies, polymerase II transcription sites, snRNP and non-snRNP splicing speckles and DNA damage foci). Notably, they were resistant to RNase treatment but disassembled upon inhibition of RNA polymerase I transcription. Moreover, inhibition of transcription by all three RNA polymerases led to the translocation of NDHII into the segregating nucleoli, namely into dense nucleolar caps marked by TLS protein. The examination of the behaviour of NDH II-truncated molecules lacking either one or both of the terminal domains revealed that N-terminal domain is indispensable for the targeting of NDHII into perinucleolar bodies and for its translocation to segregated nucleoli.

In conclusions, our work has shown that NDH II forms a novel perinucleolar compartment in which it does not colocalise with its previously described interacting partners. This might suggest a further role of NDH II in RNA metabolism apart from transcription. Identification of the components of these bodies may contribute to the completion of our view on nuclear compartmentalization and its impact on performing nuclear functions.

ABBREVIATIONS

A	absorbance
Ac	acrylamide
ActD	actinomycin D
Amp	ampicillin
APS	ammonium persulphate
ATP	adenosine triphosphate
BCA	bicinchoninic acid
Bis	N,N'-methylene bisacrylamide
bp	base pair
BSA	bovine serum albumin
C	concentration
CCD	charge-coupled device
cDNA	complementary DNA
CTD	C-terminal domain
Da	dalton
DAPCO	1,4-diazabicyclooctane
DAPI	4',6-Diamidino-2-phenylindole
DFC	dense fibrillar component
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
EtBr	ethidium bromide
FBS	fetal bovine serum
FCS2	Focht chamber system 2
FITC	fluorescein isothiocyanate
GC	granular component
Gen	geneticin

GFP	green fluorescent protein
hCMV	human cytomegalovirus
HEPES	N-(2-hydroxyethyl)piperazine - N'-(2-ethanesulfonic acid)
HSV TK	Herpes simplex virus thymidine kinase
IgG	immunoglobulin class G
IPCR	inverse polymerase chain reaction
Kana	kanamycin
kb	kilobase
LB	Luria-Bertrani
NaF	sodium fluoride
NDH II	nuclear DNA helicase II
Neo	neomycin
Ni-NTA	nickel-nitriloacetic acid
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethyleneimine
Pho	phosphate
PML	promyelocytic leukemia
PTM	posttranslational modifications
RBD	dsRNA binding domain
RGG	glycine-arginine rich
RNA	ribonucleic acid
RNA pol I	DNA-dependent RNA polymerase I
RNA pol II	DNA-dependent RNA polymerase II
RNase	ribonuclease
rpm	rotation per minute
R-SLB	reducing sample buffer
RT	room temperature
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
SLB	sample buffer

snRNP	small nuclear ribonuclear protein
SV40	Simian virus
TAE	Tris-acetic acid-EDTA
TEMED	N,N,N',N' - tetramethylethylenediamin
TFIID	transcriptional factor IID
TGM	Tris-glycin-methanol
Thr	threonine
TLS	translocated in liposarcoma
Tm	melting temperature
Tris	tris(hydroxymethyl)-aminomethan
Tyr	tyrosine
U	unit
UBF	upstream binding factor
UV	ultra violet
WB	Western blotting
wt	wild type
Xbp	number of base pairs

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1 INTRODUCTION

1.1 *Eukaryotic nucleus and its structure*

Eukaryotic nucleus is a unique highly specialised organelle with a, to date, unsolved origin. This organelle has two major functions: it stores the cell's hereditary material and it coordinates the cell's activities, which include growth, intermediary metabolism, protein synthesis, and reproduction (cell division).

Structurally, the nucleus is composed of complex "envelope" and internal structure (chromatin, nuclear matrix, nuclear bodies, and aqueous "nucleoplasm").

1.1.1 The nuclear matrix

Biochemically, nuclear matrix is defined as the components that remain insoluble after extraction of nuclei with non-ionic detergents, high salt and nucleases (Vlcek et al., 2001). Functionally, it is a structural framework proposed to watch over the cell activities such as replication and transcription, and to organize chromatin within the nucleus.

The best-characterized part of the nuclear matrix is the nuclear envelope (NE), which enwraps chromatin.

NE consists of a single contiguous membrane with outer (ONM) and inner (INM) nuclear membranes, nuclear pore complexes (NPCs) and, in metazoa, the peripheral nuclear lamina located near the INM. The nuclear lamina is a network of lamin polymers and lamin-binding proteins (integral and peripheral) that are embedded in the INM. In the nuclear interior, lamins also form stable complexes (internal lamina), the structure of which is unknown. The ONM is continuous with the endoplasmic reticulum (ER) and is covered

with ribosomes. The INM and ONM are separated by inter-membrane space, but join at sites that are occupied by NPCs, which mediate bidirectional transport of macromolecules between the cytoplasm and the nucleus.

The NE also provides anchoring sites for chromatin and the cytoskeleton. Through these interactions, NE helps to position the nucleus within the cell and chromosomes within the nucleus, and so regulates the expression of certain genes. The NE is not static; rather it is continuously remodelled during cell division. The most dramatic example of NE reorganization occurs during mitosis in metazoa when the NE undergoes a complete cycle of disassembly and reformation. Despite the importance of the NE for eukaryotic cell life, relatively little is known about the biogenesis or many of its functions.

1.1.2 Chromosome territories and interchromatin compartments

Time-lapse microscopy observations suggest that mammalian chromosomes and chromatin are rather immobile during interphase and occupy discrete regions within nuclei. These chromosome “territories” (CT) appear to have distinct borders with, however, little mixing of chromatin from adjacent territories. The molecular interactions that define chromosome shape are not yet clear. Territories do not occupy specific positions, though gene activity can influence interphase chromosome location; chromosomes with a high density of active genes tend to be located towards the nuclear centre whereas those with a low density tend to lie closer to the nuclear periphery (Bridger et al., 2000; Tanabe et al., 2002). The chromosome territories are “porous” that means that proteins of up to 500 kDa in size can gain access to internal sites in the territory.

The nucleus also contains a large chromatin-free space lined by chromatin-domain surfaces called interchromatin compartment (IC). The IC starts at nuclear pores (Visser et al., 2000), expands between CTs and into their interior, and possibly ends with its smallest branches between ~ 1 Mb and ~ 100 kb

chromatin-loop domains. A ribonucleoprotein network located in this space comprises non-chromatin domains such as speckles, Cajal and PML bodies (Matera, 1999) but chromatin loops apparently also can expand into this space (Visser et al., 2000). The IC (by definition) does not comprise the additional interchromatin space present between chromatin fibres in the interior of compact chromatin domains.

At its most expanding sites, IC forms lacunas with diameters of up to several micrometers; at its thinnest branches, its width might be few nanometres and is maintained by repulsive electrostatic forces between the apposed chromatin surfaces that line the branch (Cremer and Cremer, 2001).

It is not clear whether non-chromatin domains represent storage sites of proteins or protein complexes, which are released to sites of action, or whether these domains might directly serve as functional sites. Cremer brothers proposed that spliced RNA could be complexed with proteins and exported to the nuclear pores in the IC space, thus preventing the entangling of RNA that is produced in the interior of compact chromatin domains (Cremer and Cremer, 2001).

1.1.3 Nuclear bodies

Nuclear bodies (NBs) are a heterogeneous group of intranuclear (nucleoplasmic) structures, situated within the nuclear matrix, “scaffold”, and distinguished mainly by morphologic (ultrastructural) and antigenic criteria. NBs include several domains containing granular and fibrillar materials that are arranged as “coiled bodies”, vesicles, spheres, or doughnut-like structures. Occasionally, vacuoles and lipid-like structures can also be seen.

Despite the absence of any delineating membranes, they are considered “compartments” because (i) they contain defining subsets of resident proteins, (ii) they can be morphologically identified by light, fluorescent and electron

microscopy, and, (iii) at least some compartments can be biochemically isolated in an enriched form (Mintz et al., 1999).

In recent years much interest has been put into elucidating of their possible role in the regulation of gene expression, signalling, and other cellular functions.

1.1.3.1 Transcription sites

Recent results suggest that different polymerases are concentrated in factories that specialize in the transcription of particular classes of genes. Transcription factors are generally concentrated in discrete foci spread throughout the nucleoplasm. Their localization rarely colocalises with sites rich in nascent RNA or RNA polymerases, and so they probably mark inactive stores rather than active sites of transcription (Pombo et al., 1998). Apart from nucleoli, OPT domains are the only examples of colocalisation between high concentrations of transcription factors and active transcription (Pombo et al., 1998).

1.1.3.1.1 OPT domains

OPT domains are transcriptionally active domains rich in PTF, Oct1, TBP, Sp1 and RNA pol II. These domains are 1.0-1.5 μm in diameter and contain nascent transcripts as well as other transcription factors. They contain few, if any, factors involved in RNA processing. The OPT domain appears in G1 phase, when it often resides next to nucleoli, and it disappears during S phase.

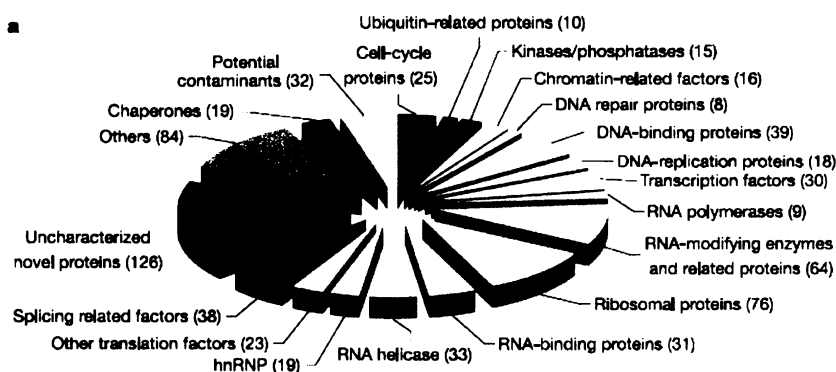
Just like the nucleoli, OPT domains were shown to associate with specific chromosomes (chr. 6 and chr. 7) and might serve as some factories involved in transcription of certain groups of genes (Pombo et al., 1998).

1.1.3.1.2 The nucleolus

The nucleolus is the most prominent nuclear structure. Most mammalian cells contain 1-5 nucleoli, each having 0.5-5 μm in diameter.

At the ultrastructural level it is differentiated into 3 (in the amniote clade) clearly identifiable regions: (i) fibrillar centres (FCs) – lightly staining regions formed by tandem arrays of rDNA genes on chromosomes 13, 14, 15, 21 and 22 and containing some nascent pre-rRNA (mainly in the cortical area) and transcription factors, such as the RNA pol I, the upstream binding factor (UBF) and the DNA topoisomerase I; (ii) dense fibillar component (DFC) - a more densely staining fibrous material, containing newly synthesized pre-rRNA and antigens such as fibrillarlin; and (iii) granular component (GC) – a single large granule-rich region, containing processed products of DFC and pre-ribosomal particles at an advanced stage of maturation. In addition, human nucleoli are generally surrounded by a shell of condensed chromatin that occasionally penetrates deeply into the organelle, reaching the FCs.

The maintenance of nucleolar structure is coupled to ongoing RNA polymerase I transcription, which upon inhibition leads to rapid and dramatic reorganisation (Olson et al., 2000). Actinomycin D (ActD), a potent, dose dependent, inhibitor of RNA pol I, II and III, is routinely used to examine the structural reorganisation of nuclear and nucleolar proteome upon transcriptional inhibition.



(from Andersen et al.: Nucleolar proteome dynamics; NATURE, VOL 433, 6 JANUARY 2005)

Recent proteomic analysis of the nucleolus (Andersen et al., 2005) has shown that still the largest category of identified nucleolar proteins represents a group

of novel and previously uncharacterized factors. The next largest category corresponds to proteins that bind to NA and/or nucleotides (previous reports indicate that DEAD box motif is quite abundant among nucleic acid binding nucleolar proteins (Andersen et al., 2002).

Inferring from the proteomic composition of the nucleolus, its functions can be proposed.

Basically, it is a site of transcription of ribosomal DNA (the genes encoding three of the ribosomal RNA species, namely, 18S, 5.8S, and 28S), the subsequent processing of the pre-rRNA, and the biogenesis of pre-ribosomal particles.

However, after years of intensive research, model of plurifunctional nucleolus has been introduced (Pederson, 1998). Following the evidence, nucleolus is also involved in much of the cell's intranuclear and nuclear-cytoplasmic transport and processing of certain tRNAs and mRNAs as well. The biosyntheses of signal recognition particle RNA and telomerase RNA involve a nucleolar stage and the nucleolus is also participating in processing of U6 RNA, one of the spliceosomal snRNAs. The nucleolus was shown to regulate the sequestration of several proteins that control cell-cycle checkpoints, including pRb1, Mdm2, Cdc14 and Pch2. Another role of the nucleolus involves a cellular response to stress conditions, such as heat or ionising irradiation.

1.1.3.2 *RNA editing*

1.1.3.2.1 Cajal (coiled) bodies

Initially described as “nucleolar accessory bodies” by Santiago Ramon y Cajal in 1903, they gained their second name due to their characteristic appearance as a tangle of coiled fibrillar strands of 40–60 nm in diameter (Monneron and Bernhard, 1969). CBs are small spherical structures, which are typically present in number of 1-5 copies per nucleus, ranging in size from 0.1-1.0 μm . At least two size classes of CBs have been characterized in living cells, the larger

(termed CB) and smaller (termed mini-CB) and they appear to arise through joining and separation events. Their occurrence is nucleoplasmic; however, they have been detected in nucleoli of certain cell lineages. They were shown to preferentially colocalise with certain gene loci (for example of histone genes, of gene clusters encoding the U1, U2 and U3 snRNA).

The nucleoplasmic-shuttling phosphoprotein, p80-coilin, has been identified as a specific marker of CBs. Furthermore, they contain a large number of components, including the spliceosomal snRNPs, U3, U7, U8 and U14 snoRNAs, basal transcription factors TFIIF and TFIIH, cleavage and polyadenylation specificity factor, and nucleolar components fibrillarin, Nopp140, and B23 protein. Also specific oncogenic proteins, for example, a member of the Ewing family of oncoproteins 'pigpen' have recently been confirmed to localise to CBs.

The function of the CBs is unknown. They might be involved in transport, maturation and assembly of snRNPs and snoRNPs (Sleeman and Lamond, 1999). The observed localisation of some pol I, II and III components to CBs indicates that CBs can also function as a platform for assembly of pol I, II and III transcriptosomes (Murphy et al., 2002).

1.1.3.2.2 Gems

Gems (gemini of Cajal bodies) are found in the nucleoplasm and are coincident with or adjacent to Cajal bodies, depending upon the cell line examined, and they have been characterized by the presence of the survival of motor neurons gene product (SMN) and an associated factor, Gemin2 (Matera, 1999). The cytoplasmic pool of SMN and Gemin2 has been implicated in the assembly of snRNPs, and their nuclear pool may play an additional role in snRNP maturation.

1.1.3.2.3 hnRNPs (heterogeneous nuclear ribonuclear proteins)

In human cells, there are more than thirty hnRNPs. They show RNA-binding specificity and presumably bind to hnRNA in a non-random fashion.

Many different functions have been proposed for them: proper presentation of pre-mRNA for the processing systems, retaining of non-spliced RNA in nucleus, anchoring RNA to the nuclear matrix, promoting the transport of mature mRNA from the nucleus to the cytoplasm. Moreover, some hnRNPs are clearly important for splicing (hnRNP A1).

1.1.3.2.4 snRNPs (small nuclear ribonuclear protein) and spliceosomes

The five major snRNP particles (U1, U2, U4/U6, U5) and the U/U5/U6 tri-snRNP (U stands for “rich in uridylic acid”) are evolutionary highly conserved and participate in the splicing of pre-mRNA. They form the core of the spliceosome.

Spliceosomes are distinct particles, 40-60 nm in size. Together with a large number of proteins, the complex catalyses removal of introns from eukaryotic mRNA precursors through two consecutive *trans*-esterification reactions. At least 30 proteins of the spliceosome fulfil a known or putative role in gene expression steps other than splicing.

Each small nuclear RNA (snRNA) is complexed with at least seven protein subunits to form small nuclear ribonuclear protein (snRNP). All of them are transcribed by RNA pol II and contain Sm site; except for U6, which is transcribed by RNA pol III and does not contain Sm site. The Sm proteins bind to the Sm site and form a highly stable snRNP core structure, which is morphologically similar to all the snRNAPs. They are recognised by anti-Sm-auto-antibodies from patients suffering from systemic lupus erythematosus (SLE).

1.1.3.3 RNA metabolism

1.1.3.3.1 Perinucleolar structures

There are three basic structures that can be summarized under the term perinucleolar structures – perinucleolar compartment (PNC), Sam68 nuclear body (SNB) and hnRNP L-enriched perinucleolar structure.

