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Elucidating the interactions of interleukin-1α with components of the eukaryotic transcription machinery

Ph.D. thesis

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Prague 2013

I declare that I worked on this thesis on my own and that I used only the sources mentioned in the References section. This thesis, nor its substantial part, has not been submitted elsewhere with the aim to obtain the same or another academic degree.

Prague, May 2013 ....

ACKNOWLEDGEMENTS

This thesis is the result of several years of my work in the Laboratory of RNA Biochemistry at the Department of Genetics and Microbiology, Charles University in Prague. Certain results have been achieved in the Childhood Leukaemia Investigation Prague (CLIP) laboratory at the 2nd Faculty of Medicine, Charles University in Prague. However, it would not be possible to accomplish this thesis without the aid of people who supported me, helped and encouraged me to explore the fascinating world of science. Therefore I would like to express my sincere thanks to following people:

Martin Pospíšek, head of the Laboratory of RNA Biochemistry and my advisor, for his patience and support over the years, as well as for encouragement even in hopeless situations. You have always been willing to help all your students, suggesting new hypotheses and ideas. Your excellent scientific approach has been very motivating for me and it was a pleasure to work in your lab.

Ladislav Burýšek and Mirka Burýšková for the possibility to work in their laboratory at the University of Ulm in 2004/2005 and for introduction to the mammalian cell culture techniques and virus infection. Many thanks for your friendly support, enthusiasm and inspirational discussions over my results.

Many thanks to Jitka Forstová, head of the Laboratory of virology at the Department of Genetics and Microbiology, for giving me the chance to work in her lab where I performed experiments on mammalian cells at the time when there was no tissue culture facility in our laboratory.

I would also like to acknowledge Zora Mělková from the Institute of Immunology and Microbiology, 1st Medical Faculty, Charles University in Prague, for enabling me to perform certain experiments involving infection with vesicular stomatitis virus.

I am grateful to Jan Trka for hiring me as a research assistant in Childhood Leukaemia Investigation Prague (CLIP) in 2007 to work on the project studying *HOX* gene expression in acute lymphoblastic leukaemia. During my experimental work there I was introduced to the quantitative real-time PCR technique and learned many interesting things about leukaemia investigation and treatment.

Many thanks to Jan Černý from the Department of Cell Biology for the help with fluorescence microscopy experiments and Tomáš Kalina from the CLIP group for the introduction to flow cytometry and subsequent data analysis with FlowJo.

My special thanks to my collaborators Vaclav Vopálenský, Tomáš Mašek and Zuzana Feketová for much advice and many inspirational conversations regarding my experiments. Furthermore I would like to thank Josef Novák for the excellent collaboration and enormous commitment to the IL-1 project, Ondřej Šebesta for the aid with fluorescence microscopy techniques, Kateřina Mocová, Silvia Mrvová, Klára Kazdová and Denisa Dolečková for their help with various experiments and our technicians Vlasta Pelechová and Petra Studničková for their technical help. Thanks also to all the other lab members that have also been very supportive. I greatly appreciate the possibility to work in this team.

Last but not least, I would like to thank my parents for all their love and encouragement.

This work was supported by by Ministry of Health of the Czech Republic (grant #NS9819-4/2008)

ABSTRACT

Interleukin-1α (IL-1α) is a pleiotropic cytokine and a key mediator of host immune response. It is synthesised as a 31-kDa precursor, that is cleaved by the cysteine protease calpain into the 17-kDa mature IL-1α and the 16-kDa N-terminal peptide of IL-1α (IL-1αNTP). Although IL-1α can be secreted, act on target cells through the surface receptor IL-1RI and trigger the signal transduction pathway, increasing evidence points toward the involvement of IL-1α in certain nuclear processes. IL-1αNTP is highly conserved among higher eukaryotes and contains a nuclear localisation sequence; indeed, both the precursor and IL-1αNTP are found in the cell nucleus. Previously, a genetic interaction of IL-1α with nuclear histone acetyltransferase (HAT) complexes has been reported from mammalian cells and, interestingly, also from the heterologous yeast model.

This thesis extends the research of the nuclear function of IL-1α and demonstrates that IL-1α physically associates with the HAT/Core module of yeast SAGA and ADA HAT complexes. Results of the HAT subunit gene knock-out experiments followed by a set of co-immunoprecipitations also suggest a novel model of the yeast SAGA complex assembly, in which ADA appears to represent only a partly functional HAT complex.

In its natural milieu of mammalian cells, IL-1α is demonstrated to co-localise with the tumour suppressor protein p53 within the cell nucleus. This interaction is further supported with a co-immunoprecipitation experiment where the IL-1α precursor binds p53. Moreover, it is shown that the subcellular localisation of IL-1αNTP can be modulated under different culturing conditions.

Finally, this thesis presents an analysis of *HOX* gene expression in immunophenotypically and genotypically defined subsets of paediatric patients with acute lymphoblastic leukaemia (ALL). The expression of 23 selected *HOX* genes in 61 ALL patients is studied and the results are compared with the levels of *HOX* gene transcription in sorted cell populations of B and T lymphocytes from healthy donors. Aberrant *HOX* gene expression patterns have been identified in the leukaemic cells compared to their closest physiological counterparts

Keywords: interleukin-1; interleukin-1α; histone acetyltransferase; SAGA; ADA; *HOX* genes; acute lymphoblastic leukaemia; heterologous expression; transcription

ABSTRAKT

Interleukin-1α (IL-1α) je pleiotropní cytokin, který hraje klíčovou roli v imunitní odpovědi organismu. Je produkován jako prekursor o molekulové hmotnosti 31 kDa, který je štěpen cysteinovou proteázou calpainem za vzniku maturovaného IL-1α (17 kDa) a tzv. N-terminálního peptidu IL-1α (IL-1αNTP; 16 kDa). Ačkoliv IL-1α může být sekretován a spouštět dráhu signální transdukce skrze povrchové receptory cílových buněk, bylo zjištěno, že IL-1α se účastní také některých procesů v buněčném jádře. IL-1αNTP je u vyšších eukaryot vysoce konzervován a obsahuje jaderný lokalizační signál, díky kterému prekursor i samotný IL-1αNTP vstupují do buněčného jádra. Při předchozím výzkumu byla popsána genetická interakce IL-1α s jadernými histonacetyltransferázovými (HAT) komplexy v savčích buňkách a překvapivě také v heterologním kvasinkovém modelu.

Tato disertační práce se dále věnuje výzkumu jaderné funkce IL-1α a přináší důkazy o fyzické interakci IL-1α s „HAT/Core“ modulem kvasinkových histonacetyltransferáz SAGA a ADA. Výsledky pokusů zahrnujících knock-out jednotlivých podjednotek kvasinkových HAT komplexů a následné koimunoprecipitace poukazují na nový model skládání SAGA komplexu, ve kterém ADA představuje pravděpodobně jen částečně aktivní HAT komplex.

V savčích buňkách jakožto přirozeném modelu pro studium IL-1α pak byla prokázána kolokalizace tohoto cytokinu s tumorsupresorovým proteinem p53. Tuto interakci potvrzuje také následná koimunoprecipitace obou proteinů. Také bylo zjištěno, že subcelulární lokalizaci IL-1αNTP lze modulovat pomocí rozdílných kultivačních podmínek.

Poslední část této práce popisuje analýzu exprese *HOX* genů u imunofenotypově a genotypově definovaných podskupin pacientů s dětskou akutní lymfoblastickou leukemií (ALL). Exprese 23 vybraných *HOX* genů byla studována u 61 pacientů a výsledky byly porovnány s úrovní transkripce *HOX* genů v populacích B a T lymfocytů získaných od zdravých dárců. Bylo zjištěno, že v leukemických buňkách dochází k aberantní expresi *HOX* genů v porovnání se zdravými buňkami.

Klíčová slova: interleukin-1; histonacetyltransferáza; SAGA; ADA; HOX geny; akutní lymfoblastická leukemie; heterologní exprese; transkripce

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1 ABBREVIATIONS

aa	amino acid
AIDS	acquired immune deficiency syndrome
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
AMPK	AMP-activated protein kinase
ARTS-1	aminopeptidase regulator of tumour necrosis factor receptor 1 shedding
ATP	adenosine triphosphate
BLPs	B-lymphocyte progenitors
bp	base pair
CBP	CREB binding protein *but also* calmodulin-binding protein
CREB	cAMP-response element binding protein
cDNA	complementary DNA; reverse transcribed mRNA
CDK	cyclin-dependent kinase
cDNA	complementary DNA
Ct	cycles to threshold
ctrl	control
Da	dalton
DAPI	4', 6-diamidino-2-phenylindole
ddH_2O	deionised distilled water
DMEM	Dulbecco´s modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EtBr	ethidium bromide
FAB	French-American-British
FCAS	familial cold autoinflammatory syndrome
FCS	fetal calf serum
FGF	fibroblast growth factor
FIL1	family of interleukin 1
FRET	Förster resonance energy transfer
Gal4BD	DNA binding domain of Gal4
gDNA	genomic DNA
GFP	green fluorescent protein
GNAT	Gcn5-related N-acetyltransferase
GST	glutathion S-transferase
HA	haemagglutinin
HAT	histone acetyltransferase
HDAC	histone deacetylase
HIF	hypoxia-induced factor
HMGB	high-mobility group box
HRP	horseradish peroxidase
HSP	heat shock protein

