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The role of Adenylate-Uridylate rich RNA-binding Factor 1 (AUF1) in thyroid carcinoma progression

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For Asia, Jagoda and my parents

Referat und bibliographische Beschreibung

AUF1 ist ein Bindungsprotein aus der Familie der heterogenen nuklearen Ribonukleoproteine D (hnRNPD), das selektiv A(denin)-U(racil)-reiche Abschnitte auf der RNA (ARE) bindet, welches die Stabilität der mRNA verschiedener Gene reguliert, u.a. Proto-Onkogene, Wachstumsfaktoren, Zytokine und Zellzyklusregulierende Gene. Studien im Tiermodell und in Zellkulturen zeigen, dass AUF1 in verschiedenen Organen wie Leber, Niere, Lymphgewebe und Melanozyten exprimiert wird. Des Weiteren konnte gezeigt werden, dass AUF1 Einfluss auf die Embryonalentwicklung, die Apoptose und die Tumorgenese hat. Es gibt noch keine Untersuchungen über die Expression in Schilddrüsenkarzinomgeweben oder die Regulation von AUF1 in Schilddrüsenkarzinom-zelllinien.

Wir konnten zeigen, dass in Schilddrüsenkarzinomzelllinien die Bildung von zellulärem AUF1 abhängig von der Proliferation dieser Zellen ist. In allen untersuchten Zelllinien wurde AUF1 sehr stark im Kern exprimiert. Das Expressionsverhältnis zwischen Kern und Zytoplasma veränderte sich jedoch während der Proliferation und des Zellzyklusses. Die Proliferations- und Zellzyklusinduktion zeigten eine erhöhte AUF1 Expression in der zytoplasmatischen Fraktion. Weitere Studien zeigten eine zytoplasmatische Erhöhung von AUF1 in sich teilenden Zellen. Immunhistochemie und Untersuchungen auf zytoplasmatischen und nuklearen Extrakten von Schilddrüsegeweben zeigten, dass die AUF1 Expression “steady state level” mit der Gewebemalignität korreliert. Statistische Analysen des Kern/Zytoplasma Verhältnisses von AUF1, zeigten die höchste AUF1 Expression in normalen und benignen Schilddrüsengeweben. Eine logarithmische Darstellung dieser Daten zeigt, dass eine Erniedrigung von diesem Verhältnis mit dem Prozess der Dedifferenzierung dieser Gewebe korreliert.

Analysen der Proteinexpression nach der Ausschaltung von AUF1 zeigten eine Erniedrigung von Zyklinen sowie einen erhöhten Spiegel von Retinoblastoma Protein und Inhibitoren von Zyklin abhängiger Kinasen. Eine Erhöhung von p21, p27, p57 und Retinoblastoma Protein durch AUF1 führte zu einer Wachstumsverzögerung in allen untersuchten Zellen. In weiteren Untersuchungen konnten wir die Korrelation zwischen der Expression von Tumorsuppressors sowie Tumorpromotoren und die AUF1-Ausschaltung zeigen. Ein Knock-down von AUF1 führte zu einer erhöhten Expression von CD82 und zu einer ernidrigten Expression von c-Myc, ENO1 und S100A4. Die Zellen, welche mit siRNA gegen ENO1 oder Retinsäure behandelt wurden, zeigten eine Erniedrigung der AUF1-Expression und Zellinvasivität.

Die hier gezeigten Daten deuten auf die wichtige Rolle von AUF1 in der Schilddrüse hin, wobei die deutlichen Unterschiede in der Expression eine mögliche Verwendung als diagnostischen Marker hinweisen. AUF1 kann die Stabilität von Faktoren regulieren, welche in die Zellproliferation, Zellmigration und Zelldifferenzierung involviert sind. Es kann postuliert werden, dass AUF1 als Regulator auf transkriptonaler- und/oder translationaler-Ebene möglicherweise zur Entstehung von Schilddrüsenkarzinomen beitragen kann.

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Abstract

AUF1/heterogeneous nuclear ribonucleoprotein D (hnRNPD) is an adenylate uridylate-rich element (ARE) binding protein, which regulates the mRNA stability of many genes related to growth regulation, such as proto-oncogenes, growth factors, cytokines and cell cycle regulatory genes. Several studies demonstrated AUF1 expression in kidneys, liver, lymphoid tissues and melanocytes, and its involvement in apoptosis, tumorigenesis and development by its interactions with AREs bearing mRNAs.

AUF1 is expressed in most thyroid carcinoma cell lines and tissues. In further study we provided evidence that AUF1 may be involved in thyroid carcinoma progression. Investigations on thyroid tissues revealed that cytoplasmic expression of AUF1 in malignant tissues was increased when compared to those in normal and benign thyroid tissues. By subcellular fractionation of thyroid tissues and immunohistochemistry we could show that cytoplasmic expression of AUF1 in benign and malignant tissues was significantly increased compared to normal thyroid tissues. Moreover, the logarithmic nuclear/cytoplasmic ratio of total AUF1 expression in normal, goiter, adenoma and follicular thyroid carcinoma decreased with tissue malignancy. In thyroid carcinoma cell lines AUF1 was mostly detectable in nucleus, however, in dividing cells its increased production was additionally observed in cytoplasm.

We found AUF1 in complexes with ARE-bearing mRNAs, previously described to be crucial for proliferation and cell cycle of thyroid carcinoma. Total or exon-selective knock-down of AUF1 led to growth inhibition accompanied by induction of cell cycle inhibitors and reduced levels of cell cycle promoters. Decrease in AUF1 production as a response to retinoic acid or AUF1-siRNA treatment correlated with down-regulation of glycolytic ENO1 and proliferation-promoting c-Myc, and reduced invasive potential of thyroid carcinoma cells.

Our data demonstrate the existence of complex network between AUF1 and mRNAs encoding proteins related to cell proliferation. AUF1 may control the balance between stabilizing and destabilizing effects which both are exerted on cell cycle machinery in thyroid carcinoma. Although we can not exclude participation of other factors, thyroid carcinoma may recruit cytoplasmic AUF1 to disturb the stability of mRNAs encoding cyclin dependent kinase inhibitors, leading to uncontrolled growth and progression of tumor cells. Thus, AUF1 may be considered as a new, additional marker for thyroid carcinoma.

Trojanowicz, Bogusz: The role of Adenylate-Uridylate rich RNA-binding Factor 1 (AUF1) in thyroid carcinoma progression. Halle, Martin-Luther-Universität, Med. Fak., Diss., 79 Pages, 2009

ABBREVIATIONS

8505	undifferentiated thyroid carcinoma cell line
AA	amino acid
Ab	Antibody
ACN	acetonitrile
APS	Ammoniumpersulfate
ARE	Adenylate Uridylate-Rich Element
ATP	Adenozyno triphosphoran
AUF1	Adenylate-Uridylate rich RNA-binding Factor 1
BC-PAP	papillary thyroid carcinoma cell line
bp	base pair
BrdU	bromodeoxy uridine
BSA	Bovine Serum Albumine
C-643	undifferentiated thyroid carcinoma cell line
CDIs	cyclin-dependent kinase inhibitors
CDKs	cyclin-dependent kinases
cDNA	complementary DNA
DAB	diaminobenzidine
DEPC	diethylpyrocarbonat
DMSO	dimethyl sulfoxide
DNA	DeoxyriboNucleic Acid
DNMTs	DNA methyltransferases
dNTP	2-deoxynucleoside 5'-triphosphates
dsRNA	double stranded RNA
DTT	dithiothreitol
E. Coli	Escherichia Coli
ECL	enhanced chemiluminiscence
EDTA	Ethylene Diamine Tetraacetic Acid
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-Linked ImmunoSorbent Assays
EtOH	ethanol
FA	Follicular Adenoma
FCS	Fetal Calf Serum
FTC	Follicular Thyroid Carcinoma
g	gram
GAM	goat anti mouse

GAR	goat anti rabbit
h	hour
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
HBSS	Hank's Balanced Salts Solution
HCl	hydrochloric acid (salt acid)
HDAC	histone deacetylases
HTh-74	undifferentiated thyroid carcinoma cell line
IgG	Immunoglobulin G
kDa	kilo Dalton
KO	knock-out
LB-A	Luria broth with ampicilin
MALDI-ToF	matrix-assisted laser desorption ionization time of flight
MetOH	methanol
mg	milligram
min.	minute
miRNA	micro RNA
ml	millilitre
mM	miliMol
mm^3	cubic millimetre
MOCK	negative control
mRNA	messenger RNA
MS	mass spectrometry
MTC	Medullary Thyroid Carcinoma
MTT	3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
$NADH_2$	nicotinamide adenine dinucleotide
$NaHCO_3$	Natrium Carbonate
ng	nanogram
NH_2	ammonium
NH_4Cl	ammonium chloride
NH_4HCO_3	ammonium hydrogen carbonate
nm	nanometre
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered-Saline
PBS-T	Phosphate-Buffered-Saline - Tween

PCR	polymerase chain reaction
Pri-miRNA	primary miRNA
PTC	Papillary Thyroid Carcinoma
RA	Retinoic Acid
RBD	RNA Binding Domain
RISC	RNA-Induced Silencing Complex
RITS	RNA-Induced Transcriptional Silencing complex
RNA	RiboNucleic Acid
RNAi	RNA interference
RT	Reverse Transcription
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
s	second
SDS	sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
Taq	Thermus aquaticus.
TBE	TRIS-Boric acid-EDTA
TCA	trichloro acetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
T_M	melting temperature
TNM	tumor-node-metastasis
Tris	tris(hydroxymethyl)aminomethane
TSG	Tumor Suppressor Genes
TWEEN 20	polysorbat 20
UTC	Undifferentiated Thyroid Carcinoma
V	volt
W	watt
WT	wild type
µg	microgram
µl	microliter

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1 Introduction

1.1 Carcinogenesis

Carcinogenesis is the process by which normal cells are transformed into cancer cells. It was demonstrated that tumors result from a subversion of diverse mechanisms controlling growth, division, and mortality of the cells. Cell division occurs in almost all tissues and under many physiological circumstances. In normal, healthy cells the balance between proliferation and programmed cell death (usually apoptosis) is maintained by tightly regulating both processes in order to ensure the integrity and proper functioning of organs and tissues. Disruption of the balance between both processes may result in uncontrolled apoptosis (i.e. neurodegenerative or autoimmune diseases) and in neoplasia.

Tumor progression is generally considered as a multi-step process that develops over time as a consequence of successive mutations. Mutations that inactivate tumor suppressor genes (TSG), activate proto-oncogenes or handicap the genes involved in maintaining the genomic stability, inhibit apoptosis and provide an uncontrolled growth advantage [1].

1.1.1 Tumor suppressor genes

TSG are defined as a class of genes encoding proteins that negatively regulate cell proliferation [2]. They exert a repressive effect on the regulation of cell cycle or promote apoptosis and sometimes do both. Loss or TSG mutation is a critical step for the development of tumors. Retrospective studies revealed that TSG could be characterized with their three cardinal properties. First, they are recessive and in tumors undergo biallellic inactivation. Second, loss or mutation in single allele increases tumor susceptibility, because additional inactivation of second allele leads to complete loss of gene function. Third, the same gene is frequently inactivated in sporadic cancers [3].

TSG are divided into 2 classes: class I, represented by DNA repair genes, which protect the genome from mutations (caretakers) and class II known as gatekeepers, which prevent cancer through direct control of cell growth. It was demonstrated that restoration of missing gatekeeper function in cancer cells lead to suppression of neoplastic growth, whereas restoration of caretaker will not affect tumor growth [4].

1.1.1.1 The Class I Tumor Suppressor Genes

The members of this class are usually responsible for repairing DNA damage and control of genomic stability. Division of somatic cells and DNA duplication are not perfect processes, and sometimes copying errors may occur. DNA repair genes encode proteins responsible for DNA proofreading. Loss or dysfunction of these genes can develop abnormalities and lead to neoplasia. In end effect oncogenes and abnormal TSG are produced.

Table 1 Representative Tumor Suppressor Genes of Class I

Gene	Function	Familial cancer association	Other major tumor types	References
MSH2 and MLH1	DNA mismatch repair	Hereditary nonpolyposis colorectal cancer (Lynch syndrome)	Endometrial, gastric, ovarian, bladder cancer	5, 6, 7, 8, , 9, 10, 11, 12
ATM	DNA damage sensor (protein kinase)	Ataxia telangiectasia (T-cell lymphoma)	Lymphoreticular malignancies	13, 14
NBS1	DNA repair, S phase check point	Nijmegen breakage syndrome (T-cell lymphoma)	Lymphoreticular malignancies	15, 16
CHK2	Protein kinase (G1 checkpoint control)	Li-Fraumeni syndrome		17
BRCA1, BRCA2	DNA repair	Familial breast and ovarian cancer		18, 19
FA genes	DNA repair, S phase check point	Fanconi Anemia	Acute myelogenous leukemia	20
VHL	E3 ligase recognition factor for HIFα	Von Hippel-Lindau syndrome	Renal cell carcinoma, cerebellar hemangiosarcoma	21, 22, 23

1.1.1.2 The Class II Tumor Suppressor Genes

The members of this class directly regulate tumour progression by inhibiting growth or by promoting cell death. The most studied genes that are disrupted in majority of human cancers are the retinoblastoma tumour suppressor gene (RB1), and the TP53 gene.

Retinoblastoma is a childhood eye cancer that results from inactivation of both wild-type copies of the retinoblastoma susceptibility gene (RB1). RB1 inactivation may result in the genesis of malignant or benign tumors [24, 25]. The product of this gene, the retinoblastoma protein, pRB, is a nuclear phosphoprotein that mediates progression through the first phase of the cell cycle, playing a major role in the control of cell division and differentiation. It was found in a mutated or inactivated form in a variety of human cancers [26-28].

TP53 tumor suppressor gene encodes a transcription factor that regulates the cell fate in response to various stresses, either genotoxic (DNA alterations induced by irradiation, UV, carcinogens, cytotoxic drugs) or not genotoxic (hypoxia, nucleotide depletion, oncogene activation, microtubule disruption, loss of normal cell contacts). P53 can induce transient or prolonged (senescence-like) cell cycle arrest, irreversible cell cycle arrest (senescence) or apoptosis. It may also promote DNA repair and inhibit angiogenesis [29, 30]. Approximately 50% of sporadic human tumors harbour somatic mutations in the p53 gene locus [31]. Despite this complexity, p53 activity has been associated with prognosis and prediction of

tumor response to various therapies. It was shown that over-expression or restoration of both, retinoblastoma protein and wild-type p53 led to growth inhibition or induction of apoptosis in different human cancer models [32-35].

Table 2 Representative Tumor Suppressor Genes of Class II

Gene	Function	Familial cancer association	Other major tumor types	References
RB	Transcriptional co-expression; blocks cell cycle by repressing E2F	Retinoblastoma	Many	36, 37, 38
P53	Transcription factor; promotes cell cycle arrest and apoptosis	Li-Fraumeni syndrome	>50% of cancers	39, 40, 41, 42, 43
INK4a (p16)	Cdk inhibitor (RB activation)	Melanoma	Many	44, 45
ARF	Mdm2 antagonist; protects p53 by inhibiting Mdm2	Melanoma	Many	46, 47
APC	Wnt/Wingless signalling; targets β-catenin for degradation	Familial adenomatous polyposis	Colorectal cancer	48, 49, 50
PTEN	Degrades 3-phosphorylated phosphoinositides, which activate growth and survival pathways	Cowden syndrome	Glioblastoma, endometrial, thyroid, and prostate cancers	51, 52, 53, 54, 55

1.1.2 Oncogenes

The oncogenes are genes encoding proteins that are capable of stimulating cellular growth and participate in the onset and development of cancer. Their precursors (proto-oncogenes) are present in eukaryotic cells, and promote the normal growth and division of cells. They become oncogenes due to mutations or increased expression [56]. Upon activation, products of oncogenes become tumor-inducing agents. There are several basic mechanisms leading to activation of proto-oncogenes:

- Chromosomal abnormality or mutation within a proto-oncogene resulting in production of abnormal protein with increased activity

- Gene duplication or amplification leading to increased number of copies of proto-oncogene in the cell. As a result oncogenes with increased stability and prolonged activity are produced
- Viral infection resulting in the control of a proto-oncogene by a more active viral promoter
- Mutations in microRNAs

In the field of clinical oncology the association between oncogene alterations in cancer cells and prognosis of patients has been extensively investigated in various types of human cancers. It was demonstrated that over-expression of *N-myc* oncogene is a prognostic factor for patients with neuroblastoma [57, 58], while increased levels of *c-erbB-2* oncogene correlates with aggressiveness of ovarian and breast cancers [59, 60]. Point mutations in *ras* oncogene, especially *K-ras* gene, were demonstrated for various human cancers, such as pancreatic, colorectal, lung adenocarcinoma and thyroid carcinoma [61-63]. Generally, oncogenes are amplified late in tumor progression and their expression correlates well with clinical parameters [64, 65].

1.1.3 Gene inactivation and genomic stability

The multistep process of cancer progression involves both genetic and epigenetic alterations that drive normal human cells into malignant derivatives. Epigenetic regulation involves heritable modifications that do not change the DNA sequence but rather provide “extra” layers of control that regulate, how chromatin is organized and genes are expressed [66]. Epigenetic gene inactivation is usually controlled by two reversible processes of DNA methylation and post-translational histone protein modifications.

Activity of many genes in also controlled on post-transcriptional level. Posttranscriptional gene regulation occurs through alterations in translational efficiency and in messenger RNA (mRNA) stability [67-69]. Stability of mRNA is mainly controlled by RNA interference processes and RNA binding proteins, which act to selectively degrade or stabilize mRNAs.

1.1.3.1 DNA methylation and histone modifications

DNA methylation is one of the most important mechanisms involved in gene silencing. DNA methylation is established and maintained by DNA methyltransferases (DNMTs) at cytosines within CpG dinucleotides clustered, within the 50 regulatory regions of most housekeeping genes. These cytosines are usually un-methylated in actively transcribed genes. In contrast, methylated cytosines are generally associated with silenced DNA. With regard to processes of carcinogenesis, it was demonstrated that promoters of many TSG are often methylated, what lead to loss of their function. Promoter methylation and especially hypermethylation was observed in different human cancers [70]. Cellular pathways affected by CpG island hypermethylation include cell cycle, apoptosis, cell adherence, DNA repair, carcinogen

metabolism and so on. For instance, genes such as APC, p16INK4a, hMLH1 or BRCA1 are silenced in many types of cancer due to CpG island hypermethylation [71, 72].

Epignetic regulation also involves modifications of histone proteins that can affect DNA organisation and gene expression. Specific enzymes, including mainly histone deacetylases (HDAC), are recruited to ensure that a specific DNA region is either accessible for transcription, or that DNA is targeted for silencing. Histone modifications are relatively complex and may include acetylation, methylation and phosphorylation [73-75].

The proper timing, establishment and maintenance of epigenetic patterns of DNA methylation and/or histone protein modifications are essential for normal development. In cancer cells both patterns can modify chromatin accessibility by transcription factors, alter gene expression and increase the risk of genomic instability driving to tumorigenetic process [76-79].

1.1.3.2 RNA interference

RNA interference (RNAi) is a mechanism of gene silencing at the stage of translation or by hindering the transcription of specific genes. In this process specific RNAi proteins are guided by double stranded RNAs (dsRNA) to the target messenger RNA (mRNA) where they cleave the target sequence into smaller parts or induce epigenetic changes to the gene like histone modification or DNA methylation. The RNAi pathway is initiated by the enzyme dicer, which cleaves long dsRNA molecules into short fragments of 20–25 base pairs. One of the two strands of each fragment, known as the guide strand, is then incorporated into the RNA-induced silencing complex (RISC) and pairs with complementary sequences. Thereafter, the catalytic component of RISC complex-argonaute induces the cleavage of the target. Another outcome possible, guided by RNA-induced transcriptional silencing (RITS) complex, is pre-transcriptional modification of heterochromatin and resulting gene silencing. However, this process is not well understood [80-83].

Generally there are two types of dsRNA leading to inhibition of gene expression. First type includes exogenous dsRNA coming from infection by a virus with RNA genome or laboratory manipulations. This type of dsRNA after enzymatic dicer modifications is called small interfering RNA (siRNA). The initiating dsRNA can also be endogenous (originates in the cell) and like above is also exported from nucleus to cytoplasm, where it is cleaved by dicer. This type of dsRNA is called microRNA (miRNA). Micro RNAs are genomically encoded non-coding RNA molecules and similar to siRNAs regulate target mRNA stability. Recent studies demonstrated that miRNAs could function as oncogenes or tumor suppressors [84].

1.1.3.3 Micro RNAs and carcinogenesis

Micro RNAs are encoded in the genome and are transcribed by RNA polymerase II (pol II) as long precursor transcripts, which are known as primary miRNAs (pri-miRNAs) of several

kilobases in length. Mature miRNAs are generated from pri-miRNAs by sequential processing steps including excising of 60-80 nt pre-miRNA (RNAse-III enzyme Drosha and its binding partner DGCR8), export to cytoplasm (exportin 5) and processing by dicer to double stranded 18-24 nt long miRNA. One of the miRNA strands remains stably associated with RISC. Subsequently, miRNA guides RISC to target mRNA, which will then be cleaved or translationally silenced [85-88].

Recent studies suggest that miRNA expression profiling can be correlated with disease pathogenesis and prognosis, and may ultimately be useful in the management of human cancer. Those miRNAs whose expression is increased in tumors may be considered as oncogenes and those with decreased as tumor suppressor genes.

Table 3 Representative cancer-related miRNAs and their potential targets

Cancer	**miRNAs involved ***	**miRNA-targeted genes**	**References**
Brain cancer	miR-21**+** , miR**-**221**+**, miR-181**-**		89, 90
Breast cancer	miR-125b**-**, miR-145**-**, miR-21**-**, miR-155**-**		91
Chronic lymphocytic leukemia	miR-15**-**, miR-16**-**	BCL2	92
Colorectal neoplasia	miR-143**-**, miR-145**-**		93
Hepatocellular carcinoma	miR-18**+**, miR-224**+**, miR-199**-**, miR-195**-**, miR-200**-**, miR-125**-**		94
Lung cancer	let-7**-**, miR-17-92**+**	RAS, MYC	95, 96, 97
Lymphomas	miR-155**+**, miR-17-92**+**	BIC	98, 99, 100
Papillary thyroid carcinoma	miR-221**+**, miR-222**+**, miR-146**+**, miR-181**+**	KIT	101, 102
Testicular germ cell tumors	miR-372**+**, miR-373**+**	LAST2	103

*represents **+** increased or **–** decreased expression of the miRNAs in these types of cancer

Currently, investigations on the function of miRNAs in cancers are based on microRNA microarray analyses. Thus, findings can be limited by the known miRNAs in human. Although up to 1000 miRNAs have been estimated in the human genome, only about 200–300 miRNAs have been currently identified in humans [104]. Although evidence shows that some miRNAs play an essential role in human cancers, the molecular mechanisms of miRNA-regulated pathogenesis are unclear. How miRNAs regulate the multistep processes of initiation, promotion, malignant conversion, progression, and metastasis of cancers awaits further investigation.

1.1.3.4 RNA binding proteins

RNA-binding proteins may regulate every aspect of RNA metabolism, including pre-mRNA splicing, mRNA trafficking, stability and translation of many genes. The dynamic association

of these proteins with RNA defines the lifetime, cellular localization, processing and the rate at which a specific mRNA is translated. One of the pathways involved in regulating of mRNA stability is mediated by AU-rich elements (AREs) binding proteins. These proteins are involved in processes of apoptosis, tumorigenesis and development, and are the main subject of this study.

1.2 Introduction to mRNA binding proteins

The growth and development of eukaryotic organisms require that gene expression is regulated. Typically regulation process is considered to occur at DNA level by differential transcription or protein level, where target proteins are selectively degradated. However, gene expression can also be regulated at the level of RNA, including alterations in translational efficiency [67] or mRNA stability [68, 69]. Stability of mRNA may influence gene expression in virtually all organisms, from bacteria to mammals. In mammalian cells, the abundance of particular mRNAs can fluctuate manyfold following a change in the mRNA half-life, without any change in transcription. The processes that regulate mRNA half-lives can, in turn, affect how a cell grows, differentiates, and responds to its environment. One of the most important mechanisms affecting mRNA half-live is regulation of its stability mainly executed by mRNA binding proteins.