Despite their different nuclear localisations, PNCs and SNBs share some common characteristics: they are both rich in RNA binding proteins and nucleic acids and are predominantly localised to the periphery of the nucleolus. They are observed mostly in transformed cells, although prevalence differs among different cell types and cell lines. Their structural integrity is influenced by the transcriptional state of the cell. When cells are treated with Pol I transcription inhibitors and nucleolar segregation is induced, both PNCs and SNBs disassemble.

PNC is an irregularly shaped structure composed of electron-dense strands ranging from 0.25 – 1.0 μm with a large variability in shape and size. It is a dynamic structure and exhibits discrete movement at the nucleolar periphery over time.

Several molecular constituents of the PNC have been described. Among them are small RNAs transcribed by RNA polymerase III [RNase MRP RNA, RNase P RNA, and hY RNAs; (Lee et al., 1996)] and three RNA binding proteins, polypyrimidine tract binding PTB/hnRNP I (Ghetti et al., 1992), CUG binding protein CUG-BP/hNab50 (Timchenko et al., 1996), and KH-splicing regulatory protein [KSRP; (Huang, 2000)].

The presence of hnRNP proteins, splicing factors, and small RNAs transcribed by RNA pol III suggests that the PNC is involved in RNA metabolism. Nascent RNA has been observed in the PNC and is most likely derived from Pol II and/or Pol III transcription.

The SNB is composed of phosphorus-rich and nitrogen-rich fibres and granules, suggesting that SNBs are abundant in nucleic acids. Basically, they contain a

unique group of RNA binding proteins, that contain a GSG (GRP33, Sam68, GLD-1) domain, also termed the STAR (signal transduction and activation of RNA) domain; namely Sam68, SLM-1 and T-STAR. The domain is postulated to be responsible for RNA binding and protein-protein interactions.

SNBs do not appear to concentrate newly synthesised RNA when examined, nor are enriched with pre-mRNA splicing factors (snRNP particles).

Sam68 binds to the RNA motif UAAA with high affinity and may shuttle between the nucleus and the cytoplasm, it has been speculated that SNBs may be involved in the transport of mRNA through the nucleus (Chen et al., 1999).

The shape and size of the hnRNP L-enriched perinucleolar structure is similar to the PNC and SNB, however, it occupies different nuclear regions. Their exact function is still unclear.

1.1.3.3.2 PML bodies

Promyelocytic leukaemia protein (PML) is a tumour suppressor that is implicated in the regulation of gene transcription, protein degradation, DNA repair, cellular growth, senescence and apoptosis [for a review, see (Dellaire and Bazett-Jones, 2004)]. PML protein is crucial for a proper assembly of large nuclear multiprotein complexes referred to as PML bodies (Ishov et al., 1999).

PML bodies are spherical domains scattered in number of 10-30 per nucleus throughout the nucleoplasm ranging from 0.3 – 1.0 μm in size. They are present in cells with different tissue origins, but PML protein level is diminished in most types of human tumours (Gurrieri et al., 2004).

In addition to the PML protein, several other proteins, including Sp100, SUMO-1, pRb, Daxx and CBP, are enriched in this nuclear domain in addition to being diffusely distributed in the nucleoplasm.

PML bodies appear to play a role in nuclear storage, growth control and apoptosis. Moreover, PML bodies might detect highly localised concentrations

of exogenously introduced specific proteins and potentially regulate their transcription (Tsukamoto et al., 2000).

1.2 *Mechanisms of protein distribution within the nucleus*

Generally, as a consequence of the state of activity and functionality, a protein has its distinct localisation within or out of the cellular environment.

Protein localisation can be determined primarily by some intrinsic “sorting signal”, coded in its primary structure. These are short sequence segments that localise proteins to intra or extra-cellular environments. They include signal peptides, membrane-spanning segments, lipid anchors, nuclear import signals, and motifs that direct proteins to organelles such as mitochondria, peroxisomes, lysosomes, chloroplasts, the Golgi apparatus, and the endoplasmic reticulum.

In addition to proteolytic cleavage or interaction with other protein partners, posttranslational modifications are heavily participated in determining the protein subcellular localisation.

1.2.1 Posttranslational modifications

I will now focus on those posttranslational modifications, which were predicted or reported for NDH II/RHA and other DexH/D box helicases.

1.2.1.1 O-GlcNAc modification

O-linked β -N-acetylglucosamine (O-GlcNAc) modification is an abundant and dynamic post-translational event that implies the attachment of the monosaccharide N-acetylglucosamine in a β -linkage to serine and threonine hydroxyl groups of nucleoplasmic lipids or proteins. Concerning the residues and proteins that are modified, the nature of the mechanism, and the existence of cycling enzymes O-GlcNAc transferase (OGT) and neutral β -N-acetylglucosaminidase [O-GlcNAcase; (Comer and Hart, 2000)] it is more analogous to phosphorylation than to classical complex O-glycosylation.

Proteins modified by O-GlcNAc include transcription factors, skeletal and signaling components, hormone receptors and a number of oncogenes and viral proteins.

Recent work has shown that the C-terminal domain of RNA pol II is modified by O-GlcNAc and that there might be a reciprocal and inhibitory relationship between this modification and phosphorylation, which is required for the elongation of RNA pol II (Comer and Hart, 2001). To date, more than 10 transcription factors have been shown to be modified by O-GlcNAc, including c-myc, Sp1 transcription factor, p53 and NF- κ B (Wells and Hart, 2003). The modification is associated with increased transcriptional activity; moreover, glycosylation of c-myc plays a role in protein stability and its subcellular localisation (Kamemura et al., 2002).

1.2.1.2 *Methylation*

Arginine methylation is a very common posttranslational modification that occurs predominantly in the nucleus and results in the addition of one or two methyl groups to the guanidine nitrogen atoms of arginine (Gary and Clarke, 1998). There are three main forms of methylated arginine identified in eukaryotes: ω -N^G monomethylarginines (MMA); ω -N^G, N^G-asymmetric dimethylarginines (aDMA); and ω -N^G, N^G-symmetric dimethylarginines (sDMA).

The addition of methyl groups to nitrogen, carbon, sulfur, and oxygen atoms is catalyzed by at least two different classes of ubiquitously expressed protein arginine methyltransferase (PRMT) enzymes. Both types catalyze the formation of MMA as an intermediate, and type I PRMTs (PRMT1, PRMT3, PRMT4, PRMT6) lead to the production of aDMA, whereas type II PRMTs (PRMT5 and PRMT7) catalyze the formation of sDMA.

The main pool of modified proteins harbors glycine and arginine-rich (GAR) motifs (Najbauer et al., 1993); these are recognized by the type I enzymes, PRMT1, PRMT3, and PRMT6, whereas PRMT4 displays a higher degree of specificity and does not methylate GAR motifs. The type II enzymes, PRMT5 and PRMT7, methylate isolated arginine residues as well as arginines within GAR motifs.

RNA binding proteins (RBPs) represent major targets for PRMTs because most hnRNPs (A1, A2, K, R, and U) harbor GAR motifs. Arginine methylation might serve as a maturation signal, as several RBPs including Sam68 are mislocalised in their hypomethylated state (Cote et al., 2003).

The role of methylation in transcriptional regulation can be inferred from the early observation on that histones are substrates of PRMT1, PRMT4, and PRMT5 (McBride and Silver, 2001). There are numerous transcription factors, including p53, YY1, and NF- κ B that contribute to the recruitment of the PRMTs to promoters (Bedford and Richard, 2005) and PRMTs have been shown to methylate transcription coactivators including CBP/p300 as well as transcription elongation factors.

Signalling pathways are governed by posttranslational modifications that alter partially protein-protein interactions. Methylated arginines have been shown to block some interactions [via SH3 domains; (Selenko et al., 2001)] and to promote others [via WW and Tudor domain (Bedford et al., 2000)].

Recently, first direct evidence of arginine methylation-dependent nuclear transport in mammalian systems has been shown. The C-terminus of RHA contains a 110-amino acid bi-directional nuclear transport domain (NTD) that is necessary and sufficient for nuclear import and export. This region harbors an NLS, as well as a nuclear export sequence. In vitro assays have shown that in the presence of arginine methylation inhibitors, the import of NTD in the

nucleus via an RGG-rich NLS is abrogated and thus arginine methylation was shown to determine its subcellular localisation (Smith et al., 2004).

1.2.1.3 *Sumoylation*

Sumoylation is a posttranslational event involving reversible covalent attachment of an ubiquitin-like protein – small ubiquitin-related modifier (SUMO) – to the ϵ -amino group of lysine residues of target proteins by a controlled enzyme pathway.

The three SUMO proteins (1–3) are 92–97 amino-acid polypeptides that resemble ubiquitin. Sumoylation resembles ubiquitylation in that it involves an E1-activating enzyme (the Aos1/Uba2 heterodimer), an E2-conjugating enzyme (Ubc9), and an E3 ligase that promotes the transfer of SUMO from the E2 enzyme to substrates. In addition to the PIASs, there are two other SUMO E3 ligases: the polycomb protein Pc2 and the nuclear pore component RanBP2. SUMO modification is reversed by SUMO-specific isopeptidases.

The biological functions of SUMO appear to be diverse, ranging from nuclear transport to signal transduction, transcription, and genome stability. Attachment of SUMO can induce a relocalization of the target protein within the cell, a conformational change, or an alteration of protein-protein interactions, but often its mode of action remains poorly understood. Despite the similar conjugation mechanism, Ub and SUMO often direct their targets to very different fates and attachment of SUMO can protect the protein from the proteolytic effect of ubiquitylation (Desterro et al., 1998).

RHA has recently been shown to be another DexH/D helicase that interacts with Ubc9 and ARIP3/PIASx; and this interaction leads to the SUMO-1 conjugation of RHA both in vitro and in vivo. The transcription activity of RHA is potentiated by this interaction; however, the catalytic activity of Ubc9 seems to be dispensable for the transcription activation activity of RHA. The N-terminal

domain of RHA, encompassing amino acid residues 1–137, is sufficient for its interaction with Ubc9 (Argasinska et al., 2004).

1.2.1.4 Phosphorylation

The attachment of the phosphate group to the Ser/Thr or Tyr residues of the target protein has been to date the most intensively examined posttranslational modification. It is believed that ~1000 protein kinases and 500 protein phosphatases are likely to be encoded by the human genome and approximately 30% of the cellular proteins contain covalently bound phosphate. Protein phosphorylation is a dynamic event, undergoing rapid turnover of the phosphate group by an antagonistic group of protein kinases and phosphatases. It is not simply used to switch the activity of a protein on and off, but can have many additional roles. It can affect the rate at which a protein is degraded, its ability to translocate from one subcellular compartment to another, to dock with other proteins or to bind divalent cations.

I will here focus on the recent knowledge on one specific class of Ser/Thr protein phosphotransferases, the PKC family, as by computer prediction (see further) many feasible PKC recognition sequences were identified.

To date (2006), at least 11 different PKC isotypes have been identified in mammals and all are believed to play distinct regulatory roles. According to their requirements for phospholipids for their activity, they can be subdivided into three subgroups: the classical or conventional PKCs (cPKCs) require phosphatidylserine (PS), Ca²⁺, and diacylglycerol (DG) or phorbol esters. Novel PKCs (nPKCs) are Ca²⁺ independent and require only DG and PS. Atypical PKCs (aPKCs) are dependent on PS, inositol lipids or phosphatidic acid.

Interestingly, all isoforms share at their N-terminus the pseudosubstrate region and can be autophosphorylated to become active.

PKC is thought to reside in cytoplasm in an inactive conformation and to translocate to the plasma membrane or cytoplasmic organelles upon cell activation by different stimuli. However, PKC is capable of translocating to the nucleus as well. There are reports showing translocation of PKC- α to the nuclear envelope and further to the interior, excluding nucleoli, whereas the δ isotype was only found in nucleoli. PKC- ϵ was localised in the pore complexes at the nuclear envelope [for a review, see (Martelli et al., 2006)].

PKC isoform substrate specificity has been attributed to distinctive compartmentalisation patterns for individual PKC isotypes. A relatively large number of binding proteins have been identified for PKC- α lamin A/C and B, C23 nucleolin, PTB-associated splicing factor (PSF), p68 RNA helicase, and the heterogeneous nuclear ribonucleoprotein (hnRNPs) proteins A3 and L (Rosenberger et al., 2004). In vivo and/or in vitro substrates for PKC isozymes moreover include histones H1, H2B, H3, DNA methyltransferase, B23/nucleophosmin, RNA pol II, CREB, p53 and many others.

It seems that nuclear PKC isozymes might be involved in the regulation of DNA replication, RNA synthesis and processing, gene expression, nucleocytoplasmic transport, and chromatin structure.

It has been shown recently that phosphorylation/dephosphorylation cycles may regulate protein function and enzymatic activities of p68 (Yang, 2004). P68 is a DEAD box RNA helicase with RNA helicase, ATPase and RNA unwinding activities. Tyrosyl phosphorylation by PKC affects these activities by impairing RNA binding property of the helicase C-terminus and so disturbing the function of the protein in the pre-mRNA splicing process (Yang, 2004). NDH II/RHA, was shown to be phosphorylated by DNA dependent protein kinase (DNA-PK), and this modification was strikingly stimulated by poly(A)-containing RNA. The phosphorylation of NDH II could be abolished by the addition of RNase A, which confirmed the RNA-dependent activity of DNA-

PK. DNA-PK also catalyzed the DNA dependent phosphorylation of NDH II that was unaffected by RNase (Zhang et al., 2004).

1.2.1.5 Acetylation

Protein acetylation is a reversible process, in which histone acetyltransferases (HATs) transfer the acetyl moiety from acetyl coenzyme A to the ϵ -amino groups of internal, highly conserved lysine residues. This modification neutralises the positively charged lysine residues of the histone N-termini; the consequence of acetylation, however, depends on where within the protein acetylation takes place.

Lysine acetylation is known to occur in histones and HMG proteins, in some nuclear import factors (importin- α family), and in over 40 sequence-specific transcription factors and affecting their DNA binding affinity, coregulator association, nuclear localisation, phosphorylation, ubiquitination and stability [for a review, see (Yang, 2004)]. In most cases, this modification potentiates the transcription.

Acetylation can regulate protein-protein interactions by generating a recognition site for the bromodomain; a protein module conserved in many proteins (Zeng and Zhou, 2002), reminding of the specific recognition of phosphorylated residues by phospho-specific binding modules, such as SH2 domains and 14-3-3 proteins.

Of the DEAD box family of RNA helicases, p68 and p72 have been shown to act as transcriptional regulators and interact with HDAC1 (an established transcriptional repression protein) in a transcription-repressive manner (Wilson et al., 2004).

Werner helicase (WRN), a member of the RecQ DNA helicase family plays a role in the cellular response to DNA damage and its DNA damage-induced

translocation from nucleoli into nucleoplasmic foci is regulated by acetylation (Blander et al., 2002).

NDH II has been shown to interact with the WRN and there is a direct protein-protein interaction mediated by the N-terminal RBD II and C-terminal RGG domain of NDH II (Friedemann et al., 2005).

1.3 Eukaryotic DNA and RNA helicases

Helicases are ubiquitous enzymes that use the energy of NTP hydrolysis to separate energetically stable duplexes of nucleic acids into single strands.

They play an important role in nearly all aspects of nucleic acid metabolism, including replication, repair, recombination, and transcription.

Despite the diversity of their functions, high sequence conservation is maintained, suggesting that all helicase genes evolved from a common ancestor.

All helicases share at least three common biochemical properties: nucleic acid binding, NTP/dNTP binding and hydrolysis, and unwinding of duplex DNA in the 3'-5' or 5'-3' direction.

Most helicases from many different organisms contain about seven to nine short conserved amino-acid sequence fingerprints (designed Q, I, Ia, Ib, II, III, IV, V and VI) called “helicase signature motifs”. These are separated by nucleotide stretches of low sequence but high length conservation. The “helicase” motifs are usually clustered in a region of 200-700 amino acids called the core region. In contrast, the N-terminal and C-terminal regions of the molecule are characterized by a high degree of sequence and length variability. It has been suggested that the divergent regions are responsible for individual protein functions, whereas the highly conserved domains are involved in ATP binding and hydrolysis or binding and unwinding of nucleic acids.

1.3.1 Classification of helicases

Based on the extent of similarity and organisation of the conserved motifs, helicases were classified into three superfamilies (SF) and two smaller families.

SF1 and SF2 are the largest and most related superfamilies and contain at least seven conserved motifs (I, Ia, II-VI). Motifs I and II are the Walker A and B

Table 1 Properties of Conserved Motifs

Motif	Known or Suggested Function
Q	unique for the DEAD-box family; ATP binding and hydrolysis (yeast); adenine recognition
I	P-loop; Walker A NTP-binding motif; binds β and γ phosphates of the ATP molecule
Ia	involved in ssDNA binding
Ib	substrate binding; not so highly conserved and may not be always present
II	Walker B NTP-binding motif; binds to β and γ phosphates through Mg^{2+} ; coupling of ATPase and helicase activity
III	coupling of NTP hydrolysis to unwinding
IV	substrate binding
V	substrate binding
VI	binds γ phosphate of the ATP molecule; is required for movement along the DNA substrate

sequences characteristic of ATPases (Walker et al., 1982), domains which are required (although not sufficient) to confirm a helicase. DNA helicases are characterized by the classic Walker motif A (G-X-X-X-X-G-K-T) and belong to the superfamily I, whereas RNA helicases show a variation of this domain (A-X-X-G-X-G-K-T) and form the SF2 (Luking et al., 1998).