IAP	inhibitor of apoptosis
IL	interleukin
IL-1αFull	precursor of interleukin-1α
IL-1αMat	mature interleukin-1α
IL-1αNTP	N-terminal part of interleukin-1α
IL-18BP	IL-18 binding protein
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
IMPs	immature myeloid progenitors
INL	IL-1αNTP-like
IP	immunoprecipitation
IRAK	IL-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
KAT	lysine or K-acetyltransferase
kDa	kilodalton; protein molecular weight unit
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MCP-1	monocyte chemotactic protein-1
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
MKK	mitogen-activated protein kinase kinase
MIP-2	macrophage inflammatory protein 2
MOZ	monocytic leukaemia zinc finger protein
MWS	Muckle-Wells syndrome
MYST	MOZ, Ybf2/Sas3, Sas2, Tip60
NLS	nuclear localisation sequence
NTP	N-terminal part
OD	optical density
PBS	phosphate-buffered saline
PCAF	p300/CREB binding protein associated factor
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PGR	predisone good responders
PMSF	phenylmethylsulphonyl fluoride
PPR	prednisone poor responders
pre-Bs	B-lymphocyte precursors
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SALSA	SAGA altered, Spt8 absent
SD	synthetic drop-out
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIGIRR	single immunoglobulin IL-1R-related molecule
SLB	sample loading buffer
SLIK	SAGA-like
ssDNA	single strand deoxyribonucleic acid
TAE	Tris-acetate-EDTA

Taf	TATA-binding protein-associated factor
TAK	transforming growth factor-β-activated kinase
TBP	TATA-binding protein
TBS	Tris-buffered saline
TEMED	tetramethylethylenediamine
TFTC	TATA-binding protein-free TAFII complex
TIFA	TRAF-interacting protein with a forkhead-associated domain
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TPI	triose-phosphate isomerase
US FDA	United States Food and Drug Administration
UTR	untranslated region
UV	ultraviolet
yGFP	yeast-enhanced green fluorescent protein

2 INTRODUCTION

Although the nucleus occupies roughly only about 10% of the eukaryotic cell volume, it is an organelle of key importance to the cellular life. This conspicuous, highly-organised cellular compartment is the site of a multitude of complex biochemical processes including DNA replication and repair, gene transcription or RNA processing. Various nuclear proteins are selectively transported into the nucleus through nuclear pores, however, until 1993, nobody knew that this also is the case of the proinflammatory cytokine interleukin-1α (IL-1α). It was the year when a nuclear localisation signal was found within the IL-1α amino acid sequence and shortly, a hunt for discovery of the role of IL-1α in the cell nucleus began.

Twenty years later, there are still many unresolved issues regarding the nuclear function of IL-1α. Certain studies reported the association of IL-1α with a nuclear protein necdin, others demonstrated that nuclear IL-1α regulates cell motility or development of sterile inflammation. Our laboratory significantly contributed to the research of nuclear IL-1α with the discovery of the interaction of IL-1α with transcriptional apparatus through nuclear histone acetyltranserase complexes. These results were obtained using heterologous IL-1α production in the yeast model and yeast thus proved to be an excellent tool for the investigation of this pleiotropic human cytokine.

The aim of this thesis was to further investigate the physical association of nuclear IL-1α with yeast histone acetyltransferase complexes SAGA and ADA and to specify the protein or module binding directly to IL-1α in the yeast cells. Moreover, I intended to study IL-1α in its natural milieu, i.e. in mammalian cells, with an emphasis on the putative interaction of IL-1α with the tumour suppressor p53. Besides the IL-1α project, carried out in the Laboratory of RNA biochemistry at the Faculty of Science, Charles University in Prague, I was involved in an analysis of expression of selected *HOX* genes in patients with acute lymphoblastic leukaemia. The results in this part of my thesis were obtained during my work in the laboratory of the Childhood Leukaemia Investigation Prague group at 2nd Faculty of Medicine, Charles University in Prague.

3 OBJECTIVES

The objectives of this Ph.D. thesis are:

PART I

- to study the subcellular localisation of the IL-1α precursor, mature IL-1α and IL-1αNTP in their natural milieu of mammalian cells
- to investigate the putative interaction of the IL-1α precursor with the tumour suppressor protein p53
- to study the subcellular localisation of IL-1αNTP and the possibility of its modulation using certain agents or culturing conditions

PART II

- to study the expression of the IL-1α precursor in the heterologous yeast model
- to investigate the subcellular localisation of the IL-1α precursor and mature IL-1α in yeast cells
- to elucidate the interactions of the IL-1α precursor with yeast histone acetyltransferase complexes SAGA and ADA using gene knock-out and subsequent co-immunoprecipitation using the IL-1α precursor as a bait

PART III

- to optimise qRT-PCR conditions of 23 *HOX* gene systems using various leukaemic cell lines
- to analyze the expression of selected *HOX* genes in 61 paediatric patients with acute lymphoblastic leukaemia
- to determine the expression of selected *HOX* genes in sorted cell populations of B and T lymphocytes from healthy donors and to compare the results with the data obtained from leukaemic patients

4 LITERATURE REVIEW

4.1 Interleukin-1

Interleukin-1 (IL-1) has been studied extensively since its discovery in 1972 and in the course of time, it has been shown to represent a multifunctional molecule with a wide range of distinct biological effects. Nowadays, IL-1 is generally recognised to be a potent proinflammatory cytokine promoting inflammation during infection, injury or stress; however, its activities are not restricted only to the immune system. IL-1 is also involved in the regulation of blood calcium levels (Boyce *et al.* 1989; Nguyen *et al.* 1991), stimulation of proliferation of various cells (Giulian and Lachman 1985; Lacey *et al.* 1987; Stepien *et al.* 1994; Tesch *et al.* 1997; Niki *et al.* 2004), promotion of angiogenesis (Salven *et al.* 2002; Carmi *et al.* 2009), regulation of blood pressure (Takahashi *et al.* 1992; Kimura *et al.* 1993) or regulation of central nervous system functions (Krueger and Obal 1993; Takahashi *et al.* 1997). In spite of its name referring to leukocytes, IL-1 affects nearly every tissue and organs and the dysregulation of IL-1 has been proved to underlie many human diseases and pathologies.

4.1.1 IL-1: a key proinflammatory mediator

IL-1 represents one of the most important mediators of the inflammatory response that induces a cascade of proinflammatory effector molecules. As a highly potent initiator of inflammation, IL-1 is the key player in orchestrating host defence responses. It is produced in response to local or systemic injury or infection and has wide-ranging effects on expression of a significant number of genes including cytokines (IL-1, IL-6, TNF), chemokines (IL-8, MCP-1), growth factors (FGF), acute phase proteins (C-reactive protein, complement factors) and other proinflammatory mediators (cyclooxygenase, inducible nitric oxide synthase) (Warner *et al.* 1987; Sironi *et al.* 1989; Ikejima *et al.* 1990; Kunz *et al.* 1994; Zheng *et al.* 1995; Dinarello 1996; Cronauer *et al.* 1999; Okuno *et al.* 2002). The systemic effects of IL-1 include fever, hypotension and neutrophilia; locally, IL-1 promotes infiltration of circulating

lymphocytes to the inflammed tissue, induces tissue remodelling and enhances wound healing (Goldring and Krane 1987; Postlethwaite *et al.* 1988; Sauder *et al.* 1990; Kubota *et al.* 2002; Sato *et al.* 2005). IL-1 also stimulates and activates T and B lymphocytes, NK cells and macrophages (reviewed in (Dinarello 1988) and (Dinarello 1996)).

The production of IL-1 is stimulated by microbial products such as LPS or flagellin (Wyant *et al.* 1999), proinflammatory cytokines such as IL-1 (Elias *et al.* 1989) or IL-18 (Puren *et al.* 1998), other inflammatory substances and acute phase proteins like C-reactive protein (Tilg *et al.* 1993), complement factors (Haeffner-Cavaillon *et al.* 1987) or urate crystals (Di Giovine *et al.* 1987).

The major interleukin-1 producing cells are macrophages, but many other cells, e.g. neutrophils, lymphocytes, dendritic cells, keratinocytes, endothelial cells, hepatocytes, fibroblasts or muscle cells, have been shown to synthesise IL-1. Main target cells of IL-1 actions are primarily cells of the immune system such as monocytes, lymphocytes, granulocytes, dendritic cells, but this cytokine can affect many other cells like epithelial cells, fibroblasts, endothelial cells, liver cells or smooth muscle cells (Zucali *et al.* 1986; Kupper *et al.* 1988; McMahon *et al.* 1997; Beasley and Cooper 1999; Kojima *et al.* 2009; Kojima *et al.* 2011).

Although the proinflammatory activities of IL-1 contribute to pathogen clearance and eradication of infection, excessive IL-1 expression can result in tissue damage and development of various pathological processes. It is now generally accepted that IL-1 plays a role in development of rheumatoid arthritis, spondyloarthritis (Monnet *et al.* 2012), Alzheimer disease (Nicoll *et al.* 2000; Rainero *et al.* 2004) and associated vascular dementia (Yucesoy *et al.* 2006), diabetes mellitus (Bendtzen *et al.* 1986; Mandrup-Poulsen *et al.* 2010), psoriasis (Mee *et al.* 2006), periodontitis (Lopez *et al.* 2005), systemic sclerosis (Kawaguchi 1994; Kawaguchi *et al.* 2006), autoimmune encephalomyelitis (Sutton *et al.* 2006) and cerebral infarction (Um *et al.* 2005). Increasing evidence also points towards involvement of IL-1 in a wide range of cardiovascular diseases (reviewed in (Vicenova *et al.* 2009)).