1.2.1 Structure of mRNA and the role of 3'-UTR in regulation of mRNA stability

Degradation and destabilization of messenger RNA is regulated by both numerous *cis*-elements in the RNA structure, which vary in location and different *trans*-acting factors [105, 106].

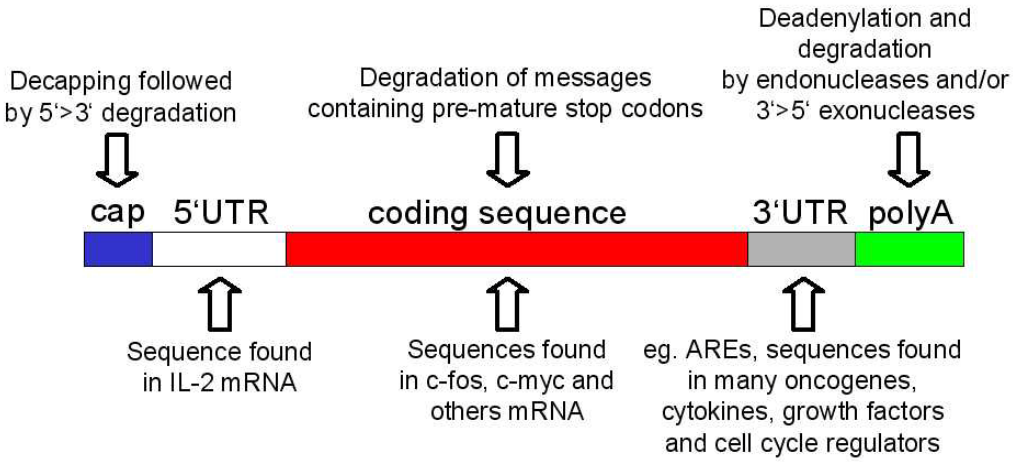

Fig. 1: Structure of mRNA with indication of decay processes and signals for decay. Stability of mRNA is determined by numerous *cis*-acting elements. Indicated above are decay processes that act on all mRNAs.

The cap structure is an important *cis*-element and determinant of the stability of all messages. Experimentally it was shown that removal of the cap structure leads to mRNA degradation in a 5'>3' fashion. Studies in yeast have identified a decapping enzyme DCP1 [107], cytoplasmic 5'>3' exonuclease XRN1 [108] and two distinct proteins that interact directly or indirectly with DCP1 and DCP2 [109]. Higher eukaryotic homologs of both decapping enzyme and the 5'>3' exonucleases still remain to be identified.

Poly(A) tail, located at the opposite end of mRNA, plays also an important role in its stability. Shortening of poly(A) tail is the rate-limiting step in the turnover of many mRNAs. In higher eukaryota de-adenylating actions are dependent on activity of two nucleases- DAN (deadenylation nuclease) [110] and PARN (poly(A)-specific ribonuclease) [111].

The degradation of mRNAs can also be signalled by sequence elements in the 5' untranslated region (UTR), coding sequence and/or the 3' UTR. 3'UTR is often related to mRNA decay. It is a target for many hormones, cyclic nucleotides and proteins that alter the stability of specific mRNAs through interaction with adenylate-uridylate rich elements (AREs) [112-114].

1.2.2 Classification of AREs

AREs are the best-studied instability elements in mammalian messages [115]. AREs consist of multiple stretches of adenylate and uridylate residues and are present in the 3' UTRs of many mRNAs, including those of growth factors, cytokines and lymphokines. Because of the importance of proteins encoded by ARE-containing mRNAs, this element is considered as pivotal gene regulatory target in vertebrate cells. Currently, AREs have been assigned to three classes based on sequence and decay characteristics. Class I AREs, such as the *c-fos* ARE, contain one to three scattered copies of the pentamer AUUUA embedded within U-rich regions. Class II AREs, like the GM-CSF ARE, consist of at least two overlapping copies of a critical nonamer UUAUUUA(U:A)(U:A) also in the context of a U-rich region. Class III AREs, an example of which is the *c-jun* ARE, lack the hallmark AUUUA pentamer but signal degradation with U-rich (and possibly other unknown) sequences. mRNAs containing class III AREs, like those containing class I AREs, exhibit degradation intermediates with 30–60 nucleotides of their poly(A) tail remaining. Detectable degradation intermediates of messages containing class II AREs are poly(A)$^-$ [116].

1.2.3 ARE binding proteins

There exists substantial evidence that ARE-mediated mRNA stability is subject to regulation. Cell stress, stimulation and neoplastic transformation have all been shown to regulate stability of ARE-containing mRNAs [117-119]. Control of stability of many labile mRNAs is one of the critical mechanisms involved in post-transcriptional gene regulation in mammalian cells. Complex differences between normal and neoplastic cells are often related to

differential mRNA expression, including genes that are essential to the cell division, immune response and tumorigenesis [120]. This regulation is largely exerted through the interaction of RNA-binding proteins with AREs contained in their 3'UTR. AREs are found in many transcripts encoding proto-oncogenes (c-fos, c-jun, c-myc, egr-1), growth factors (VEGF, EGF, IGF, TGF), cytokines (interleukins, interferons) and cell cycle regulatory proteins (p16, p21, p27, p53, cyclin A, B1, D1, E2, Cdk 2 and 6). Their list has considerably increased with genome sequencing programs [121-123]. Many RNA binding proteins (RBPs) have been described that selectively recognize these sequences and modulate their translation and/or stability [124], including Hu proteins (HuB, HuC, HuD and **HuR**), BRF1, TIAR, TIA-1, KSRP, TTP and **AUF1** [125-131].

1.2.4 AUF1 and HuR

Previous studies demonstrated at least 14, apparently distinct proteins that selectively bind AREs sequences. However, only two of these proteins, **AUF1** and **HuR**, have been demonstrated to alter the stability of ARE-containing mRNAs *in vivo*. Both proteins exert opposite effects on target mRNAs. AUF1 is often related to degradation of target mRNAs while HuR is known to promote stabilization of several transcripts by enhancing their stability, altering their translation, or performing both functions [132, 133].

1.2.4.1 Structure

AUF1 (hnRNPD) is expressed as a family of four protein isoforms designated by their apparent molecular masses as p37, p40, p42 and p45, which arise by differential splicing of a single transcript. [134]. HuR is a 36 kDa ubiquitously expressed member of the Hu family of RNA-binding proteins related to Drosophila ELAV family. Both proteins interact with target mRNAs through their RNA binding domains (RBD). All AUF1 isoforms contain two while HuR contains three RBDs. It was demonstrated that presence of all three RBDs is required for effective interaction between Hur and target mRNAs. In transient transfection assays, deletion of third RBD alone abolishes HuR's ability to stabilize ARE-containing reporter mRNAs [135]. The family of AUF1 proteins distinguishes a 37 kDa (p37AUF1) core protein, a 40 kDa protein (p40AUF1) containing an N-terminal 19 amino acid insertion (exon 2), a 42 kDa protein (p42AUF1) exhibiting a C-terminal 49 amino acids insertion (exon 7), and a 45 kDa protein (p45AUF1) with insertions of both exon 2 and exon 7. Presence or absence of these alternatively spliced exons confers distinct biological properties to individual AUF1 isoforms. Presence of exon 7 not only affects nucleo-cytoplasmic distribution [136, 137], but also blocks ubiquitination of p42AUF1 and p45AUF1 [138]. In contrast, the lack of exon 7 targets p37AUF1 and p40AUF1 to the ubiquitin proteasome pathway, where both isoforms serve as substrates in decay reaction. This results in rapid and selected decay of ARE-mRNAs [139, 140]. Importantly, absence of exon 2 in p37AUF1 and p42AUF1 is associated

with high affinity binding of these isoforms [141].The smallest AUF1 isoform p37 posses the strongest mRNA binding affinity, which for other isoforms decreases in following rank of order: p37>p42>p45>p40.

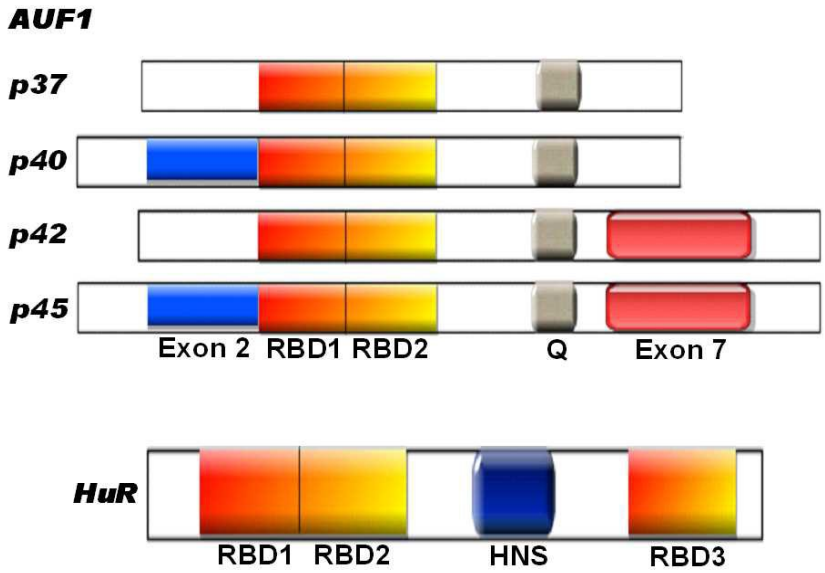

Fig. 2: Structure of AUF1 and HuR; RBD1-3, RNA binding domains; Q, glutamine rich element; HNS, HuR nucleo-cytoplasmic shuttling sequence; Exon 2, 19 amino acids; Exon 7, 49 amino acids.

1.2.4.2 Developmental expression

AUF1 and HuR RNAs are expressed early in development [142]. First studies concerning expression of AUF1 and HuR throughout development, are based on mice models. High levels of AUF1 proteins were found in lymphoid tissues, such as spleen and thymus, and lower levels in brain and fetal liver. In adult liver AUF1 was undetectable. In spleen and thymus extracts, isoforms p40AUF1 and p45AUF1 were more abundant than p37AUF1. Isoforms p45AUF1 and p40AUF1 were most abundant in brain and in fetal liver, respectively. Both mentioned organs lacked expression of p37AUF1. Expression pattern of HuR is very similar to that of AUF1. It was abundant in lymphoid tissues and fetal liver and its expression decreased with liver differentiation process [143].

1.2.4.3 Role and Function

AUF1 is reported to bind both single stranded DNA and RNA, while HuR is demonstrated to bind RNA only [144, 145]. Both proteins are able to bind AREs of all classes (I, II and III) and their over-expression noticeably influences the degradation and stability of ARE containing mRNAs. It was reported that increased level of AUF1 in human erythroleukemic K562 cells, especially isoforms p37AUF1 and p42AUF1, induced ARE-directed mRNA degradation [146]. Investigations performed on HuR revealed that this protein, when up-regulated, exerts quite opposite effects in comparison to AUF1. In transient transfection experiments HuR

appeared to act by protecting the body of the message from degradation, rather than slowing the rate of deadenylation in over-expressing cells. HuR-mediated stabilization was particularly observed for messages containing AREs of class I and II, and to a lesser extent class III [147, 148]. The triggering of AUF1-mediated degradation or HuR-induced stabilization is consistent with changes of cellular localisation of both proteins. Previous studies demonstrated that blocking of ARE-mediated mRNA decay by heat shock, down-regulation of the ubiquitin-proteasome pathway or by inactivation of the E1 ubiquitinating enzyme all resulted in hnRNPD movement to the nucleus of human HeLa cells [139]. However, the cellular factors and/or events involved in regulating these different activities for AUF1 remain to be defined.

HuR like AUF1 is predominantly nuclear and shuttles between the nucleus and the cytoplasm via a novel shuttling sequence, HNS, located in the hinge region between its second and third RBD [149]. HuR's ability to shuttle has led to the suggestion that HuR may initially bind mRNAs in the nucleus and accompany them into the cytoplasm, providing ongoing protection from the degradation machinery. It is worth noticing that AUF1 is also able to shuttle between nucleus and cytoplasm, however sequence or factors involved in this process are still unknown.

Previous reports demonstrated that both AUF1 and HuR are involved in processes of apoptosis, tumorigenesis and development by its interactions with AREs bearing mRNAs.

1.2.4.4 Target mRNAs for AUF1 and HuR

AUF1 and HuR were shown to bind target transcripts on both distinct, non-overlapping sites, and on common sites in a competitive fashion. In the nucleus, both proteins were found together within stable ribonucleoprotein complexes; in the cytoplasm, HuR and AUF1 were found to bind to target mRNAs individually, HuR co-localizing with the translational apparatus and AUF1 with the exosome [150].

Influence of HuR on target mRNAs stabilization and translation is robustly linked to its cytoplasmic localisation. By specific interaction with AREs bearing mRNAs, HuR is able to modify their stability or translation, or perform both functions subsequently [151-153]. The main HuR's targets include following mRNAs: cyclin A1, cyclin B1, c-fos, VEGF, TNF-a, b-catenin, c-myc, cyclooxygenase-2, myogenin, MyoD, GM-CSF, interleukins, p21, p27, p53, and hsp70.

AUF1 appears to enhance target mRNA decay, a process that is closely related to the ubiquitination and targeting of AUF1 to the proteasome [154]. Like HuR, AUF1 target mRNAs also encode mitogenic, immune response, cancer-associated, stress response, and cell cycle regulatory proteins such as c-fos, c-jun, c-myc, egr-1, interleukins, p21, hsp70, MnSOD, catalase, cyclin D1, and cdc25 [69, 155-157].

HuR targets (total: 201 genes)		AUF1 targets (total: 194 genes)		HuR and AUF1 targets (total: 267 genes)		
BAD	(1.10)	HNRPDL	(1.09)	TP53	(2.00)	(1.00)
XRCC3	(1.11)	ANXA5	(1.63)	MAX	(1.78)	(1.04)
STAT3	(1.35)	HNRPA2B1	(1.72)	JUNB	(1.25)	(1.06)
GSK3B	(1.54)	H3F3B	(1.75)	TPM2	(1.30)	(1.08)
HDAC7A	(1.62)	PPP1CA	(1.84)	CASP3	(1.28)	(1.09)
TUBB2	(1.64)	COX10	(1.85)	MAP2K2	(3.95)	(1.09)
RNASEH1	(1.89)	MTF1	(2.00)	RAB10	(1.26)	(1.14)
CCNF	(2.03)	TXN	(2.01)	SFN	(2.07)	(1.15)
ATF5	(2.26)	EEF1A1	(2.04)	PVT1	(1.31)	(1.23)
ID4	(2.39)	HCS	(2.20)	RAC1	(2.10)	(1.24)
HSPA8	(2.54)	DATF1	(2.23)	EIF4E	(2.79)	(1.28)
HIF1A	(2.86)	CDK7	(2.30)	ANXA2	(2.68)	(1.30)
TIMP3	(3.75)	HPRT1	(2.42)	HNRPF	(1.16)	(1.31)
MCM2	(3.93)	EIF4A2	(2.50)	E2F6	(1.03)	(1.34)
ACTG2	(4.07)	XRCC5	(2.62)	E2F3	(1.56)	(1.37)
PPP1CB	(4.86)	FANCC	(2.95)	HNRPA1	(1.33)	(1.42)
ACTB	(4.94)	CXCL5	(3.02)	CALM2	(2.12)	(1.67)
MTA1	(5.08)	NPM1	(3.09)	ACTG1	(5.69)	(1.76)
ETF1	(5.31)			ARF1	(1.32)	(1.84)
				EIF1AY	(1.65)	(1.99)
				POU2F1	(1.31)	(1.99)
				PTMA	(4.13)	(2.04)
				CCND1	(1.90)	(2.27)
				HNRPC	(2.17)	(2.42)
				HNRPAB	(1.35)	(2.62)
				BTF3	(2.62)	(2.69)
				EIF1A	(2.40)	(2.96)

Fig. 3: Representative mRNA targets for HuR and AUF1 according to [150]. Values in brackets (ratios) were calculated by comparing HuR or AUF1 signals to corresponding IgG controls (only ratios ≥ 1 were considered). Full target list is available under http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1361.

1.2.4.5 AUF1, HuR and cancer progression

AUF1 was first described as an activity that could accelerate degradation of c-myc mRNA in a cell-free system [158]. However in cells capable of proliferation such as fetal hepatocytes and adult lymphocytes, where AUF1 levels are much higher compared to adult hepatocytes, it could protect c-myc mRNA from decay [143]. Current data in transgenic mice showed that over-expression of AUF1 followed by deregulation of c-myc, c-fos and c-jun, led to development of sarcomas with strong cyclin D1 expression. Accumulation of this cell cycle regulator favours increased cell division and tumorigenesis, what has been described in various neoplasias [159, 160]. Non-small-cell lung carcinoma cells treated with prostaglandin A_2, an experimental anti-cancer agent, revealed decreased levels of cyclin D1, whose mRNA turnover was regulated by AUF1 [161]. In malignant melanoma cells, reduced levels of AUF1 promoted IL-10 over-expression, which led tumor cells to evade the immune system and protected them from apoptosis [162]. Another involvement of AUF1 during apoptosis was demonstrated in leukemia cells exposed to UVC irradiation, where binding of hnRNPD to bcl-2 mRNA enhanced its turnover [163]. In senescenced cells, which exhibit higher levels of cyclin D1 and p21, and reduced levels of cyclin A1, B1, and c-fos, AUF1 was identified as a critical mediator of senescence events connected with p16 mRNA turnover [164]. Currently

published studies demonstrated AUF1 as a translational promoter of c-Myc mRNA. Knock-down of AUF1 in K562 (human chronic myeloid leukemia), THP-1 (human promonocytic leukemia), HeLa (human cervical carcinoma) and HT-29 (human colon carcinoma) cell lines led to decrease in cytoplasmic and total cellular MYC protein, and subsequent reduction of cell proliferation [165].

Recent studies examining AUF1 expression in murine lung tumors revealed that its cytosolic expression was increased in benign and malignant neoplasia, and correlated with growth rate both *in vitro* and *in vivo* [166]. Similar results were demonstrated for HuR, another ARE binding protein, where it's relative cytoplasmic abundance in colon was lowest in the normal mucosa, moderately higher in adenomas, and highest in carcinomas [167]. Other reports demonstrated HuR as a regulator of cyclin A1 and cyclin B1 mRNA stability during cell proliferation. In human colorectal carcinoma RKO cells, HuR was almost exclusively nuclear during early G1, but increasing in the cytoplasm during late G1, S and G2, what correlated with stability of mRNAs encoding cyclins A1 and B1, and increased proliferation of RKO cells [168].

1.3 Benign and malignant diseases of thyroid gland

The thyroid gland consists of two lobes lying on either side of the ventral aspect of the trachea. Each lobe is about 4 cm in length and 2 cm thickness connected together by a thin band of connective tissue called the isthmus. Weighing approximately 20 g, it is one of the largest classical endocrine glands in the body and receives a high blood flow from the superior thyroid arteries (arising from the external carotids) and the inferior thyroid arteries (arising from the subclavian arteries). The functional unit of the thyroid gland is the follicle, a roughly spherical group of cells arranged around a protein-rich storage material called colloid. The follicular cells are orientated with their bases near the capillary blood supply and the apices abutting the colloid [169].

1.3.1 Benign thyroid goiter

The term non-toxic goiter refers to enlargement of the thyroid which is not associated with overproduction of thyroid hormone or malignancy. Experimental studies have shown that numerous cytokines and growth factors can affect thyroid function and these may have clinical implications. They may exert overall inhibitory effects on the hypothalamic-pituitary-thyroid axis and could account for some of the effects seen in non-thyroidal illness. Cytokines may also be involved in the etiology of autoimmune responses and it is noteworthy that the thyroid gland produces more cytokines than any other endocrine gland. Like cytokines, growth factors are present at all levels of the axis and there is evidence that insulin-like growth factor potentiates TSH action on thyrocytes. This could account for the thyroid

enlargement and growth of some nodules. These may be “hot” (i.e. take up radioactive iodine and show increased thyroid hormone synthesis) or “cold” (i.e. non-functional). Goiter is often merely a symptom of a more serious thyroid dysfunction such as:

- Hyperthyroidism, an overactive thyroid gland caused by:
    - Graves' disease (~80%) - autoimmune with stimulating antibodies to the TSH receptor
    - Toxic multinodular goiter (~ 15%)
    - Toxic adenoma (‘hot’ nodule, ~ 2%)
    - Thyroiditis (~ 1%)
    - TSH secreting pituitary tumor (<0.01%)
    - Trophoblastic tumors (<0.001%)
    - Thyrotoxicosis factitia (<1%)
    - Thyroid follicular carcinoma (<0.01%)

- Hypothyroidism, an underactive thyroid gland caused mainly by:
    - Hashimoto's disease - autoimmune thyroid destruction
    - Primary (atrophic) hypothyroidism (Probably endstage Hashimoto's disease)
    - Post-radioiodine therapy which destroys thyroid tissue
    - Post-surgery of the gland
    - Thyroiditis (non-lymphocytic)
    - Impaired T4 synthesis due to genetic defect
    - Antithyroid drugs
    - Loss of function TSH receptor mutations
    - Thyroid hormone resistance

- Other forms of thyroiditis (De Quervain's or Riedel's thyroiditis)

1.3.2 Thyroid carcinoma

Tumors of the thyroid gland may be primary (arising from the cells within the thyroid gland) or secondary due to malignant cells which have spread from other tissues. The majority of primary tumors arise from epithelial cells of the thyroid gland and are, therefore, termed adenomas if benign, and carcinomas if malignant.

Those arising from parafollicular cells (also termed C cells; produce hormone calcitonin) are called medullary thyroid carcinomas (MTC). About 25% of MTC is genetic in nature and is classified as familial MTC (caused by a mutation in the RET proto-oncogene). MTC which occurs by itself it is termed as sporadic and when it coexists with tumors of the parathyroid

gland and medullary component of the adrenal glands (pheochromocytoma) it is called multiple endocrine neoplasia type 2A (MEN2A).

The epithelial cell tumors are sub-classified as papillary (PTC), follicular (FTC) or undifferentiated (UTC), according to their histological appearance. PTC and FTC are found more often in women (2 to 4 fold more often than men), aged 45–50 years. In areas with adequate iodine intake, the commonest tumor is papillary, accounting for some 80% of all tumors. Where iodine intake is low there is a relative increase in follicular and anaplastic carcinoma, though no overall increase in frequency. The prognosis of PTC is usually optimistic with long-term survival rates of more than 90%. FTC predicts more aggressive behaviour with recurrences or/and distant metastases to liver, lung and bones. Poorly differentiated thyroid tumors (UTC, anaplastic) are much less common, metastasize early, and have a much poorer prognosis with a 5 years survival rate lower than 5%. A number of factors, both genetic and environmental have been implicated in the etiology of epithelial tumors [170-174].

Etiological factors in thyroid cancer:

Growth factors: the role of such known thyroid growth factors as TGF-α, EGF, VEGF and IGF-1 in neoplasia remains uncertain.

Oncogenes: **RET** is a gene coding for a tyrosine kinase receptor for neurotrophic growth factor. It is not normally expressed in thyroid follicular cell tumors. The ret genes express C-terminal fragments of the receptor which leads to dysregulating signalling. There are at least 10 forms of RET oncogenes which have been designated as RET/PTC1, RET/PTC2, RET/PTC3…RET/PTC10 (where PTC stands for papillary thyroid carcinoma). The rearrangements of ret are particularly seen in patients who have had tumors after irradiation e.g. papillary tumors post-Chernobyl. RET is also a factor in medullary cell carcinoma of the thyroid gland.

RAS is a membrane associated monomeric G protein involved in signal transduction processes. Activating mutations of RAS genes are found with a similar frequency in follicular adenomas and carcinomas.

p53 is a tumor-suppressor gene. Mutations of p53 are seen in undifferentiated thyroid carcinoma.