SF3 consists of helicases of small DNA and RNA viruses and their prophage remnants in cellular genomes (Iyer et al., 2004). Only three sequence motifs were identified, contained within a limited ~ 100 amino acid region: Walker A and B motifs, and a novel motif C. Later, another conserved motif was noted, designated B', sandwiched between motifs B and C (Koonin, 1993).

Family 4 contains helicases related to bacterial DnaB protein (Ilyina et al., 1992). All of these helicases are functionally and physically associated with DNA primases. They contain five conserved motifs (I, Ia, II-IV).

The bacterial transcription termination factor Rho, a DNA-RNA helicase, falls into the last family. Interestingly, it shows significant sequence similarity to proton-translocating ATPases, demonstrating the first example of apparent evolutionary relationship between a helicase and a group of non-helicase NTPases.

The chromosomal replicative helicases in prokaryotes and (probably) eukaryotes, the hexameric helicases, are evolutionary too distinct to be substantial for the sake of this work [for a review, see (Patel and Picha, 2000)].

Recent work has shown that some helicases, such as the RuvB branch migration enzyme and minichromosome maintenance (MCM) proteins, belong to the AAA+ (ATPases associated with a variety of cellular activities) family of chaperone-like ATPases rather than any of the previously described helicase superfamily.

1.3.2 DExH/D box RNA helicases

Due to the sequence of motif II, some RNA helicases of the SF2 are called DEAD-box helicases. In this motif they share a unique conserved sequence DExH/D (where x stands for any amino acid) crucial for the helicase activity of the protein.

DExH/D helicases that have been purified possess ATPase, RNA binding and unwinding activity and some of them also annealing enzymatic properties.

1.3.2.1 ATPase activity

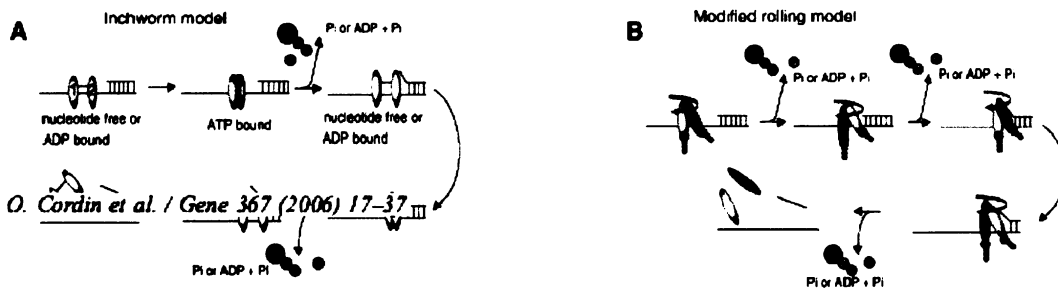
The affinity for ATP is rather low, the K_m ATP of DExH/D helicases range from 80 μ M (Lorsch and Herschlag, 1998) to over 1 mM (Yu and Owttrim, 2000). Nevertheless, a direct evaluation is hazardous because none of these proteins were tested in the context of an assembled complex where they can interact with their biological partners. The ATPase activity of all the DEAD-box proteins was shown to be stimulated upon RNA binding.

1.3.2.2 RNA binding ability

DExH/D box helicases have shown very little substrate specificity and usually bind in a sequence-independent manner with greater affinity to ssRNA than to dsRNA. Putative substrates for the best characterised RNA helicases contain duplex regions that are generally below 10 bp (what is roughly the size of the binding site). Interactions involve primarily base stacking and sugar-phosphate backbone. However, continuous dsRNA is rare in biological systems and so, compared to DNA helicases, processivity of RNA helicases is rather low. The helicase core may lack substrate specificity, some specificity can be provided by domains or regions located in the amino-terminus and carboxyl-terminus ends of the protein.

1.3.2.3 Unwinding activity

Unwinding activity has been shown for only a subset of the DExH/D proteins. A helicase can unwind the substrate in both 3'-5' and 5'-3' directions. Recent models that are at the time widely accepted are the "inchworm" model and the "active rolling" model (see below).



1.3.2.4 Annealing activity

For some helicases of this RNA helicase family also the strands annealing activity was reported. This was the case of highly related p68 and p72 helicases (Rossler et al., 2001) and the RNA Helicase II/Gu (Valdez et al., 1997). Interestingly, its RNA annealing activity resides in its glycine-arginine-rich carboxyl-terminus domain instead of the own helicase core (Valdez et al., 2002).

1.3.2.5 Functions and interactions of DExH/D helicases

RNA helicases serve the cell as some "RNA chaperones" as they are capable of regulating RNA metabolism, including unwinding DNA-RNA intermediates and dsRNA molecules.

Functions of DExH/D helicases can further be exerted on a wide range of ribonucleoprotein substrates as they are able to disrupt or rearrange RNA-protein interactions, moreover, independently of duplex unwinding (Fairman et al., 2004).

1.3.2.5.1 Transcription and translation

Only a few of DEAD-box proteins can be found associated with transcription; Ddx20/DP103 was shown to be essential for repression of transcription (Gillian and Svaren, 2004).

Maleless protein [Mle; (Lee et al., 1997)], a *Drosophila* homologue of human RNA helicase A is a member of the “compensasome”, a protein complex binding to X chromosome of *Drosophila* males. It is necessary for dosage compensation (e.g. for two-fold transcription of single male X chromosome compared to female X chromosome).

Two RNA helicases, eukaryotic Initiation factor 4A (eIF4a) and Ded1 have been so far identified to be essential in cap-dependent translation initiation (Linder, 2003).

1.3.2.5.2 RNA splicing

There are at least eight RNA helicases that play essential roles in different steps of pre-mRNA splicing in *S. cerevisiae* (Prp 2, 5, 16, 22, 28, 43, Sub5, Brr2). Some of their homologues so far identified in humans, are hypothesized to be involved in both spliceosome and non-snRNP mediated RNA splicing.

1.3.2.5.3 RNA decay and mRNA export

Nonsense-mediated decay is a surveillance mechanism that degrades spliced mRNA harbouring a premature termination codon. RNA helicases are components of RNA degradation complexes or exosomes that degrade RNAs in the cell. The helicases implicated involve RhlB in *E. coli* (Py et al., 1996), Suv3 in *S. cerevisiae* mitochondria (Margossian et al., 1996) and Dob1 and Ski2 in *S. cerevisiae* exosomes (de la Cruz et al., 1998). The export of mRNA through the nuclear pore also requires dedicated proteins to bind mRNA. To name some of them, in yeast these are Dbp5 and Sub2 (with a human homologue UAP56).

1.3.2.5.4 Processing of miRNAs and siRNAs

Micro RNAs (miRNAs) and small interfering RNAs (siRNAs) play a role in a wide variety of cellular functions, including gene silencing and developmental regulation of gene expression, and few DExH/D helicases (Dicer, Gemin3) are involved in the processing of their double stranded RNA precursors.

1.3.2.5.5 Viral replication

Two viral RNA helicases that have been well studied are vaccinia virus NPH-II, and HCV NS3 (Hepatitis C Virus Non-Structural protein 3), most of the viral helicases are included in the SF3 helicase superfamily.

1.3.2.5.6 Ribosome biogenesis

In *S. cerevisiae*, 14 out of the 24 known DEAD-box proteins are required for the formation of 40S or 60S ribosomal subunits (de la Cruz et al., 1999). In *E. coli* only five DEAD-box proteins were identified, and at least three of them seem to be involved in ribosome biogenesis: SrmB, CsdA and probably DbpA.

1.3.3 Nuclear DNA helicase II

Nuclear DNA helicase II (NDH II), alternatively named RNA helicase A (RHA), is a DExH/D box RNA helicase capable of unwinding both DNA and RNA (Zhang and Grosse, 1994). Originally purified from calf thymus, NDH II is highly conserved among man (Lee and Hurwitz, 1993), cow (Zhang et al., 1995), mouse (Lee et al., 1998), worm (Wilson et al., 1994), and fruit fly (Kuroda et al., 1991).

1.3.3.1 Characteristics and Domain Structure

Human NDH II gene is localised on the chromosome 1q25, its pseudogene on chromosome 13q22 (Lee et al., 1999). Full-length human NDH II has molecular weight of 142 kDa and localises to nucleus in human cells. The staining pattern of NDH II in interphase cells is homogenous, nucleoplasmic with apparent exclusion from the nucleoli. Murine NDH II, on the contrary, shows distinct

nucleolar staining pattern, predominantly in dense nucleolar compartment (DFC; see Chapter 1.1.3.1.2), often accompanying UBF. Possible explanation so far suggested is the different domain structure (see Discussion) but, however, nucleolar localisation of NDH II can also be observed under certain conditions in human primary cells [transcriptional arrest after reaching confluence; (Fuchsova and Hozak, 2002)]. As early as in prophase, when chromosomes start condensing and nuclear envelope is disrupted, NDH II migrates to cytoplasm (Zhang et al., 1999b).

As a member of DExH/D box helicases, NDH II consists of 7 conserved helicase domains in the helicase catalytic core (see Table 1): the classical Walker-type nucleotide binding sites A and B in motif I, DEIH signature in motif II, and lysine-rich nuclear localisation signal (NLS) between motifs III and IV. Moreover, NDH II, as many RNA helicases, has additional domains flanking the core as well. At the N-terminus, there are two double-stranded RNA (dsRNA)-binding domains (dsRBD); and an arginine-glycine-rich domain (RGG box) is found at the C-terminus of the molecule.

As the name suggests, dsRBDs bind double-stranded RNA and – at least *in vitro* – RNA-DNA hybrids, both forming A-form helices, which differ from the typical dsDNA B-form helix in that the minor groove is shallow and broad and the major groove is narrow and deep. They have been reported to interact with as little as 11 bp of dsRNA, independently of nucleotide sequence arrangement. They are approximately 70 amino acids in length and can be divided into two groups according to the level of homology they possess to a defined consensus sequence. Type A dsRBDs show strong homology to the entire length of the consensus sequence while type B dsRBDs (also referred to as half domains) are highly conserved only at their C-termini and generally bind dsRNA poorly, if at all. dsRBDs mediate protein interactions in RNA interference, mRNA elongation, editing, stability, splicing, and/or export. dsRBDs can act as nuclear

localization signals (NLSs) as well as be involved in mediating nuclear export, this activity is however independent of RNA binding (Liu and Samuel, 1996). Another function of dsRBDs is their ability to mediate protein dimerisation (Doyle and Jantsch, 2003).

The GAR domain contains one or more RG or RGG motifs, which are formed by stretch of arginines and glycines. RGG tri-repeats are found in several RNA-binding proteins (Varani and Nagai, 1998) and as possible substrates of type I protein methyltransferases they can be monomethylated or asymmetrically dimethylated [N_GN_G-dimethylarginine, referred to as aDMA; (Gary and Clarke, 1998)]. The GAR domain can function in protein-protein interactions, and it can bind nucleic acids non-specifically. The GAR domain of pea nucleolin is a DNA helicase that catalytically translocates in the 5'-3' direction and possesses DNA unwinding and ATPase activities (Nasirudin et al., 2005). Further possible roles of GAR domain still need to be elucidated.

Murine NDH II has an unusually elongated RGG-box at the C-terminus of the molecule, but the species-specific function of this has not yet been shown. However, this may be the decisive point that determines its different subnuclear (nucleolar) localisation in murine cells as mentioned before.

1.3.3.2 Interactions and predicted functions of NDH II

In vitro, NDH II binds equally well to ssRNA, dsRNA and with only a slightly decreased affinity to ssDNA, while dsDNA is obviously not its substrate. NDH II was also shown to be a poly(A)-binding protein (Zhang et al., 1999b).

1.3.3.2.1 Transcription

Role of NDH II in transcription can be deduced from the finding that NDH II is a bridging factor between the transcriptional co-activator CBP/p300 and RNA polymerase II (Nakajima et al., 1997); for this interaction its N-terminal domains are responsible (Nakajima et al., 1997). NDH II stimulates transcription via the

interaction with the transcription co-activator, the breast cancer-specific tumor suppressor protein BRCA1 (Anderson et al., 1998). It also forms a component of the NF- κ B transactivation complex – “enhanceosome” – together with TLS/FUS, CBP/p300, RNA pol II and general transcription factor (Tetsuka et al., 2004). NDH II binds to the small nuclear ribonucleoprotein (snRNP)-associated proteins for their recruitment to the RNA polymerase holoenzyme.

NDH II is also able to bind dsDNA, where it co-operates with DNA topoisomerase II α for transcriptional regulation (Zhou et al., 2003). In its N-terminal domain also resides the ability to bind promoter of p16^{INK4a} and to induce its modest up-regulation (Myohanen and Baylin, 2001).

The view of NDH II involvement in transcription can further be supported by the fact that NDH II was shown to interact in a transcription-dependent manner with PML nuclear bodies (Fuchsova et al., 2002), where, according to some reports, transcription of IFN- α -inducible genes may take place.

1.3.3.2.2 RNA metabolism

There are some studies suggesting an involvement of NDH II in the RNA metabolism as well. NDH II has been isolated as a component of human prespliceosomes (Hartmuth et al., 2002) and its subnuclear localisation is similar to pre-mRNA and mRNA binding proteins including the hnRNP proteins (Zhang et al., 1999b). Moreover, NDH II was shown to directly bind to filamentous actin (F-actin) in the nucleus (Zhang et al., 2002). Recently, NDH II has been found to be associated with the dsDNA-end binding protein Ku antigen within heterogeneous ribonucleoprotein complexes formed after ActD treatment, containing γ H2AX as well (Mischo et al., 2005). The helicase also influences retroviral RNA splicing or transport, leading to an overall stimulation of the transcription level of retroviral RNAs (Li et al., 1999) and facilitates its shuttling through the nuclear pore complexes (Tang and Wong-Staal, 2000).

2 MATERIALS AND METHODS

2.1 *Chemicals and equipment*

2.1.1 Chemicals

acetic acid glacial	Lachema
acrylamid	Amresco
actinomycin D	Fluka
agar	Fluka
agarose	Sigma
ampicillin	Fluka
APS	Merck
ATP	Sigma
BCA protein solution A	Pierce
BCA protein solution B	Pierce
bovine serum albumin	Sigma
bromphenol blue	Serva
BSA	Sigma
CaCl ₂	Penta
chlorophorm	Serva
chromatographic papers	Whatman
DAPCO	Sigma
Dapi	Sigma
dithiothreitol	Fluka
DMSO	Sigma
dNTP	Fermentas
EDTA	Serva
EGTA	Sigma
ethanol	Sigma
ethidium bromide	Sigma
FBS	Gibco
fetal bovine serum	Gibco
formaldehyd 20%	Sigma
gentamycin	Sevapharma
glucose	Lachema

glutamin	Sevapharma
glycerol	Sigma
glycine	Serva
HCl	Lachema
HEPES	Sigma
imidazole	Sigma
isoamylalcohol	Sigma
isopropanol	Sigma
kanamycin	Fluka
KCl	Lachema
leupeptin	Serva
L-glutamine	PAN Biotech
methanol	Kulich
MgCl ₂	Sigma
Mowiol	Hoechst
NaCl	Fluka
NaF	Sigma
NaHCO ₃	ÚMG Dejvice
NaOH	Lachema
Ni-NTA magnetic agarose beads	QIAGEN
nitrocellulose membrane	PALL Corp.
N'N'-methylen-bis-acrylamid	Amresco
non-fat dried milk	Promil
Nonidet NP-40	Fluka
Pefabloc	Roche Diagnostics
PEI	Sigma
pepstatin	Serva
PfuULTRA DNA polymerase	Stratagene
phenol	Sigma
Ponceau S	Bio-Rad
potassium acetate	Fluka
protein A agarose beads	Roche Diagnostics
restriction enzymes	Fermentas
RNase A	Boehringer Mannheim
RPMI 1640	ÚMG Dejvice
SAP	Fermentas
SDS	Serva

sodium azide	Sigma
sodium orthovanadate	Sigma
sucrose	Serva
SuperSignal West Femto Maximum Sensitivity Substrate	Pierce
SuperSignal West Pico Trial Kit	Pierce
T4 DNA ligase	Fermentas
TEMED	Sigma
Tris	ICN
Triton X-100	Sigma
trypan blue	Sigma
trypsin	Sigma
tryptone	Oxoid
Tween-20	Serva
X-ray films	Foma Bohemia
yeast extract	Fluka
β -mercaptoethanol	Sigma