4.1.2 The two IL-1 forms and the IL-1 family

Interleukin-1 was first described in 1972 as a lymphocyte-activating factor (Gery and Waksman 1972), although the same molecule was isolated already in 1940 and named endogenous pyrogen. It was not until 1985 that scientists discovered that there are actually two forms of IL-1 (March *et al.* 1985). Interleukin-1 is a term for two distinct but related proteins, interleukin-1α (IL-1α) and interleukin-1β (IL-1β), encoded by two separate genes. The tertiary structure of both molecules is similar, a β-barrel composed from 12 antiparallel β-strands (Priestle *et al.* 1988; Finzel *et al.* 1989; Veerapandian 1992) (Figure 1). IL-1α, IL-1β and other proteins with structural and evolutionary relationship to IL-1 are classified into the so-called IL-1 family.

The original members of the IL-1 family are IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). Nowadays, the IL-1 family has diverged into a group of fascinating pleiotropic molecules and comprises 11 members with both proinflammatory and anti-inflammatory activities (Table 1). Except for IL-18 and IL-33, all of the IL-1 family member genes are located on the long arm of chromosome 2. According to the intron-exon organisation, it is thought that most IL-1 family members emerged as a result of a duplication of an ancestral gene (Eisenberg *et al.* 1990). In the case of the IL-1F5 IL-1 family member, duplication of the IL-1Ra gene has presumably taken place (Mulero *et al.* 1999).

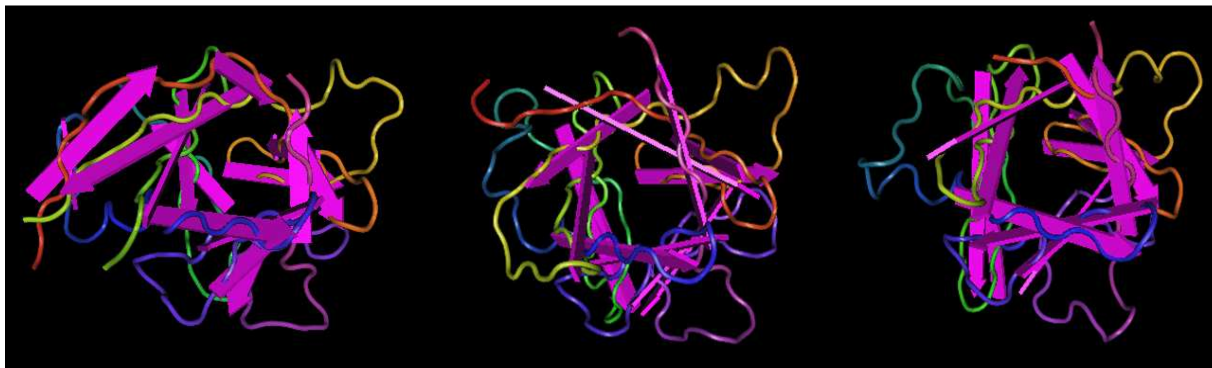


Figure1: Crystal structure of IL-1α (left), IL-1β (middle) and IL-1Ra (right). These three molecules share a similar tertiary structure forming a β-barrel composed from 12 antiparallel β-strands (Priestle *et al.* 1989; Graves *et al.* 1990; Vigers *et al.* 1994).
Source: MMDB database, http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml (Chen *et al.* 2003); MMDB ID 58059 (IL-1α), 58048 (IL-1β) and 1337 (IL-1Ra). Edited with Cn3D 4.1 (Wang *et al.* 2000).

IL-18, structurally related to IL-1 (Kato *et al.* 2003), binds the IL-18 receptor and this event results in NF-κB activation (Matsumoto *et al.* 1997; Torigoe *et al.* 1997). IL-18 exhibits potent immunomodulatory properties. Formerly known as the IFN-γ-inducing factor, it induces IFN-γ (Nakamura *et al.* 1989) and is crucial for pathogen and virus clearance (Fujioka *et al.* 1999; Sugawara *et al.* 1999; Wei *et al.* 1999; Kawakami *et al.* 2000; Mori *et al.* 2001). However, due to its proinflammatory properties, IL-18 has been found to be implicated in autoimmune diseases such as rheumatoid arthritis (Gracie *et al.* 1999; Yamamura *et al.* 2001) or psoriasis (Gracie *et al.* 2003). The natural inhibitor neutralising IL-18 activities is the 40-kDa IL-18-binding protein (IL-18BP). Given that IL-18BP is induced by IFN-γ in various cells (Muhl *et al.* 2000; Paulukat *et al.* 2001), it is suggested that IL-18BP serves as a negative feedback inhibitor of the IL-18-mediated immune response. Recombinant IL-18-binding protein has been therapeutically administered to the patients with rheumatoid arthritis and psoriasis and has been shown to be safe for clinical application (Tak *et al.* 2006).

IL-1F5 and IL-1F7 (named also IL-37) probably act as anti-inflammatory cytokines and especially the latter recently emerged as a key anti-inflammatory molecule (Nold *et al.* 2010). IL-1F5 has been shown to attenuate IL-1 and LPS-induced inflammatory responses in a IL-4-dependent manner (Costelloe *et al.* 2008). While IL-1F5 anti-inflammatory activities are mediated through activation of receptor SIGIRR (Costelloe *et al.* 2008), IL-37 interacts with the IL-18 receptor (Kumar *et al.* 2002), can bind to IL-18BP and enhance the ability of IL-18BP to neutralise IL-18-induced IFN-γ (Bufler *et al.* 2002). In RAW macrophages overexpressing IL-37, levels of proinflammatory cytokines such as IL-1α, MIP-2, TNF-α and IL-6 are significantly reduced (Sharma *et al.* 2008). In hepatocytes, IL-37 has been shown to decrease production of proinflammatory cytokines and chemokines during inflammatory liver injury (Sakai *et al.* 2012). IL-37 was also able to reduce the expression of colonic TNFα and IL-1β and to protect mice from experimental colitis (McNamee *et al.* 2011).

IL-1F6, IL-1F8 and IL-1F9 are proinflammatory cytokines signaling through the IL-1R-related protein 2 (IL-1Rrp2) and IL-1 receptor accessory protein that leads to the activation of NFκB (Debets *et al.* 2001; Towne *et al.* 2004). All three cytokines have been demonstrated to increase the production of proinflammatory mediators IL-6 and IL-8 (Towne *et al.* 2004; Magne *et al.* 2006) and to contribute to the pathogenesis of psoriasis (Johnston *et al.* 2011)

IL-33, being the most recently added IL-1 family member, represents a molecule that may ressemble to IL-1α in some aspects. IL-33 has been shown to bind the ST2 receptor and to activate the MAP kinase pathway resulting in phosphorylation of NF-κB (Schmitz *et al.* 2005). However, this molecule can also be found in the cell nucleus associated with heterochromatin and mitotic chromosomes and it exhibits potent transcriptional repressor properties (Carriere *et al.* 2007). IL-33 promotes the Th2 response and induces the Th2 -associated cytokines such as IL-5 and IL-13 and treatment of mice with IL-33 induces splenomegaly, eosinophilia and raises serum IgE levels (Schmitz *et al.* 2005). This may be the rationale for the involvement of IL-33 in the development of allergy (Ohno *et al.* 2012; Nakae *et al.* 2013). The processing of IL-33 has not been fully clarified yet since the IL-33 precursor is cleaved by caspase-1 *in vitro* (Schmitz *et al.* 2005); however, *in vivo*, proteolytic cleavage of the IL-33 precursor seems to be catalyzed by calpain (Hayakawa *et al.* 2009). Cleavage of IL-33 by caspase-3 inactivates its cytokine function while, interestingly, the translocation of IL-33 into the nucleus is not abrogated (Ali *et al.* 2009).

The most studied IL-1 family members, i.e. IL-1α, IL-1β and IL-1Ra as well as the IL-1 receptors, will be discussed in separate chapters.

name	synonyme	receptor	action
IL-1F1	IL-1α	IL-1RI, IL-1RII	proinflammatory
IL-1F2	IL-1β	IL-1RI, IL-1RII	proinflammatory
IL-1F3	IL-1Ra	IL-1RI, IL-1RII	antagonist
IL-1F4	IL-18	IL-18R	proinflammatory
IL-1F5	FIL1δ, IL-1Hy1, IL-1H3, IL-36Ra, IL-1RP3	IL-1Rrp2, SIGIRR	anti-inflammatory
IL-1F6	FIL1ε, IL-36α	IL-1Rrp2	proinflammatory
IL-1F7	FIL1ξ, IL-1H4, IL-37, IL-1RP1	IL-18R	anti-inflammatory
IL-1F8	FIL1η, IL-1H2, IL-36β	IL-1Rrp2	proinflammatory
IL-1F9	IL-1H1, IL-36γ, IL-1RP2	IL-1Rrp2	proinflammatory
IL-1F10	IL-1Hy2	IL-1RI	antagonist?
IL-1F11	IL-33	T1/ST2	Th2 response

Table 1: Current members of the IL-1 family (adapted from (Busfield *et al.* 2000; Carmi *et al.* 2009; Dinarello 2009; Dinarello *et al.* 2010; Boraschi *et al.* 2011) and other sources).