Thyroid irradiation: external irradiation dose-dependently increases the incidence of thyroid cancer and is marked in younger patients. Therapeutic doses of radioiodine do not appear to result in an increased risk of thyroid malignancy.

Other: familial cases of thyroid cancer have been reported in familial adenomatosis coli, Gardner's disease and Cowden's syndrome. There is controversy over the association with certain histocompatibility antigens.

1.3.3 Current molecular factors and markers in thyroid carcinogenesis

Improved understanding of the genetic events associated with thyroid carcinogenesis and progression to more aggressive forms, may lead to the identification of more reliable tumor-specific prognostic markers. Currently it is thought that PTC and FTC arise independently of one another, whereas there is some evidence to suggest a progression from FA to FTC. Genomic rearrangements that result in activation of the RET proto-oncogene were the first recognized molecular events found to be common in PTC, but not FTC, particularly in individuals exposed to ionizing radiation. RAS mutations have been detected in PTC, but are more commonly detected in FA and FTC, and have been thought to be among the earliest events in cancer progression. More recently somatic mutation of the BRAF gene has been found to be an even more common genetic event accompanying the development of PTC. Current evidence indicates that mutations affecting RAS, BRAF and RET are non-overlapping in individual PTC. More recent work has indicated that a chromosomal rearrangement resulting in a fusion gene between the thyroid-specific transcription factor PAX8 gene (2q13) and the PPARγ gene (3p25) may be involved in FA to FTC progression. This rearrangement is found infrequently in FA [175-178].

Table 4 Markers that have been studied for the detection of benign and malignant thyroid cancers.

Benign or Early Stage Thyroid Carcinoma Markers	**Early and Late Stage Thyroid Carcinoma Markers**
Thyroid Peroxidase (TPO) Thyroglobulin (Tg) TSH Receptor (TSHR) Na Iodide Symporter (NIS) TTF-1	RET/PTC RAS BRAF PAX8/PPARγ Mucin (Muc1) Proliferating Cell Nuclear Antigen (PCNA) Leu-M1 Antigen p53 DNA methylase Telomerase Focal Adhesion Kinase (FAK) Galectin-3 Ki-67 (MIB1) Oncofetal Fibronectin

According to cancer stem cell hypothesis for thyroid carcinogenesis, genetic alterations and abnormal differentiation of thyroid stem cells may facilitate the increasing heterogeneity and progression of thyroid carcinoma, leading to well-differentiated tumor (FTC or PTC), advancing to poorly differentiated or undifferentiated tumor (UTC). Cancer stem cells may originate from normal stem cells or normal mature cells in consequence of dedifferentiation. Given that mature cells have a very limited life-span, it is likely that cancer stem cells originate from stem cells with self-renewal ability. Thyroid stem cells contain a pool of cells in various stages of differentiation, including thyroid stem cells with long-term self-renewal, bipotential stem cells, progenitors of follicular cells and progenitors of C cells. All mentioned cells are similar to haematopoietic stem cells and during embryogenesis are present in ultimobranchial body and thyroid diverticulum. It was demonstrated that these cells harbour some characteristics of stem cells and express carcinoembryonic antigen (CEA), cytokeratin and p63, and lacks terminal differentiation markers such as thyroglobulin (TG) and calcitonin (CT). Also thyroid transcription factor 1 (TTF1), thyroid-stimulating hormone receptor (TSHR) and pair box protein 8 (Pax8), which classically regulate normal thyroid development and folliculogenesis, were found to be changed in thyroid cancer stem cells [179-185].

Investigations performed in our group revealed that CD97, a dimeric glycoprotein belonging to the secretin receptor superfamily, might play an important role in the dedifferentiation of thyroid tumors. In normal thyroid tissue, no CD97 immunoreactivity could be found, whereas in differentiated thyroid carcinomas, CD97 expression was either lacking or low. Undifferentiated thyroid carcinomas revealed high CD97 expression [186, 187]. Also expression and activity of different proteins including telomerase activity, E-cadherin, maspin,

APN, PPARgamma expression, were demonstrated to serve as useful markers for thyroid carcinoma differentiation and progression [188-193].

More recently we also found that Raf-1 kinase inhibitory protein (RKIP) and ENO1 may be involved in processes of thyroid tumorigenesis. We demonstrated that reduction of RKIP expression is a highly predictive factor for thyroid carcinoma patients with lymph node and distant metastasis [194]. Also follicular thyroid carcinoma cells responded to retinoic acid pre-treatment, an anti-proliferative and re-differentiation agent in the therapy of thyroid carcinoma, with reduced ENO1 expression and decreased invasiveness [195].

1.4 Aim of this study

AUF1/heterogeneous nuclear ribonucleoprotein D (hnRNPD) was identified as protein which regulates the mRNA stability of many genes related to growth regulation, such as proto-oncogenes, growth factors, cytokines and cell cycle regulatory genes. Several studies demonstrated AUF1 expression in kidneys, liver, lymphoid tissues and melanocytes, and its involvement in apoptosis, tumorigenesis and development by its interactions with AREs bearing mRNAs. Until now, no AUF1 expression was demonstrated in thyroid tissues, especially in thyroid carcinoma.

In view of its involvement in processes of carcinogenesis and regulation of stability of many cytoplasmic mRNAs, I investigated the role of AUF1 in thyroid carcinoma progression. Most importantly, I aimed to define AUF1 as a novel molecular diagnostic/ prognostic marker for human thyroid carcinoma and whether its knock-down suppress invasive ability of thyroid carcinoma. To better understand its function, I performed several biochemical assays, demonstrating the influence of AUF1 on ARE-regulated cell cycle modulators, previously described to be crucial for thyroid carcinoma progression. I also determined whether AUF1 shuttling from nucleus to cytoplasm correlated with growth rate and malignancy of thyroid carcinoma cell lines and tissues, respectively. Finally I investigated the effect of transient and stable AUF1 knock-down on invasive potential of thyroid carcinoma cell lines.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals and biochemicals

Table 5 List of chemicals and biochemicals used in this study

Buffers	**Contents**
Cell culture	
Phosphate buffered saline (PBS)	137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4 x 7 H_2O; 1.4 mM KH_2PO_4; pH 7.4
Hank's Balanced Salts (HBSS)	Invitrogen, Karlsruhe
RNA/DNA Analysis	
10xTBE	890 mM Tris-Base; 890 mM, Boric acid; 20 mM EGTA; pH 8.0
10x PCR Buffer	Amersham, Uppsala, Sweden
10x Taq-Gold Buffer	Amersham
RNA measuring buffer	0.1%TRIS/HCl (1 M) pH 7.5 in DEPC H_2O
Protein Analysis	
Total lysis buffer 1	5 M NaCl, 1 M TRIS/ HCL pH 7.5, 0.5 M EDTA pH 8.0, 0.5 M EGTA pH 7.5, Triton-100
Total lysis buffer 2	20 mM HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM DTT
Hypotonic buffer A	10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$
Extraction buffer C	20 mM HEPES [pH 7.9], 0.45 M NaCl, 1 mM EDTA
Loading buffer	0.5 M TRIS/HCl pH 6.8, Glycerin, 10% SDS, Bromophenol Blue, Aqua bidest
Native loading buffer	0.5 M TRIS/HCl pH 6.8, Glycerin, Bromophenol Blue, Aqua bidest
Tris/HCl buffers	50 mM Tris/HCl; pH 7.5
Western-blot running buffer (10x)	3% TRIS, 14.4% Glycine, 0.6% SDS
Western-blot transfer buffer	1.4% Glycine, 0.3% TRIS, 20% Methanol
TBS/T	10 mM Tris-Base; 0.5 M NaCl; pH7.5;/0.1% TWEEN20
PBS/T	PBS/0.1% TWEEN20
Stripping solution	0.2 M Glycine pH 2.5, 0.05% Tween 20
SDS wash-out buffer	2.5% Triton X-100 in bidestillated H_2O

Chemicals	**Origin**
Cell culture	
G418- Sulfate (Geneticine)	Invitrogen
HCl	VWR, Darmstadt
Natrium Carbonate	Merck, Darmstadt

Chemicals	Origin
PBS	AppliChem GmbH, Darmstadt
Non-silencing siRNA	Qiagen, Hilden
Retinoic Acid	Sigma-Aldrich, Steinheim
3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide	Sigma-Aldrich
Natrium Carbonate ($NaHCO_3$)	Merck
Lipofectamine 2000	Invitrogen
Fetal Calf Serum (FCS)	BioWest, Nuaille, France
Natrium Carbonate	Merck
Trypsin/EDTA	Invitrogen
RNA/DNA Analysis	
Trizol reagent	Invitrogen
Chlorophorm	Merck
Isopropyl alcohol	Merck,
Ethanol	Merck
RNAse free Water	Qiagen
RNAse out	Invitrogen
Random primer	Invitrogen
AmpliTaq polymerase -Gold	Roche, Penzberg
Taq polymerase	Amersham
Agarose	Roche
GoTaq (Hot Start)	Promega, Leiden, Netherlands
Ethidium Bromide	Serva, Heidelberg
peQ Universal Agarose	PeQLab Biotechnology, Erlangen
100 bp DNA Ladder	Invitrogen
1000 bp DNA Ladder	Invitrogen
Sepharose A	Sigma-Aldrich
Protein Analysis	
Acrylamide	Roth, Karlsruhe
TRIS	Amersham
HCl	VWR
PlusOne^{TM} SDS	Amersham
Ammoniumpersulfate (APS)	Pharmacia Biotech, Freiburg
TEMED	Biorad, Muenchen
Glycine	Serva
TWEEN 20	Serva
Broad range protein marker	Promega
High-Range Rainbow Molecular Weight Markers	Amersham

Chemicals	Origin
Bovine Serum Albumin	Sigma-Aldrich
Milk powder	Sucofin, Trade Service International, Zeven
X-ray film (Hyperfilm)	Amersham
EDTA	Merck
Protease inhibitor cocktail	Roche
Acrylamide	Amersham
Dithiothreitol (DTT)	Carl Roth
HCl	VWR
Hydrogen peroxide solution, 30%	Merck
Methanol	VWR
Natrium Carbonate	Merck
Triton X-100	Sigma-Aldrich
Xylol	Roth
Isopropanol	Merck
Proteinase K	Applied Biosystems, Darmstadt

Staining Solutions	Origin
Toluidin blue solution	Amersham
Coomassie Blue R250	Amersham
Mayer's Hemalaun	Merck
Eosine	Merck
Bromophenol Blue	Amersham

Media in human cell culture	Origin
DMEM/ F12	Invitrogen
RPMI	Invitrogen
OPTIMEM	Invitrogen
Media in bacterial cell culture	**Origin**
Luria-Broth	Invitrogen
SOC	Invitrogen

Primer	Primer sequence	bp	T_M (°C)	Polymerase
AUF1	S-5'-TAA-GAA-CGA-GGA-GGA-TGA-AGG-3', AS-5'-TTC-CCA-TAA-CCA-CTC-TGC-T-3'	603, 663, 753, 810	58	Taq Gold
p21	S-5'-AAG-ACC-ATG-TGG-ACC-TGT-CA-3' AS-5'-GGC-TTC-CTC-TTG-GAG-AAG-AT-3'	169	58	GoTaq (Hot Start)
p27	S-5'-ATG-TCA-AAC-GTG-CGA-GTG-TCT-3', AS-5'-TCT-GTA-GTA-GAA-CTC-GGG-CAA-3'	269	60	GoTaq (Hot Start)
p57	S-5'-TCG-CTG-CCC-GCG-TTT-GCG-CA-3' AS-5'-CCG-AGT-CGC-TGT-CCA-CTT-CGG-3'	289	72	GoTaq (Hot Start)
cyclin A1	S-5'-GCC-TGG-CAA-ACT-ATA-CTG-TG-3' AS-5'-CTC-CAT-GAG-GGA-CAC-ACA-CA-3'	194	60	GoTaq (Hot Start)
cyclin B1	S-5'-CGG-GAA-GTC-ACT-GGA-AAC-AT-3' AS-5'-AAA-CAT-GGC-AGT-GAC-ACC-AA-3'	177	56	GoTaq (Hot Start)
cyclin D1	S-5'-TGC-GAG-GAA-CAG-AAG-TGC-GAG-3' AS-5'-TGG-CAC-AGA-GGG-CAA-CGA-AG-3'	370	64	GoTaq (Hot Start)
cyclin D3	S-5'-GAC-CTG-GCT-GCT-GTG-ATT-GC-3' AS-5'-TCT-GTA-GGA-GTG-CTG-GTC-TGG-C-3'	407	64	GoTaq (Hot Start)
cyclin E1	S-5'-GAC-CGG-TAT-ATG-GCG-ACA-CAA-G-3' AS-5'-TTC-AAG-GCA-GTC-AAC-ATC-CAG-G-3'	360	64	GoTaq (Hot Start)
Rb1	S-5'-CAG-ATG-CAA-TTG-TTT-GGG-TG-3' AS-5'-TGA-ATG-GGC-AGT-CAA-TCA-AA-3'	346	56	GoTaq (Hot Start)
18S	S-5'-GTT-GGT-GGA-GCG-ATT-TGT-CTG-G-3' AS-5'-AGG-GCA-GGG-ACT-TAA-TCA-ACG-C-3'	344	60	Taq
CD9	S-5'-TGC-ATC-TGT-ATC-CAG-CGC-CA-3' AS-5'-CTC-AGG-GAT-GTA-AGC-TGA-CT-3'	800	60	Taq
CD82	S-5'- GCA GTC ACT ATG CTC ATG G-3' AS-5'-TGC TGT AGT CTT CGG AAT G-3'	598	58	Taq
ENO1	S-5'-GGC-TAC-ACT-GAT-AAG-GTG-G-3' AS-5'GAG-GAG-CTG-GTT-GTA-CTT-G-3'	530	60	Taq
AUF1 insert (2x 60nt)	S-5'-GAT-CCC-CGA-TCC-TAT-CAC-AGG-GCG-ATT-TCA-AGA-GAA-TCG-CCC-TGT-GAT-AGG-ATC-TTT-TTA-3' AS-3'-GGG-CTA-GGA-TAG-TGT-CCC-GCT-AAA-GTT-CTC-TTA-GCG-GGA-CAC-TAT-CCT-AGA-AAA-ATT-CGA-5'	60	90>10 (step cooling)	

siRNA	Sequence
Non-silencing	5’-AAT-TCT-CCG-AAC-GTG-TCA-CGT-3’
ENO1	5’-AAC-CAG-CTC-CTC-AGA-ATT-GAA-3’
AUF1 exon 2	5″-GCA-GCG-ACG-GCA-CAG-CGG-G-3’
AUF1 exon 3	5’-GAU-CCU-AUC-ACA-GGG-CGA-U-3’
AUF1 exon 7	5’-CUG-GAA-CCA-GGG-AUA-UAG-3’

Vector	Origin
pSUPER.neo+GFP	Oligoengine, Seattle, USA

Antibodies	Origin
AUF1	Millipore, Amsterdam Zuidoost, Netherlands
p53 PAb1801	Abcam, Cambridge, UK
p21 6B6	BD Pharmingen, Heidelberg
p27 F-8	Santa Cruz, Heidelberg
p57 H-91	Santa Cruz
cyclin A1 H-432	Santa Cruz
cyclin B1 GNS1	Santa Cruz
cyclin D1 C-20	Santa Cruz
cyclin D3 C-16	Santa Cruz
cyclin E1 HE12	Santa Cruz
Rb1 4H1	Cell Signaling Technology, Frankfurt am Main
ENO1 C-19	Santa Cruz
c-Myc 9E10	Santa Cruz
B-actin AC15	Sigma-Aldrich
goat anti-rabbit sc-2004	Santa Cruz
goat anti-mouse sc-2005	Santa Cruz
donkey anti-goat sc-2020	Santa Cruz
Alpha-tubulin	Sigma-Aldrich
CD 82 (KAI 1) sc-17752	Santa Cruz

Kits	Origin
Pierce Western Blotting substrate Reagents	Perbio science, Bonn
LSAB-Kit-plus	Dako, Glostrup, Denmark
Kodak developing solution	Kodak, Norderstedt
Qiaprep Spin mini-prep kit	Qiagen
Gel extraction kit	Qiagen
ATP assay (cell vitality assay)	Promega

2.1.2 Instruments

Table 6 List of instruments used in this study

Cell culture	**Source**
Cell incubator, Herasafe	Heraeus Instruments, Hanau
Safety cabinet, HS 12	Heraeus Instruments
Cool centrifuge, Hettich POTANTA/RP	Heraeus Instruments
Water bath box, WB14	Memmert GmbH, Schwabach
Light (Fluorescence) Microscope, Axiovert 25	Karl Zeiss, Jena
Plastic flasks	Greiner Bio One, Frickenhausen

Protein Analysis	**Source**
Cryotom (HM 560)	MICROM, Walldorf
Trans-Blot Cell	BIO-RAD Laboratories, Muenchen
Mini-Protean II device	BIO-RAD Laboratories
WK230 LAUDA cooling system	Boehringer Ingelheim, Heidelberg

RNA/DNA Analysis	**Source**
Horisontal Gel Electrophoresis system	BIO-RAD Laboratories
Homogenizer (MICRO-DISMEMBRATOR S)	B. Braun Biotech International, Melsungen
3 TRIO-Thermocycler	Biometra, Goettingen
UV-Transluminator	Biometra
Table microcentrifuge	Denver Instruments, Goettingen
Thermomixer 5436	Eppendorf, Hamburg
HIGH-SPEED-centrifuge	Heraeus Instruments
Kodak scan camera, Image station 440 CF	Kodak

Measurements	**Source**
Spectrophotometer UV 1602	Shimadzu, Duisburg
Spectra Rainbow ELISA	TECAN, Mainz
Sirius luminometer	Berthold Detection Systems, Oak Ridge, USA
Precision balance	Sartorius, Goettingen
pH-meter	InoLab (WTW), Weilheim

Computational analysis	**Source**
CLC RNA Workbench software	CLC bio, Katrinebjerg, Denmark

2.1.3 Cell lines

Table 7 Cell lines and cell culture media used in this study

Cell type	Appellation	Reference
Thyroid	FTC-133	supplied by Prof. P. Goretzki, established from a lymph node metastasis of a follicular thyroid carcinoma from a 42-year-old male; 90% DMEM/ F12+ 10% FBS
	FTC-236	supplied by Prof. P. Goretzki , established from a lymph node metastasis of a follicular thyroid carcinoma, from which the FTC 133 cell line had been established
	FTC-238	supplied by Prof. P. Goretzki, established from a lung metastasis of a follicular thyroid carcinoma from a 42-year-old male; 90% DMEM/ F12+ 10% FBS
	BC-PAP	DSMZ, Braunschweig, Germany established from the tumor tissue of a 76-year-old woman with metastasizing papillary thyroid carcinoma 90% RPMI 1640 + 10% FBS
	8505C	DSMZ, Braunschweig, Germany, established from undifferentiated thyroid carcinomas of a 78 year old female patient; 90% DMEM/ F12+ 10% FBS
	C-643	supplied by Dr. N.E. Heldin, Univ. Uppsala, Sweden, established from undifferentiated thyroid carcinoma; 90% DMEM/F12+ 10% FBS
	HTh74	supplied by Dr. N.E. Heldin, Univ. Uppsala, established from undifferentiated thyroid carcinoma; 90% DMEM/F12+ 10% FBS
	SW1736	supplied by Dr. N.E. Heldin, Univ. Uppsala, Sweden, established from undifferentiated thyroid carcinoma; 90% DMEM/F12+ 10% FBS

2.1.4 Tissues

Thyroid tissue specimens from 58 patients were investigated in the present study. Tissues of all patients had been obtained after surgery performed between 1994 and 2001 at the Department of General, Visceral and Vascular Surgery, Martin Luther University Halle-Wittenberg, Halle/ Saale, Germany. Tumor tissues were staged according to the Tumor-Node-Metastasis (TNM) staging classification (UICC-AJCC 1997). The specimens were cryopreserved in liquid nitrogen after resection. The study was approved by the ethical committee of the Martin Luther University, Faculty of Medicine, and all patients gave written consent.

Table 8 List of thyroid tissues employed in this study as classified by pTNM status.

No.	Tissue	pTNM	No.	Tissue
1	FTC	pT4NxM1	30	Normal
2	FTC	pT2N0Mx	31	Normal
3	FTC	pT3NxM1	32	Normal
4	FTC	pT4N1M1	33	Goiter
5	FTC	pT4N0Mx	34	Goiter
6	FTC	pT3NxM0	35	Goiter
7	FTC	pT2N1Mx	36	Goiter
8	FTC	pT2N0	37	Goiter
9	FTC	pT3N0M0	38	Goiter
10	FTC	pT3N0M1	39	Goiter
11	FTC	pT4N0M0	40	Goiter
12	FTC	pT3N1M0	41	Goiter
13	FTC	pT3NoMo	42	Goiter
14	FTC	pT4N1M0	43	Goiter
15	PTC	pT2N0M0	44	Goiter
16	PTC	pT4N1M0	45	Goiter
17	PTC	pT2N0M0	46	Adenoma
18	PTC	pT4N0Mx	47	Adenoma
19	PTC	pT1N1M0	48	Adenoma
20	PTC	pT1N0M0	49	Adenoma
21	PTC	pT4N1Mx	50	Adenoma
22	PTC	pT4N1Mx	51	Adenoma
23	UTC	pT4NxMx	52	Adenoma
24	UTC	pT3N0M1	53	Adenoma
25	UTC	pT4NxMx	54	Adenoma
26	UTC	pT3N1Mx	55	Adenoma
27	UTC	pT4N2Mx	56	Adenoma
28	UTC	pT4	57	Adenoma
29	UTC	pT3N1M1	58	Adenoma

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Routine culturing of mammalian cells

All investigated cell lines were maintained at 37°C and 95% humidity in the presence of 5% CO_2. For sub-culturing of the cells, the medium was removed, and the cells were washed with 1 x HBBS medium. Then the medium was removed and 4 ml of 1x Trypsin-EDTA solution was added per 75 cm^2 culture flasks (middle size). The flasks were incubated for several minutes at 37°C. Detached cells were moved into falcon tubes containing growth medium with FCS. After centrifugation step (1200 rpm, 5 min), the cells were resuspended and splitted in ratio 1:50. For specific experiments, the cells were counted in Neubauer chamber.

2.2.1.2 Cells freezing and defrosting

Cells from 125 cm^2 culture flask were trypsinized, centrifuged and counted. $5x10^6$ cells were resuspended in 1 ml freezing medium (Fetal Calf Serum and DMSO; 1:9) and sequentially frozen in -20°C for 24 hrs, then -80°C for 24 hrs, and finally stored in liquid nitrogen. Cells were defrosted in 50 ml Falcon tubes containing pre-warmed growth medium. After centrifugation step the supernatant was discarded and cell pellet was resuspended in fresh culture medium.

2.2.1.3 Retinoic acid pre-treatment

The human follicular thyroid carcinoma cell lines FTC-133 and FTC-238 were cultured in DMEM/F12 medium, supplemented with 1.125 g/l sodium carbonate and 10% fetal calf serum (FCS). For treatment with RA, $8x10^5$ cells were plated in 75 cm^2 flasks and cultured to 80% confluency. The day before treatment, growth medium was replaced with serum-free medium. After 24 h, the cells were treated with 1 µM RA dissolved in ethanol for 24 h, 48 h and 72 h in a standard humidified incubator (37°C, 5% CO_2). Untreated control cells were cultured in medium with the same concentration of ethanol but without RA. Medium was replaced daily.

2.2.1.4 Generation of shRNA transfectants

For shRNA experiments, FTC-133 cells were transfected with 1 µg of pSUPER.neo+GFP vector bearing sequence targeting exon 3 used in transient RNAi experiments. Lipofectamine 2000 was used as a carrier. Control cells received plasmid alone. FTC-133 transfectants producing shRNAs targeting AUF1 and control cells were selected employing 800 µg/ml of geneticin. Knock-down efficiency was assessed by western blot analysis.

2.2.1.5 Cryopreservation and homogenization

Resected human thyroid tissues were snap frozen in liquid nitrogen and stored in -80°C untill use. Cryo tissues were homogenised with homogeniser for RNA and protein analysis. Additionally frozen sections at 6 µm were cut on a cryostat for further immunohistochemical staining.