2.1.2 Buffers and media

- SOC medium (per 1000 ml):
 - 20 g tryptone; 5 g yeast extract; 0.5 g NaCl
 - dissolve, add 1 ml of 250 mM KCl, adjust pH with NaOH to pH 7.0 and autoclave, prior to use add 0.5 ml of filtered 2M MgCl₂
- LB (Luria-Bertrani) medium (per 1000ml):
 - 10 g tryptone; 5 g yeast extract; 10 g NaCl
 - dissolve and autoclave
- LB (Luria-Bertrani) agar (per 200 ml, cca 10 dishes):
 - 2 g tryptone; 1 g yeast extract; 2 g NaCl; 3 g agar
 - dissolve and autoclave; after cooling pour into Petri dishes (cca 20 ml per dish)
- RPMI
 - 5x RPMI 1640, 10% FBS, gentamicin (40 µg/ml), 0.15% NaHCO₃, 4 mM L-glutamine, 0.011% sodium pyruvate, 2.5 mg/ml glucose, 10 mM HEPES, pH 7.2
- 1x PBS
 - 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄·12H₂O, 1.5 mM KH₂PO₄
- Reducing Sample buffer (4x R-SLB)
 - 250 mM Tris (pH 6.8), 40% glycerol, 8% SDS, 400 mM DTT, 0.2% bromphenol blue
- Sample buffer for BCA (4x SLB I)
 - 250 mM Tris (pH 6.8), 40% glycerol, 8% SDS
- 10x SLB II
 - 1 M DTT, 0.5% bromphenol blue
- SDS-buffer 1 (separation, lower)
 - 1.5 M Tris (pH 8.8), SDS 4 mg/ml
- SDS-buffer 2 (stacking, upper)
 - 0.5 M Tris (pH 6.8), SDS 4 mg/ml
- 30% acrylamide-0.8% N-N'-methylenebisacrylamide (30% Ac-Bis)
 - 30 g acrylamide, 0.8 g N-N'-methylenebisacrylamide
 - H₂O ad 100 ml

- 10x Running buffer for SDS-PAGE
 - 250 mM Tris, 1.92 M glycine, 0.1% SDS
- TGM buffer
 - 192 mM glycine, 25 mM Tris, 5% methanol
- 50x TAE buffer
 - 40 mM Tris, 20 mM glacial acetic acid, 0.5 M EDTA, pH 7.5
- Lysis buffer A
 - 200 mM NaCl, 20 mM Tris, 0.5% Nonidet P-40, 2 mM EDTA, 10 mM EGTA, pH 7.4
- Lysis buffer B
 - 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.05% Tween-20, pH 8.0
- Fixative solution
 - 4% formaldehyde, 0.1% Triton X-100/PBS
- PB buffer for RNase treatment
 - 100 mM KAc, 10 mM Na₂HPO₄, 30 mM KCl, 1 mM MgCl₂, 1 mM ATP, 1 mM DTT

2.1.3 Equipment

ABI Prism 310 sequenator	Applied Biosystems
BR4i centrifuge	Jouan
CO2 incubator IGO 150	Jouan
confocal microscope SP AOBS	Leica
confocal microscope TCS SP	Leica
EPS 3501 XL power supply	Amersham Biosciences
FastBlot	Biometra
flow box MSC 12	Jouan
Gene Pulser TM electroporator	Bio-Rad
horizontal electrophoresis	Thermo EC
inverse fluorescence microscope	Nikon
MiniProtean 3 electrophoresis	Bio-Rad
PowerPac 300 power supply	Bio-Rad
PTC-200 gradient cyler	MJ Research Inc.
shaker/incubator 37C	Lab. Companion
sonicator U50 control	IKA Labortechnik
spectrophotometer Anthelie light 5	Secomam
UV illuminator	Syngene

2.1.4 Antibodies

2.1.4.1 Primary antibodies

- bovine NDH II: rabbit serum (Dr. Zhang, Germany)
- TLS: mouse hybridoma supernatant (Dr. Ron, USA)
- UBF: human autoimmune serum (Dr. Grummt, Germany)
- C23-nucleolin: mouse monoclonal IgG (Santa Cruz Biotechnology, USA)
- DFC: human autoimmune serum (Dr. Hernandez-Verdun, France)
- fibrillarin: mouse monoclonal antibody (Dr. Baran, ČR)
- GC: human autoimmune serum (Dr. Brengon)
- γ H2AX: mouse monoclonal IgG (Upstate, USA)
- B23-nucleophosmin: goat polyclonal IgG (Santa Cruz Biotechnology, USA)
- RNA pol I: rabbit polyclonal antibody (Dr. Grummt, Germany)
- RNA pol II CTD: mouse monoclonal IgG (Dr. de Lanerolle, USA)
- Sm domain: human autoimmune serum (Dept. of Health and Human Services, Atlanta, USA)
- SC-35 (non snRNP splicing factor): mouse monoclonal IgG (Sigma-Aldrich, USA)
- TFIID (TBP): rabbit polyclonal IgG (Santa Cruz Biotechnology, USA)
- PML: mouse monoclonal IgG antibody (Santa Cruz Biotechnology, USA)
- GFP: rabbit polyclonal antibody (Molecular Probes, USA)
- phospho-Ser (for WB): mouse monoclonal IgM (Alexis, USA)
- phospho-Tyr (for WB): rabbit polyclonal antibody (Dr. Hořejší, ČR)
- phospho-Thr (for WB): mouse monoclonal IgG1 (Santa Cruz Biotechnology, USA)

2.1.4.2 Secondary antibodies

- Cy5 – conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA)
- Cy3 – conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA)

- Cy5 – conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, USA)
- FITC – conjugated anti-rabbit IgG (Sigma-Aldrich, USA)
- Cy3 – conjugated anti-goat IgG (Jackson ImmunoResearch Laboratories, USA)
- Cy5 – conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, USA)
- GAM: “goat anti-mouse” antibody conjugated with horseradish peroxidase for protein detection on Western blot (Bio-Rad Laboratories, USA)
- GAR: “goat anti-rabbit” antibody conjugated with horseradish peroxidase for protein detection on Western blot (Bio-Rad Laboratories, USA)

2.2 *Working with bacteria*

2.2.1 Fundamentals for work with bacteria

Work was done in flow-boxes or in a sterile ambient.

All used equipment was sterile - autoclaved plastic, glass and solutions (121°C, 20 min).

Prepared liquid media were stored at 4°C or frozen and agar plates were kept at 4°C upside-down.

The pH of the solutions (buffers, media) was adjusted with 10 M NaOH or concentrated HCl.

2.2.1.1 Selective media

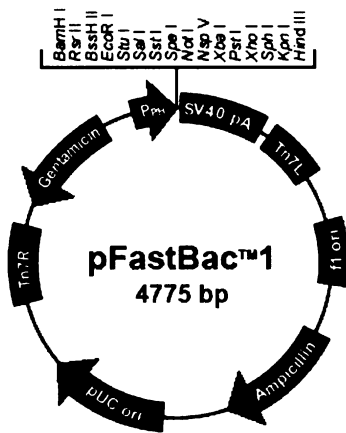
When preparing selective medium (for bacteria with antibiotic resistance), ampicillin or kanamycin were added to a cooled (~40°C) autoclaved solution in a final concentration 100 µg/ml; or 30 µg/ml, respectively.

2.2.1.2 Used bacterial strains of E.coli

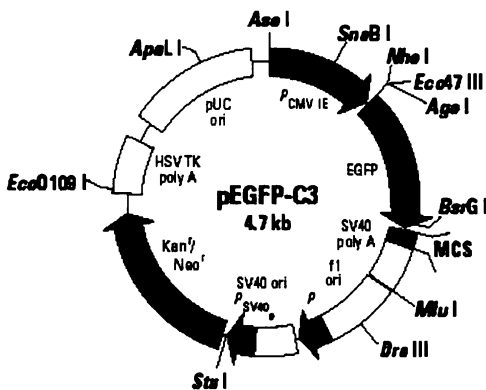
- DH5 α (Invitrogen, USA): F- ϕ 80lacZ Δ M15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoAsupE44 thi-1gyrA96 relA1 λ -

2.2.1.3 Used bacterial plasmids

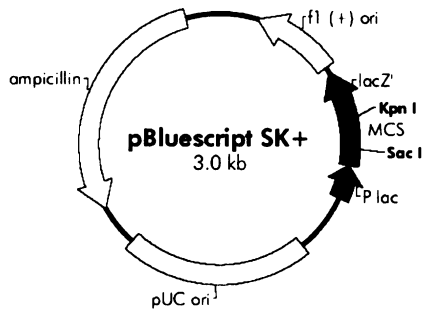
- pFastBac1 (Invitrogen, GeneBank: AY598466.1) 4.775 kb, Amp^r/Gen^r, multiple cloning site, pUC origin of replication, polyhedrine promoter, SV40 polyadenylation signal



- pEGFP-C3 (Clontech Laboratories, GenBank: U55761): 4.7 kb, enhanced green fluorescent protein gene (EGFP), Kana/Neo^r, multiple cloning site, SV40 origin of replication, hCMV immediate early promoter, HSV TK polyadenylation signal



- pBluescript SK(+) (Stratagene): 3.0 kb, Amp^r, multiple cloning site, f1 and pUC origin of replication, lac promoter, β -galactosidase α -fragment



- pBluescript-NDH II (Dr. Zhang, Jena, Germany): original plasmid pBluescript SK+ with cDNA of bovine NDH II

2.2.2 Cultivating bacteria

2.2.2.1 In liquid media

For good aeration, the culture bottle should have a volume at least five times bigger than the volume of the containing medium. Antibiotics were added as described.

The bacterial culture was added either as a liquid growing culture or as a colony picked up by a sterile pipette tip or a sterile toothpick.

Incubation was done at 37°C at vigorous shaking until the optimal density was reached.

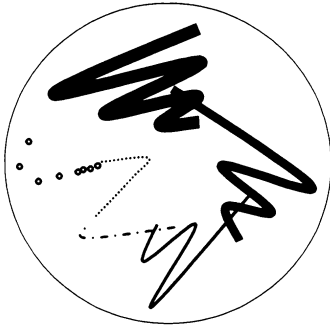
2.2.2.2 On solid media

An adequate volume of a liquid bacterial culture (100-200 µl) was poured onto agar plates and spread across the whole agar surface.

The plates were kept at 37°C upside-down overnight.

If single colonies did not occur, smaller volume of the bacterial culture was applied onto the plates or a greater dilution of the bacterial culture was used.

Eventually, a following method of spreading over the agar plate might have been used:



The streak was begun at one edge of the plate. The side of the toothpick containing cells was pressed to the surface of the agar plate and quickly pulled back and forth across a part of the surface. The streaks should lie near one another but should not cross over previous streaks. The same step was repeated in new territory of the agar plate with a fresh toothpick starting by crossing over the branch of the preceding streak.

2.2.3 Transformation of competent bacteria

2.2.3.1 Preparation of chemically competent bacteria (CaCl₂ method)

20 ml of LB medium was inoculated with 0.2 ml of an overnight grown culture of DH5 α . Cells were incubated at 37°C at vigorous shaking until reaching the OD₆₀₀ 0.4 –0.6.

Culture was collected into 50 ml polypropylene centrifuge tubes and after centrifugation (4000 rpm, 7 min, 4°C), the bacterial pellet was resuspended in 10 ml of ice-cold sterile 100 mM MgCl₂. The cell suspension was incubated for 1 hour on ice and then centrifuged again (4000 rpm, 5 min, 4°C). Cells were resuspended in 1 ml of ice-cold sterile 100 mM CaCl₂ and sterile glycerol was added to a final concentration of 20 %.

Aliquots of the bacterial suspension (of 100 μ l) were frozen in liquid nitrogen and stored at -70°C .

2.2.3.2 Preparation of electrocompetent bacteria

20 ml of liquid LB medium was inoculated with a colony of DH5 α grown on agar plate. Cells were incubated overnight at 37°C at vigorous shaking. Next day, 1000 ml of LB medium were inoculated with 10 ml of this pre-culture and incubated at 37°C at vigorous shaking until OD_{600} 0.5 – 0.7 was reached.

At that time, the culture was cooled on ice for 20 min and then centrifuged (4000 rpm, 5 min, 4°C). Pellet was resuspended in 1000 ml of ice-cold sterile 10% glycerol and the suspension was incubated on ice for 20 min.

After centrifugation (4000 rpm, 5 min, 4°C), the pellet was resuspended in 100 ml of ice-cold sterile 10% glycerol and again incubated for 20 min on ice.

After the last centrifugation (4000 rpm, 5 min, 4°C), the pellet was resuspended in 4 ml of ice-cold sterile 10% glycerol and 100 μ l-aliquots of the cell suspension were made.

These were frozen in liquid nitrogen and stored at -70°C .

2.2.3.3 Transformation of competent bacteria by heat shock

Primarily, the aliquots of chemically competent cells were thawed on ice and transformation tubes (Falcon #2059) were pre-chilled.

DNA was put on the wall of the transformation tube and 100 μ l of competent cells were added so that the drop of the DNA was washed down with the cells and resulting mixture was incubated for 30 min on ice.

Next, cell suspension in cap tubes was heat-shocked in the 42°C water bath for 45-50 sec (time is important, should not get over 60 sec and below 40 sec) and then put back on ice for 2 min.



0.9 ml of SOC medium at RT was added into the tube and incubated at 37°C for 1 hour at vigorous shaking.

100-200 µl of the cells were plated on agar plate and incubated at 37°C overnight, upside down. Rest of the transformation suspension was stored at 4°C.

Next day, after counting the colonies, the transformation efficiency (colony forming units per µg of DNA) was determined. If we did not get any colonies, the rest of the transformation suspension was centrifuged and supernatant, except 100-200 µl of SOC medium, was discarded. The remaining suspension was plated on a fresh agar plate.

2.2.3.4 Transformation of electrocompetent bacteria by electroporation

The aliquots of electrocompetent cells (100 µl for one transformation reaction) were thawed on ice and plasmid DNA or ligation mixture (1-5 µl) was added. The mixture was put into chilled electroporation cuvettes (Bio-Rad, gap width 0.2 cm). Electroporation was done using the Bio-Rad electroporator under following conditions: 2500 V, 200 Ω, 25 µF.

Immediately after electroporation, 1 ml of LB medium was added to the cuvette; the mixture was put into cap tubes and incubated at shaking for 1 hour at 37°C.

Cells were then briefly centrifuged, inoculated on an agar plate with appropriate selective antibiotics and incubated at 37°C for 16-24 hours.

2.3 *Working with cell lines*

2.3.1 Fundamentals for work with cell cultures

Work was done in sterile flow-boxes.

All used equipment was sterile - autoclaved plastic, glass and solutions (121°C, 20 min).

Prepared liquid media, antibiotics and add-ins were stored at 4°C or frozen.

2.3.2 Used mammalian cell lines

- H1299: human cell line derived from lymph node metastasis of non-small cell lung cancer which lack expression of p53 protein due to a homozygous partial deletion; cultivated in RPMI medium

2.3.3 Cultivating of cell lines

All cell lines were cultivated in CO₂ incubators at 37°C and 5% CO₂ atmosphere until reaching the needed stage of confluency.

2.3.4 Passaging of cell lines

Cell passages were performed in order to provide the cell culture with fresh medium and relieve of toxic or harmful products of their metabolism.

The spent medium was aspirated and the dish was washed once with an equal volume (or greater) of PBS. Enough trypsin (0.05% trypsin-0.52 mM EDTA was applied to cover the dish bottom surface (approximately 0.5 ml for a 60 mm dish), an excess of trypsin was aspirated and plates were returned into a CO₂ incubator for 1-2 min.

Next, 0.7-1 ml of fresh medium was added, the cell clumps were broken up by vigorous pipetting and swished all around the dish with a circular stream, avoiding bubbles as much as possible till reaching an even suspension of single cells.

Finally, the suspension of cells was transferred to an equal volume of medium in a new dish.

<u>Dish</u>	<u>surface area [cm²]</u>	<u>volume of medium[ml]</u>
96-well	~0.2	0.100
24-well	2.0	0.500
35 mm	9.5	2.0-2.5
60 mm	21	5.0
100 mm	56	10.0-12.5

2.3.5 Freezing of cell aliquots

To the trypsinised cells (see above), 0.7 ml of fresh medium was added and the cell clumps were broken up by vigorous pipetting until reaching a homogenous suspension of cells.

0.6 ml of the suspension was put into a cryotube, where already DMSO and FBS were added to a final concentration of 10 % and 40 %, respectively.

Suspension was thoroughly mixed and put directly into an isopropanol-containing box for protracted freezing.

2.3.6 Thawing of cell aliquots

The cryotube was put directly from -70°C into a 37°C water bath for 1-2 min until the suspension was fully thawed. Then, the cell suspension was transferred into 5 ml of fresh medium in an 15 ml Falcon tube and mixed carefully.