4.1.3 IL-1 receptors and their function

There are two types of receptors binding both IL-1α and IL-1β. The IL-1 signaling pathway is triggered by the 80-kDa IL-1 receptor type I (IL-1RI) that possesses a characteristic cytosolic Toll-IL-1R (TIR) domain and three extracellular immunoglobulin-like domains (Ig domains). The cytosolic domain itself doesn´t exhibit the kinase activity (Sims *et al.* 1989), the signaling is activated after the binding of either IL-1α or IL-1β. IL-1 receptor accesory protein (IL-1RAcP), an IL-1RI homologue that has however no affinity for IL-1α or IL-1β, represents a coreceptor crucial for the IL-1RI signaling (Greenfeder *et al.* 1995; Korherr *et al.* 1997; Wesche *et al.* 1997; Cullinan *et al.* 1998). Recently, TILRR, a novel IL-1RI co-receptor, has been shown to potentiate IL-1RI activities via conformational changes of IL-1RI and adapter protein recruitment (Zhang *et al.* 2010; Zhang *et al.* 2012).

In contrast to IL-1R type I, the 60-kDa IL-1R type II (IL-1RII) functions as a decoy receptor that is unable to trigger the signal transduction pathway (Colotta *et al.* 1993), because its short (29 amino acids) cytoplasmic domain lacks the Toll-IL-1R domain (McMahan *et al.* 1991). IL-1RII is able to form a complex with IL-1 and therefore it acts as a negative regulator of the IL-1 signaling. However, IL-1RII also binds IL-1RAcP (Lang *et al.* 1998) and makes it unavailable for functional IL-1 receptors. This represents another way to downregulate the IL-1 signaling that cannot be overcome by excess of extracellular ligand.

Furthermore, proteolytic cleavage of IL-1RII by aminopeptidase ARTS-1 (aminopeptidase regulator of tumour necrosis factor receptor 1 (TNFR1) shedding) generates 45-47-kDa soluble IL-1RII (sIL-1RII) that binds and neutralises IL-1 (Cui *et al.* 2003b). Interestingly, in addition to TNFR1 and IL-1RII, ARTS-1 also mediates shedding of the IL-6 receptor (Cui *et al.* 2003a) and may therefore contribute to the attenuation of the inflammatory response. In addition to the extracellular IL-1RII, a recent study reported that intracellular IL-1RII is able to bind the IL-1α precursor and to inhibit its proinflammatory activity in necrotic cells (Zheng *et al.* 2013).

The importance of IL-1RII and its negative regulatory function can be exemplified by pox viruses that acquired this receptor (gene B15R in vaccinia) (Spriggs *et al.* 1992) deletion of which affects virus virulence (Alcami and Smith 1992). Similar protein is expressed also by variola, cowpox virus and ectromelia virus

causing mousepox; however, these IL-1-binding proteins differ in several amino acid residues (Smith and Alcami 2000).

The IL-1 signaling pathway is very similar to that triggered by the Toll-like receptors (TLR) involved in the innate immunity (Figure 2). Both IL-1R and TLR contain the evolutionary conserved intracellular TIR domain that is crucial for interactions of the receptors with adaptors mediating the signal from outside. However, according to (Bahi *et al.* 2003), both TIR domain-containing receptors evolved separately.

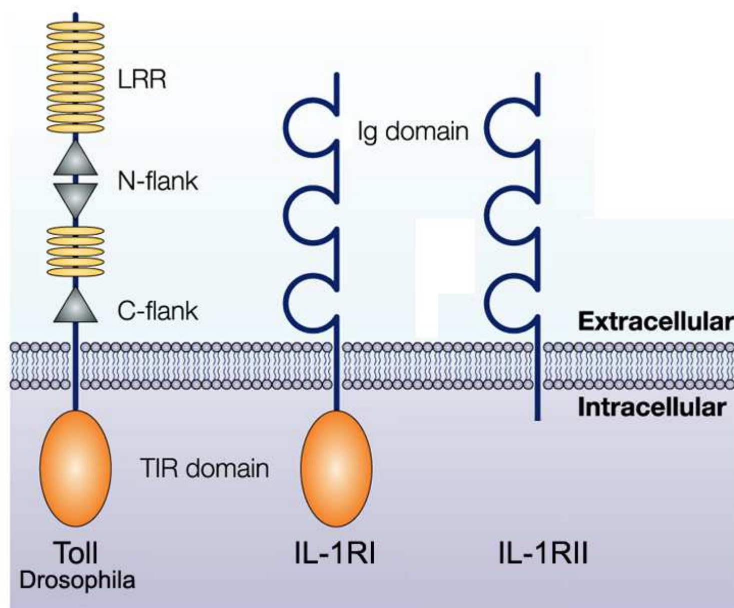


Figure 2: Structure of *Drosophila* Toll receptor and mammalian IL-1 receptors. The extracellular domain of Toll contains leucine-rich repeats (LRR) flanked by cysteine-rich motifs (known as the N- and C- flanks). The IL-1 receptors interact with IL-1 by extracellular Ig domains, but only IL-1RI shares with Toll the TIR domain that mediates the IL-1 signaling pathway. Adapted from (Lemaitre 2004).

4.1.4 Mechanism of IL-1 action and the signal transduction pathway

Extracellular interleukin-1 triggers a complex network of signaling pathways that target expression of a significant number of genes. The IL-1 signaling pathways can be affected and modulated by a variety of regulatory proteins and the abundance of different negative regulators contribute to the tight regulation of the IL-1 action in the organism (Figure 3).

Following IL-1 binding, the extracellular Ig domains of the IL-1 receptor appear to wrap around the IL-1 molecule (Vigers *et al.* 1997). IL-1RAcP, an IL-1RI homologue that has however no affinity for IL-1α or IL-1β, is recruited to the IL-1/IL-1RI receptor complex (Greenfeder *et al.* 1995) followed by adaptor MyD88 (Muzio *et al.* 1997), protein Tollip (Burns *et al.* 2000) and kinases IRAK (IL-1 receptor-associated kinase)-1 (Croston *et al.* 1995) and IRAK-4 (Li *et al.* 2002b; Lye *et al.* 2004). The binding of MyD88 to IL-1RI is enhanced by its co-receptor TILRR (Zhang *et al.* 2010). Upon IL-1 binding to the receptor, IRAK-1 becomes phosphorylated on Thr-209 and Thr-387 (supposedly by IRAK-4) and autophosphorylated within the so-called ProST region (proline-, serine-, threonine-rich) (Croston *et al.* 1995; Yamin and Miller 1997; Kollewe *et al.* 2004) and interacts with TRAF6 (tumour recrosis factor receptor-associated factor) (Cao *et al.* 1996) probably through TIFA protein (TRAF-interacting protein with a forkhead-associated domain) (Takatsuna *et al.* 2003; Ea *et al.* 2004). IRAK-1, IRAK-4 and TRAF6 then leave the IL-1 receptor. Pellino-1, a homologue of the drosophila adaptor protein Pellino, is recruited (Jiang *et al.* 2003) and thus a new intermediate complex IRAK-1-IRAK-4-TRAF6-Pellino-1 is formed. Subsequently, IRAK-4 and Pellino-1 leave the complex and TRAF6 becomes activated by polyubiquitination (Wang *et al.* 2001a). At the cytoplasmic membrane, another complex is formed that consists of IRAK-1, TRAF6, TAK1 (transforming growth factor-β-activated kinase) and two TAK1-binding proteins TAB1 and TAB2 (Ninomiya-Tsuji *et al.* 1999; Takaesu *et al.* 2001) or alternatively TAK1, TAB1 and TAB3 (Cheung *et al.* 2004). Afterwards, phosphorylated IRAK-1 is ubiquitinated and targeted to degradation in proteasomes, TAB2 becomes phosphorylated and TAB1 mediates the activation of TAK1 by autophosphorylation (Kishimoto *et al.* 2000). Phosphorylation of TAK1 and TAB2 then facilitates the translocation of this complex from the membrane to the cytosol (Jiang *et al.* 2002).

As a result of all these events, NF-κB inhibitor (IκB) becomes phosphorylated on serine residues -32 and -36 by MEKK1 kinase (Lee *et al.* 1997), covalently modified by TRAF6-mediated and Ubc13/Uev1A-dependent ubiquitination at lysine-63 (Deng *et al.* 2000) and degraded in proteasomes. NF-κB transcription factor is thus made available and enters the nucleus where it activates transcription of target genes. Alternatively, the signal triggered by IL-1 is mediated by MAP kinases and AP-1 since the TAK1 kinase is able to activate both MKK4-JNK and MKK6-p38 pathway as well (Yamaguchi *et al.* 1995).