2.2.2 RNA/DNA analysis

2.2.2.1 RNA extraction from cells

Total RNA from cell lines was isolated using TRIZOL reagent according to manufacturer's instructions. Briefly, 1 ml of TRIZOL reagent was added directly to monolayer of adherent cells in middle size flasks (75 cm^2) and incubated at room temperature (RT) for 5 min. Total content of the flasks was transferred into 2 ml tubes and after addition of 0.2 ml chlorophorm shaked by hand and incubated 2-3 min at RT. The samples were then centrifugated at 12000 x g at 4°C for 15 min in order to separate the upper RNA-containing phase

(transparent-aquatic). Remaining lower-phenol and interphase comprise DNA, proteins and salts, and were discarded. RNA-containing phase was transferred into fresh 2 ml tubes. The RNA was precipitated by addition of 0.5 ml of isopropanol. After incubation for 10 min at RT, the samples were centrifuged at 12000 g at 4° C for 10 min. The supernatant was removed and remained RNA pellet was washed twice with 1 ml 75% EtOH. After this procedure the pellet was air-dried, resuspended in RNAse-free water, at 55°C for 5 min and stored at -80°C. RNA concentration was measured using spectrophotometer at wave-lengths between 260 and 320 nm.

2.2.2.2 RT-PCR analysis

1 μg of total RNA was used as template for first strand cDNA synthesis employing Superscript reverse transcriptase kit and 500 ng/ml of oligo d(T) primers. Total RNA (1 μg) was diluted in DEPC-water till 10 μl end volume and denaturated in 95°C for 3 min. To such prepared RNA 15 μl reaction mix (2,7 μl DEPC-water, 5,0 μl 5x First Strand Buffer, 2,5 μl 0,1 M DTT, 3,0 μl Random primers, 1,0 μl 12,5 mM dNTP, 0,3 μl superscript II and 0,5 μl RNAse out) was added, mixed and incubated at 42°C for 45 min and 95°C for 3 min. The samples were stored at -20°C.

PCR reaction was performed as 25 μl solution containing 16.8 μl dH_2O, 2.5 μl 10x PCR buffer, 3.0 μl dNTP mixture (100 uM), 0.25 μl sense primer (10 pmol/ml), 0.25 μl antisense primer (10 pmol/ml), 0.2 μl polymerase (AmpliTaq-5 U/μl, TaqGold-5 U/μl) and 2 μl cDNA sample. For reactions with GoTaq polymerase, composition of PCR mix was as follows: 12 μl dH_2O, 10 μl 5x Flexi buffer, 8 μl 25 mM $MgCl_2$, 7.5 μl dNTP mixture (200 μM), 4 μl sense primer (10 pmol/ml), 4 μl antisense primer (10 pmol/ml), 0,5 μl GoTaq polymerase (5 U/μl) and 2 μl cDNA sample. Amplificated PCR products were analysed on 2% agarose gels and stained with ethidium bromide. For image analyses Kodak Image System 440 cf (Eastman Kodak, New York, USA) was used. PCR conditions of specific genes are listed in table 5.

2.2.2.3 mRNA half-life

FTC-133, BC-PAP and 8505C cell lines were treated with 1 μg/ml anisomycin for 15, 30, 60 and 120 min. Total RNA was isolated at each stimulation point. Half-life of specific mRNAs was investigated by PCR with specific primers, visualised on 2% agarose gels and evaluated employing Kodak Image Software.

2.2.2.4 RNAi

For RNAi analysis, 200 nM siRNA targeting ENO1 (5'-AAC-CAG-CUC-CTC-AGA-AUU-GAA-3') and 100 nM siRNAs targeting specific AUF1 exons were used. The siRNA sequence targeting exon 2 was 5'GCA GCG ACG GCA CAG CGG G, exon 3 5' -GAU-CCU-AUC-ACA-GGG-CGA-U-3' and exon 7 5'UG GAA CCA GGG AUA UAG. Non -silencing,

randomized sequence 5'-AAU UCU CCG AAC GUG UCA CGU-3 not matching any known human gene was used at 100 nM as a control. siRNAs were transfected in the presence of serum free OptiMEM medium using Lipofectamine 2000 (Invitrogen) as the carrier. Analyses were performed 72 h after transfection.

2.2.2.5 AUF1-shRNA construct

Empty pSUPER.neo+GFP was multiplied using competent E.coli cells transformed with 50 ng of vector on ice for 30 min. Induction of heat shock exactly at 42°C for 45 sec. and cooling down on ice for 2 min, placed vector inside bacteria. Transformed bacteria were incubated in SOC medium at 37°C in shaker for 30 min and then were spread on LB medium-agar-ampicilin plates. After 16 h at 37°C, single colonies containing vector were picked up and further multiplied in liquid LB-ampicilin medium. Vector was extracted from bacteria using Qiaprep Spin mini-prep kit. For cloning of shRNA sequence targeting AUF1, two single 60-oligonucleotide sequences were annealed into one dsDNA and ligated into empty pSUPER.neo+GFP vector previously linearized with BglII and XhoI restriction enzymes. Linearized vector was purified on 1% agarose gel and by employing Gel extraction kit. Competent bacteria cells were then transformed with ligation mix containing AUF1-shRNA vector diluted 1:10 with dH_20. Single colonies from LB plates were then multiplied and tested for the presence of insert (restriction analysis and sequencing).

2.2.3 Protein analysis

2.2.3.1 Total protein extraction and western blot analysis

Total cell lysates for western blot were obtained employing total lysis buffers 1 and 2. Protein concentration was measured using Bradford method. Protein extracts were resolved on 10% and 12% polyacrylamide-SDS gels (SDS-PAGE). To determine the size of proteins, Rainbow Marker was run in separate line of each gel. Electrophoresis was performed at 40 mA for about 2 h at RT. Proteins were transferred onto PVDF membrane in wet mini-Transblot cell at 17 V overnight or for 2 h at 1 A, both at 6°C and stained with Ponceau staining solution. Blocking was performed in 5% non-fat milk powder or 5% BSA in 1xTBS /Tween20 (0.1%) for 1 h. After 3x washing with 1xTBS/Tween20, the membranes were incubated overnight with specific primary antibodies (Table 5). Immunoreactive bands were visualised by exposing X-ray film and developed using Kodak detection kit. Densitometric data were obtained using Kodak Digital science 1D software

2.2.3.2 Subcellular fractionation

FTC-133, BC-PAP and 8505C cell lines and homogenized thyroid tissues were washed with PBS, incubated in hypotonic buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl2) supplemented with inhibitors (protease inhibitor cocktail, Roche, Mannheim, Germany) and

lysed by addition of buffer A containing 2.5% Nonidet P-40 plus inhibitors. Nuclei were pelleted (3,500 rpm, 4 min, 4°C), and supernatants saved, freeze-thawed five times, and centrifuged (10 min, 3,500 rpm, 4°C). Cytosolic fractions were subjected to an additional step of high-speed centrifugation (14,000 rpm for 60 min at 4°C) and any pelleted material was discarded. For preparing nuclear fractions, nuclear pellets were incubated in extraction buffer C (20 mM HEPES [pH 7.9], 0.45 M NaCl, 1 mM EDTA) plus inhibitors and centrifuged (10 min, 14,000 rpm, 4°C), and supernatants were saved. Total protein extracts were obtained with lysis buffer containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM DTT and protease inhibitors.

Total, cytosolic and nuclear protein extracts from thyroid cell lines were subjected to western blot procedure. Alpha-tubulin and GAPDH served as cytoplasmic, p53 as nuclear and beta-actin as total protein control, respectively.

For proliferation experiments selected cell lines, FTC-133, B-CPAP and 8505C, representing three different types of thyroid carcinoma, were cultured in serum-free media for 3 days, then released by serum addition (10%) and tested at 0 h, 6 h, 12 h and 24 h for AUF1 expression in cytoplasm and nucleus.

2.2.3.3 Messenger Ribonucleoprotein Immunoprecipitation (mRNP)

Cytoplasmic lysates from FTC-133, BC-PAP and 8505C cells were supplemented with RNAse OUT, protease inhibitors and then for preclearing incubated with A–Sepharose beads only for 20 min. Protein concentration was measured with Bradford assay. For immunoprecipitation of endogenous RNA-AUF1 complexes, A-Sepharose beads were precoated either with non-immune rabbit IgG or specific AUF1 antibody for 3 h. Precleared lysates (1.5 mg) were incubated with precoated beads overnight. Half of the beads were washed, mixed with loading buffer, boiled and centrifuged. Supernatants were analysed for the presence of AUF1 by western blotting. The other half of the beads were used for RNA extraction followed by RNAse-free DNAse I and proteinase K digestion. RNA was extracted using Trizol and overnight isopropanol precipitation. RT-PCR was performed to examine RNAs associated with AUF1. RT-PCR products were visualized by 2% (w/v) agarose gel electrophoresis.

2.2.3.4 Immunohisto-/-cytochemistry

Thyroid carcinoma cell lines FTC-133, BC-PAP and 8505C were seeded on thermanox plates and let grown in normal medium for 3 days. Freshly cut cryo-embedded serial 6 µm sections of all thyroid tissues and thyroid carcinoma cell lines were washed with PBS and fixed in a 1:4 mixture of 3% H_2O_2 in ice cold 90% methanol for 20 min. After 2 times washing with PBS, cells were incubated overnight at 4ºC with the rabbit polyclonal antibody against AUF1 diluted 1:1000 with Dako Antibody Diluent. Negative control sections were exposed to

the secondary antibody only and processed as described below. After 3×10 min washing in PBS, cells were incubated for 30 min with a 1:1000 dilution of biotinylated goat anti-rabbit secondary antibody followed by incubation with an avidin–biotin-peroxidase complex. After 3×10 min washing in PBS, specific immunostaining was visualized with diaminobenzidine (DAB) chromogenic solution (1:50). Finally, cells were lightly counterstained with Mayer's hematoxylin and photographed under light microscope. Fluorescent AUF1 staining was performed on microscope slides. Briefly, the cells were fixed with 4% formaldehyde solution in PBS for 10 min. After 3×10 min washing in PBS, the cells were incubated with previously mentioned AUF1 antibody overnight. Goat anti-rabbit IgG rhodamine-conjugate antiserum was used to visualize fluorescent AUF1 staining. All immunostained tissue sections were examined by two independent reviewers using an Axioplan light microscope.

2.2.4 Cell activity assays

2.2.4.1 MTT test

In 96-well plates, the cells were seeded and cultured with DMEM-F12 medium supplied with 10% FCS. The next day, the cells were transfected with 100 nM siRNAs (targeting AUF1 exons and non-silencing) diluted in OptiMem without serum. Lipofectamine 2000 was used as a carrier. After 24 h, transfection medium was replaced and the cells were grown for additional 48 h. For MTT assay, cells were then stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for 4 h at 37°C and shortly incubated with DMSO. Thereby, a colored formazan salt develops depending on the availability of mitochondrial $NADH_2$ only in living, but not dead cells. Optical density was measured with Tecan Elisa Reader. For FTC-133 stably expressing AUF1 targeting shRNAs and RA pre-treatments, MTT assay was performed for 24, 48 and 72 hours in serum free conditions.

2.2.4.2 Luminometric ATP assay

For ATP assays, substrate (100 µl) was added to control and treated wells and incubated with the cells on a shaker and on the bench top for 2 min and 10 min, respectively. Luminescence was measured with a Sirius luminometer.

2.2.4.3 Motility assay

Motility of FTC-133 and FTC-238 cells pre-treated with RA was evaluated in 24-well Transwell chambers. The upper and lower culture compartments were separated by polycarbonate filters with 8 µm pore size. To investigate the effect of RA on the motility of differentiated thyroid carcinoma cells, FTC-133 or FTC-238 were pre-treated with 1 µM RA (dissolved in ethanol) for 72 h and then plated at 1 x 104 cells/well in DMEM/F12 medium without FCS. Control cells were pre-treated with medium containing the same concentration of ethanol but without RA. The cells migrated from upper to lower compartment for 24 hours

in a 5% CO_2 atmosphere at 37°C. After a 24 hour motility period, cells remaining on top of the filter were wiped off with cotton swabs and those cells that had traversed the membrane pores to the lower surface of the membrane were washed with chilled PBS, incubated for 5 min in 1:1 PBS/methanol and 15 minutes in methanol before staining with 0.1% toluidin blue in 2.5% sodium carbonate. Migrated cells were counted by light microscopy in four separate high-power fields per filter.

2.2.4.4 Statistical analysis

Diagrams were created with SPSS software and Microsoft Excel, and all experimental parameters were calculated for statistical significance using Kruskal-Wallis H test. Differences between groups were determined by the Mann-Whitney test (U test) features of SPSS 12.0 software for Windows. Differences between other experimental data were evaluated using t-Student's test. P-values of < 0.05 were considered to indicate statistical significance.

2.2.4.5 Computational analysis

Identification and analysis of AU-rich elements within cell cycle and proliferation related mRNAs were performed by employing CLC RNA Workbench software.

3 Results

3.1 Expression of AUF1 in thyroid cell lines and tissues

First analyses of AUF1 expression were performed on the RNA level. We could show that all investigated thyroid carcinoma cell lines expressed AUF1 mRNA, however with various levels of the different isoforms (Fig. 4).

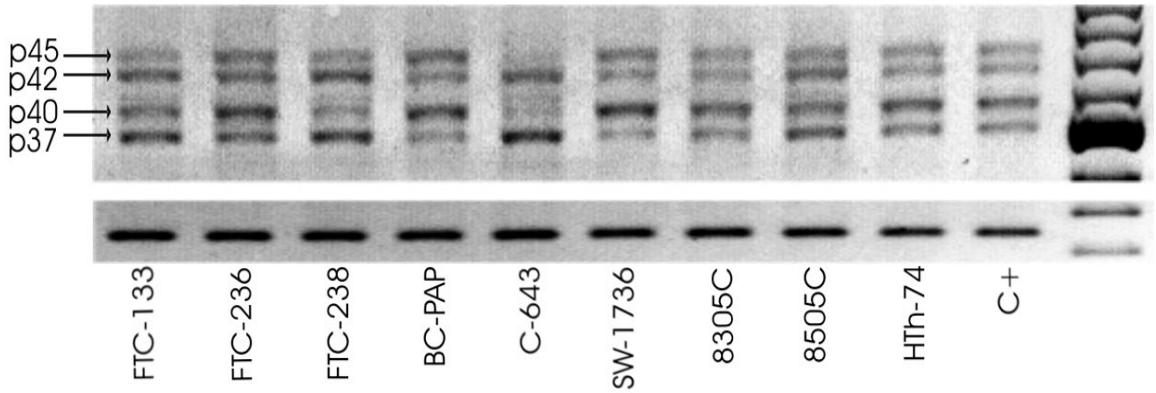

Fig. 4: AUF1 mRNA expression in human thyroid cancer cell lines; the primers used detected 4 different AUF1 isoforms: p45, p42, p40 and p37, respectively. The BHY cell line (human oral squamous carcinoma), showing strong AUF1 mRNA expression, was used as a positive control (C+). 18S transcripts (lower panel) were used to judge for equal cDNA amounts

In order to answer the question whether the cellular localisation of AUF1 (hnRNPD) correlates with neoplasia, normal, benign and malignant thyroid tissues were subjected to immunohistochemistry and subcellular protein fractionation. Immunohistochemical studies revealed that cytoplasmic expression of AUF1 in tissues from malignant thyroid diseases was increased when compared with benign and normal thyroid tissues (Fig. 5). More pronounced data were obtained from fractionation experiments and western blot analysis (Fig. 6). We could demonstrate that strongest cytoplasmic AUF1 expression was observed in adenoma (n=10), PTC (n=10) and UTC (n=10) tissues, while the lowest in goiter (n=10) and normal (n=3) tissues, respectively. The lowest nuclear hnRNPD expression was detected in FTC (n=10) and UTC, while the strongest in PTC and normal tissues respectively.

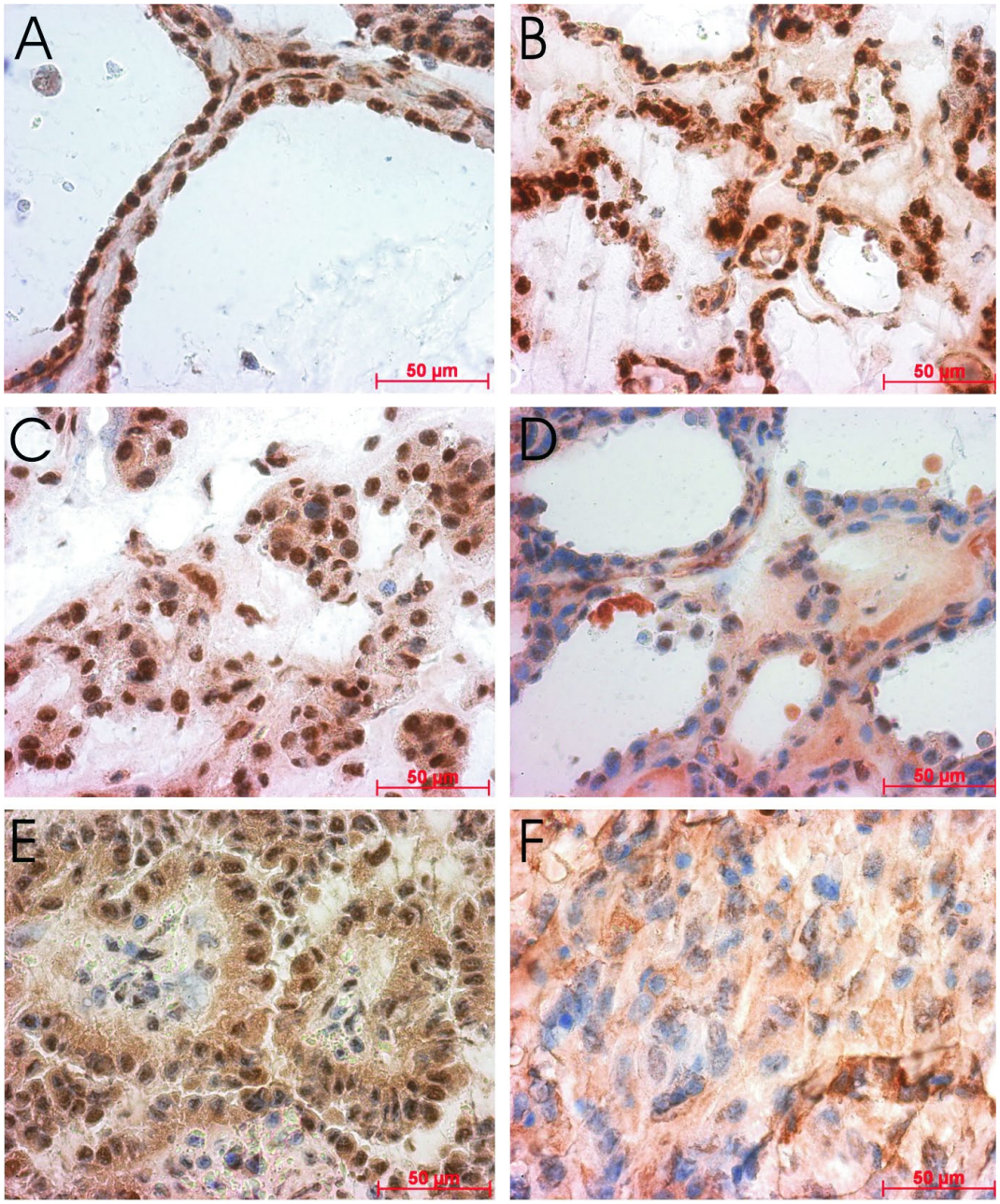

Fig. 5: Immunohistochemistry performed on thyroid tissues. Freshly cut serial 6µm cryosections of normal (A), goiter (B), adenoma (C), FTC (D), PTC (E) and UTC (F) tissues were subjected to AUF1 immunodetection. Strong, nuclear AUF1 immunoreactivity is visible in benign thyroid tissues.

Analysis of AUF1 expression evaluated as ratio between nuclear and cytoplasmic fractions revealed that its expression in goiter, adenoma and FTC tissues was significantly lower, when comparing to normal thyroid tissues and decreased with tissue malignancy (Fig. 7).

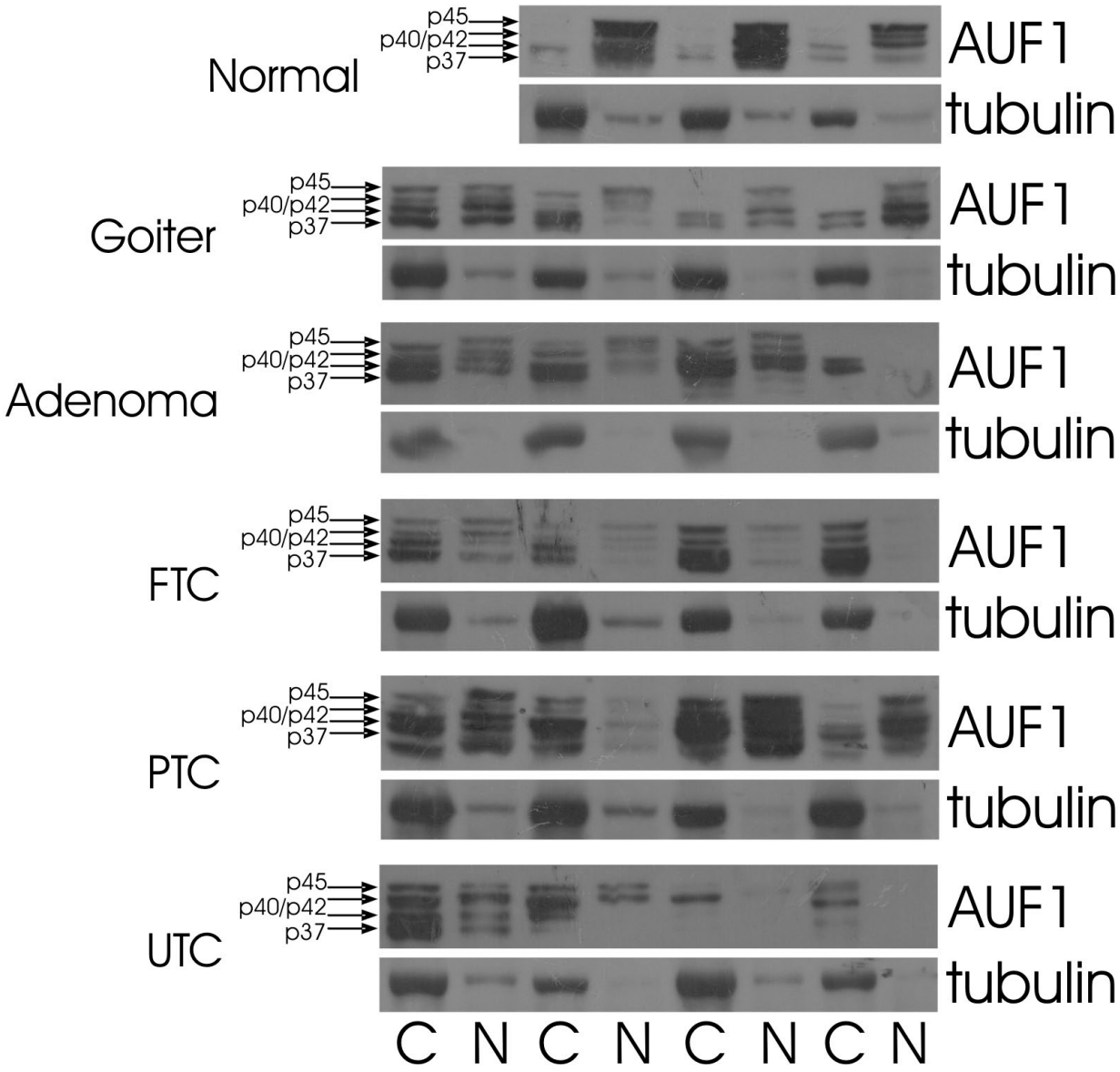

Fig. 6: Using Western analysis AUF1 protein levels were determined in cytoplasmic (C) and nuclear (N) extracts of human thyroid tissues. Normal, goiter, adenoma, FTC, PTC and UTC tissues were subjected to subcellular protein fractionation and western analysis with AUF1 antibody. Alpha-tubulin served as cytoplasmic protein control.