The cells were centrifuged at 1500 rpm for 5 min at RT, the supernatant aspirated, leaving 500-1000 µl for resuspending the cells. Next, the suspension was transferred in a Petri dish with an adequate volume of fresh medium.

2.3.7 Transfection of mammalian cells

2.3.7.1 Transfection using PEI

Transfection was performed in the 24-well plates. Cells should have reached 60-70 % confluence when starting the transfection procedure.

2 hours prior to transfection, culture was supplied with an equal volume of fresh 10% FBS-containing medium.

For each well, two separate mixes were prepared: 50 µl of plasmid DNA (at different final concentrations)/150 mM sterile NaCl and 50 µl of 1x sterile PEI/150 mM sterile NaCl, and left at RT for 15 min. Then, these two suspensions were mixed together and left to stand at RT for next 15 min. Meanwhile, 10% FBS medium was removed and replaced with half volume of serum-free medium.

100 µl of the DNA/PEI/NaCl solution was added to cell culture in serum-free medium and put into a CO₂ incubator.

2-3 hours after transfection, depending on how the cells withstood the serum deprivation and PEI, half volume of 20% FBS medium was added to cells.

6 hours after transfection, the transfection medium was removed and replaced with fresh 10% FBS medium.

2.3.7.2 Transfection using LipofectamineTM2000

Transfection was performed in 24-well plates. Cells should have reached 60-70% confluency when starting transfection procedure.

For each transfection, 1 µg of DNA in 50 µl of serum-free medium in a separate tube, and 1 µl of Lipofectamine in 50 µl of serum-free medium in another tube was diluted. The medium must not have contained antibiotics, as they destroy Lipofectamine particles and cause cell death.

The two solutions were combined with gentle mixing and incubated for 15 min at RT.

Cells were rinsed once with serum-free medium and 0.4 ml of serum-free medium was added to the tube of DNA and Lipofectamine before overlaying the mixture onto the rinsed cells.

Cells were incubated for 6-10 hours, then fresh complete medium was added. The medium was changed again 18-24 hours after the start of transfection.

Effectivity of the transfection was established 24 hours after transfection using fluorescence microscopy.

2.4 Preparation of tagged recombinant proteins

2.4.1 Cloning

2.4.1.1 Plasmid DNA isolation from bacteria

3 ml of the liquid bacterial culture were subsequently centrifuged in a 1.5 ml eppendorf tube (8.300 rpm, 8 min, 4°C). The rest of the culture was stored at 4°C. The medium was aspirated and the bacterial pellet was left at -20°C for 1-3 hours to facilitate the bacterial lysis.

100 µl of P1 solution with RNase A (100 µg/ml) at RT was then added to the pellet and bacteria were resuspended by vigorous vortexing. Next steps were performed on ice to prevent degradation events after bacterial wall disruption (not necessary).

200 µl of P2 solution then was added, mixed by inverting the tube 5 times (NOT vortexed) and incubated on ice for 5 minutes.

Next, 150 µl of ice cold P3 were added, the lysate was mixed by inverting the tube 5 times and incubated on ice for another 5 minutes.

Resulting precipitate of proteins was centrifuged at 14 000 rpm, 10 minutes at 4°C.

Next, the supernatant containing plasmid DNA was either first subjected to phenol-chloroform extraction or directly precipitated with ethanol.

For phenol-chloroform purification, equimolar volume of Tris-equilibrated phenol (pH 8.0) was added to the supernatant, inverted 30 times and spun down (2 min, 5000 rpm, RT). The phenol fraction was discarded and this step was repeated again with another volume of phenol, phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1).

After last chloroform extraction, the upper fraction containing DNA was collected into new eppendorf tube and precipitated with either isopropanol (0.7 volumes) or with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ice-cold 96% ethanol. The suspension was put into -20°C and then centrifuged at 14 000 rpm, 15 min, 4°C. The supernatant was discarded; the pellet of precipitated DNA was rinsed with ice-cold 70% ethanol and air-dried. Finally, the DNA was dissolved in sterile dH₂O.

2.4.1.1.1 Characterisation of DNA by spectrophotometer

Concentration of purified DNA was measured spectrophotometrically as a function of the absorbance of the solution measured at 260 nm (A_{260}) and then calculated using the formula:

$$C_{\text{dsDNA}} = A_{260} \times 50 \mu\text{g/ml} \times \text{dilution factor}$$

Its purity was determined by measuring the A_{260}/A_{280} ratio; values should have reached about 1.8; lower values indicated contamination with aromatic substances (phenol) or proteins, higher with RNA.

2.4.1.1.2 Characterisation of DNA by agarose gel electrophoresis

Horizontal agarose gel electrophoresis was performed to establish the proper size and conformation of isolated DNA.

Agarose gel was prepared as a solution of agarose powder in 1x TAE buffer at final concentrations of 0.8 – 2 %. The suspension was heated until the powder completely dissolved and after cooling down to approximately 40°C it was poured into the electrophoretic tray with combs of appropriate size and number and was left to solidify.

Then, the gel was submerged with TAE buffer and samples containing 1x loading buffer (Fermentas) were loaded into the gel wells. The electrophoretic separation was performed at 70-90 V Bio-Rad PowerPac 300 power supply. The separation was stopped as soon as the bromophenol blue reached 2/3 of the gel. DNA was visualised after the incubation of the gel in TAE buffer containing 20 µg/ml ethidium bromide (15 min) using GENEGenius Bioimaging system (SynGene) and GeneSnap software (SynGene) for image editing.

2.4.1.2 Restriction analysis

Restriction analysis of the DNA was performed to either determine appropriate length and sequence of the DNA or to cut DNA into suitable fragments that were further subjected to cloning.

Restriction cleavage was done at 37°C for 1-3 hours in volumes depending on the final employment of the product (10 µl for DNA verification; 100 µl for DNA isolation from the gel); always altering the original schedule:

- DNA: maximum of 50 ng in one reaction (for verification, not limited when for cloning)
- restriction enzyme (1 U for digest of 1 µg of substrate DNA in 16 hours)
- 1x appropriate restriction enzyme buffer
- H₂O

2.4.1.3 DNA sequencing

DNA sequencing was performed by Dr. Felsberg (Institute of Microbiology, ASCR, Prague) using ABI PRISM 310 sequenator.

Obtained sequences were analysed by BioEdit and SeqMan (Lasergene) software.

2.4.1.4 Isolation of DNA fragments from the gel

Agarose gel with wide combs for accomodation of great volume of sample, pre-stained with EtBr (20 µg/ml), was prepared. DNA was separated under low voltage (70 V) to prevent band diffusion. As soon as the bands were distinctly separated, bands of DNA of appropriate size were cut off with clean scalpel under the UV illumination. DNA was isolated using the Zymoclean colony purification kit according to the manufacturers protocol (Zymo Research).

2.4.1.5 Dephosphorylation and ligation

Prior to ligation, the purified DNA fragments had to be dephosphorylated in order to prevent plasmid self-circularisation during ligation. Eventually, dephosphorylation could have been performed before the isolation of DNA from the gel to get rid of the phosphatase which was in some cases reported to inhibit the ligation reaction. The dephosphorylation was performed for 30 min at 37°C and phosphatase was inactivated for 15 min at 65°C.

- 1 U of shrimp alkaline phosphatase (SAP) dephosphorylates 1 pmol of 5' ends of plasmid DNA
- The amount of the 5' ends in the DNA solution was calculated as

$$= \frac{2 \times 10^6 \times \mu\text{g}(dsDNA)}{X_{bp} \times 660Da}$$

Ligation mixture was prepared according to the following protocol:

- DNA: the amount of overall DNA should not have exceeded 200 ng in one reaction mixture. The DNA of vector and insert was basically of the same size and thus the DNA(vector)/DNA(insert) = 100 ng/100 ng
- 1x Ligation buffer containing ATP: common restriction buffers are also compatible with T4 ligase when adding ATP in final concentration of 0.25-1 mM
- T4 DNA Ligase: about 1 μ l for cohesive or blunt ends per reaction; time of the reaction can be reduced to as little as 10 min at 22°C when joining sticky ends at this amount of enzyme. Theoretically as little as 0.01 U should be sufficient for cohesive ends ligation
- H₂O up to 10 μ l

The ligation reaction was thoroughly mixed, kept at RT for 1-2 hours and after that was either directly transformed into bacteria or kept at -20°C for further use.

2.4.2 Deletion mutagenesis

Inverse PCR (IPCR) originally serves for rapid *in vitro* amplification of DNA sequences that flank a region of known sequence. The method uses the polymerase chain reaction (PCR), but the primers are oriented in a reverse direction of the usual orientation. In our case, this method was used to create deletion mutants lacking a certain domain using pair of primers oriented „out of“ this domain into the plasmid.

After IPCR, the nonmutated parental plasmids, which are methylated during growth *in vivo* in a Dam⁺ host, were digested by Dpn I restriction enzyme (recognition site: 5'-Gm6ATC-3'), while PCR-generated linear plasmid DNA remains undigested.

This linear DNA is then end-polished with cloned PfuUltra™ DNA polymerase to remove extended bases placed on the 3' ends of the PCR products by DNA polymerase, and ligated to create a circular molecule of plasmid containing a partially deleted cDNA of our interest.

2.4.2.1 Inverse PCR

PCR reaction was prepared according to the following protocol:

Name	Sequence	No. of bases	Tm	GC%
NDH II3315	5'-CATGGAGGCTTTGGTTGTTGAA-3'	22	47.9	45.5
EGFP_N_seq	5'-CGTCGCCGTCAGCTCGACCAG-3'	22	69.6	72.7
NDH II_128	5'-TAACCTTCCACCTGAACCTC-3'	20	58.4	50.0
RGG_R	5'-Pho-ATATCCGCTTCCATTGTCG-3'	19	55.2	47.4
RGG_F	5'-Pho-TAATGTCAGTTCTGCGGTCC-3'	20	58.4	50.0
RBD_I_R	5'-Pho-ACACCAGGCATACAGAAAAT-3'	20	53.2	40.0
RBD_I_F	5'-Pho-AAGAGTGAAGAAGTTCCAGC-3'	20	55.3	45.0
RBD_II_R	5'-Pho-ATTTTCCAAGGTCCAGTTT-3'	19	50.2	36.8
RBD_II_F	5'-Pho-CAAACATCATTCAAGAGC-3'	19	50.2	36.8
DEIH_core_F	5'-Pho-GAGGGGTAACTCGGAGCAA-3'	20	59.4	55.0
DEIH_I/IIa_R	5'-Pho-GGGTGTAGGTCATCGGGG-3'	18	60.5	66.7

- ▣ DNA: 10-100 ng of purified template plasmid DNA per reaction
- ▣ primers: both forward and reverse primers had 5'-Pho in order to facilitate the recircularisation of product plasmid; added in an amount of 100 ng (0.2-0.5 mM) per primer per reaction
- ▣ dNTPs: 25 mM each dNTP
- ▣ 1X *PfuUltra*™ buffer
- ▣ *PfuUltra*™ DNA polymerase : 2.5 U per reaction for vector targets up to 15 kb
- ▣ deionised distilled H₂O up to 25 µl

The components of the PCR reaction were gently but thoroughly mixed and put in the Gradient cycler PTC-200. The PCR cycling parameters were for each reaction designed as following:

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 min
2	20	95°C	30 sec
		primer Tm – 5 °C	30 sec
		72 °C	1 min per kb
3	1	72°C	10 min

2.4.2.2 Digesting, polishing and ligation of the product

Directly to the 25 µl of PCR reaction, 1 µl of the *Dpn I* restriction enzyme (10 U/µl) and 0.5 µl of *PfuUltra*TM DNA polymerase (2.5 U/µl) was added and, after gentle mixing, the reaction mixture was put in the Progene cyclor and incubated at 37°C for 30 min and at 72°C for additional 15 min.

Next, to ligate the ends of newly synthesised plamids, 1 µl of T4 DNA ligase (Fermentas) was added to the PCR reaction and left at 37°C for 1 hour.

After ligation, the product was subjected to transformation. The amount of DNA used in the transformation reaction was titrated individually for each reaction.

2.5 *Analysis of protein expression*

2.5.1 SDS-PAGE and Western blot detection

2.5.1.1 Isolation of nuclei from human cells

All steps were performed on ice. Protease and phosphatase inhibitors were added to all applied solutions in a final concentration of 10 mM NaF, 1 mM sodium orthovanadate, 1 mM Pefabloc SC, 1 µg/ml leupeptin, 1 µg/ml pepstatin.

Cells grown on 100-mm dishes were rinsed with ice-cold PBS and 0.5 ml of ice-cold Lysis buffer was added to cover the plate. Cells were scraped and collected to an eppendorf tube. The suspension was then pulled through the insulin needle to facilitate the plasma membrane disruption and the process was controlled visually in the microscope using 0.4% tryptan blue.

The cell lysate was then centrifuged at 5000 rpm, 5 min, 4°C and 60 µl of the 4x SLB I was added to the pellet of cell nuclei. After short incubation (1-2 min) at

99°C the sample was sonicated (amplitude 40-50%, 3 x 5 sec) and either subjected to measuring protein concentration or frozen in -20°C.

2.5.1.2 Determining protein concentration using BCA method (Pierce)

Standards in concentrations of 25 – 2000 µg/ml BSA diluted in H₂O were used for calibration. Samples were diluted 1:25, 2:25 and 4:25 in duplicates in H₂O.

200 µl of BCA protein solution B was mixed with 10 ml of BCA protein solution A and 500 µl of combined solution were added to all standards and samples and blank, and vortexed. Incubation was performed at 37°C for 30 minutes. After cooling the tubes, absorbance was measured spectrophotometrically at 562 nm. Protein concentration of the sample was determined by calculation from the standard curve.

2.5.1.3 SDS – PAGE

Standard 7% separation SDS-gel and 4% stacking gel were prepared according to following table:

Volume	20 ml
Percentage	7%
Water	10.3 ml
"Lower" buffer	5 ml
30% Ac-Bis	4.7 ml
10% APS	0.09 ml
TEMED	0.008 ml

Volume	7.5 ml
Percentage	4%
Water	4.5 ml
"Upper" buffer	1.875 ml
30% Ac-Bis	0.975 ml
10% APS	0.0675 ml
TEMED	0.006 ml

Samples were combined with 10x SLB II and boiled at 95°C for 1 min. Next, samples were loaded into the gel wells (50 µg of proteins per lane) and an electrophoretic separation was performed at 50 V for first 30 min to sharpen the bands in the stacking gel; then at 35 mA with limitation of 150 V until the tracking line of bromphenol blue in the sample reached bottom of the gel.

2.5.1.4 Western blotting

Proteins separated in SDS-PAGE were transferred onto a nitrocellulose membrane using a semi-dry blot (FastBlot, Biometra).

Both chromatographic papers and membrane were humidified with TGM buffer and the transfer was performed for 30 min, 5 W.

2.5.1.5 Antibody detection

After Western blotting, free binding sites on the membrane without bound proteins were blocked in 5% milk in PBS/0.05% Tween-20 for 45 min and then incubations with primary and secondary antibodies (in 1% milk/0.1% sodium azide) followed. For anti-phospho antibodies 5% BSA and 1% BSA instead of milk was used for blockation and dilution of primary antibody; respectively. After each of them, the membrane was washed 3x5 min with PBS/0.05% Tween-20 to dispose of unbound and/or non-specifically bound antibodies.

Finally, the membrane was incubated with substrates and immunocomplexes were detected using ECL (SuperSignal West Femto Maximum Sensitivity Substrate; SuperSignal West Pico Trial Kit) and exposed to an X-ray film.

2.5.2 Immunoprecipitation

All steps were performed on ice in order to prevent protein degradation after cell lysis. In all used solutions, proteinase inhibitors were added to a final concentration 1 mM Pefabloc SC, 1 µg/ml leupeptin, 1 µg/ml pepstatin.

Cells were rinsed with ice-cold PBS, then 0.5 ml of ice-cold Lysis buffer A was applied to cover the dish surface and the cells were scraped into an eppendorf tube. Lysis was performed for 10-15 min until cells were completely lysed as confirmed visually in the microscope.

After centrifugation (14 000 rpm, 10 min, 4°C) the supernatant was collected into a new eppendorf tube and an appropriate volume of immunoprecipitating

antibody was added. A 2-hour incubation at 4°C on a rotary incubator followed.

Next, Protein A agarose bead suspension was added to lysate and the mixture was incubated at 4°C on a rotary incubator for 1 hour. The bead suspension was washed 3x5 min with 0.5 ml of Lysis buffer to remove unbound protein and antibody. After a final short spin, 50 µl of Lysis buffer and Sample buffer were added to the beads and the sample was boiled at 99°C for 2 min.

Next, either SDS-PAGE was performed or the sample was kept frozen at -20°C until further use.

2.5.3 Co-precipitation using Ni-NTA agarose beads

All steps were performed on ice in order to prevent protein degradation after cell lysis. In all used solutions, proteinase inhibitors were added to a final concentration of 1 mM Pefabloc SC, 1 µg/ml leupeptin, 1 µg/ml pepstatin.