Treatment of cells with extracellular IL-1 induce expression of a large number of transcripts, e.g. NF-κB, c-Jun, c-Fos, IL-1α and β, IL-2, IL-6, IL-8, TNF, cyclooxygenase-2, C-reactive protein, serum amyloid A, inducible nitric oxid synthase, complement proteins, hepatocyte growth factor, hypoxia-induced factor 1, heat-shock protein hsp70, plasminogen activator inhibitor type 2 and many others (Warner *et al.* 1987; Weinstein and Taylor 1987; Dalton *et al.* 1989; Curran *et al.* 1990; Ikejima *et al.* 1990; Tosato and Jones 1990; Cruz *et al.* 1991; Kumar *et al.* 1992; Munoz *et al.* 1992; Stylianou *et al.* 1992; Tamura *et al.* 1993; O'Neill 1995; Zhang *et al.* 1995; Dinarello 1996; Huang *et al.* 1998; Thornton *et al.* 2000; Jura *et al.* 2008).

Negative regulation of the IL-1 pathway can occur at multiple levels. Outside the cytoplasmic membrane, the functional concentration of IL-1 can be decreased by action of IL-1RII, an inactive receptor which lacks the TIR domain and does act as a kind of decoy target for IL-1, preventing its binding to IL-1RI. Furthermore, IL-1Ra that is unable to trigger the IL-1R-mediated pathway can competitively occupy the IL-1 receptors and therefore is able to abrogate the signaling. Inside the cell, an intracellular form of IL-1RII can associate with the IL-1α precursor and prevent its cleavage by calpain. Given that cleaved IL-1α is significantly more active than the precursor and can be secreted from the cells, this mechanism contributes to keeping the IL-1α activity under tight control (Zheng *et al.* 2013).

Two naturally occuring variants of IRAK but lacking the kinase activity, IRAK-M and IRAK-2, can also inhibit the IL-1/TLR signaling (Kobayashi *et al.* 2002; Janssens and Beyaert 2003). Regulation of the IRAK kinase activity is further provided by the mechanism of alternative splicing. IRAK-1b, an alternatively spliced version of IRAK-1, is not efficient in NF-κB activation and does not become phosphorylated and degraded in response to IL-1. This prolonged stability of IRAK-1b may lead to the replacement of IRAK-1 by IRAK-1b in the signaling complex and attenuate the IL-1 signaling pathway (Jensen and Whitehead 2001). Another splice variant of IRAK1 lacking exon 11, IRAK-1c, associates with MyD88, Tollip and TRAF6 but acts as a negative regulator by its inability to be phosphorylated by IRAK-4 and subsequently autophosphorylated (Rao *et al.* 2005). Further suggested negative regulators of the IL-1 signaling are Smad6, PP2Cβ-1 and PP2Cε. Binding of Smad6 to Pellino-1 prevents its involvement in the signaling (Choi *et al.* 2006). TAK-1-mediated signaling can be inhibited via TAK-1 dephosphorylation by PP2Cβ-1

isoform of protein phosphatase PP2C (Hanada *et al.* 2001). Another member of the PP2C family, PP2Cε, might be involved in the attenuation of TAK-1 function as well (Li *et al.* 2003).

Finally, certain studies confirmed the involvement of microRNAs in the posttranscriptional regulation of TRAF6 and IRAK-1. 3´-UTR of both RNA molecules contain multiple target sequences for miR-146 and both genes are regulated by miR-146 *in vivo* (Taganov *et al.* 2006). Furthermore, two isoforms of miR-146, miR-146a and miR-146b, are rapidly induced by IL-1β and contribute to the negative regulation of IL-1 signaling by downregulating the release of IL-1-induced chemokines RANTES and IL-8 (Perry *et al.* 2008) via NF-κB and MAP kinase pathways (Perry *et al.* 2009).

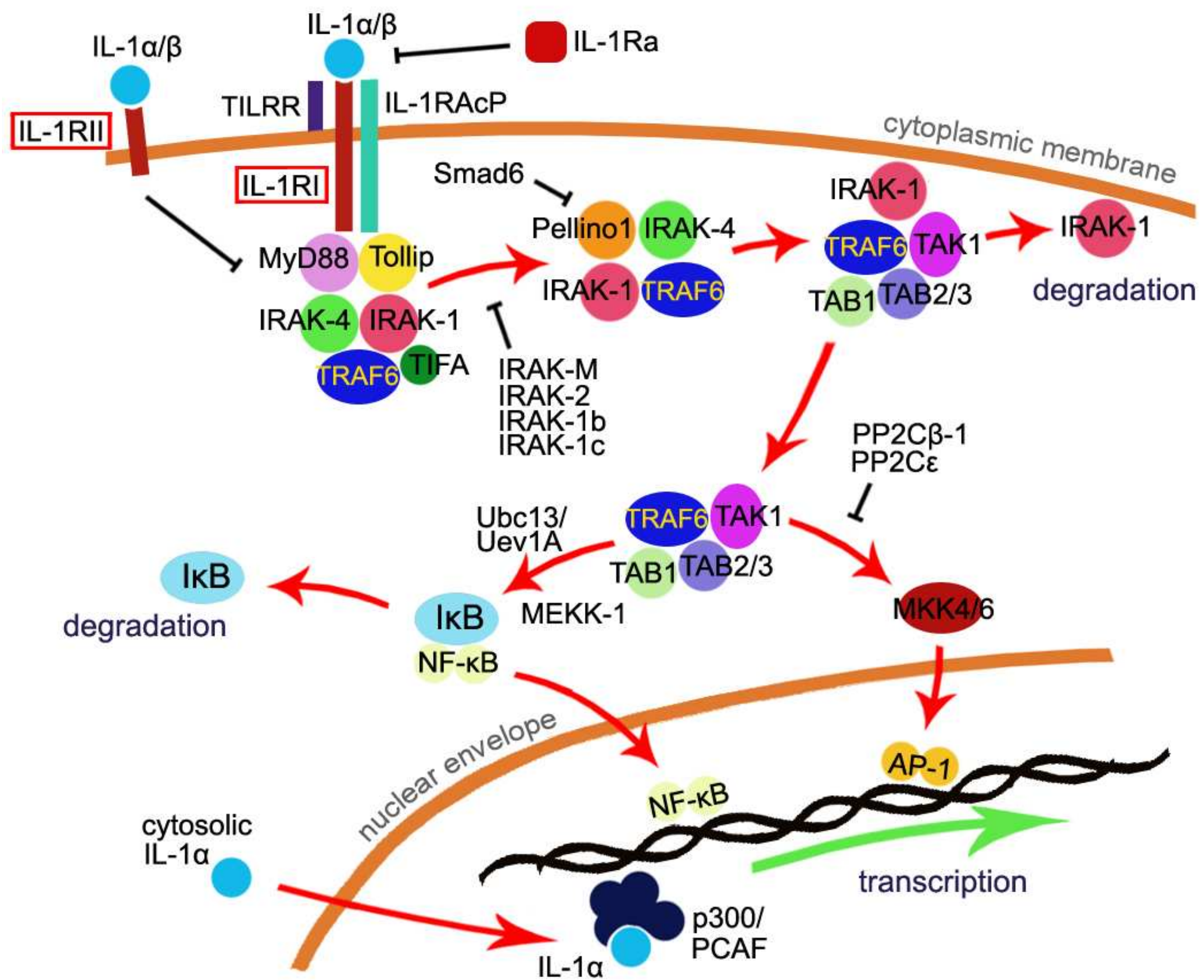


Figure 3: The IL-1 signaling and its negative regulation. Adapted from (Vicenova *et al.* 2009).

4.1.5 IL-1α and IL-1β: two strikingly different molecules

In spite of the similarity in protein structure and ability to induce inflammation, IL-1α and IL-1β differ significantly in certain features and seem to play partly different roles in the organism. In particular, the striking difference in regulation, processing, tissue-specific expression, subcellular localisation and compartmentalisation of IL-1α and IL-1β isoforms suggests the differential function and physiological role of these molecules (Kavita and Mizel 1995; Beissert *et al.* 1998; Nakae *et al.* 2001; Song *et al.* 2003).

Both IL-1 forms are produced in consequence of stress or cell injury as 31-kDa precursors which undergo proteolytical cleavage by specific proteases in the process of maturation. The precursor of IL-1α is cleaved by Ca^{2+}-dependent protease calpain (Kobayashi *et al.* 1990) that generates 17-kDa mature IL-1α and the 16-kDa so-called N-terminal part of IL-1α (IL-1αNTP) while the processing of the IL-1β is mediated by caspase-1 (also referred to as the IL-1β-converting enzyme) (Black *et al.* 1989). Unlike IL-1β, both the IL-1α precursor molecule (amino acids 1-271) and the mature protein (amino acids 113-271) are biologically active and able to bind to membrane receptors (Mosley *et al.* 1987), although the cleaved form of IL-1α is ~50 times more active than the precursor (Zheng *et al.* 2013). IL-1α is constitutively expressed by many cell types under physiological conditions and its synthesis is stimulated during inflammation, on the contrary, IL-1β is not produced unless the cell receives an inflammatory signal and also its cleavage by caspase-1 is a tightly regulated process. Moreover, while mature IL-1β is released from cells via an ATP-dependent non-classical secretory pathway including $P2X_7$ receptors and pannexin-1 (Ferrari *et al.* 1997b; Pelegrin and Surprenant 2006), the secretion of IL-1α seems to employ a different pathway dependent on copper ions, protein product of the S100A13 gene (Mandinova *et al.* 2003; Prudovsky *et al.* 2003; Mohan and Yu 2011) and in certain cases surprisingly also on IL-1β (Fettelschoss *et al.* 2011); nevertheless, the IL-1α secretion is rather a rare event and the protein is usually not detected in body fluids of healthy individuals (Dinarello 1996).