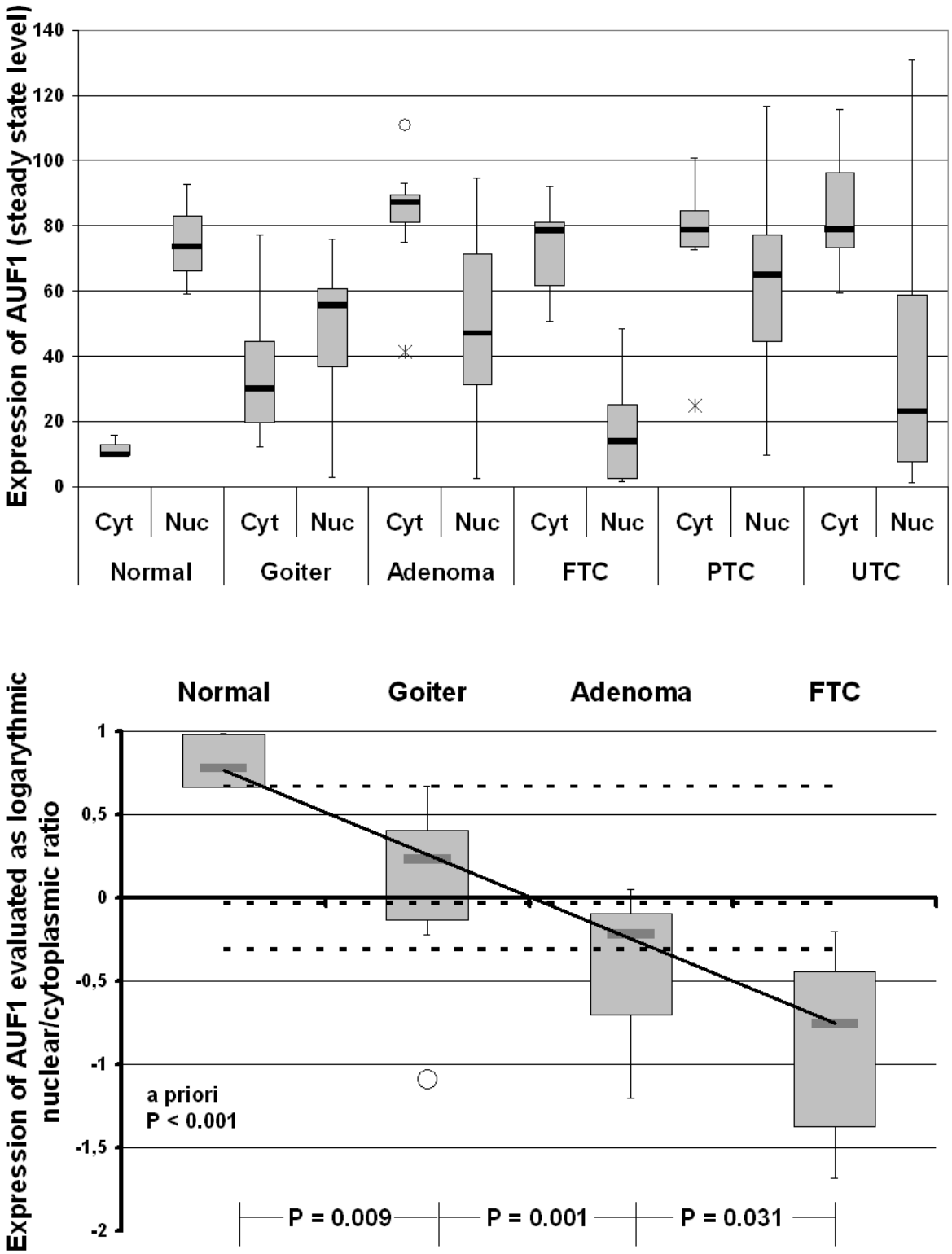

Fig. 7: Semi-quantitative evaluation of AUF1 protein expression in thyroid tissues. Increased cytoplasmic AUF1 expression is noticeable in benign and malignant tissues as compared to normal thyroid extracts (upper panel). Logarithmic nuclear/cytoplasmic ratio of AUF1 expression in goiter, adenoma and FTC tissues was significantly lower, when comparing to normal thyroid tissues and decreased with tissue malignancy (lower panel).

3.2 Elevated cytoplasmic AUF1 levels correlated with increased proliferation of thyroid carcinoma cell lines

In order to investigate the potential role of AUF1 in the proliferation of thyroid carcinoma cells, serum starved and serum stimulated FTC-133, BC-PAP and 8505C cells were examined for AUF1 expression in cytoplasm and nucleus.

We could show that the cellular distribution of AUF1 protein is related to proliferation of thyroid carcinoma cell lines. AUF1 was mostly nuclear in all investigated cell lines and its expression in cytoplasm and nucleus was growth dependent. The highest nuclear and lowest cytoplasmic AUF1 levels were noticed in starved cells, after 3 days of serum removal. In FTC-133 and BC-PAP cells p37AUF1 was exclusively expressed in nucleus and absent in cytoplasm. Addition of serum led to increased cytoplasmic level of p37AUF1 and p40/p42AUF1 isoforms, especially visible after 24h. Interestingly, in 8505C established from undifferentiated thyroid carcinoma, this effect was less pronounced and AUF1 expression was only slightly elevated during cell proliferation (Fig. 8).

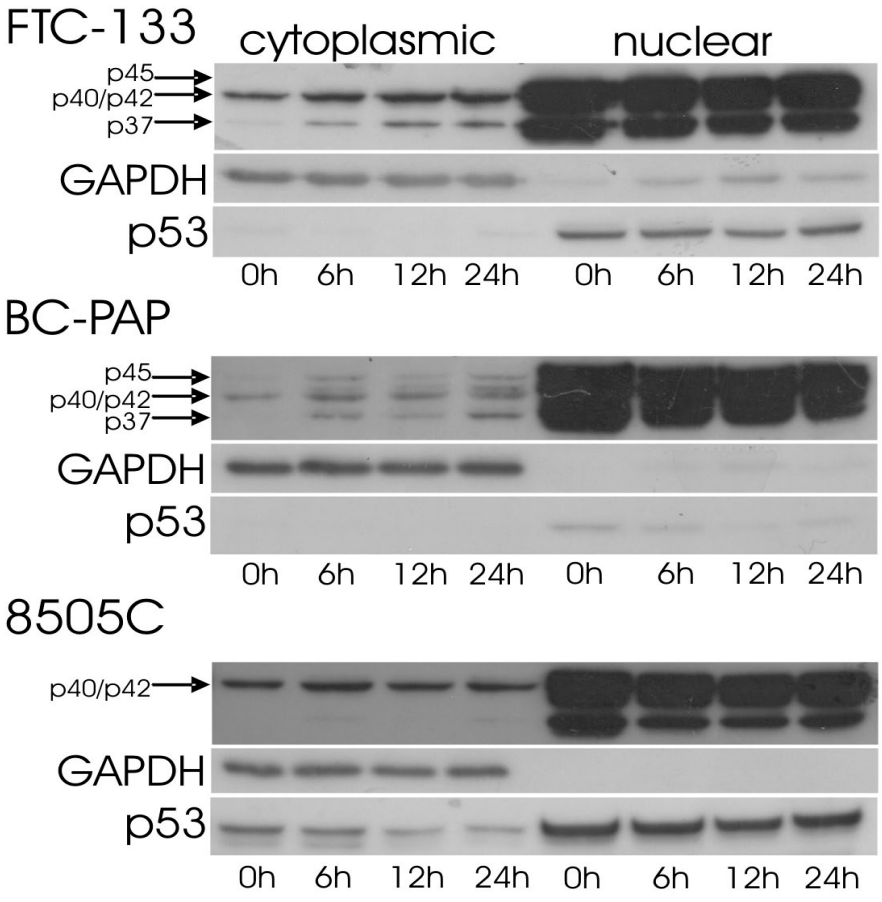

Fig. 8: Analysis of cytoplasmic and nuclear AUF1 proteins in thyroid carcinoma cell lines. Western blot analysis performed on fractionated FTC-133, BC-PAP and 8505C thyroid carcinoma cells. The cells were serum starved for 3 days and then serum stimulated for the times indicated; Protein levels for GAPDH and p53 served as loading controls for cytoplasmic and nuclear proteins, respectively. Increasing cytoplasmic AUF1 expression is noticeable as the thyroid cells proliferate.

Further studies concerning the possible role of AUF1 and its cytoplasmic/nuclear distribution were performed employing immunocytochemistry. Thyroid carcinoma cell lines were seeded on thermanox plates, fixed and then stained with AUF1 anti-serum. Microscopic investigations were mainly focused on cells undergoing cell division and showing strong AUF1 staining. We could demonstrate that dividing thyroid carcinoma cell lines revealed elevated cytoplasmic AUF1 levels as compared to surrounding cells (Fig. 9 a,b). Thus, this observation indicates that increased AUF1 shuttling from nucleus to cytoplasm may promote proliferation of thyroid carcinoma cells.

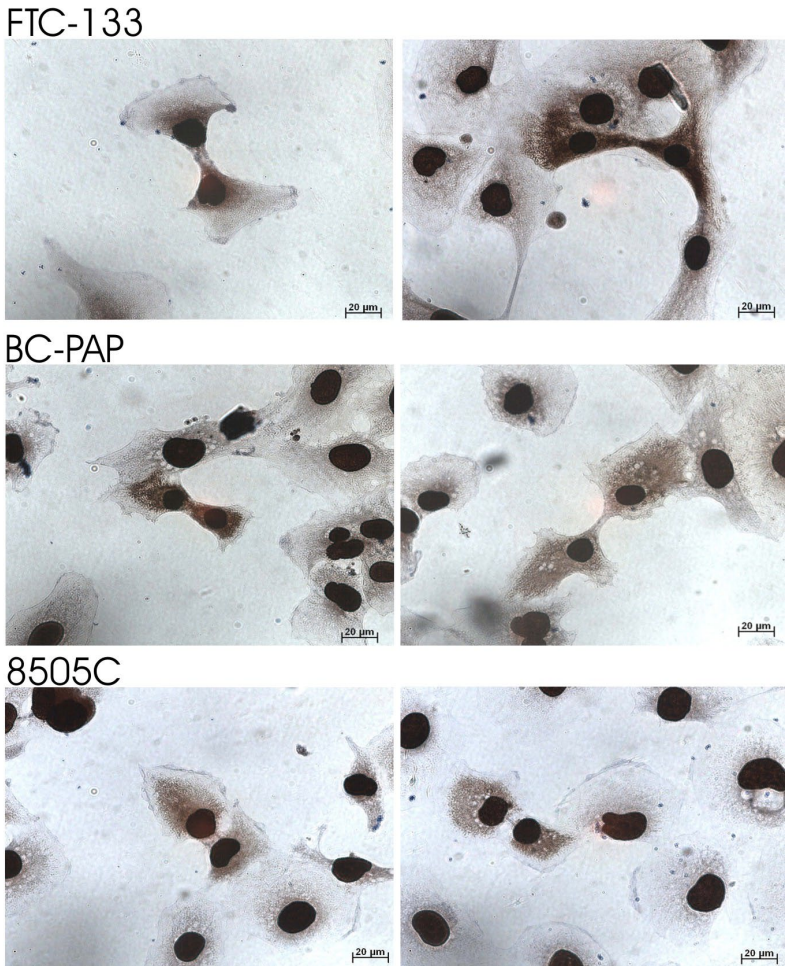

Fig. 9a: Immunocytochemical analysis of AUF1 expression in thyroid carcinoma cells. Stronger cytoplasmic AUF1 immunoreactivity is observed in FTC-133, BC-PAP and 8505C currently undergoing cell division.

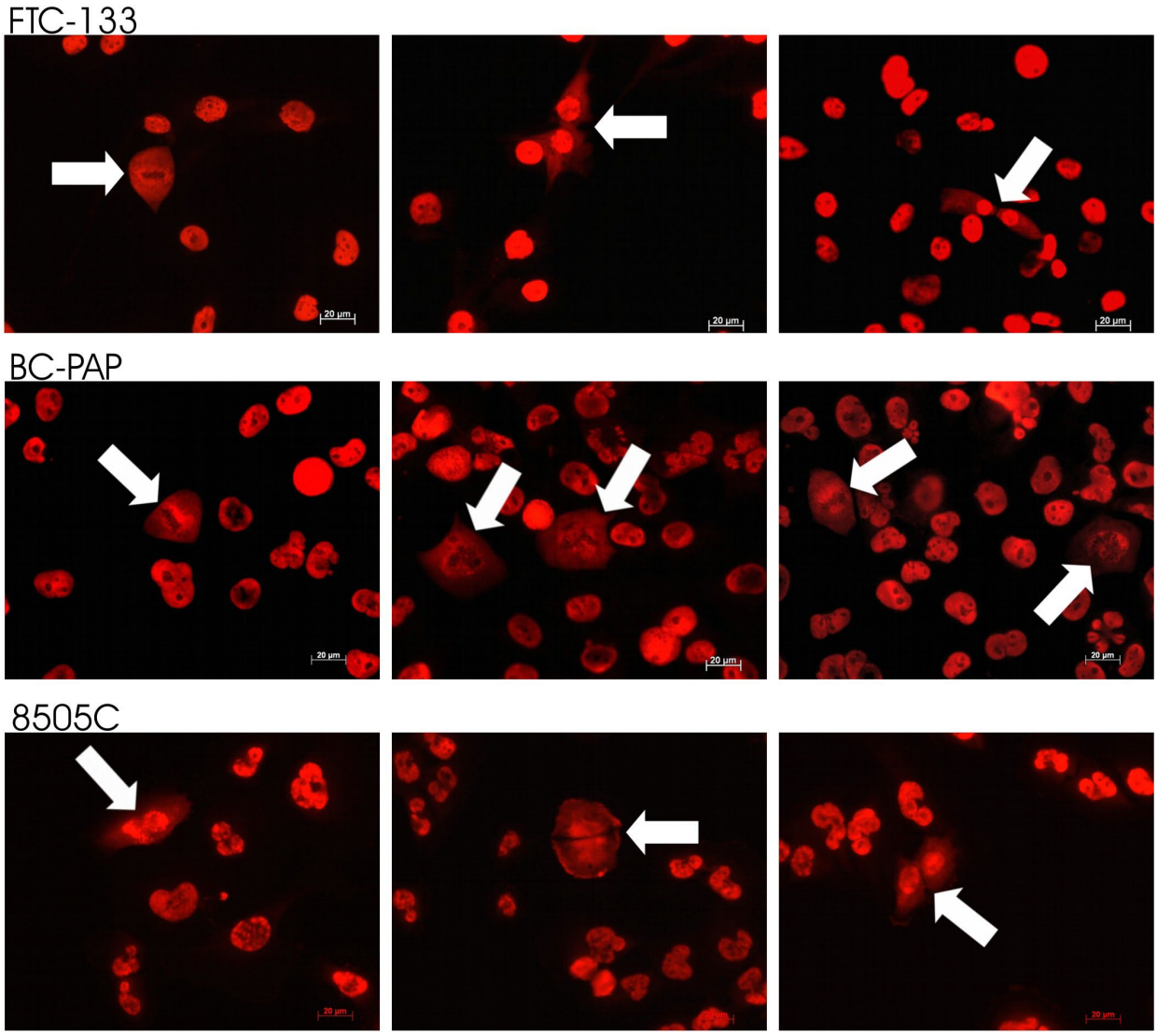

Fig. 9b: Immunofluorescent analysis of AUF1 expression in thyroid carcinoma cells. Cytoplasmic AUF1 immunoreactivity is visible only in dividing FTC-133, BC-PAP and 8505C cells.

3.3 AUF1 binds mRNAs of common cell cycle regulators

Cytoplasm of thyroid carcinoma cells is a rich source of many labile mRNAs, which could be potential targets for AUF1. Additionally many of these mRNAs encode proteins related to cell cycle or cell proliferation. Computational analysis revealed the presence of many AUUUA repeats within their 3' UTR. Some of them like in cases of cyclin A1, cyclin B1 and Rb1 were also detected out of 3' UTR. Rb1, p27 and cyclin D1 mRNAs were the richest in AUUUA motifs (Table 9).

Table 9 Features of ARE-containing mRNAs related to cell cycle and proliferation investigated in this study; + indicates the presence of A- or U-rich regions

ARE sequence	Length of 3'UTR (nt)	Adenine (%)	Uridine (%)	No. of AUUUA motifs	No. of UUAUUUA (U/A)(U/A) nonamers	No. of AUUUA motifs out of 3'UTR	Presence of A- or U-rich regions
p21	1552	20	26	3	0	0	+
p27	1345	34	33	7	0	0	+
p57	716	29	27	3	0	0	+
cyclin A1	218	35	32	1	0	2	+
cyclin B1	623	39	29	4	0	2	+
cyclin D1	3208	29	25	7	0	0	+
cyclin D3	1052	24	25	2	0	0	+
cyclin E1	543	24	28	0	0	0	+
Rb1	1820	30	38	12	1	3	+
p53	1208	32	19	0	0	0	+

In order to investigate which mRNAs are associated with AUF1 in thyroid carcinoma cells, we performed immunoprecipitation (IP) reactions to isolate mRNA subsets bound to AUF1 using specific antibody. The nonspecific association of mRNAs with IP reagents was determined by parallel incubations with IgG (Fig. 10, upper panel). Detection of mRNAs in each thyroid cell line was achieved by reverse transcription (RT) followed by RT-PCR and agarose gel electrophoresis. As shown in Fig. 10 (lower panel), AUF1 antibody precipitated several mRNAs while control IgG did not precipitate these RNAs. We found that p21 and p27 mRNAs were associated with AUF1 in all cell lines investigated, however the highest signal intensities were observed in FTC-133. Unfortunately, despite repeated attempts, we did not find p57 to be associated with AUF1. Further analysis of AUF1 targets, revealed its presence in complexes with cyclins. IP reactions from FTC-133 and BC-PAP revealed relative high amounts of cyclins A1 and D1, while in undifferentiated thyroid carcinoma cell line 8505C these mRNAs were not detectable. Cyclin B1 was found to be slightly bound to AUF1 in all cell lines investigated, while cyclin E1 could only be weakly amplificated in FTC-133. We did not find any cyclin D3 expression in all IP reactions. It is worth to notice that mRNAs of almost all cyclins were not associated with AUF1 in 8505C representing undifferentiated thyroid carcinoma cells. However whether AUF1 is involved in progression of differentiated thyroid carcinoma only requires further clarification.

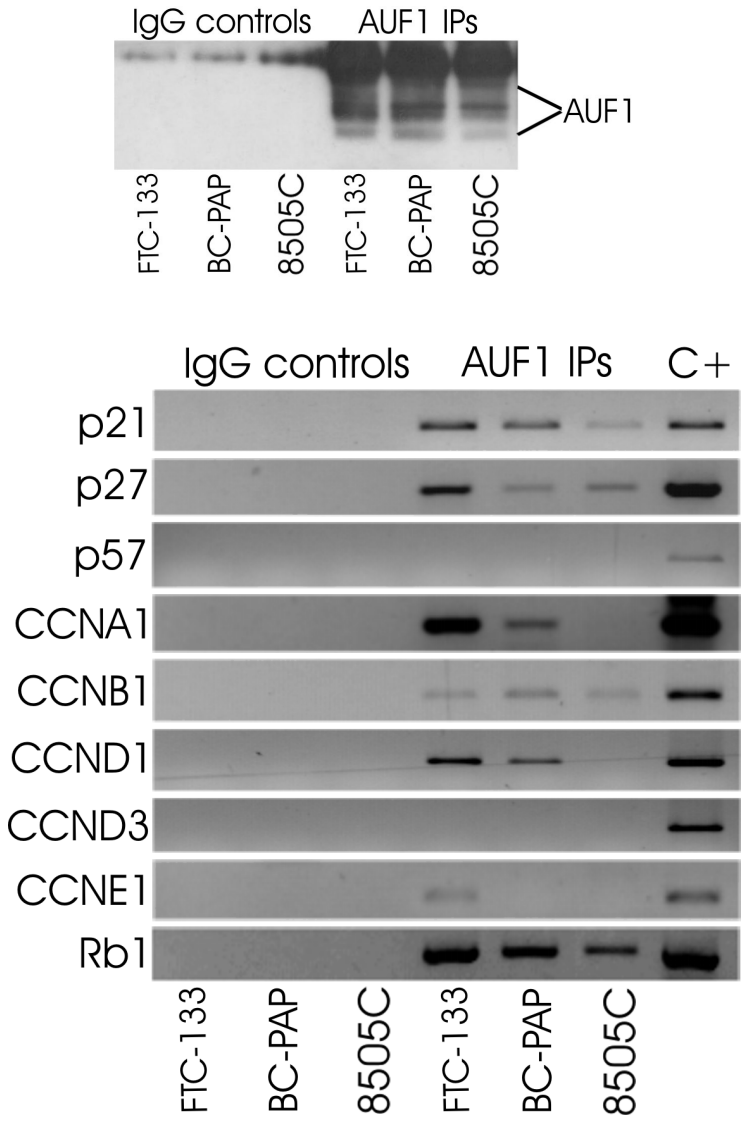

Fig. 10: Immunoprecipitation of AUF1-mRNA complexes in thyroid carcinoma cell lines. Cytoplasmic protein lysates were incubated with A-Sepharose beads precoated with AUF1 antibody. AUF1-mRNA complexes were separated from each other and analysed employing western blot and RT-PCR, respectively.
Upper panel: Western blot analysis demonstrated AUF1 in cytoplasmic extracts of all cell lines investigated. Beads precoated with normal rabbit IgGs served as negative controls.
Lower panel: RT-PCR amplification of cell cycle regulators found to be associated with cytoplasmic AUF1. RNA released from AUF1 was reversely transcribed and then amplified with primers specific for selected mRNAs. We found AUF1 in complexes with p21, p27, cyclin B1 and Rb1 in all cell lines analysed. Cyclin A1 and D1 could be amplified in both differentiated thyroid carcinoma cell lines, while cyclin E1 was found in FTC-133 cells only. The presence of p57 and cyclin D3 mRNA in complexes with AUF1 could not be detected.

3.4 AUF1 targets have different mRNA half-life

In order to investigate the mRNA half-life of potential AUF1 targets in cytoplasm of thyroid carcinoma cell lines, we performed anisomycin treatment. Anisomycin is known as a translation inhibitor inducing mRNA accumulation in cytoplasm. As demonstrated in Fig. 11, AUF1 targets varied in mRNA stability.