Cells were rinsed with ice-cold PBS, then 0.5 ml of Lysis buffer B was applied to cover the dish surface and the cells were scraped into an eppendorf tube. Lysis was performed for 10-15 min until cells were completely lysed as confirmed in the microscope.

After centrifugation (14 000 rpm, 10 min, 4°C) the supernatant was collected into a new eppendorf tube and appropriate volume of 5% Ni-NTA agarose bead suspension (Qiagene) was added. Incubation was performed for 1 hour at 4°C on a rotary incubator. After a short spin, 50 µl of the Elution buffer were added to the pellet of agarose beads with bound protein. After a short incubation on a rotator, the mixture was spun down and the eluate was collected. 4x R-SLB was added to the eluate and the sample was boiled at 99°C for 2 min.

Next, either SDS-PAGE was performed or the sample was frozen at -20°C until further use.

2.5.4 Indirect immunofluorescence staining

The cells grown on coverslips were rinsed twice with PBS and fixed in Fixative solution for 20 min at RT. After washing fixed cells 3x5 min with PBS, 1 hour incubation with primary antibody was performed.

After washing cells 3x5 min with PBS/0.05% Tween-20, 45 min incubation with secondary antibody followed.

Eventually, third staining (20 min at RT with 1 μ M TOTO3) was performed.

Finally, after washing the cells 3x5 min with PBS/0.05% Tween-20, coverslips were mounted in Mowiol + 2.5% DAPCO + 1 μ g/ml DAPI, coated with polish and kept in a dark and cold place.

Immunofluorescence was visualized using confocal laser scanning microscope LEICA TCS SP. Image files were processed with the Adobe Photoshop 7.0 software.

2.5.4.1 RNase treatment

For a RNase treatment, cells were fixed with 2% PFA, 0.2% Triton X-100 in PB buffer (20 min, on ice), then subjected to nuclease digestion with RNase A (1 mg/ml, 30 min, 37°C) and after washing with PB two times, immunostaining was performed as described previously.

To all applied solutions, proteinase inhibitors were added to a final concentration of 1 mM pefabloc SC, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin.

2.5.5 Live cell imaging

2.5.5.1 Using Spectral Confocal Microscope Leica TCS SP

Live cell observation was done in plastic Petri dishes for cell cultivation. The dish was assembled into the adapter with heated table and temperature was maintained at 37°C. The medium was CO₂-independent (Gibco), but the cells

were kept under normal atmosphere. The submersible objective was used (Leica; 40x) and scans were collected in the frequency of 6 minutes. The pictures were processed using Leica and Adobe Photoshop 7.0 software.

2.5.5.2 Using Spectral Confocal Microscope Leica SP AOBS

Living cell studies were performed using an FCS2 live cell chamber. Transfected cells were grown on customized coverslips (diameter 32 and 40 mm) that were assembled into the FCS2 chamber, covered with 1 ml of suitable medium (RPMI), where temperature was maintained at 37°C, and cells were kept in the atmosphere containing 5% CO₂. The chamber was directly mounted onto the specimen stage of an inverted epifluorescence microscope (Leica) equipped with a cooled CCD camera. The scans were performed each 6 minutes in order not to damage the cell with laser irradiation and not to bleach out EGFP. Pictures were processed with Leica and Adobe Photoshop 7.0 software.

3 RESULTS

3.1 *Preparation of EGFP-NDH II fusion construct*

I started working on my project with the goal to prepare a vector containing NDH II fused with EGFP at the N-terminus.

After successful cloning of NDH II full-length cDNA (Dr. Zhang, Germany) into EGFP-C3 expression vector (Clontech), transfected H1299 cells did not show any sign of detectable expression of EGFP-NDH II.

Then, sequencing showed that the assumed “NDH II” cDNA was actually a combination of bovine and human NDH II, probably a preliminary cloning intermediate obtained while attempting to get full-length cDNA from the cDNA library.

I then received another plasmid with a cDNA that matched the published sequence of NDH II (GenBank Accession Nb: Y_10658) and started again from the beginning.

Both plasmids were transformed into DH5 α bacteria using heat shock and positive clones selected during incubation on Amp⁺, respectively Kan⁺ agar plates. DNA was isolated using the QIAfilter™ Plasmid Maxi Kit (QIAGEN) for isolation of plasmid DNA.

Both plasmids were cut by BamH I; restriction of pFastBac1-NDH II resulted in 2 fragments – full length NDH II cDNA and a linearised pFastBac1; pEGFP-C3 was with BamHI linearised in its multicloning site (see Chapter 2.2.1.3).

NDH II cDNA and linearised pEGFP-C3 were ligated and the resulting pEGFP-NDH II construct was transformed into DH5 α by electroporation. Positive clones were isolated, and the plasmid DNA was purified using the QIAfilter™ Plasmid Maxi Kit (QIAGEN). The obtained DNA was cut with restriction

enzymes (BamHI, HindIII, EcoRI) in order to determine the proper orientation of the insert and length of the construct. Furthermore, the fusion area of the molecule was sequenced in order to determine whether no frameshift mutation occurred.

3.2 *Preparation of cell line stably expressing EGFP-NDH II*

Next, DNA was transfected into H1299 cells using PEI transfection protocol, as described (see Chapter 2.3.7.1).

Transfected cells were selected for first month with geneticin (50 µg/ml – 200 µg/ml) and cloned with cloning circles.

The integrity of expressed fusion protein was confirmed by Western blotting and by indirect immunofluorescence.

Western blotting analysis with anti-NDH II antibody confirmed presence of two NDH II molecules differing by the mass corresponding to EGFP (~26 kDa), in control cells only endogenous (~140 kDa) protein was expressed (see Figure 1A). The anti-GFP staining gave no specific signal in control cells, whereas in transfected cells it recognised a band migrating at ~ 170 kDa (see Figure 1A).

EGFP-NDH II fluorescence signal in control cells showed an expected nucleoplasmic staining out of nucleoli that colocalised to the staining pattern of the anti-NDH II antibody (see Figure 1B).

The antibody staining, however, showed some disadvantages when compared to EGFP. The images obtained with the antibody were smeared and, moreover, when staining of NDH II in the nucleolar “caps” (see further) after transcription inhibition was performed, the antibody clearly had problems to penetrate into the dense nucleolar material and stain the antigen properly (see Figure 2A).

To conclude, stably transfected cell line of H1299 cells expressing EGFP-NDH II was successfully established.

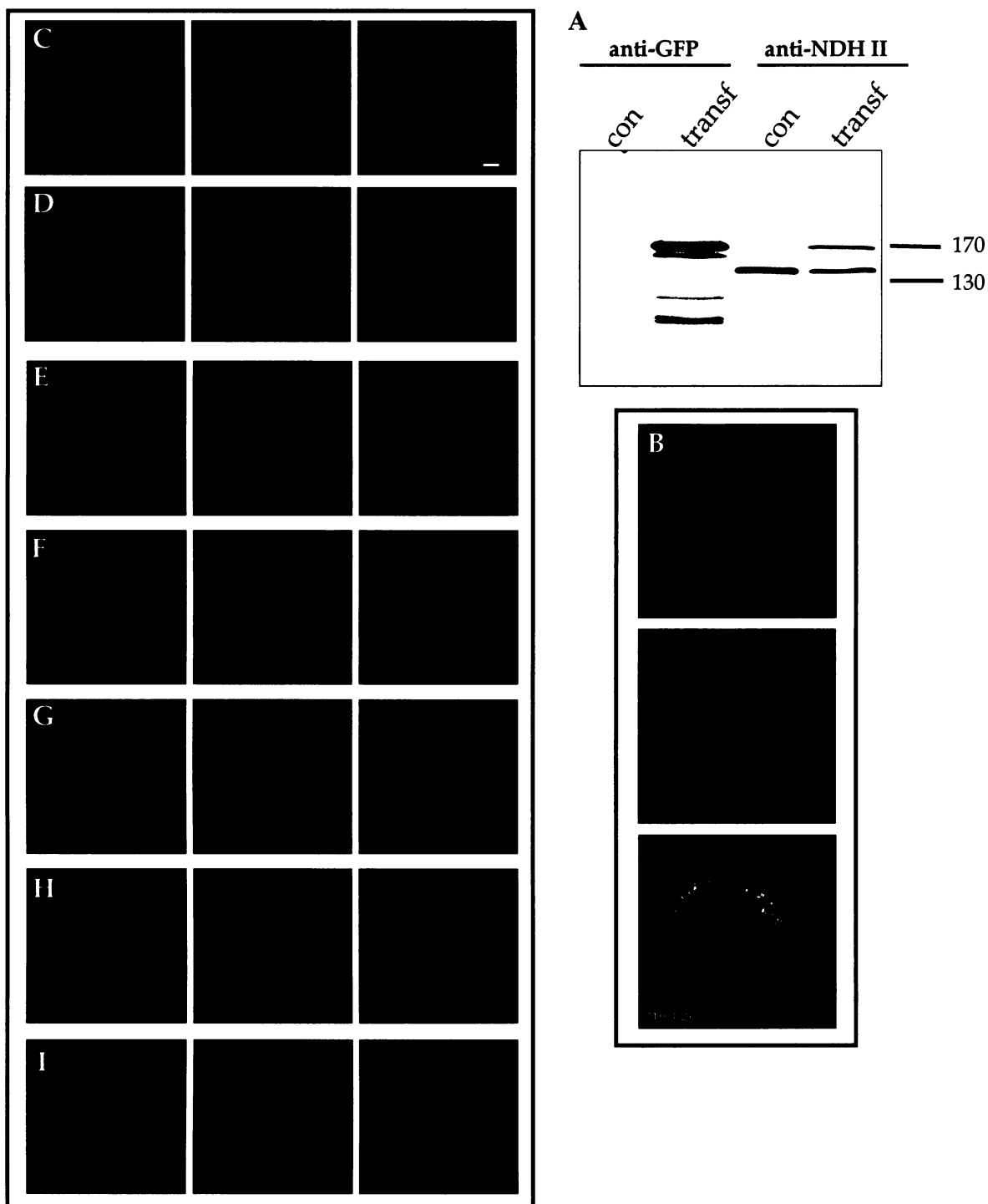


Fig. 1: Perinucleolar bodies analysis.

(A) Both EGFP-NDH II fusion protein and endogenous NDH II are expressed in transfected cells (transf) and are detected at the expected size by Western blot using antibodies anti-NDH II and anti-GFP. (B) The nucleoplasmic localization of EGFP-NDH II (green) is indistinguishable from endogenous NDH II (merge), yet, in non-transfected cells stained with the anti-NDH II antibody, no "perinucleolar bodies" are observable (red). (C- I) overlap of NDH II (green) in control cells with (C) PML, (D) RNA pol II, (E) TBP, (F), SC-35, (G) Sm, (H) γ H2AX, (I) TLS (all red). Right-hand column shows the merged output. Bars, (B) 5 μ m, (C- H) 2 μ m.

3.3 *NDH II colocalised to perinucleolar bodies*

After the transfection, I noticed that besides nucleoplasmic staining, intensively stained speckles are found close to nucleoli (see Figure 1B). These were only observed in cells transfected with fusion EGFP-NDH II, the antibody, for some reason, failed to recognise the structures in untransfected cells (see Figure 1B).

The bodies were always localised adjacent to the nucleoli, in a number of 1-3 per nucleus. They were present in ~85 % of cells, unrelated to the level of confluence of the cell culture. During mitosis, the bodies underwent a cycle of dissolving and reconstruction; during interphase, however, they showed no sign of intense movement along the nucleolus or further to the nucleoplasm, as confirmed by live cell microscopy. Their size and shape differed between the cells according to an unknown condition. Upon selective inhibition of enzymes of posttranslational modifications, their occurrence was not influenced and their number did not change (see Figure 3F). RNase treatment also had no observable effect on their occurrence or their appearance what might suggest that their scaffold is not formed by RNA. However, any treatment that affects the transcriptional level of the cell, although results in an unaltered nucleoplasmic localisation of NDH II, dissolved the bodies (low ActD treatment (5-40 nM), wash-out of 400 nM ActD; data not shown).

It is generally believed that the main function of NDH II lies within the RNA pol II transcription pathway (see Chapter 1.3.3.2.1), and so I examined, whether these NDH II-containing “perinucleolar bodies” would colocalise with some proteins involved in transcription – RNA pol II, PML, and TBP, a marker of the OPT domain (see Chapter 1.1.3.1.1). Double immunofluorescence studies showed no colocalisation of these antigens with the “perinucleolar bodies”. In addition, NDH II may be involved in RNA splicing, therefore, I co-stained the EGFP-NDH II expressing cells with antibodies against the snRNP marker Sm and a non-snRNP splicing factor SC-35. No colocalisation of both antigens with

NDH II was observed. Moreover, these “perinucleolar bodies” were neither containing damaged DNA as shown by staining with the antibody against γ H2AX, a marker of single and double stranded DNA breaks.

Thus, with these experiments we were unable to identify possible components of NDH II-containing “perinucleolar bodies”. We observed no colocalisation with some of the previously described NDH II interacting partners (RNA pol II, PML and γ H2AX).

3.4 NDH II is translocated into nucleolar caps after ActD treatment

Nucleolus, when transcription is disabled, forms distinct nucleolar “caps” that differ according to the contents and microscopic appearance [see Figure 2B; (Malatesta et al., 2000; Vera et al., 1993)]. “Dark nucleolar caps” (DNCs) have a concave base and appear to be pressed onto the surface of the nucleolar body. The less frequent “light nucleolar caps” (LNCs) have a convex appearance without a clear margin between them and the nucleolar body, therefore seeming protruding slightly to the nucleolar body. In the segregated nucleolus, the FC is removed from the DFC and forms “fibrillar caps” that cannot be seen by light microscopy but are in close association with LNCs. The remaining granular component is called the “central body” (Shav-Tal et al., 2005). A number of nucleolar as well as nucleoplasmic proteins translocates into these caps as reviewed in following Table 2:

(according to Shav-Tal et al., Molecular Biology of the Cell, Vol. 16, 2395–2413, May 2005)

DNC	LNC	FC	Central body	Cajal body	PML body
PSF	Fibrillarin	UBF	p14(ARF)	p80 coilin	PML
p54nrb	Nopp 140	TBP			Sp100
TLS/FUS	gar1				
EWS	ALL-1				
U1-70K	p110				
cdk2					
hnRNP K					
p68 helicase					
p220NPAT					
TAFII70					
CstF-64					
hnRNP F					
hnRNP H					
MPP10					
Pre-rRNA	U14snoRNA		MRP RNA	U93 scaRNA	
	E3 snoRNA				
	U3 snoRNA				
	U6 snRNA				

Using EGFP-NDH II transfected cells I was able to note the delocalisation of NDH II as soon as after 2 hours after applying ActD at a dose of 400 nM, which is reported to block RNA polymerase I, II and III transcription (Journey and Goldstein, 1961). I observed that EGFP-NDH II translocated into distinct nucleolar caps and I performed a number of colocalisation experiments to find out, to which of the recently described caps NDH II is targeted.

Deducing from the results obtained formerly in our laboratory that NDH II and PML are interacting partners in dependence on transcriptional activity of the cell (Fuchsova et al., 2002), I supposed that NDH II should colocalise with PML protein also under conditions of transcriptional arrest in unique PML-containing nucleolar caps. This expectance however was not confirmed as PML was detected in small structures adjacent to large nucleolar caps containing NDH II (see Figure 2G).

To find out, whether the NDH II-containing “caps” could be LNCs or FCs, I performed double and triple immunofluorescence studies with UBF and fibrillarin. As a marker of DNC I chose TLS.