There is also a striking difference in the subcellular localisation of both IL-1 forms. Due to the presence of a nuclear localisation sequence (NLS) in the N-terminal part of the IL-1α precursor (Wessendorf *et al.* 1993), unprocessed IL-1α as well as IL-1αNTP are commonly found in the cell nucleus. In contrast, IL-1β is not

a nuclear protein; although there has been some evidence of IL-1β presence in the cell nucleus, this IL-1 form has no functional NLS that would drive active nuclear import of IL-1β (Luheshi *et al.* 2009b; Ross *et al.* 2013).

Treatment of cells with both IL-1 forms showed that IL-1α and IL-1β exert differential biological effects in osteoclast formation and activity (Trebec-Reynolds *et al.* 2010), on Leydig cell function (Calkins *et al.* 1990), IL-6 and chemokine production in primary neurones (Tsakiri *et al.* 2008), thyroid epithelial cell growth (Rasmussen *et al.* 1990), fibrosarcoma cell invasiveness (Song *et al.* 2003), T-cell stimulation (Boraschi *et al.* 1990) or on eosinophil oxidative metabolism (Whitcomb *et al.* 1989).

4.1.6 Current knowledge of the biological function of interleukin-1α

As it was already mentioned, extracellular 17-kDa mature IL-1α is able to bind IL-1 receptors and trigger the IL-1 signaling pathway. Mutation analysis has shown that regions important for the IL-1α interaction with IL-1RI and IL-1RII are situated within the C-terminal part of the protein (Gayle *et al.* 1993). Conversely, the post-translational modifications such as phosphorylation and myristoylation occur within the N-terminal domain of IL-1α (Stevenson *et al.* 1993; Watanabe and Kobayashi 1995). Human IL1A gene located on chromosome 2 (Modi *et al.* 1988) is transcribed into mRNA containing 7 exons; an unusual splice variant of IL-1α mRNA lacking exon 5 that contains the calpain cleavage site has been found in rat testis (Sultana *et al.* 2000). A similar alternatively spliced IL-1α mRNA has also been reported from canine, feline and porcine LPS-stimulated lung macrophages (Straubinger *et al.* 1999).

The transcription of IL-1α proceeds constitutively in certain cell types or can be induced by bacterial LPS, chemicals including phorbol esters or benzo(a)pyrene, concanavalin A, various types of injury or infection of macrophages with intracellular parasites such as *Leishmania major* (Dinarello 1996; Bersudsky *et al.* 2000; Hawn *et al.* 2002; Ueng *et al.* 2005; Luheshi *et al.* 2011). Under hypoxic conditions, the transcription of the *IL1A* gene is controlled by the hypoxia inducible factors HIF-1α and HIF-1β (Rider *et al.* 2012).

As it was already mentioned, the IL-1α precursor is usually cleaved by the cysteine protease calpain at arginine 112. Interestingly, in addition to calpain, IL-1α can be cleaved by the cytotoxic lymphocyte-derived protease granzyme B. The cleavage site is located at aspartate 103 and following cleavage with granzyme B, IL-1α exhibits enhanced bioactivity as shown by boosted antibody responses (Afonina *et al.* 2011).

The secretion of IL-1α from the cells has been a matter of controversy ever since. Unlike IL-1β, that acts as a classical cytokine, being secreted in response to stress, infection or injury and acting on target cells through the membrane receptors, most authors state that IL-1α predominantly remains cell-associated. Cells constitutively producing IL-1α (keratinocytes, epithelial cells..) rarely secrete it. The secretion of IL-1α appears to represent a rare event and certain hypotheses suggest that extracellular IL-1α is released from dying cells, serving thus as a danger signal, alerting the immune system in the case of non-infectious cell or tissue damage. For this type of molecules, a term „alarmin“ has been proposed by Joost Oppenheim in 2006 (Bianchi 2007).

However, certain studies describe the mechanism of IL-1α secretion. It has been shown to proceed along a non-conventional pathway that is convergent, yet distinct of the release pathway of the fibroblast growth factor 1 (FGF1) (Landriscina *et al.* 2001; Tarantini *et al.* 2001). Mature IL-1α and the Ca^{2+}-binding protein S100A13 associate near the inner plasma membrane and form a heterotetrameric Cu^{2+}-dependent structure (Mandinova *et al.* 2003). This complex binds specifically acidic phospholipids in the plasma membrane, transclocates across the membrane probably due to a conformational change and outside the cell it dissociates (Kathir *et al.* 2007; Mohan and Yu 2011).

In certain cases, the IL-1α secretion appears to be dependent on inflammasomes and caspase-1, employing the pathway typical for IL-1β. This can be explained by the physical association of IL-1α with IL-1β and piggy-backing on its transport from the cytoplasm outside the cell (Fettelschoss *et al.* 2011; Gross *et al.* 2012; Yazdi and Drexler 2012). Certain cell types (e.g. NIH/3T3 or U937) have been shown to release mature IL-1α in response to temperature stress (Tarantini *et al.* 2001; Mandinova *et al.* 2003).

Nevertheless, according to most literature sources, the essential part of the IL-1α biological function consists in its role within the cell. IL-1α can act intracellularly

and this mechanism of action independent on cell-surface IL-1 receptors is characterised as „intracrine". Interestingly, extracellular IL-1α has been found to be internalised and targeted to the Golgi apparatus in a complex with IL-1RI (Praast *et al.* 1995). Other groups reported the association of IL-1α with IL-1RII inside human fibroblasts (Kawaguchi *et al.* 2006), Jurkat cells and macrophages (Zheng *et al.* 2013).

Furthermore, a membrane-associated form of the IL-1α precursor (Kurt-Jones *et al.* 1985) binding the membrane exterior via a lectin-like interaction (Brody and Durum 1989) can be induced by treatment of various cells with IFN-γ or LPS (Inamura *et al.* 1989; Okubo *et al.* 1989; Gueniche *et al.* 1994; Kaplanski *et al.* 1994; Apte *et al.* 2006; Fettelschoss *et al.* 2011). The membrane IL-1α precursor is biologically active and can activate IL-1 receptors on adjacent cells in a juxtacrine manner (Kaplanski *et al.* 1994).

The functions attributed to intracellular IL-1α include induction of IL-6 and PDGF-A (Kawaguchi *et al.* 1999), induction of IL-8 during chlamydial infection (Cheng *et al.* 2008) or following infection by *Yersinia* (Bose *et al.* 2012), effects on oncogenicity and metastatic activity of transformed cells (Lakatosova-Andelova *et al.* 2009), contribution to cerebral ischemic injury (Luheshi *et al.* 2011), enhancement of fibrosis (Kawaguchi *et al.* 2004) or modulating the antiviral function of IFN-γ (Hurgin *et al.* 2007).

Intracellular IL-1α also seems to be involved in cell senescence. Treatment of endometrial stromal fibroblasts with IL-1α accelerates age-related changes and this effect can be reversed by transfection of the cells with intracellular IL-1 receptor antagonist (Rinehart *et al.* 1999). Experiments aimed at antisense RNA-mediated degradation of the IL-1α mRNA showed that decrease of intracellular IL-1α resulting from the antisense RNA treatment prevented senescence of endothelial cells (Maier *et al.* 1990). Senescent cells exhibit increased levels of IL-1α mRNA and protein and presumably, intracellular IL-1α contributes to the inhibition of growth and the formation of the senescent phenotype of endothelial cells *in vitro* (Garfinkel *et al.* 1992). While IL-1α is upregulated in senescent cells, mRNA levels for IL-1β and IL-1Ra remain unaltered (Mariotti *et al.* 2006).

Although IL-1α is generally not secreted as often as IL-1β, several recently published papers drawed the attention also to the interaction of extracellular IL-1α with its membrane receptors. More precisely, treatment with soluble IL-1α was shown

to be important for growth of prostate tissues and critical for the development of the prostate (Jerde and Bushman 2009). IL-1α together with its receptor and MyD88, adapter involved in IL-1 signaling pathway, is also crucial for the induction of sterile inflammatory response that occurs in response to sterile cell death (Chen *et al.* 2007; Luheshi *et al.* 2011; Rider *et al.* 2011) or after release of IL-1α-containing apoptotic bodies from endothelial cells (Berda-Haddad *et al.* 2011).

Furthermore, several lines of evidence suggest that secreted IL-1α may be involved in cancer. Extracellular IL-1α can stimulate pancreatic carcinoma cells in an autocrine manner to develop a metastatic phenotype (Sawai *et al.* 2003; Melisi *et al.* 2009), promote invasivity of lung endothelial cells (Yasuda *et al.* 1999) and contribute to the melanoma resistance to the cytotoxic T-cells (Kholmanskikh *et al.* 2010). IL-1α of pancreatic ductal adenocarcinoma cell origin also enhances the formation of an inflammatory tumour environment that is beneficial for tumour survival (Tjomsland *et al.* 2011).

In response to *S. aureus* infection, IL-1α but not IL-1β secretion stimulates chemokine secretion in an autocrine manner (Olaru and Jensen 2010). IL-1α has also been found to be released by platelets in the brain and to induce inflammatory activation of endothelial cells in the brain; IL-1α, but not IL-1β, can thus be involved in pathogenesis of inflammatory-mediated brain injury (Thornton *et al.* 2010).