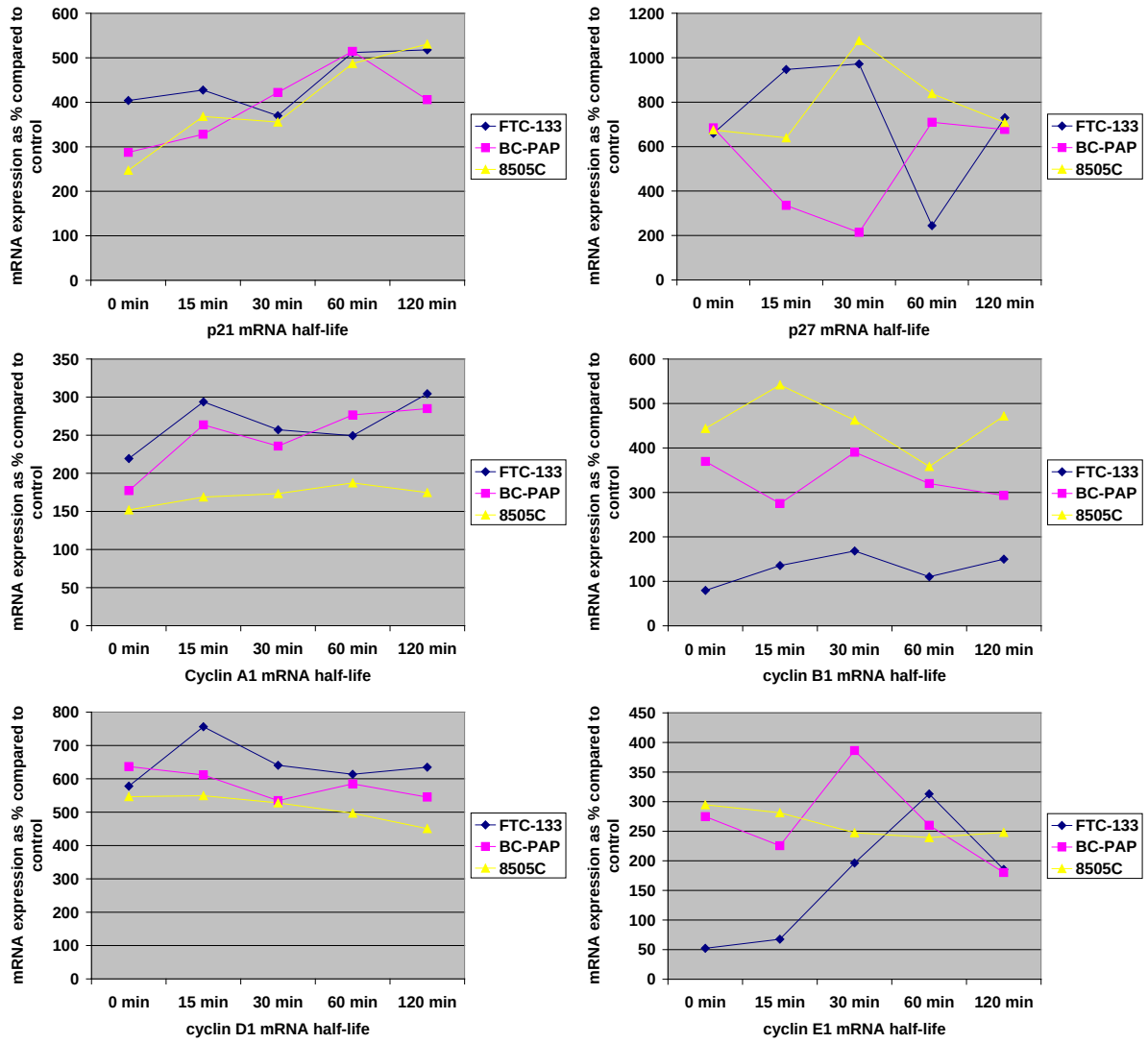

Fig. 11: Half-life of mRNAs detected as potential AUF1 targets. FTC-133, BC-PAP and 8505C cell lines were incubated with translation inhibitor-anisomycin. Cytoplasmic accumulation of mRNAs was investigated with primers specific for cyclins and cyclin dependent inhibitors.

Out of all CDIs investigated, p21 demonstrated the highest mRNA stability, (reaching about 120 min in all three cell lines), offering AUF1 relative long cytoplasmic availability for decay actions. Stability of second member of CIP/KIP family of inhibitors p27, revealed its increasing mRNA stability in FTC-133 and 8505C cells, reaching about 30 min. Investigations on BC-PAP demonstrated very short p27 mRNA half-life, occurring as instable shortly after anisomycin addition. Out of all cyclins analysed, cyclin A1 mRNA revealed the longest half-life, reaching similar stability as p21 of about 120 min in all cell lines investigated. In case of cyclin B1, its mRNA was stable for only about 15 min. Induction of cytoplasmic accumulation of cyclin D1 mRNA was achieved in FTC-133 cells for 15 min only, while in other cell lines was unstable and decreased with anisomycin incubation time. The highest stability of cyclin E1 was observed in FTC-133 (about 60 min) and BC-PAP (about 30 min) cell lines.

3.5 Selective AUF1-exon down-regulation decreased proliferation of thyroid carcinoma cell lines

The function of AUF1 in thyroid carcinoma progression is complicated to investigate by the existence of four isoforms and additionally by the fact that both destabilizing and stabilizing effects of AUF1 on ARE-containing mRNAs have been reported [131, 196]. To differentially decrease selected AUF1 isoforms we employed siRNAs targeting exon 3 contained in all four isoforms, exon 2 in isoforms p40AUF1 and p45AUF1, exon 7, which is present in isoforms p42AUF1 and p45AUF1. In order to knock-down isoforms p40AUF1, p42AUF1 and p45AUF1, without affecting p37AUF1, we used a combination of siRNAs targeting exon 2 and exon 7. First, using western blot, we evaluated whether the selected siRNAs specifically decreased corresponding isoforms (Fig. 12). As expected designed siRNAs specifically targeted AUF1 isoforms, however RNA interference effects were weaker in BC-PAP cell line comparing to FTC-133 or 8505C.

We could demonstrate that targeting of exon 2 decreased expressions of p40AUF1 and p45AUF1. However, due to tight and cumulative localization of AUF1, detection of isoform p40AUF1 was obscured. Interference of exon 3 led to reduced expression of all AUF1 isoforms and employing siRNA targeting exon 7 led to down-regulation of two upper isoforms p42AUF1 and p45AUF1. Finally, combination of two siRNAs interfering exons 2 and 7 specifically reduced 3 AUF1 isoforms p40AUF1, p42AUF1 and p45AUF1, while isoform p37AUF1 remained unchanged. In order to investigate the influence of AUF1 knock-down on proliferation of thyroid carcinoma cell lines, FTC-133, BC-PAP and 8505C with selectively decreased AUF1 isoforms, were evaluated employing MTT assay.

As shown in Fig. 13, each of siRNAs tested reduced proliferation of the cells. However, the most pronounced reduction was observed after silencing of all four AUF1 isoforms (by ~58% for FTC-133, ~56% for BC-PAP and ~76% for 8505C). These results are consistent with the previous studies demonstrating similar effects of AUF1 knock-down on proliferation of K562 (human chronic myeloid leukemia), THP-1 (human promonocytic leukemia), HeLa (human cervical carcinoma) and HT-29 (human colon carcinoma) cell lines [165]. Silencing of p45AUF1, p42AUF1 and p40AUF1 isoforms simultaneously or separately induced weaker reduction in proliferation rates when comparing to experiment with silencing of exon 3, where additional knock-down of isoform p37AUF1 was induced. Thus, this observation suggests that silencing of p37AUF1 corresponds to stronger reduction in proliferation of thyroid carcinoma cell lines. Therefore, further experiments were performed by employing knock-down of exon 3 only.

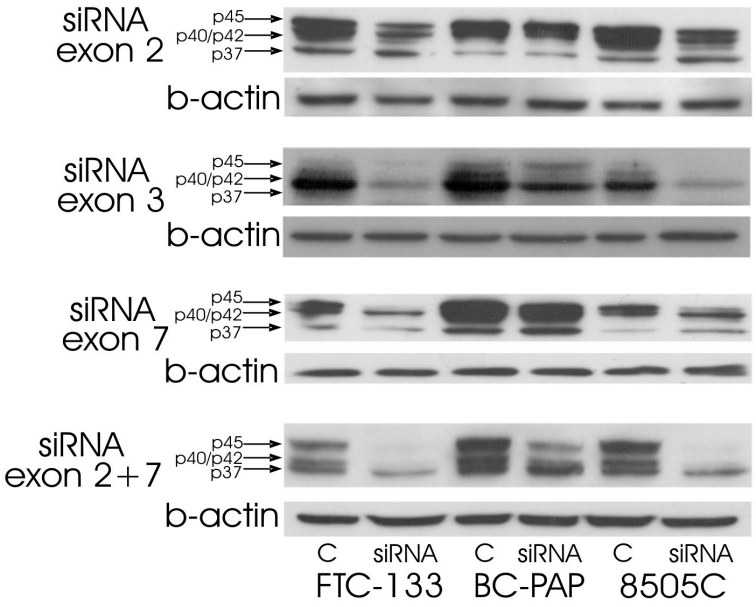

Fig. 12: Selective knock-down of AUF1 isoforms studied by RNA interference. FTC-133, BC-PAP and 8505C cells were incubated with siRNAs targeting different AUF1 exons. Specific silencing of total AUF1 was mediated by knock-down of exon 3 contained in all four isoforms, exon 2 in isoforms p40AUF1 and p45AUF1 and exon 7 in isoforms p42AUF1 and p45AUF1. Combination of siRNAs targeting exon 2 and exon 7, respectively, led to silencing of isoforms p40AUF1, p42AUF1 and p45AUF1 while p37AUF1 remained unaffected. Expression analysis was performed 72 h after transfection and representative western blotting results are shown; Detection of beta-actin was used as protein loading control.

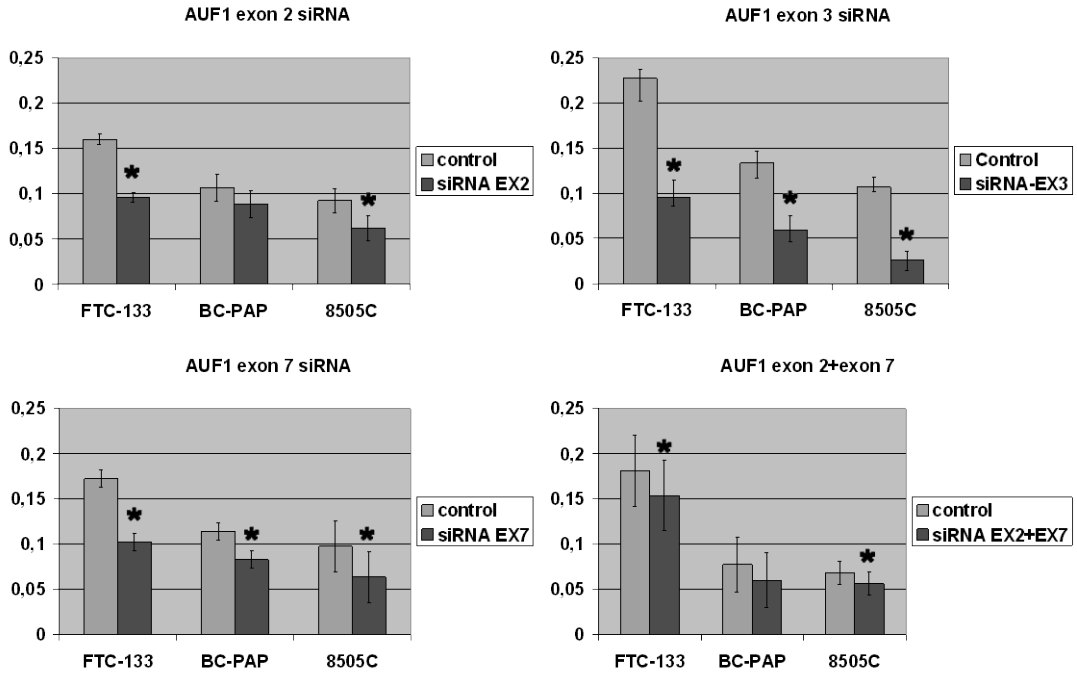

Fig. 13: Proliferation of thyroid carcinoma cell lines after selective knock-down of AUF1 isoforms. FTC-133, BC-PAP and 8505C cells were evaluated for their growth properties employing MTT assay. Selective or total AUF1 silencing noticeably decreased proliferation of the cells, however the most pronounced growth inhibition was observed after interference of all AUF1 isoforms (* $p<0.05$).

In order to verify the results of transient reduction of all AUF1 isoforms, DNA sequence corresponding to siRNA used against exon 3 was cloned into shRNA expressing vector and transfected into FTC-133 cell line. As shown in Fig. 14, all clones (A13, A17 and A21) of FTC-133 stably expressing shRNAs clearly demonstrated knock-down of all AUF1 isoforms when comparing to wild type cells or EGFP control (E19).

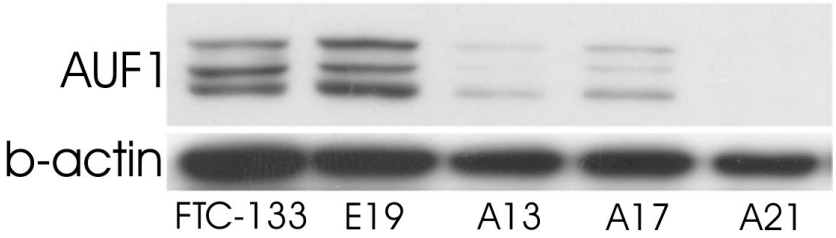

Fig. 14: FTC-133 cells transfected with shRNA vector noticeably reduced AUF1 expression comparing to EGFP controls or wild type cells. Protein expression was determined using western blot with specific anti-AUF1 serum. Beta-actin served as normalizing marker.

Additionally, the same clones tested for their growth properties revealed reduced proliferation rates as demonstrated by MTT (Fig. 15).

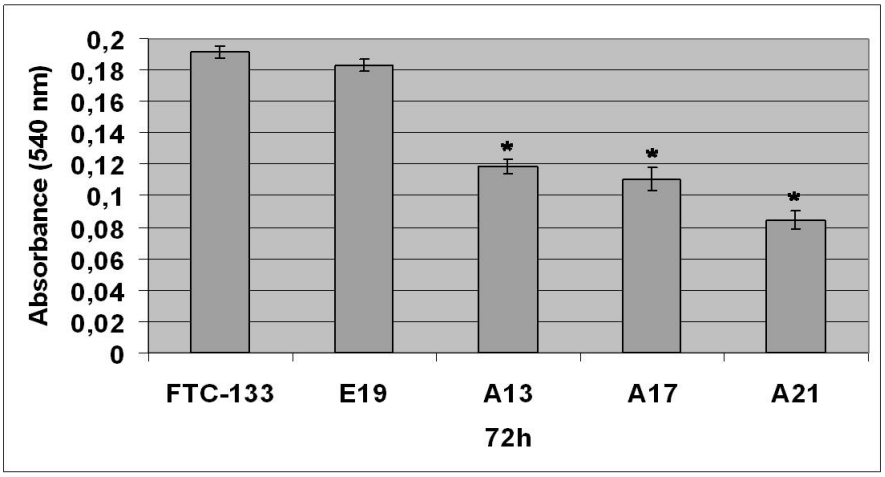

Fig. 15: Growth properties of FTC-133 cell line stably over-expressing shRNAs targeting all AUF1 isoforms. FTC-133 wild type cells, EGFP controls and three AUF1-shRNA clones (A13, A17 and A21) were employed for MTT assay. Evaluation performed after 72 h revealed significantly decreased proliferation rates of all AUF1-shRNA transfectants as compared to corresponding controls (* $p<0.05$).

3.6 Decreased proliferation rates of thyroid carcinoma cell lines affected the expression of cyclin-dependent kinase inhibitors

We also found that in thyroid carcinoma cell lines AUF1 was able to bind mRNAs of Kip/Cip family of CDK inhibitors p21 and p27, however, no binding of p57 was detectable. Kip/Cip family comprises p21, p27 and p57, inhibitors that are able to inhibit all cyclin-CDK complexes. In most cell systems their expression was induced by anti-proliferative signals and led to cell cycle arrest [197-200].

Protein expression analysis performed on Kip/Cip family of CDK inhibitor, revealed that transient interference of all AUF1 isoforms led to changed protein expression pattern of p21, p27 and p57 in all three cell lines analyzed (Fig. 16). Investigations of p21 demonstrated its increased production in AUF1-siRNA treated FTC-133 and 8505C cell lines. In slowly growing BC-PAP cells representing papillary thyroid carcinoma, p21 protein was only slightly elevated after AUF1 silencing.

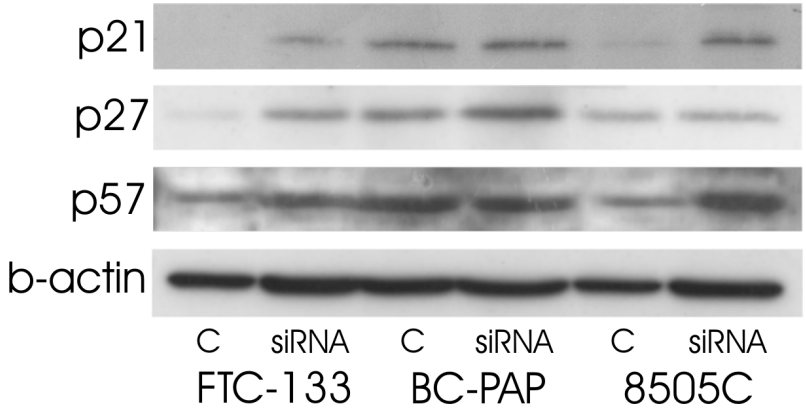

Fig. 16: Analysis of cyclin-dependent kinase inhibitors after silencing of all AUF1 isoforms. Protein analysis was performed with specific antibodies raised against p21, p27 and p57. AUF1 knock-down led to increased p21 production in FTC-133 and 8505C cells, while in BC-PAP its production was only slightly elevated. Increased levels of p27 were observed in differentiated thyroid carcinoma cells FTC-133 and BC-PAP, while p57 was up-regulated in 8505C cells only.

Investigations of p21 performed on mRNA level revealed its presence in all cell lines. What more silencing of AUF1 led to elevation of p21 mRNA in FTC-133 and noticeably in 8505C. In case of BC-PAP its mRNA expression was also only slightly elevated (Fig. 17).

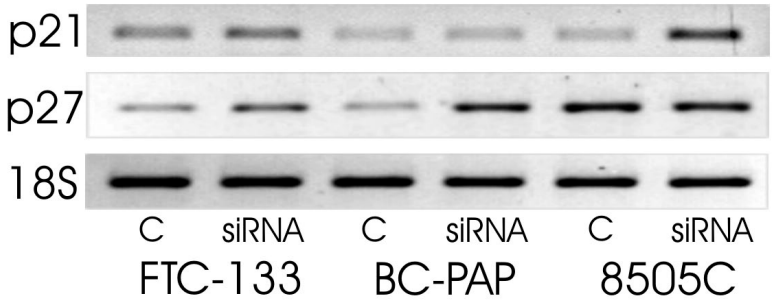

Fig. 17: Expression of CDIs performed on transcriptional level. RT-PCR analysis of p21 revealed its up-regulation in all cell lines investigated. Expression of p27 mRNA was increased in FTC-133 and BC-PAP cells, while in 8505C cells remained without changes. As AUF1 does not bind p57 mRNA, its analyses was omitted.

The second mRNA of Kip/Cip family demonstrated to be a target for AUF1 was p27. Expression of p27 in control FTC-133 and BC-PAP cells was comparable to that of p21 experiment. Employing of siRNA targeting AUF1 noticeably increased p27 expression in both cell lines investigated. Interestingly, AUF1 knock-down showed no effect on p27 protein

expression in 8505C. Similar effects could be demonstrated for transcriptional level, where p27 mRNA correlated with protein expression (Fig. 17).

The last CDK inhibitor we investigated was p57. All three thyroid cell lines revealed its presence on protein level, however no AUF1-p57 mRNA binding was observed in our mRNP experiments and p57 mRNA analysis was omitted. Protein expression analyses revealed that silencing of AUF1 did not induce noticeable regulation of p57 in FTC-133 and BC-PAP. The only one cell line we could demonstrate up-regulation of p57 protein was 8505C. However, lack of AUF1 association with p57 mRNA suggests that increased p57 protein expression in this cell line was indirect effect of AUF1 knock-down (Fig. 16).

3.7 AUF1 knock-down led to dysregulation of cell cycle-related proteins

To obtain additional information concerning the regulation of cell cycle related-proteins, further investigations were performed on cyclins A1, B1, D1, D3 and E1, previously reported to be increased in extracts from human cancers, and related to thyroid carcinoma [201-205]. Western blot analysis performed after AUF1 knock-down, revealed decreased levels of cyclins crucial for cell proliferation (Fig. 17).

We could demonstrate that cyclin A1 strongly expressed in control FTC-133 was noticeably reduced after employing siRNA targeting AUF1. Similar results were obtained for BC-PAP and 8505C cells. However controls of both cell lines expressed cyclin A1 much weaker when comparing to FTC-133 cells. Surprisingly, cyclin A1-AUF1 complexes in 8505C cells could not be detectable; nevertheless siRNA mediated silencing of AUF1 led to decreased level of cyclin A1 in this cell line.

The next cell cycle regulator analysed was cyclin B1. We showed that cyclin B1 mRNA was associated with AUF1 in all cell lines investigated. However, on protein level it was expressed in control 8505C cells only and AUF1 knock-down noticeably decreased cyclin B1 production in this cell line.

Expression of cyclin D1 was found to be reduced in all cell lines analysed, while expression of cyclin D3 was down-regulated in FTC-133 and 8505C. In BC-PAP cells its expression remained strong and unchanged after siRNA treatment. Interestingly, cyclin D3 could not be amplified in mRNP reactions, nevertheless was regulated in siRNA experiments.

Expression of cyclin E1 was only slightly decreased in FTC-133 cells and also in this cell line mRNA of cyclin E1 was found to weakly associate with AUF1.

We could also demonstrate that AUF1 knock-down affected production of two tumor suppressor genes, p53 (wild type) and retinoblastoma protein Rb. Expression of p53 was noticeably decreased in FTC-133 and 8505C cell lines, while production of retinoblastoma

protein was increased. Interestingly, both in control and siRNA treated BC-PAP cells, expression of p53 and retinoblastoma protein was not detectable (Fig. 18).

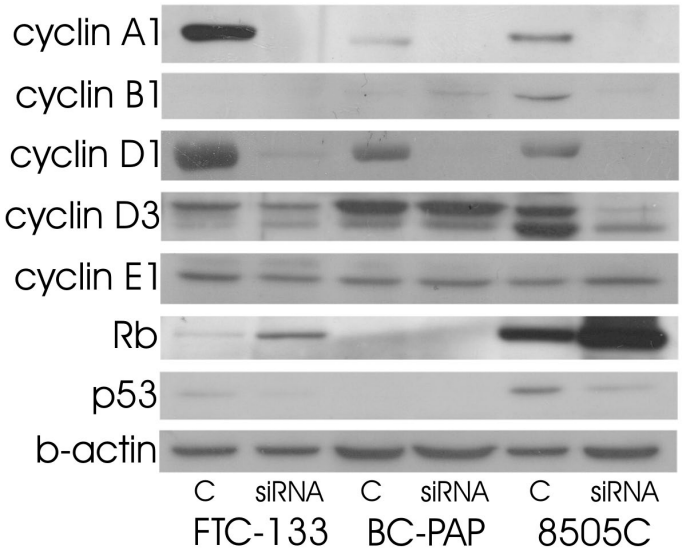

Fig. 18: Protein expression analysis of selected cell cycle regulators in thyroid cancer cell lines. 20µg of total protein lysates were separated by PAGE, blotted onto PVDF membranes and incubated with specific antibodies for cyclin A1, B1, D1, D3, E1, p53 and retinoblastoma protein (Rb). β-actin served as protein loading marker.

Further analysis performed on FTC-133 transfectants with reduced AUF1 expression revealed similar expression pattern of cell cycle related proteins demonstrated in transient AUF1 experiments. We could show that stable AUF1 knock-down led to down-regulation of cyclins A1, D1, D3, E1 and p53, while expression of retinoblastoma protein was elevated comparing to EGFP controls or wild type cells (Fig. 19). Expression of cyclin B1 was omitted as control FTC-133 and siRNA treated cells lack its expression.

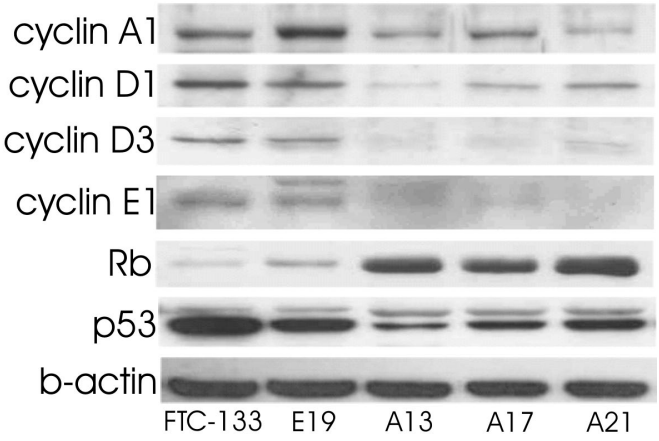

Fig. 19: Protein expression analysis of selected cell cycle regulators in FTC-133 transfectants. 20 µg of total protein lysates were separated by PAGE, blotted onto PVDF membranes and incubated with specific antibodies for cyclin A1, B1, D1, D3, E, p53 and retinoblastoma protein (Rb). β-actin served as protein loading marker.