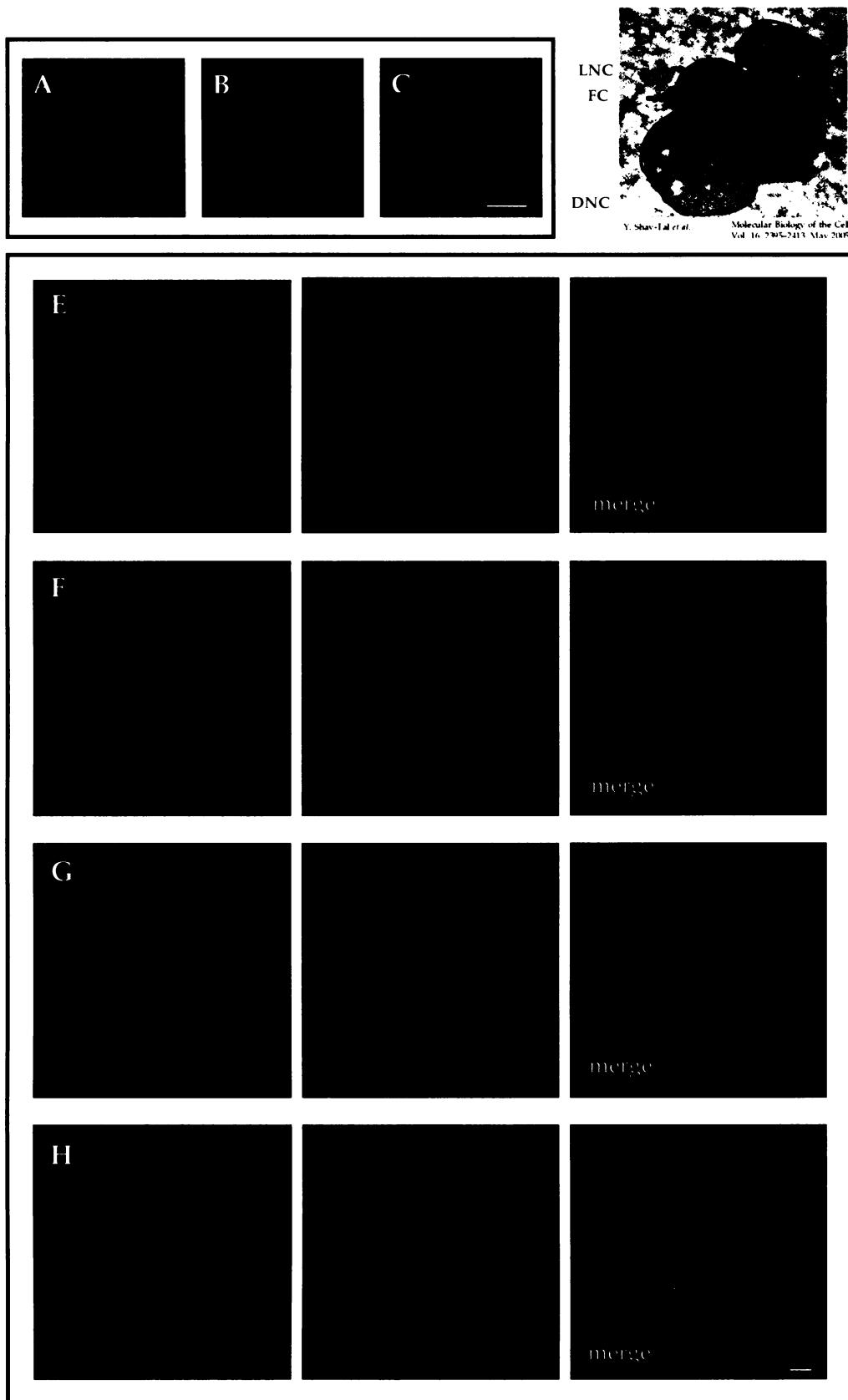


Fig.2: NDH II translocates into DNC upon transcription inhibition

(A-C) (A) The anti-NDH II antibody (red) does not penetrate into the dense protein-NA material of segregated nucleolus. (B) Translocation of the exogenous NDH II (green) is, however, not impaired (D) Ultramicroscopic appearance of individual nucleolar "caps" (from Shav-Tal et al., 2005). (E-H) Overexpressed EGFP-NDH II (green) after transcription inhibition forms "caps" are adjacent to (E) fibrillarin (e.g. FC), (F) UBF (e.g. LNC), (G) PML (e.g. PML speckles), but colocalise with (H) TLS (all red). Bars, (A-H) 2 μ m.

Colocalisation of NDH II with neither UBF nor fibrillarin was found. Although both of them were shown to form “caps”, these were always adjacent to “caps” containing NDH II and showed no signs of overlap (see Figure 2E, F).

The colocalisation with TLS was more arguable. There were cells that showed clear colocalisation (see Figure 2H), however, in the majority of cells, TLS did not translocate to the nucleolar caps, or, it formed caps that were adjacent to NDH II.

A commonplace possibility that the antibody is not working properly can of course serve as a plausible explanation of this doubtful result.

A pleasant way of solving this is to use some different marker of the DNC. Unfortunately, these antibodies at the time of my writing were not at my disposal.

3.5 Preparation of cell lines expressing truncated EGFP-NDH II

To find out, whether for the transcription-inhibition-dependent translocation of NDH II its terminal domains could be responsible, I prepared 3 cell lines of H1299 cells expressing truncated EGFP-NDH II proteins.

First, primers had to be designed for site-directed mutagenesis by inverse PCR within the plasmid. I have designed 5 pairs of primers for subsequent deletion of various terminal domains of the molecule. All of these pairs were checked for primer dimmers and hairpins using the NetPrimer software (see Table, p.53).

I managed to amplify 4 of them, unfortunately, one gave rise to a weak band which I was unable to isolate. The three other primer pairs worked well and I prepared three deletion mutants which, following the preceding description, were transfected into H1299 cells, this time using Lipofectamine reagent and selected to obtain clones with stable expression of truncated EGFP-NDH II

protein. They were yet confirmed only by restriction analysis of the plasmid DNA.

The deletion mutant lacking C-terminal RGG-rich tail (Δ RGG) showed in control cells clear nucleoplasmic localisation with exclusion from nucleoli at intensities comparable to the full length NDH II. The “perinucleolar bodies” were also formed, adjacent to nucleoli as in H1299-EGFP-wtNDH II. However, different behaviour of Δ RGG mutant was observed when transcription was inhibited. After ActD treatment, only a slight portion of the mutant protein localised to nucleolar caps. The staining of the “caps” was weak compared to the nucleoplasm and occurred in a low number of cells (see Figure 4C). RGG domain itself, however, does not seem to be essential for NDH II nuclear localisation, rather it may serve as a co-partner of some different domain of the NDH II molecule or NDH II-interacting partner.

Second mutant, lacking the dsRBD at the N-terminus of the molecule (Δ RBDI/II), showed in untreated control cells nucleoplasmic localisation with clear distinct borders around nucleoli but the intensity of the staining was very low, suggesting that this truncated form of NDH II was either less stable or more quickly degraded. This suggests a crucial role of the dsRBD domain in stability and/or functionality of the protein. Interestingly, in response to ActD, Δ RBDI/II mutants did not translocate into nucleolar “caps”, although these were formed (as confirmed by staining with other antigen markers; data not shown). The localisation of Δ RBDI/II remained nucleoplasmic (see Figure 4D). This might suggest, that the binding properties of the N-terminal domain of the protein are crucial for transcription-inhibition dependent localisation of NDH II. Basically the same was observed for the core-NDH II mutant lacking both N- and C-terminal domains. Localisation of control cells was nucleoplasmic, clearly out of nucleoli, upon transcription inhibition the pattern remained the

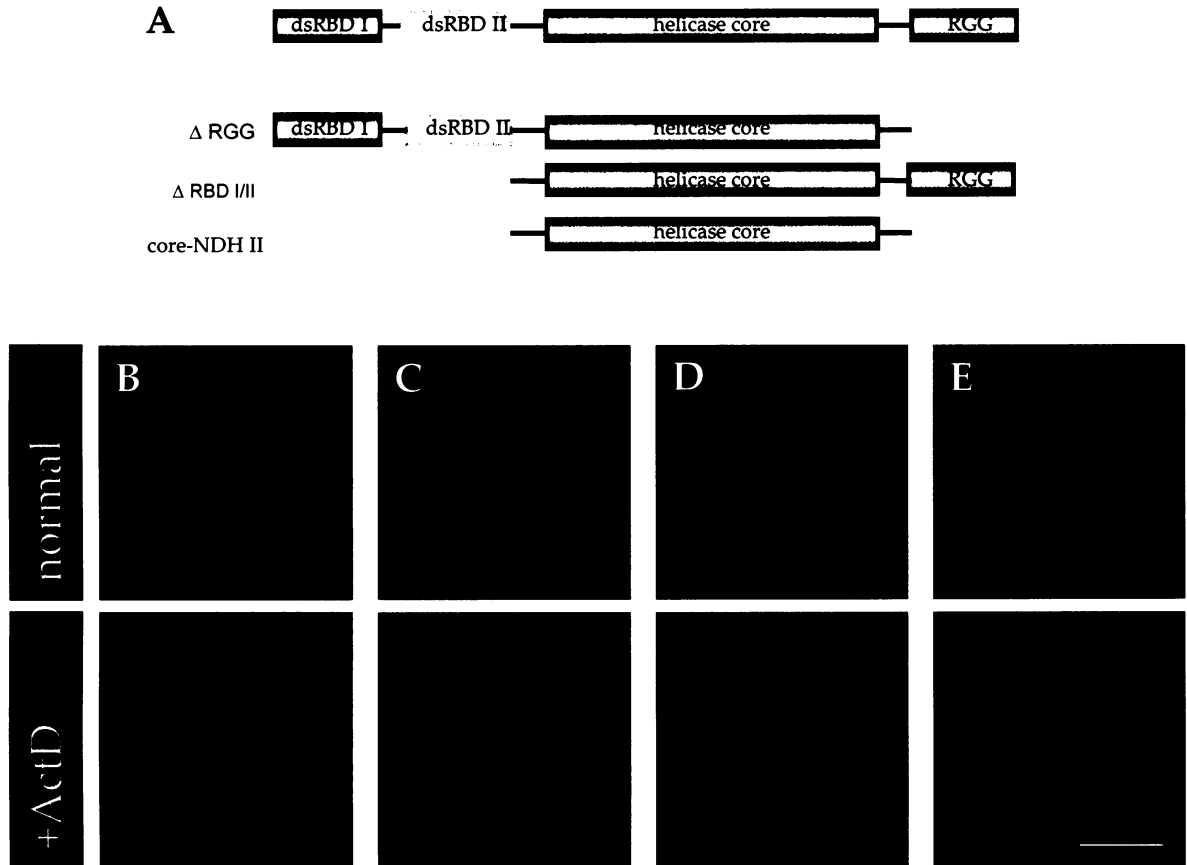


Fig 3: Deletion of either of the terminal domains of NDH II causes reorganization of NDH II in the nucleus
(A) A scheme representing the truncated NDH II molecules. **(B-D)** Different intensities of nucleolar and nucleoplasmic stainings are observed for wtNDH II **(B)** and its mutants. **(C)** The Δ RGG forms “perinucleolar bodies” in control cells. After ActD treatment, only a small fraction of the protein localises into nucleolar caps. **(D)** The Δ RBD I/II does not constitute perinucleolar bodies and upon transcription inhibition it does not translocate to segregating nucleoli. **(E)** The core-NDH II follows the same mode of localisation in both control and treated cells as the Δ RBD I/II mutant. Bars, 5 μ m.

same. Similarly to Δ RBD I/II mutants, core-NDH II mutants did not stain any “perinucleolar bodies” in control cells (see Figure 4E).

In addition, when the intensity of the cytoplasmic staining was compared between cell lines expressing mutants, Δ RGG mutants, just as the EGFP-wtNDH II did not show any cytoplasmic localisation. In mutants lacking the N-terminal domain, however, a very weak diffuse cytoplasmic staining could have been observed. We might conclude that the properties or the conformation of NDH II N-terminus is a more determining factor for NDH II subcellular localisation than its C-terminal domain. This can be due to its established binding properties or its posttranslational modification. To determine the latter possibility, further experiments were performed.

3.6 *The effect of inhibitors of PTM enzymes on NDH II localisation*

As reviewed in Chapter 1.2, posttranslational modifications can often determine subnuclear localisation of a protein.

In order to limit possible modifications that need to be tested, computer prediction analyses were performed that stem from the recognition sequence of a particular modification enzyme and predict the probability of a certain modification from the amino acid sequence of the target protein.

3.6.1 Acetylation

>Sequence - netAcet 1.0 prediction

Seq-Position-Residue	Score	Acetylation predicted
Sequence-2-G	0.466	no

Acetylation of NDH II was not confirmed by a prediction programme but concluding from the fact that the N-terminal acetylation occurs on approximately 80–90% of the different varieties of (predominantly cytosolic)

mammalian proteins (Polevoda and Sherman, 2003) and that its prediction is extremely difficult to approach owing to lack of data and a clear consensus motif (Polevoda and Sherman, 2003), I decided to test the possibility that NDH II might be posttranslationally modified by acetylation.

Moreover, Werner helicase, an interacting partner of NDH II (Friedemann et al., 2005), is confirmed to be acetylated and this modification is reported to be responsible for determining its subcellular localisation (Blander et al., 2002). NDH II itself interacts with CBP, a CREB-binding protein, which possesses a histone acetyltransferase activity and functions as a molecular platform for transcriptional activators.

I used trichostatin A [Sigma; 500 ng/ml; 24 hours; Yoshida et al., 1990; Hoshikawa et al., 1994], an inhibitor of histone deacetylases and anacardic acid [Alexis; 20 μ M; 24 hours; (Balasubramanyam et al., 2003)], a cell permeable non-competitive inhibitor of p300 and PCAF histone acetyltransferase activities.

However, treatment of the cells with these inhibitors showed no observable effect on formation of “nucleolar caps” (see Figure 3F).

3.6.2 Methylation

Arginine methylation activity is associated with transcriptional activation and so is the so far proposed function of NDH II.

Methylation was predicted using MeMo: Methylation Modification Prediction Server (2.0), and a number of prospective recognition sites for protein methyltransferases was discovered:

Residue	Position	Flanking sequences			
R	141	VPGPTWDRGANLKDY	R	1242	RGPSGGYRGSGGFQR
R	410	QNSVVIIRGATGCGK	R	1249	RGSGGFQRGGGRGAY
R	461	AERVAFERGEEPGKS	R	1253	GFQRGGGRGAYGTGY
R	503	RKLEAGIRGISHVIV	R	1265	TGYFGQGRGGGGY
R	1175	DNGSGYRRGGSSYSG	K	152	LKDYYSRKEEQEVQA
R	1219	AGVGGGYRGSRRGGF	K	264	EAYSGLTKKKEGETV
R	1223	GGYRGSRRGGFRGNS	K	265	AYSGLTKKKEGETVE
R	1227	GVSRRGGFRGNSGGDY	K	266	YSGLTKKKEGETVEP
R	1235	GNSGGDYRGPSSGGYR	K	1011	CYHKEKRKILTTEGR

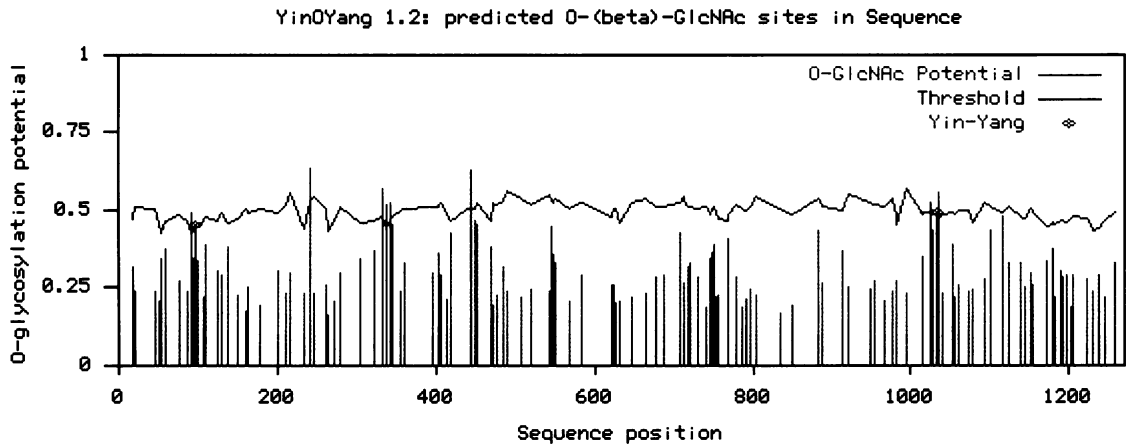
I have used the protein arginine methyltransferase inhibitor AMI-1 [Calbiochem; 100 μ M; 24 hours; (Cheng et al., 2004)], which is reported to prevent *in vivo* arginine methylation of cellular proteins and is cell-permeable.

Although RHA has been described to serve as a substrate for arginine methylation (Smith et al., 2004), according to my obtained results, it is improbable that this modification is responsible for transcription-inhibition-dependent translocation of NDH II into nucleolar caps.

3.6.3 O- β -GlcNAc glycosylation

This specific type of O-glycosylation was reported to be a highly dynamic event and shown to modify a number of nuclear transcription factors (see Chapter 1.2.1.1). NDH II, as a possible transcription co-factor, might serve as a possible substrate for O- β -GlcNAc transferase.

Moreover, when examining the computer prediction analysis using the NetOGlyc 3.1 programme, few possible recognition sites for this enzyme have been found in NDH II amino acid sequence. In addition, these sites may serve as so-called “YinYang” sites that means, they can be both phosphorylated and glycosylated at one amino acid residue what serves as a dynamic regulation mechanism for the protein response to various stimuli.



Recently, role of O- β -GlcNAc glycosylation for regulation of nuclear proteins is becoming heavily appreciated; however, it does not seem to be involved in relocalisation of NDH II in response to ActD treatment. Using alloxan [Sigma; 5 mM; 24 hours; (Konrad et al., 2002)], as an only so far identified inhibitor of O-GlcNAc transferase, I was not able to register any observable impact on NDH II translocation into nucleolar “caps” after transcription inhibition.