Therefore, IL-1α can play the role of a classical proinflammatory cytokine acting through its membrane receptors and triggering the signaling pathway leading to NF-κB or AP-1. However, the low occurence of secreted IL-1α and the non-classical way of its release, the absence of a secretory sequence and the presence of a nuclear localisation signal suggests the possible function of IL-1α in the cell nucleus.

4.1.7 Nuclear function of IL-1α: a dual-function cytokine

Although IL-1α was first found in the nucleus at the end of 1980s (Grenfell *et al.* 1989; Curtis *et al.* 1990), its nuclear activities have been studied extensively after the discovery of a nuclear localisation sequence within the IL-1α precursor in 1993 (Wessendorf *et al.* 1993). In the course of time, it has become evident that in addition to the extracellular function of mature IL-1α, the IL-1α precursor exerts certain activities in the cell nucleus. The first report of a nuclear IL-1α function was in 1994

when Maier *et al.* stated that the inhibitory effect of IL-1α on cell proliferation could only be attributed to the nuclearly localised IL-1α precursor and not to the mature IL-1α (Maier *et al.* 1994). Few years later, it was shown that the IL-1α precursor, in contrast to mature IL-1α, was able to regulate the motility of human umbilical vein endothelial cells *in vitro* (McMahon *et al.* 1997). Further studies characterised the nuclear interaction of IL-1α with the growth suppressor protein necdin (Hu *et al.* 2003), transactivation of transcription via interaction with histone acetyltransferase complexes (Buryskova *et al.* 2004), activation of NF-κB and induction of proinflammatory mediators independent on surface IL-1 receptors (Werman *et al.* 2004).

Although IL-1α is the acidic form of IL-1 with pI=5,0 and therefore is less likely to possess DNA-binding activities, it has been shown to be a chromatin-associated factor that is retained in apoptotic cells so that no inflammation is induced by extracellularly released protein. Conversely, necrotising cells release the IL-1α precursor that triggers a robust inflammatory response with recruitment of infiltrating neutrophils and macrophages (Cohen *et al.* 2010). A different situation comes up in the microglial cells, where necrotic cell death triggers immobilisation of IL-1α in the nucleus (Luheshi *et al.* 2009a). This may represent a protective mechanism preventing the release of proinflammatory mediators in the brain, regarded as a partially immune-privileged organ.

Within the nucleus, IL-1α has also been reported to be localised in the interchromatin regions referred to as the nuclear speckles, where pre-mRNA splicing and processing factors can be found (Luheshi *et al.* 2009a).

Interestingly, the action of IL-1α, being a proinflammatory cytokine with the receptors on the cell surface, in the regulation of nuclear processes, is not a unique mechanism in host defence. A small group of „dual-function“ proteins that can trigger signaling through a cell membrane receptor but act also within the cell nucleus has been described (Carriere *et al.* 2007). Presently, this group of proteins include IL-1α, high-mobility group box 1 (HMGB1), IL-16, IL-33 and IL-37.

HMGB1 has been found associated with nuclear chromatin (Agresti and Bianchi 2003) and outside the cells acts as a proinflammatory cytokine (Wang *et al.* 1999a; Wang *et al.* 1999b), contributing to the production of IL-1β and IL-18 (He *et al.* 2012).

IL-16 was originally described as a cytokine acting as a chemoattractant for $CD4^+$ T-cells (Berman *et al.* 1985), but later, a nuclear localisation sequence was

identified within the N-terminal domain of the IL-16 precursor (Zhang *et al.* 2001) and it has been suggested that the nuclearly localised precursor of IL-16 contributes to the regulation of cell cycle progression (Wilson *et al.* 2002; Zhang *et al.* 2008c).

IL-33 is a cytokine from the IL-1 family acting also as a nuclear transcriptional repressor (Carriere *et al.* 2007; Gadina and Jefferies 2007). It is particularly expressed in the high endothelial venules, where it was first identified (Baekkevold *et al.* 2003). IL-33 is primarily associated with the augmentation of Th type 2 immune responses, but can also promote Th1 immunity under certain conditions (Smith 2011). Recently, it has been reported to be involved in the amplification of the local allergic inflammatory response (Su *et al.* 2013).

IL-37 appears to be a key anti-inflammatory molecule that is expressed in five splice variants, one of which (IL-37b) has been shown to translocate to the nucleus upon stimulation with LPS (Sharma *et al.* 2008; Ross *et al.* 2013). Nevertheless, IL-37 is probably released from cells upon a proinflammatory stimulation (Boraschi *et al.* 2011).

The dual functionality of these cytokines may potentially represent a residual ancient mechanism of host defence consisting in regulating directly gene expression in response to stress (Werman *et al.* 2004). Consistent with this theory, neither IL-1α, HMGB-1, IL-16, IL-33 nor IL-37 possess a conventional signal peptide that would determinate these molecules to be secreted via the classical endoplasmic reticulum-Golgi route (March *et al.* 1985; Ferrari *et al.* 1994; Baier *et al.* 1997; Schmitz *et al.* 2005). This could be a mechanism protecting the organism from uncontrolled secretion of these molecules (Prudovsky *et al.* 2008). Other possibility is, according to (Rao *et al.* 2007), that detection of intracellular molecules outside the cells may represent an ancient pathway of recognition of cell injury.

4.1.8 The N-terminal part of IL-1α

The N-terminal part of IL-1α resulting from the IL-1α precursor cleavage is evolutionary so well conserved among different vertebrate species (Buryskova *et al.* 2004) that it probably doesn´t represent only a byproduct of IL-1α maturation. Instead, a biological role of IL-1αNTP in the cell nucleus has been suggested (Stevenson *et al.* 1997; Pollock *et al.* 2003; Buryskova *et al.* 2004). Similarly as the

IL-1α precursor, IL-1αNTP translocates to the cell nucleus due to the presence of a NLS and can be found in the interchromatin regions (Pollock *et al.* 2003). The peptide can probably also associate with nuclear chromatin (Cohen *et al.* 2010), but is unable to trigger the inflammatory pathway through the IL-1 receptors (Gayle *et al.* 1993; Labriola-Tompkins *et al.* 1993).

Different biological functions have been assigned to the nuclearly localised IL-1αNTP so far. Stevenson *et al.* found that mesangial cells overexpressing IL-1αNTP develop malignant transformation to a spindle cell-type tumour (Stevenson *et al.* 1997). In a screening aimed at the identification of IL-1αNTP binding proteins, HAX-1 (antiapoptotic HS-1-associated protein X-1) has been found to interact with IL-1αNTP (Yin *et al.* 2001). This interaction seems to occur within three different segments of IL-1αNTP including NLS and is important for the transport of the IL-1α precursor to the cell nucleus (Kawaguchi *et al.* 2006). Another study from Dr. Lovett´s laboratory showed that IL-1αNTP can induce apoptosis in various tumour cells and interacts with certain proteins involved in splicing (Pollock *et al.* 2003). In our laboratory, we have previously shown that IL-1αNTP functionally interacts with histone acetyltransferase complexes (Buryskova *et al.* 2004).

Despite of the wide range of actions of IL-1αNTP on various cellular processes, up to the present, no biological effects have been attributed to the N-terminal propiece of IL-1β.

4.1.9 Interleukin-1β

The second IL-1 form, IL-1β, shares only limited sequence similarity with IL-1α, 26% at the protein level and 45% at the nucleic acid level. Similarly as IL1A, the IL1B gene encoding IL-1β is located on chromosome 2 (Webb *et al.* 1986). IL-1β mRNA is ubiquitously expressed, but not always translated. Usually, a secondary stimulus is needed to the efficient IL-1β production and release (Schindler *et al.* 1990; Hogquist *et al.* 1991; Perregaux *et al.* 1992; Perregaux and Gabel 1994).

Only mature IL-1β is released from producing cells and acts as a proinflammatory cytokine although small amounts of the IL-1β precursor can be secreted by murine macrophages (Beuscher *et al.* 1990). Activation of caspase-1, enzyme cleaving the IL-1β precursor into its mature form, takes place through the

inflammasome. Several inflammasome types with a varying subunit composition have been described but one of the best characterised is the NLRP3 inflammasome. This protein complex is composed from the NLP family member NLRP3 (also known as NALP3 or cryopyrin), the adaptor molecule ASC, a CARD domain-containing protein cardinal and caspase-1 (Agostini *et al.* 2004). The NLRP3 inflammasome can be activated by various stimuli including bacterial products (muramyl dipeptide, bacterial RNA), exogenous dust components (asbestos, silica) or by deposits of monosodium urate and calcium pyrophosphate dihydrate crystals (Martinon *et al.* 2004; Kanneganti *et al.* 2006; Martinon *et al.* 2006; Dostert *et al.* 2008). The activated inflammasome complex then facilitates caspase-1 self-cleavage and subsequent processing of the IL-1β precursor. However, to a minor extent, IL-1β can be processed aslo by other proteases such as proteinase 3, elastase, granzyme A, cathepsin D or collagenase (Hazuda *et al.* 1990; Irmler *et al.* 1995; Coeshott *et al.* 1999).