3.8 AUF1 is able to affect factors related to tumor growth and progression

3.8.1 Decreased AUF1 levels affected RNA-stabilizing protein HuR

AUF1 is one of the proteins regulating the stability of ARE-bearing mRNAs. It was demonstrated, that also other ARE binding protein HuR, can compete with AUF1 for binding to the same mRNAs. HuR is a known mRNA stabilizing protein and its knock-down was reported to correlate with decreased proliferation and tumor growth *in vivo* [167]. In order to investigate relations between AUF1 and HuR in thyroid carcinoma, FTC-133, BC-PAP and 8505C cells were incubated with siRNA targeting all AUF1 isoforms. Thereafter, protein lysates were incubated with specific HuR anti-serum. The same procedure was applied to proteins obtained from FTC-133 transfectants stably expressing AUF1-shRNAs. As shown in Fig. 20, AUF1 knock-down led to reduction of HuR protein in FTC-133 and 8505C cells, and FTC-133 transfectants. In BC-PAP, which is slowly growing cell line, expression of HuR was not affected after employing AUF1-targeting siRNA.

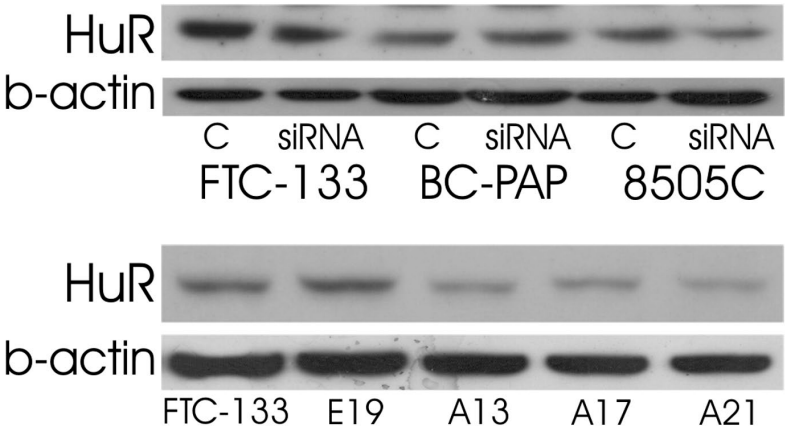

Fig. 20: Expression of HuR after employing siRNA targeting AUF1. Western blot analysis was performed on thyroid cancer cell lines (upper panel) and FTC-133 transfectants (lower panel). B-actin served as normalizing marker.

3.8.2 AUF1 knock-down correlated with elevated levels of TSG

Our published data demonstrated that expression of several tumor suppressor genes (TSG) and tumor promoting factors, may serve as novel biomarkers for thyroid carcinoma. We found that down-regulation of CD9, CD82 and RKIP may reflect an increased *in vivo* metastatic potential of thyroid cancer cells [194, 206]. Inversely, elevated expression of S100A4 and ENO1 was correlated with increased proliferation and metastatic potential of thyroid carcinoma [195].

The influence of AUF1 on above mentioned factors was investigated by employing RNAi. We demonstrated the presence of cross-link between AUF1 and CD9, CD82, S100A4 and ENO1, as these proteins were affected after AUF1 knock-down. RNAi studies revealed that

AUF1 knock-down led to increased expression of two tumor suppressor genes CD9 and CD82. Subsequently, decreased expression of tumor promoting S100A4 and ENO1 was observed (Fig. 21). We found CD82 to be elevated in FTC-133 and BC-PAP cells. 8505C cells representing aggressive undifferentiated thyroid carcinoma did not express this protein both in control and siRNA treated cells.

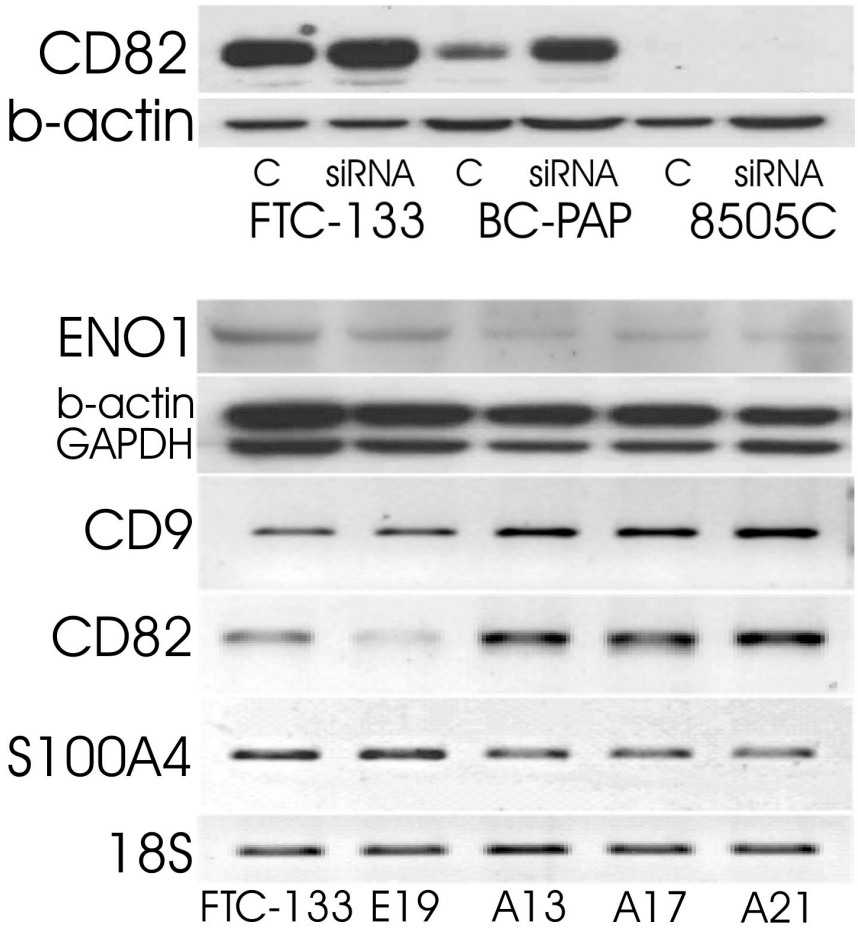

Fig. 21: Upper panel: expression of CD82 in thyroid carcinoma cell lines. Lower panel: expression of CD9, CD82, ENO1 and S100A4 in transfectants expressing AUF1 targeting shRNAs. Increased levels of tumor suppressors (CD9 and CD82) and subsequent decrease of tumor promoters (ENO1 and S100A4) are visible. 18S and B-actin served as normalizing markers.

3.8.3 AUF1, HuR and ENO1 responded to RA therapy

RA therapy was successfully used for treatment and chemoprevention of solid cancers [207, 208] including thyroid carcinomas [209]. Cell culture experiments in thyroid carcinoma cell lines showed that RA treatment affects thyroid-specific functions, cell-cell or cell-matrix interaction, differentiation markers, growth, and tumorigenicity [210]. RA has an anti-proliferative effect on the follicular thyroid carcinoma cell lines FTC-133 and FTC-238. Furthermore, pre-treatment of these cell lines with RA resulted in decreased *in-vitro* proliferation rates and reduced tumor cell growth of xenotransplants [211].

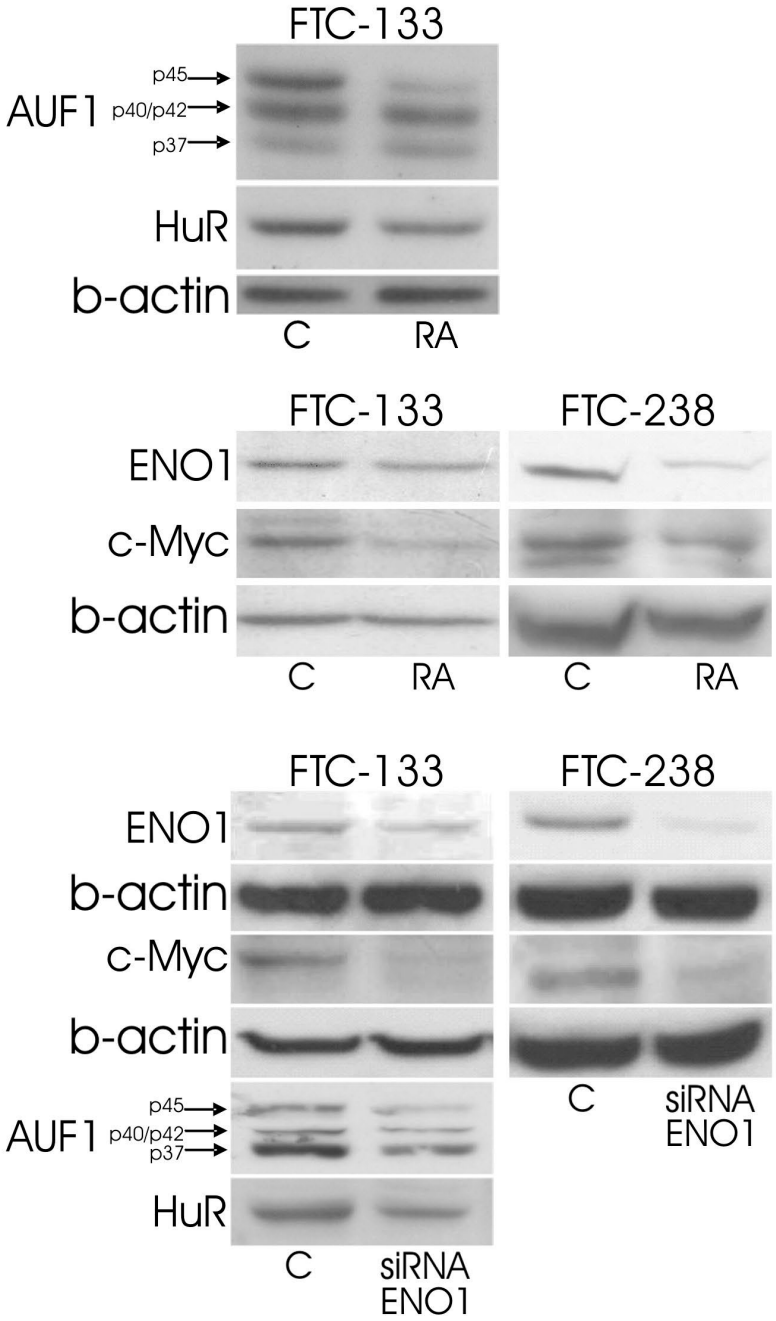

Fig. 22: Expression of AUF1 and HuR after RA pre-treatment and ENO1-siRNA employment. Upper and middle panels: Thyroid carcinoma cells pre-treated with RA revealed decreased levels of AUF1, HuR, ENO1 and proliferation-promoting c-Myc: Lower panel: ENO1 knock-down led to reduced levels of AUF1, HuR and c-Myc. B-actins served as normalizing markers.

In addition to previously published data we found that RA pre-treatment led to down-regulation of AUF1 and HuR. Moreover two others tumor promoting factors ENO1 and c-Myc were also decreased upon RA incubation (Fig. 22, upper and middle panels). In order to further investigate the correlation between AUF1 and ENO1, we applied siRNAs. As demonstrated in Fig. 22, lower panel, also ENO1 knock-down led to reduction of AUF1, HuR and c-Myc. C-Myc is known proliferation promoter and is over-expressed in many cancers. To verify whether down-regulation of above mentioned proteins correlated with reduced invasiveness of thyroid carcinoma cells, we performed ATP, MTT and motility assays. We

could show that both RA pre-treatment and ENO1 knock-down led to growth inhibition and decreased metastatic potential of thyroid carcinoma cells (Fig. 23 and Fig. 24).

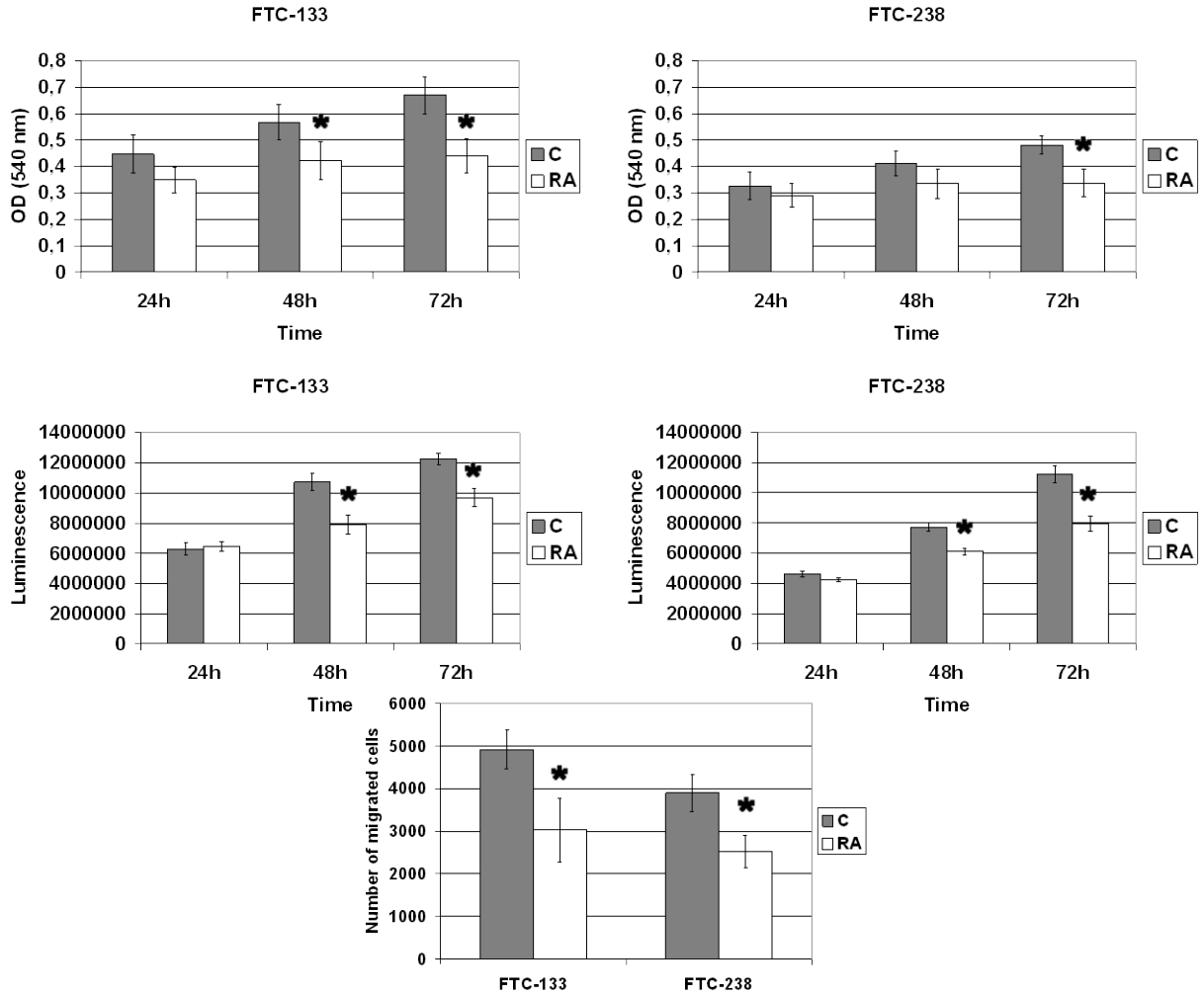

Fig. 23: Growth, vitality and invasiveness of follicular thyroid carcinoma cell lines FTC-133 and FTC-238 upon RA treatment. Upper panel: proliferation of the cells as evaluated by MTT assay. Middle panel: vitality of the cells as measured by intracellular ATP levels. Lower panel: migration of RA pre-treated cells. Both cell lines responded with significantly decreased intracellular ATP levels and the number of migrated cells (* $p<0.05$).

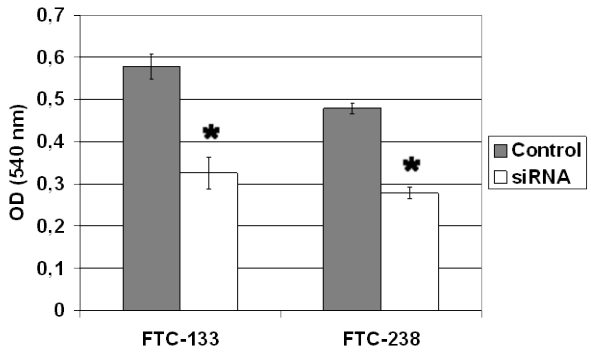

Fig. 24: FTC-133 and FTC-238 cells were treated with ENO1-derived siRNA (targeting common c-Myc binding domain) or with a scrambled, non-silencing siRNA control used at 200 nM each. The cells treated with ENO1 targeting siRNA displayed ~44% and ~42% reductions in growth, respectively (* $p<0.05$).

4 Discussion

We found in this study that all investigated thyroid carcinoma cell lines and tissues expressed AUF1, however with varying levels of different AUF1-isoforms. Investigations performed on protein level demonstrated that AUF1 is present in the cytoplasm and nucleus, and its cellular shift is cell proliferation-related. Moreover, we found that alterations in AUF1 level affected the expression of CDKs and CDIs, previously described to be crucial for thyroid tumorigenesis. These observations raise several questions about the possible role of AUF1 in thyroid carcinoma progression.

4.1 AUF1 and cell proliferation

Investigations performed on thyroid carcinoma cell lines demonstrated that AUF1 expression and localization are modulated during cell division and proliferation. We could show that as the thyroid carcinoma cell lines proliferate, AUF1 is shuttled from nucleus to cytoplasm. In our studies all AUF1 isoforms were mostly nuclear reaching their highest level after serum starvation. Subsequent serum addition led to AUF1 shuttling from nucleus to cytoplasm of thyroid carcinoma cell lines. We observed that this translocation was more pronounced for cell lines established from differentiated thyroid carcinoma when comparing to undifferentiated thyroid carcinoma cell lines. Moreover, we found that dividing and proliferating thyroid carcinoma cell lines revealed elevated cytoplasmic AUF1 levels as compared to surrounding cells. These observations support our hypothesis that (a) AUF1 shuttling from nucleus to cytoplasm may promote thyroid carcinoma progression; (b) AUF1 may be involved in thyroid differentiation process.

Involvement of ARE-binding proteins in cell cycle and proliferation of cancer cell lines was already demonstrated on colorectal carcinoma RKO cells. In those studies expression of ARE-stabilizing protein HuR was investigated throughout cell division cycle. It was demonstrated that this protein regulates cyclin A1 and cyclin B1 mRNA stability during cell proliferation. In RKO cells synchronized by serum starvation, HuR was almost exclusively nuclear during early G1, but increasing in the cytoplasm during late G1, S and G2, what correlated with stability of mRNAs encoding cyclins A1 and B1, and increased proliferation [168]. Similar to our observations concerning cytoplasmic shift of AUF1, also here increased proliferation of RKO cells was associated with cytoplasmic HuR accumulation. Moreover, we demonstrated that siRNA derived AUF1 knock-down, resulted in HuR down-regulation and decreased proliferation rates of thyroid carcinoma cells. Given that accessibility of many labile mRNAs is relative high in cytoplasm, translocation of HuR or AUF1 may be related to function of these proteins, respectively. Previous studies demonstrated that cytoplasmic AUF1 activity (a) is polysome associated; (b) takes place outside stress granules; (c) can be regulated in HuR dependent manner [212]. However, there is no data how such an activity

correlates with neoplasia. We showed that all investigated thyroid carcinoma cell lines revealed high proliferation rates and already expressed AUF1 mRNA. Thus, its role was studied by RNA interference.

We could show that selective or total knock-down of AUF1 isoforms decreased cell proliferation. What more we found that most pronounced growth inhibition was obtained after knock-down of p37AUF1 as compared to selective or simultaneous silencing of other AUF1 isoforms. p37AUF1 isoform has the strongest affinity for AREs binding *in vitro* and its over-expression was previously reported to induce tumors with high cellularity, rich vascularization and commonly observed mitosis. Overall tumors revealed increased expression of class I (c-myc, c-fos, cyclin D1) and class III (c-jun) ARE-containing mRNAs [213]. Other studies demonstrated that increased levels of p37AUF1 resulted in up-regulation of eIF4E protein expression [214]. It worth to notice that eIF4E contains ARE and its over-expression leads to malignant transformation and tumorigenesis in animal models [215]. Our results suggest that also in thyroid carcinoma, out of four isoforms investigated, p37AUF1 plays the most important role as tumor promoter.

We found that down-regulation of AUF1 led to decreased production of cyclins A1, B1, D1, D3 and E1, and additionally elevated levels of retinoblastoma protein and down-regulation of wild type p53. Previous studies reported increased levels of those cyclins in most malignant thyroid tissues and their important role in thyroid carcinogenesis [216, 217]. Other reports demonstrated that wild type p53 has been related to a poor clinical outcome in thyroid cancer [218-220]. Also other tumor suppressor, retinoblastoma protein were also demonstrated to play a pivotal role in thyroid cell differentiation and transformation. Its interaction with oncoprotein E1A leads to inhibition of differentiation of thyroid epithelial cell lines. Furthermore, it was shown that retinoblastoma protein is required for the expression of the thyroid differentiation markers thyroglobulin (Tg) and thyroperoxidase (TPO) [221]. Immunohistochemical studies performed on thyroid tissues revealed that malignant thyroid neoplasms lacked retinoblastoma protein immunoreactivity when compared to benign thyroid lesions [222]. Moreover, retinoblastoma protein reconstitution in various human cancer cell lines suppressed tumorigenicity both *in vitro* and *in vivo*, while retinoblastoma knock-down led to increased proliferation rates of lung cancer cells [223, 224].

We demonstrated that increased expression of cyclin dependent kinase inhibitors p21, p27 and p57, correlated with reduced growth rates of thyroid carcinoma cell lines after AUF1 knock-down. We found that p21 mRNA and protein were up-regulated in all thyroid carcinoma cell lines analysed. Transcriptional regulation of p21 expression has previously been shown to be positively regulated by three tumor suppressor genes, p53 [225], BRCA-1 [226] and Rb1 [227]. We found that AUF1 knock-down followed by up-regulation of p21 expression led to decreased p53 level and increased production of Rb protein. These

observations suggest that in thyroid carcinoma p21 activity may also be regulated p53 independently and be mediated by Rb protein. Both proteins were already related to thyroid carcinoma progression. It was demonstrated that progressive lost of p21 and Rb protein was observed in differentiated and most malignant, poorly differentiated or undifferentiated thyroid tissues as compared to benign thyroid lesions [228]. We also showed that AUF1 interference led to up-regulation of p27 in differentiated thyroid carcinoma cell lines FTC-133 and BC-PAP, while p57 was up-regulated in 8505C cells only, representing undifferentiated thyroid carcinoma. Reduced expression of these proteins was reported in most poorly differentiated and undifferentiated thyroid carcinomas, and the level of p27 and p57 proteins decreased during tumor development and progression [229-232]. Additionally down-regulation of p27 was noted in PTCs with tendency to lymph node metastases [233, 234].