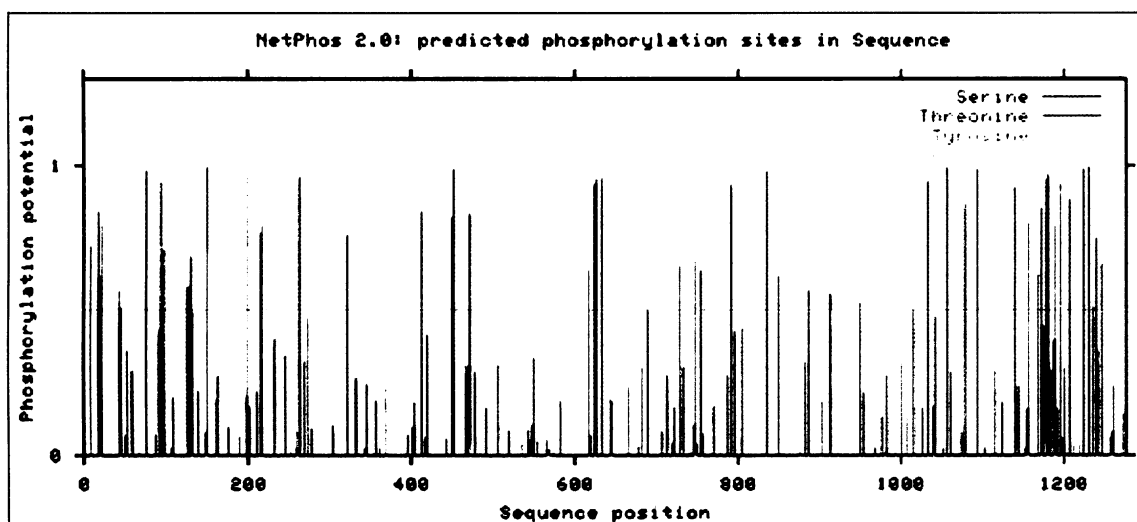
3.6.4 Phosphorylation

Phosphorylation is a notorious posttranslational modification that occurs on Ser, Tyr, or Thr residues of the protein (see Chapter 1.2.1.4). NDH II has been shown to directly interact with DNA-PK and was confirmed to be its substrate *in vitro* (Zhang et al., 2004).

For the “*in silico*” analysis I used NetPhos 2.0 and NetPhosK 1.0 programmes and they confirmed that NDH II molecule contains a number of convenient consensus sequences, however, predominantly for the protein kinase C.

For the confirmation of phosphorylation of NDH II I performed Western blotting with anti-phosphoSer, anti-phosphoTyr and anti-phosphoThr antibodies, but the pattern of the cell nuclei lysate was too complex to evaluate specifically NDH II phosphotylation (data not shown). It will be necessary to perform immuno- or co-precipitation of NDH II before the staining with the anti-phospho antibodies.

However, my attempts to use the anti-NDH II and anti-GFP antibody for immunoprecipitation of NDH II from cell lysate were unsuccessful, since no NDH II could have been isolated. I also tried coprecipitation of the fusion protein using the 6xHis anchor at the N-terminus of the molecule and Ni-NTA Agarose beads but also this procedure was unable to precipitate NDH II. Possible reasons for this setback are that the fusion protein might be too large to bind to the Ni-NTA agarose beads in sufficient amounts but also the 6xHis anchor might be hidden within the protein structure under native conditions and Ni ions are then unable to access it.



Phosphorylation sites predicted: Ser: 26 Thr: 13 Tyr: 15

NetPhosK 1.0

Site	Kinase	Score
T-263	PKC	0.93
S-449	PKA	0.87
T-713	PKC	0.85
S-731	PKC	0.83
T-750	PKC	0.87
S-920	PKC	0.82
S-1171	PKC	0.80

3.6.5 Sumoylation

Sumoylation pathway cannot be inhibited by any small molecule regulator but the addition of SUMO (SUMO is 10 kDa but runs on an SDS-PAGE gel as 20 kDa) changes the mobility of the target protein on SDS-PAGE and can be observed as an additional band of a “larger” protein in the gel. NDH II was shown to interact with Ubc9, an E2-conjugating enzyme of the sumoylation pathway (Argasinska et al., 2004). In agreement with this observation, using SUMOplot prediction programme, three highly probable sites of sumoylation were recognized.

Nevertheless, I did not succeed in finding a SUMO-modified isoform of NDH II in the Western blot.

No.	Pos.	Group	Score				
1	K76	VRINE I K SE EVPAF	0.94				
2	K365	QISMD L K NE LMYQL	0.91	4	K1037	FSSQD M K YP SPFFV	0.69
3	K120	PPHLA L K AE NNSEV	0.91	5	K857	LGRIL A K LP IEPRF	0.69

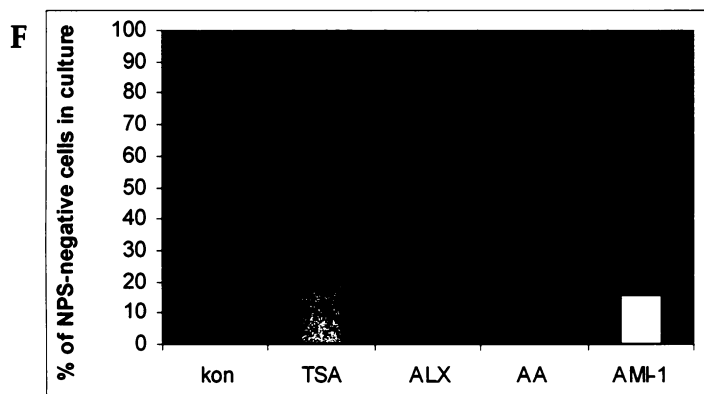
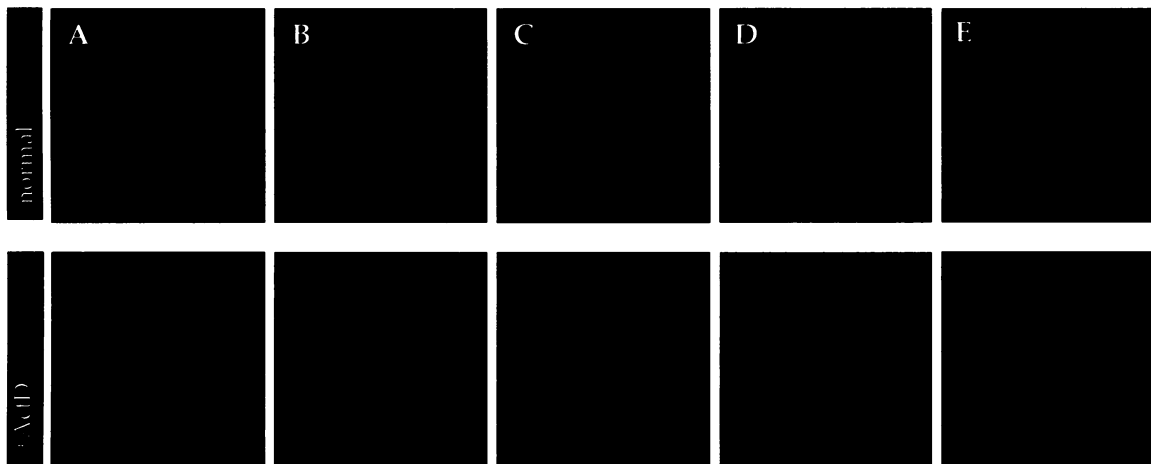


Fig. 3: Analysis of PTM inhibition on NDH II nuclear localisation

(A) In untreated cells, NDH II possesses its nucleoplasmic localization, after ActD treatment, the translocation to segregating nucleoli follows. (B-E) None of examined inhibitors of PTM enzymes changed the intensity or the staining pattern of NDH II in control cells and in cells treated with ActD 400nM. (B) TSA 500 ng/ml; 24 hours. (C) anacardic acid 20 μ M; 24 hours. (D) alloxan 5mM; 24 hours. (E) AMI-1 100 μ M; 24 hours. (F) Number of cells lacking NPS upon inhibition of PTM did not change. Bars, 5 μ m.

4 DISCUSSION

In my work I have tried to identify, whether either of the terminal domains of NDH II protein may serve as a decisive factor in determining its subcellular localisation.

I approached this problem by constructing EGFP tagged wtNDH II and its truncated forms. Using the anti-NDH II antibody was inadequate for this purpose as our numerous studies have confirmed its decreased ability to penetrate dense nuclear structures and lowered specificity for a native protein *in vivo*.

Importantly, for the first time reported, NDH II was found to localise to distinct perinucleolar structures, for which we assigned working term “NDH II perinucleolar structures (NPS)”.

In order to establish, whether these structures resemble any of the previously described nuclear compartments, I performed number of immunofluorescence experiments with nuclear proteins involved in transcription, RNA metabolism and DNA damage recognition. NDH II was, however, not found to colocalise with any, although some of them has been reported to interact with NDH II *in vitro* [RNA pol II (Anderson et al., 1998), γ H2AX (Mischo et al., 2005), PML (Fuchsova et al., 2002)].

Thus, the protein composition of these bodies could not be determined yet. In future, it is of our particular interest to examine whether these NPS could have a possible connection to Cajal body and Sam68 perinucleolar compartment, as their nuclear appearance may suggest.

NDH II, despite its name, is a RNA helicase that was shown to bind RNA as well as DNA. Since NPS does not seem to colocalise with DAPI (DNA) staining, I examined RNA as a possible scaffold of these structures. However, in contrast

to perinucleolar compartment [PNC, (Huang, 2000)], RNase treatment of cells prior to fixation had no effect on their nuclear occurrence. On the other hand, inhibition of RNA pol I alone (low doses of ActD; 5-10 nM) dissolved the NPS what might indicate some functional relationship to active RNA pol I transcription machinery and ribosomal RNA. Therefore, the nature of scaffold network of the NPS still remains to be elucidated.

Our results suggest that in transfected cells the overexpressed EGFP-NDH II localises into distinct nuclear compartments that show clear signs of specific response to external and internal stimuli (cell cycle progression, transcription inhibition).

Identification of a scaffold network of the NPS still needs to be subjected to further experiments.

We then aimed to be determined whether some of the terminal domains of NDH II are crucial for NPS formation. Notably, deletion mutants lacking the N-terminal domain (core-NDH II, Δ RBD I/II) were unable to localise to the perinucleolar compartments and only showed homogeneous nucleoplasmic distribution. The dsRBDs, as main interaction domains of the protein, are involved in NDH II binding to BRCA1, CBP and RNA pol II and are crucial for the function of NDH II as a transcriptional coactivator. It has been proposed that the binding to RNA pol II might partially influence NDH II subcellular localization (Fujita et al., 2005). In our case, however, in core-NDH II and Δ RBD I/II mutant, RNA pol II binding site was preserved and it only lack part of the BRCA1 and complete CBP binding site. Thus, the interaction with CBP may be responsible for localisation of NDH II into NPS. However, some other, yet unidentified, protein partner may serve this function as well. Unfortunately, both immuno- and co-precipitation studies that could help us to confirm this prediction, failed, as we were unable to find an immunoprecipitating antibody

and NDH II did not bind in native conformation to Ni magnetic beads via its His-tag.

We further hypothesised that some PTM of the molecule may serve as a factor responsible for its localisation. Therefore, I performed *in vivo* inhibition of acetylation/deacetylation, β -O-glycosylation and methylation, but none of these had an apparent effect on the presence and pattern of NPS. Experiments were performed under conditions that were reported to be functional *in vivo* (Balasubramanyam et al., 2003; Cheng et al., 2004; Hoshikawa et al., 1994; Konrad et al., 2002; Yoshida et al., 1990); nevertheless, it cannot be warranted that the inhibitors were effective also under our conditions since no appropriate positive markers for PTM inhibition were used.

It is important to mention that murine NDH II/RHA, according to Zhang et al. (Zhang et al., 1999a), is a nucleolar protein, on the contrary to human NDH II/RHA that is prevalently nucleoplasmic. The extended RGG box of murine NDH II is speculated to be the determining factor of its specific localisation. We expected that its deletion should then lead to diminished nucleolar targeting; in our mutants lacking the C-terminal domain, however, the effect was just opposite. The intensity of nucleolar localisation was enhanced in comparison to control cells expressing EGFP-wtNDH II. Moreover, a speckled nucleolar staining was observed, what might suggest a specific binding to some nucleolar compartment.

It was reported previously that “NDH II highly accumulates in the nucleolus and shows predominant association with subdomains in DFC and in a portion of GC attached to DFC” (Fuchsova and Hozak, 2002). I used fibrillarlin as a marker of DFC and followed identical cultivation and staining procedures. However, in the H1299-EGFP-wtNDH II, I identified DFC as a distinct but adjacent compartment not containing NDH II. Concerning the GC, after AMD treatment it dissolved into nucleoplasm, building up a complementary staining

pattern to that of NDH II. Despite these discrepancies, my results can be well-founded by the recent study of Shav-Tal (Shav-Tal et al., 2005) describing in detail nucleolar segregation of proteins of different subnuclear localization. He showed that upon transcription inhibition, fibrillarin segregates into so called fibrillar “caps”, while certain nucleoplasmic proteins form dense nucleolar “caps”, localising adjacent to the previous ones. Moreover, B23 as a GC marker, upon transcription inhibition does not localise into nucleolar caps (Shav-Tal et al., 2005), which also is in agreement with my observations.

In addition, I found that upon transcription inhibition NDH II formed nucleolar caps that were adjacent to UBF-containing and PML-containing clusters and colocalised with TLS, an authenticated component of dense nucleolar caps. The later colocalisation, although described in our laboratory previously (Fuchsova and Hozak, 2002), was in my experiments not unanimous. The colocalisation was only proven in a small percentage of cells. In the majority of cells examined, TLS did not localise to nucleolar caps at all; or, if it did, it stained different compartments than NDH II. In the report of Zinszner (Zinszner et al., 1994; Zinszner et al., 1997) and Shav-Tal et al. (Shav-Tal et al., 2005), TLS was unexceptionably found in DNCs and thus we might place the lack of colocalisation with NDH II on debit of the antibody.

As both of these proteins show nucleoplasmic staining, also in control cells their colocalisation can be observed. TLS was shown to be involved in NF- κ B-mediated transcription as a coactivator (Uranishi et al., 2001) and to associate with RNA pol II (Yang et al., 2000) what are functions strikingly similar to those of NDH II. In my further studies, I would like to confirm the interaction between these two nuclear proteins, although it would also be appealing to try the immunofluorescence analysis with some different antigens of the DNC. We will eventually try colocalisation of NDH II with p80-coilin, a marker of the last identified type of nucleolar caps, and if these experiments fail to give positive

results, we might deduce of having identified a novel nucleolar structure arising in association with segregating nucleolus.

Interestingly, the mutants lacking C-terminal domain of NDH II showed decreased intensity of translocation into segregated nucleolus. However, it was the Δ RBD I/II and core-NDH II mutants, in which the ability of the protein to form nucleolar caps was completely impaired. From this we might conclude that it is the N-terminal domain that is the determining factor crucial for the translocation of NDH II. Due to the properties of the RGG domain, its cooperating function for the N-terminus or some other interacting partners can be suggested.

In conclusions, our results suggest that in transfected cells the overexpressed NDH II localizes into distinct nuclear compartments that show clear signs of specific response to external and internal stimuli (cell cycle progression, transcription inhibition).

We may infer that the identification of proteins and nucleic acids that colocalise with NDH II in perinucleolar region (if there are some) might serve us as a guide to elucidating NDH II role in the metabolism of RNA that is not yet well understood.

Identification of further proprieties and components of these bodies may also contribute to completion of our view on nuclear compartmentalization and its impact on performing nuclear functions.

5 CONCLUSIONS

The cDNA coding NDH II was cloned into an EGFP-expressing vector whereby creating fusion EGFP-wtNDH II protein. Using site-directed mutagenesis, three of its truncated forms lacking either or both of the terminal domains were generated. Next, human cell lines stably expressing full length EGFP-NDH II and its truncated constructs were established.

In addition to nucleoplasmic staining, EGFP-NDH II was found to localise into previously non-observed nuclear bodies adjacent to nucleoli, which we termed NDH II perinucleolar structures (NPS). N-terminal Δ RBD domain of NDHII was identified as necessary for the localization of EGFP-NDHII to NPS.

Upon transcription inhibition with ActD, NDH II translocated into dense nucleolar caps, accompanying TLS. Using the deletion mutants we identified the N-terminal domain of NDH II to be crucial for this nucleolar localisation after ActD treatment, while C-terminal domain was not essential for both forming NPS and targeting NDH II to the segregated nucleolus.

Using specific inhibitors of PTM we were unable to observe any visible effect neither on occurrence and appearance of NPS nor on the translocation of NDH II into nucleolar caps.

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