In conclusion, all these observations suggest that decreased AUF1 levels, which lead to dysregulation of cell-cycle related proteins and growth inhibition, could have beneficial effects on thyroid carcinoma. Although we can not exclude participation of other factors, we provide first evidence that increased AUF1 shuttling from nucleus to cytoplasm may promote thyroid carcinoma progression. Additionally, AUF1 can regulate mRNA stability and/or translation of several key factors participating in the control of cell proliferation, highlighting the importance of post-transcriptional events in thyroid carcinoma

4.2 Involvement of AUF1 in target mRNA interactions

We found that all mRNAs analysed, out of p53, contained at least one AUUUA repeat within their 3'UTR. Furthermore many of these mRNAs were bound by AUF1, however, not all were stabilized or up-regulated when AUF1 were knocked-down. Stability of cyclin dependent kinase inhibitors p21 and p27 was generally longer than cyclins alone. Exception here was cyclin E1, which however showed relative long mRNA half life, but its interaction with AUF1 was very weak. Nevertheless we found that AUF1 is directly involved in regulation of p21, p27 and Rb1 mRNAs stability. These findings are further supported by the fact, that AUF1 was able to bind all of them, its knock-down led to increased translation of those proteins and finally, as demonstrated above to decreased proliferation of thyroid carcinoma cell lines (Results 3.3, 3.5-3.7). We found that also p57 is somehow involved in inhibition of thyroid carcinoma progression. Its 3'UTR contains 3 repeats of AUUUA sequence and translation of p57 mRNA was increased upon AUF1 inhibition in undifferentiated thyroid carcinoma cell line 8505C. On the other hand we did not identify p57 to interact with AUF1 what suggest its indirect influence and probably involvement of other ARE-binding protein(s). Also cyclin D3, which mRNA contains 2 AUUUA repeats, was not bound by AUF1; but down-regulated upon AUF1 knock-down. mRNAs of other cyclins analysed interacted with AUF1, but they were surprisingly destabilized and down-regulated upon AUF1 inhibition. There are several

possible scenarios in order to explain behaviour of these particular mRNAs in AUF1-mediated actions. mRNAs of p21, p27 and Rb1 may contain an additional signature motifs particularly recognized by AUF1 and AUF1-interacting proteins. These mRNAs are further processed by NSEP-1, which has endoribonuclease activity or degradated by ubiquitin-proteasome pathway [138, 139, 235]. Additionally destabilizing affinity of AUF1 for mRNAs of p21, p27 and Rb1 could be higher than that of stabilizing proteins like HuR. We demonstrated that AUF1 knock-down led to decreased levels of this stabilizing protein, what may additionally amplify destabilizing AUF1 actions. In case of cyclins, it is possible that AREs are not the only one binding place for AUF1 and it can interact with determinants localized within the cyclins coding region. Such an additional interaction can temporarily change the AUF1 function and depending on interaction affinity may lead to short time stabilization of cyclins mRNAs [236]. AUF1 binding to coding determinants may block translation initiation of cyclins mRNA, leading to blockage of deadenylation and accumulation of these mRNAs. It is also possible that AUF1-mediated mRNA turnover is controlled by additional decay complex or mechanisms. Some reports postulate the existence of at least two distinct mechanisms controlling AUF1-related mRNA degradation [196]. In case of cyclins, action of only one of such mechanisms may be not sufficient to disturb their stability. We also can not exclude participation of other ARE-binding proteins, like in case of p53. mRNA sequence of p53 lacks canonical AU motifs, but contains A- and U-rich sequences within its 3'UTR. We found that AUF1 is not involved in direct regulation of mRNA stability of this protein, as AUF1 knock-down led to subsequent p53 and HuR down-regulation. We speculate that p53 mRNA stability could be regulated in HuR-dependent manner, what is with agreement of previous observations. They demonstrated that in RKO cells p53 cytoplasmic mRNA is stabilized exclusively by HuR upon UVC treatment. No involvement of AUF1 and other ARE-binding proteins like TTP, TIAR or TIA-1 was observed [153].

In summary, we demonstrated that the stability of many ARE-containing mRNAs may be differentially regulated by one particular ARE binding protein. Understanding how thyroid carcinoma cells achieve distinct regulation of stability of various ARE-containing mRNAs remains an open and interesting question.

4.3 Response to RA pre-treatment

Cell culture experiments in thyroid carcinoma cell lines showed that RA treatment affects thyroid-specific functions, cell-cell or cell-matrix interaction, differentiation markers, growth, and tumorigenicity. We demonstrated that RA has an anti-proliferative effect on the follicular thyroid carcinoma cell lines FTC-133 and FTC-238. Furthermore, pre-treatment of these cell lines with RA results in decreased *in-vitro* proliferation rates and reduced tumor cell growth of xenotransplants [211].

In addition to previous studies we showed that RA treatment of the human follicular thyroid carcinoma cell lines FTC-133 and FTC-238 causes the down-regulation of ENO1 what correlated with both reduction of cell invasiveness and decrease in tumor promoting protein c-Myc [195]. Subsequently we observed reduced levels of AUF1 and other ARE-binding protein HuR upon RA treatment.

The up-regulation of glycolytic enzymes, including ENO1, appears to be a common strategy in carcinoma of diverse origin, including thyroid oncocytoma [237]. However, involvement of two ARE-binding proteins in RA-mediated actions is novel.

We found that down-regulation of ENO1, AUF1 and HuR coincided with a decrease of c-Myc suggesting an oncogene suppressive effect by which RA can impair thyroid carcinoma growth. The same effects were observed after employing specific siRNA targeting ENO1. The RA-induced down-regulation of c-Myc and resulting decrease in tumor cell proliferation is a common phenomenon described previously in lung cancer and myeloid cell lines [238-241]. However, information on the potential role of ENO1, HuR or AUF1 is lacking.

RA therapy administrated for differentiated thyroid carcinoma is well tolerated with few side effects. We found that 1 µM (300 ng/l) RA used in our study, led to reduced invasiveness of two follicular carcinoma cell lines, accompanied by decrease in ENO1, AUF1 and HuR, as well as c-Myc production. This could be partially explained by reduced glucose metabolism and tumor size, previously observed as favourable response to RA therapy [242]. Whatmore involvement of HuR and AUF1 suggest the influence of RA on ARE-regulated mRNA stability.

Basal levels of natural RA (*all-trans*) in human serum are in the nanomolar range of 2-10 nM [243]. Administration of synthetic or therapeutic levels of RA (e.g. 13 *cis* RA) frequently used in cancer such as leukemia [244, 245] or dermatology therapy [246] easily reach the low micromolar levels (0.5 to 5 µM) depending on time, duration and dose of treatment. In addition various retinoid metabolites are formed in the low to high nanomolar range. Therefore as demonstrated before [247, 248], 1 µM concentrations of RA in human serum are easily to reach in therapeutic protocols.

In summary, we identified ENO1, HuR and AUF1 proteins as novel targets and/or executioners of the RA action, reducing the invasiveness of the human follicular thyroid carcinoma cell lines FTC-133 and FTC-238. RA-induced reduction of the key glycolytic enzyme, proliferation-promoting c-Myc and two ARE-binding proteins, may serve as an additional predictive parameter of successful redifferentiation and anti-tumor therapy.

4.4 Participation of other factors in AUF1-mediated tumor progression

We found that AUF1 knock-down correlated with increased levels of tumor suppressive CD9 and CD82, and decreased levels of tumor promoting ENO1 and S100A4. Subsequently we observed reduced invasive potential of tumor cells, what suggest a beneficial effect of decreased AUF1 levels on thyroid carcinoma progression. Computational analysis of CD9, CD82, ENO1 and S100A4 mRNAs revealed that none of them contained the AUUUA, UUAUUUA(U/A)(U/A) or A-or U-rich motifs in their 3' UTRs. Based on those and previous observations we excluded their possible interaction with AUF1 and speculated that AUF1-mediated effects may be induced indirectly. Out of all tumor suppressor genes investigated in this study, we demonstrated a direct interaction between Rb1, p21 and p27 mRNAs and AUF1 protein. Whatmore, we showed that up-regulation of those proteins upon AUF1 knock-down led to decreased expression of cyclins and reduced proliferation rates of thyroid carcinoma cells. Furthermore, we demonstrated that these alterations may exert an indirect, AUF1-mediated influence on CD9, CD82, ENO1 and S100A4.

We demonstrated that AUF1 knock-down correlated with increased levels of CD9. The implication of CD9 in cancer development and progression has recently received much attention. An inverse correlation between its expression in primary tumors and the potential as tumor suppressor were established for patients suffering from melanoma, colon, lung and breast cancers. It was demonstrated that over-expression of this tetraspanin led to inhibition of motility and metastatic potential of the cells originated from these cancers [249-255]. Suppressive activity of CD9 could be partially explained by its interactions with different integrins. However, in this study we did not investigate the influence of AUF1 on CD9-mediated expression of these adhesion molecules.

The second tumor suppressor we investigated is CD82. It is expressed in many human tissues and strikingly, decrease in CD82 mRNA and protein expression is associated with the advanced stages of many malignancies [206, 256-263]. Recent studies suggest that complex mechanisms underlie CD82 loss of function, including altered transcriptional regulation, splice variant production and post-translational protein modifications. We demonstrated that AUF1 inhibition in differentiated thyroid cells, representing follicular and papillary thyroid carcinomas, led to CD82 up-regulation both at mRNA and protein levels. In undifferentiated thyroid carcinoma cells 8505C, CD82 was not detectable both in controls and siRNA treated cells. However, whether CD82 function and/or expression is/are not influenced by AUF1 in undifferentiated thyroid carcinomas only requires further clarification.

Previously we demonstrated ENO1 as a promoter of follicular thyroid carcinoma progression. Its reduction both with RA or ENO1-targeting siRNAs correlated with decreased invasive potential of thyroid carcinoma cells. Whatmore decrease in ENO1 level reflected in reduction

of c-Myc, AUF1 and HuR proteins. Similar reduction in cell aggressiveness was observed after direct inhibition of AUF1 or HuR by employing specific siRNAs.

In order to examine the relation between reduction of those proteins and invasiveness of the cells, we investigated the expression of S100A4, known as molecular marker for metastatic potential. We found decreased levels of this protein in all shRNA transfectants with induced AUF1 knock-down. Moreover as expected, AUF1-mediated decrease in S100A4 expression led to reduced growth of shRNA-transfected thyroid carcinoma cells. In addition to our data demonstrated for thyroid carcinoma, an increase in S100A4 expression has been correlated with a worse prognosis for patients with colorectal, gallbladder, bladder, oesophageal, non-small-cell lung, gastric, medulloblastoma, pancreatic and hepatocellular cancers [264-269]. S100A4, like AUF1 or HuR, has no known enzymatic activity and its actions depend on both calcium binding and dimerization. It has been proposed that S100A4 affects cell motility through its interaction with non-muscle myosin at the leading edge of motile cells [270]. S100A4 has also been reported to interact *in vitro* with actin filaments, non-muscle tropomyosin and possibly with tubulins [271-273]. These reports further suggest its possible involvement in the regulation of cell motility and cytoskeleton rearrangements. S100A4 may also bind other than cytoskeletal proteins. It was demonstrated that tumor suppressor p53 and liprin β1 are partners for S100A4 [274, 275]. It is worth to notice that in our studies AUF1-mediated decrease in S100A4 correlated with reduction of p53. However, whether AUF1 may interact with S100A4 or other proteins investigated in this study remains an open question.

It is known that AUF1 itself does not posses ribonuclease activity and AUF1-mediated degradation of target mRNAs requires engagement of other proteins. Previous reports identified the ubiquitin-conjugating enzyme E2I and three RNA binding proteins: NESP-1, NSAP-1 and IMP-2, as AUF1 interacting proteins. What is more important, NSEP-1 revealed an endoribonuclease activity, what could be possible explanation for the role of AUF1 in the regulation of mRNA stability [235]. The other mechanism consistent with AUF1-mediated gene regulation and tumor progression may occur in a manner similar to degradosome of *E. coli*. The degradosome consists of a high molecular weight complex of proteins, including glycolytic enzyme enolase [276]. Enolase itself was not identified as a component of eukaryotic mRNA degradation machinery, but other, also glycolytic enzyme lactate dehydrogenase, was identified as an AUF1 interacting protein [277]. It is possible that lactate dehydrogenase may mediate effects on mRNA turnover in a manner analogous to enolase in *E. coli*. In our studies we did not investigate whether enolase is an interacting partner for AUF1. However we demonstrated that AUF1 knock-down led to decreased levels of enolase, what reflected in reduced metabolic potential of the cells, as demonstrated by MTT assay.

The metabolic function of both enzymes is well known, but their role in mRNA degradation is not yet clear.

The ability of lactate dehydrogenase and enolase to affect mRNA turnover suggest their function beyond metabolism of tumor cells. Of particular note, elevated levels of enolase and lactate dehydrogenase are frequently detected in human cancers [278-286]. Thus, their over-expression may confer neoplastic growth advantage, either through its enzymatic or gene regulatory function.

4.5 Clinical significance of AUF1 expression in thyroid carcinoma

First studies concerning the possible role of AUF1 in processes of carcinogenesis, were obtained by employing mouse model *in vivo*. To address this hypothesis, the tumors were induced by male A/J mice, which are highly susceptible to lung carcinogenesis. Normal and carcinogenic lung tissues were harvested for cytosolic protein isolation or immunocytochemistry with AUF1 and HuR antibodies. Compared with normal peripheral lung tissue, expression of AUF1 and HuR was significantly increased in lung tumor tissues. Immunohistochemical data performed on normal tissues revealed that nuclear staining of both proteins was more intense than in cytoplasm. Contrary to normal tissue data, intensity of cytoplasmic AUF and HuR expression increased dramatically in malignant tissues (166).

Similar results were demonstrated for HuR by comparing pairs of human normal and malignant tissues of the stomach, lung, colon, thyroid and kidney. HuR staining was found to be weak to moderate in all normal tissues investigated and was almost exclusively nuclear. The increase of HuR's cytoplasmic staining was observed in malignant specimens and was most pronounced for colon tissues. Its relative cytoplasmic abundance in colon was lowest in the normal mucosa, moderately higher in adenomas, and highest in carcinomas [167, 287]. Up-regulation of HuR was also demonstrated in other malignant cells representing squamous cell carcinoma and acute myelogenous leukemia as compared to normal skin fibroblast [288].

Other reports demonstrated that over-expression of p37AUF1 isoform led to dysregulation of several target mRNAs and promoted tumorigenesis *in vivo*. AUF1 transgene mice revealed atrophy of the spleen and thymus. Tumors were observed in 50% of sick or dead AUF1 over-expressing mice. All tumors were strongly vascularized with sarcoma-like pattern and commonly observed mitosis [213].

We found that alterations in AUF1 expression and localisation are crucial for thyroid carcinoma progression. We could show that increased cytoplasmic expression of AUF1 correlated with neoplasticity, what is in agreement with previous studies [166]. By employing subcellular fractionation and immunohistochemistry on human thyroid tissues, we demonstrated that AUF1 is expressed both in cytoplasm and nucleus, however, on different

levels. The lowest cytoplasmic AUF1 level was detectable in normal and benign thyroid tissues, but the highest in adenoma and malignant tissues, including FTC, PTC, and UTC. Such a cellular translocation of AUF1 was already demonstrated for mammary gland tissue. In this study cytoplasmic localisation of AUF1 was demonstrated to be critically linked to its function. It was shown that AUF1 movement from cytoplasm to nucleus correlated with mammary gland differentiation. Subsequently HuR levels remained unchanged [289]. These observations suggest that AUF1 absence in cytoplasm is required to change the stability of ARE-containing genes during induction of milk production and growth arrest. It is worth to notice that in this study decreased proliferation rates were due to lesser AUF1 expression in cytoplasm and subsequently increasing levels in nucleus. As compared to our results where cytoplasmic shift of AUF1 correlated with tissue malignancy, its translocation to nucleus may have beneficial effects on thyroid carcinoma progression. We demonstrated that normal thyroid tissue lacks cytoplasmic AUF1, which is accumulated in nucleus. This situation changes as thyroid tissue differentiates towards malignant phenotype and ratio between AUF1 expression in nucleus and cytoplasm decrease with tissue malignancy. More importantly, analysis of cellular AUF1 expression in thyroid tissues allowed us to discriminate between follicular adenoma and FTC tissues. We found that logarithmic nuclear/cytoplasmic ratio of AUF1 expression in goiter, adenoma and FTC tissues was significantly lower, when comparing to normal thyroid tissues and decreased with tissue malignancy.

Most of investigated thyroid nodules are considered to be benign and the best indication for surgical intervention is to exclude the diagnosis of carcinoma. Fine needle aspiration (FNA) followed by cytological examination is currently the best initial diagnostic method for examination of thyroid nodules. It provides useful information; however, it can not discriminate between benign and malignant follicular thyroid tumors. Patients with diagnosed follicular thyroid disease are often advised to undergo surgical treatment to provide more reliable diagnostic information and further suitable additional treatment. The presence of carcinoma is an indication for second operation, usually complete thyroidectomy. In end effect, inadequate clinical information make patients to decide preoperatively whether undergo thyroid lobectomy or total thyroidectomy [290]. These observations indicate that there is a clear need to develop more suitable initial diagnostic tests and biomarkers allowing differentiation of benign and malignant thyroid tumors. We demonstrated that cellular localisation of AUF1 is a useful predictive and diagnostic indicator for thyroid nodules.

Its increased cytoplasmic expression directly correlated with tissue malignancy. What is more important, we found that AUF1 is an accurate biomarker to discriminate between goiter, follicular adenoma and FTC. Similar studies were also performed in other research groups, demonstrating diagnostic values of several other than AUF1 biomarkers. These studies utilized a combination of parallel expression of several biomarkers or investigated gene

expression patterns by employing microarrays. It was demonstrated that galectin-3 and matrix metalloproteinase inducer EMMPRIN were significantly increased in FTC lesions as compared with follicular adenomas. Out of five proteins tested these two revealed the highest sensitivity and specifity [291]. In similar studies, HMGA2 (high mobility group A2) protein has been shown to be significantly over-expressed in most of follicular carcinomas [292]. Other studies revealed that heat shock protein gp96, protein disulfide isomerase A3 and calreticulin, which were under-abundant in FTC, had the best predictive values [293]. Also several other biomarkers like S100A6, integral membrane protein 1 (ITM1) and autotaxin were found to be useful in diagnosis of suspicious thyroid nodules [294-297].

5 Perspectives

We identified AUF1 as a novel player in thyroid carcinogenesis that may affect the complex network of tumor suppressors and tumor promoters. Whatmore by employing subcellular fractionation and immunohistochmistry on thyroid tissues we found that cytoplasmic expression of AUF1 increases with tissues malignancy. Furthermore, statistical analysis of logarithmic nuclear/cytoplasmic ratio of total AUF1 expression in normal, goiter, adenoma and follicular thyroid carcinoma decreased with tissue malignancy. Investigations *in vitro* revealed that total AUF1 knock-down had the most growth-suppressive effects on thyroid carcinoma cells.

Hence, the expression of AUF1 either as standalone parameter or combined with previously described, is a promising biomarker to improve preoperative decisions and diagnosis, and therapies of thyroid nodules. Fine needle aspiration of suspicious thyroid nodules and subsequent immunostaining of patient's material with AUF1 antibody, could be a supportive technique to discriminate between normal, benign and carcinogenic thyroid tissues. The next question raised in this study concerns the detection of AUF1 in patient's serum and whether AUF1 levels may serve as discriminative parameter between normal and cancer patients. There is also a clear need to establish specific antibodies rose against single AUF1 isoforms and especially therapeutic agents inhibiting isoform p37AUF1 found to have the most tumor-promoting effects. Inhibition of AUF1 exerts beneficial effects on thyroid carcinoma progression; however in the future its role should be investigated in other kinds of cancer.

6 Summary

In this study we demonstrated that AUF1 is a novel player in thyroid carcinoma progression. For the first time we identified AUF1 as a post-transcriptional regulator affecting mRNAs of proteins related to cell-cycle and cell proliferation, and crucial for thyroid carcinoma.

We found that most thyroid carcinoma cell lines and tissues express AUF1, however with differential isoform distribution. In thyroid carcinoma cell lines AUF1 was mostly detectable in

nucleus, however, after induction of proliferation and in dividing cells, its increased production was additionally observed in cytoplasm. We detected here AUF1 in complexes with ARE-bearing mRNAs, encoding proteins involved in malignant transformation, also thyroid tumorigenesis. Total or exon-selective knock-down of AUF1 led to growth inhibition accompanied by induction of cell cycle inhibitors and reduced levels of cell cycle promoters. Decrease in AUF1 production as a response to retinoic acid or AUF1-siRNA treatment correlated with down-regulation of glycolytic ENO1 and proliferation-promoting c-Myc, and reduced invasive potential of thyroid carcinoma cells.

Investigations on thyroid tissues revealed that cytoplasmic expression of AUF1 in malignant tissues was increased when compared to those in normal and benign thyroid tissues. By subcellular fractionation of thyroid tissues and immunohistochemistry we could show that cytoplasmic expression of AUF1 in benign and malignant tissues was significantly increased compared to normal thyroid tissues. Moreover, the logarithmic nuclear/cytoplasmic ratio of total AUF1 expression in normal, goiter, adenoma and follicular thyroid carcinoma decreased with tissue malignancy.

We demonstrated AUF1 as one of the important players in complicated cell-cycle machinery and progression of thyroid carcinoma. This complex network between cytoplasmic mRNAs and cell behaviour, proliferation and transformation may be partially controlled by AUF1. Although we can not exclude participation of other factors, AUF1 may control the balance between stabilizing and destabilizing effects which both are exerted on thyroid cells. Malignant transformation and especially thyroid carcinoma may recruit cytoplasmic AUF1 to disturb the stability of mRNAs encoding cyclin dependent kinase inhibitors, leading to uncontrolled growth and progression of tumor cells. Thus, AUF1 may be considered as a new, additional marker for thyroid carcinoma.

7 Literature

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8 Thesis

1. AUF1/heterogeneous nuclear ribonucleoprotein D (hnRNPD) is an adenylate uridylate-rich elements (ARE) binding protein, which regulates the mRNA stability of many genes related to growth regulation, such as proto-oncogenes, growth factors, cytokines and cell cycle regulatory genes.

2. Studies on animal model demonstrated expression of AUF1 in kidneys, liver, lymphoid tissues and melanocytes, and its involvement in apoptosis, tumorigenesis and development.

3. AUF1 was detectable in all benign and malignant thyroid tissues investigated. In malignant thyroid tissues the cytosolic AUF1 levels were higher than those in the nucleus.

4. Logarithmic nuclear/cytoplasmic ratio of AUF1 expression in normal, goiter, adenoma and follicular thyroid carcinoma tissues decreased concordant to reduced differentiation of the tissues. Decreased levels of nuclear AUF1 correlated with malignancy in thyroid tumors.

5. Elevated levels of cytoplasmic AUF1 correlated with increased proliferation of thyroid carcinoma cells FTC-133, BC-PAP and 85050C. The highest cytoplasmic AUF1 levels were observed during cell division.

6. AUF1 binds ARE-bearing mRNAs of cyclins and cyclin-dependent kinase inhibitors related to thyroid carcinoma progression.

7. Knock-down of AUF1 resulted in significantly decreased proliferation rates and elevated levels of cyclin-dependent kinase inhibitors and retinoblastoma protein. Isoform AUF1p37 exerted the strongest effects on proliferation of thyroid carcinoma cells.

8. Thyroid carcinoma cells with siRNA-reduced AUF1 levels revealed induction of tumor suppressors and decreased levels of tumor promoters.

9. Employment of differentiation and therapeutical agent retinoic acid led to decreased levels of AUF1 accompanied by significantly reduced invasivity of FTC-133 cells. This correlated with reduction of glycolytic enzym ENO1 and oncogene c-Myc.

10. AUF1 may control the balance between stabilizing and destabilizing effects both of which are exerted on proliferation and/or cell cycle machinery. Thyroid carcinoma may recruit cytoplasmic AUF1 to disturb the stability of mRNAs encoding cyclin dependent kinase inhibitors, leading to uncontrolled growth and progression of tumor cells.

11. AUF1 may be considered as a new, additional marker for thyroid carcinoma.

Curriculum Vitae

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Academic Background

1996-2001	Study of biotechnology at the University of Life Sciences Poznan (Poland) finished with degree of Magister Engineer of Biotechnology (5 years)
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Work Experience

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Membership in Scientific Organisations

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2003	MALDI ToF and QToF mass spectrometry certificates Experiments on laboratory animals: course and certificate

Statement of authorship

I declare that this doctoral thesis has been composed by myself, and describes my own work, unless otherwise acknowledged in the text. All references and verbatim extracts have been quoted, and all sources of information have been specifically acknowledged. This work has not been submitted for any other degree.

Bogusz Trojanowicz

Publications and scientific activity

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