The most rapid IL-1β release occurs in the LPS-stimulated macrophages additionally stimulated with extracellular ATP (Griffiths *et al.* 1995; Ferrari *et al.* 1997a; Laliberte *et al.* 1999). The secretion of IL-1β proceeds along a non-conventional secretory pathway and is mediated by the $P2X_7$ receptor and pannexin-1 (Ferrari *et al.* 1997a; Solle *et al.* 2001; Pelegrin and Surprenant 2006).

IL-1β can be released from many cell types including endothelial, neuronal and glial cells, immune cells such as monocytes, macrophages and mast cells, keratinocytes, smooth muscle cells, fibroblasts, synoviocytes, microglia and astrocytes (Miyasaka *et al.* 1988; Sisson and Dinarello 1988; Dalton *et al.* 1989; Beuscher *et al.* 1990; Dinarello 1996; Copray *et al.* 2001; Rothwell 2003; Clark *et al.* 2006; Carta *et al.* 2011).

IL-1β is the most important factor in a number of chronic and acute diseases predominantly with an inflammatory background. It has been associated with rheumatoid arthritis (Eastgate *et al.* 1988; Rooney *et al.* 1990), Alzheimer´s disease (Vasilakos *et al.* 1994; Holden and Mooney 1995), periodontitis (Lopez *et al.* 2005), autoimmune encephalomyelitis (Sutton *et al.* 2006), psoriasis (Mee *et al.* 2006), diabetes mellitus (Holden and Mooney 1995; Larsen *et al.* 2007), Parkinson´s disease (Mattila *et al.* 2002), coronary artery disease (Waehre *et al.* 2004) or gastric inflammation (Tu *et al.* 2008) or carcinoma (Yamanaka *et al.* 2004). A study addressing the role of IL-1β in melanoma cells demonstrated that secreted IL-1β

together with IL-1α downregulates the expression of melanocyte differentiation antigens that may enable the melanoma cells to escape the immune surveillance of cytotoxic T-cells (Kholmanskikh *et al.* 2010). Targeting IL-1β may also inhibit pathological angiogenesis, not only related to cancer (Carmi *et al.* 2009).

4.1.10 The IL-1 receptor antagonist is a potent inhibitor of inflammation

The proinflammatory effects mediated by IL-1 can be abolished by a particularly effective natural inhibitor, IL-1Ra. Structurally similar to IL-1, IL-1Ra can bind the extracellular Ig domain of the IL-1 receptor and block both IL-1α and IL-1β activity. IL-1Ra is unable to recruit IL-1RAcP (Greenfeder *et al.* 1995) and therefore does not trigger the IL-1 signaling pathway. IL-1Ra was the first naturally occurring specific receptor antagonist of any cytokine or hormone-like molecule that was described. Four structural variants of IL-1Ra have been reported (Carter *et al.* 1990; Eisenberg *et al.* 1990; Muzio *et al.* 1995; Malyak *et al.* 1998). The 22-kDa glycosylated soluble form (sIL-1Ra) is secreted from various cells whereas three intracellular unglycosylated forms of IL-1Ra (18-kDa icIL-Ra1, 25-kDa icIL-Ra2 that has only been predicted so far and 16-kDa icIL-1Ra3) remain in the cytoplasm (Malyak *et al.* 1998; Arend 2002). These three variants result from the alternative splicing or alternative translation initiation of mRNA encoded by the IL-1RN gene. Soluble IL-1Ra binds the IL-1 receptor without activating the signaling pathway while the role of intracellular IL-1Ra forms remains unclear.

Soluble IL-1Ra is an acute phase protein that is induced by IL-1, IL-6 or LPS (Andersson *et al.* 1992; Gabay *et al.* 1997). It is produced predominantly in monocytes, macrophages and neutrophils and its synthesis is upregulated during the host response to infection and acute or chronic inflammation. In contrast, intracellular icIL-1Ra is expressed constitutively in keratinocytes and epithelial cells (Andersson *et al.* 1992) suggesting an autocrine function of the intracellular IL-1Ra variants. Type 1 icIL-1Ra has been demonstrated to interact with the third component of the COP9 signalosome CSN3 and to inhibit the activity of CSN-associated kinases as well as the IL-1α-mediated release of IL-6 and IL-8, displaying thus unique anti-inflammatory activities inside the cells (Banda *et al.* 2005). ,Furthermore, (Dewberry

et al. 2008) reported that type 1 icIL-1Ra promotes endothelial cell proliferation by modulating CDK2 activity and retinoblastoma (Rb) protein phosphorylation.

Given that IL-1Ra exhibits exceptional anti-inflammatory properties by blocking the inflammation induced by IL-1, the antagonist molecule apparently has a great therapeutic potential in the treatment of various inflammation-linked diseases and pathological processes. Nowadays, the recombinant non-glycosylated form of IL-1Ra, anakinra (brand name Kineret), is widely used for treatment of rheumatoid arthritis and is tested also for IL-1 inhibition during acute gout (So *et al.* 2007), systemic onset juvenile idiopathic arthritis (Pascual *et al.* 2005), adult-onset Still's disease (Fitzgerald *et al.* 2005), diabetes mellitus (Tellez *et al.* 2005) and familial cold autoinflammatory syndrome (Metyas and Hoffman 2006); results of several studies suggest that anakinra treatment may be effective in treatment of certain cardiovascular disorders (Nakano *et al.* 2001; Lim *et al.* 2002; Murtuza *et al.* 2004).

Besides of anakinra, novel anti-IL-1 molecules that can serve as therapeutic agents emerged in the last years. Rilonacept is a so-called IL-1 trap composed from the IL-1 receptor and IL-1 receptor accessory protein fused to the Fc part of human IgG1 (Economides *et al.* 2003). This protein construct binds both IL-1α and IL-1β and in February 2008 it was approved by the US FDA (United States Food and Drug Administration) for clinical use. Nowadays it is used for treatment of auto-inflammatory cryopyrin-associated periodic syndromes, i.e. familial cold autoinflammatory syndrome (FCAS) or Muckle-Wells syndrome (MWS) (Church and McDermott 2009). Another compound, canakinumab (ACZ885 or Ilaris), is a monoclonal IL-1β antibody that does not recognise IL-1α, approved by the US FDA in June 2009. In addition to the rheumatoid arthritis treatment, it is tested for therapy of MWS and FCAS. The advantage of Ilaris is that the dosing schedule is once every eight weeks (source: Novartis website, www.novartis.com) while anakinra has to be administered daily to the patients. For certain diseases such as rheumatiod arthritis, IL-1Ra gene therapy could be promising (Evans *et al.* 2005). Finally, VX-765, a caspase-1 inhibitor that reduces the production and release of IL-1β (Wannamaker *et al.* 2007), is currently being tested in patients with treatment-resistant epilepsy (source: Vertex Pharmaceuticals website, www.vrtx.com).

4.2 Histone acetyltransferases

4.2.1 Histone acetylation and enhanced transcription

In the nucleus of eukaryotic cells, DNA is highly compacted and organised into chromatin fiber, being associated with both histone and non-histone proteins. Within the nucleosome, the elementary subunit of chromatin, 147 base pairs of DNA is wrapped 1,6 times around octamers of histone proteins H3, H4, H2A, and H2B (each of these is present in two copies), with histone H1 serving to clamp DNA to the nucleosomes and thus taking part in higher-order chromatin folding. In this compact, tightly coiled form, DNA is inaccessible to the transcription machinery. Histones are small basic proteins with a globular domain and flexible amino-terminal tails that extend outwards from the nucleosome core. The evolutionary well conserved histone tails are subject to a wide range of post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination or sumoylation (Shiio and Eisenman 2003) that govern chromatin dynamics and structural flexibility, important for its function in gene expression regulation. Most of the modifications occur at the tail of histone H3 and H4. According to a study by Arya and Schlick (2006), modification of the tail of H3 and H4 is important for mediating internucleosomal interactions, H2A and H2B tail modifications induce formation of kinks and bends of the chromatin fiber and H3 tail is likely to be involved in screening electrostatic interactions in non-nucleosomal DNA (Arya and Schlick 2006). The specific pattern of post-translational histone tail modification seems to represent a unique mechanism of transcription state regulation referred to as the „histone code“.

For many years, acetylation of lysines of histone proteins has been associated with activation of transcription (Allfrey *et al.* 1964; Wade *et al.* 1997; Grant *et al.* 1998c). This effect was explained simply by the fact that histone acetylation neutralises their positive charge and loosens the interactions of histone proteins with negatively charged DNA. Indeed, acetylated oligonucleosome complexes adopt more extended conformation *in vitro* (Garcia-Ramirez *et al.* 1995). More recently, another hypothesis emerged based on the observation that acetylated lysines are recognised and bound by proteins containing a bromodomain (Dhalluin *et al.* 1999; Kasten *et al.* 2004), a highly conserved region shared by a variety of DNA-binding proteins (Haynes *et al.* 1992). According to this hypothesis, acetylation of histones creates

new binding sites recruiting the bromodomain-containing transcription activators. Most probably, both effects of histone acetylation may be physiologically relevant (Shogren-Knaak *et al.* 2006). Conversely, hypoacetylation of histones characterises inactive or silenced chromatin regions (Braunstein *et al.* 1993).

4.2.2 Histone acetyltransferases: mechanism of action and biological function

Acetylation of lysine residues on nucleosomal histones is a post-translational process catalyzed by multisubunit histone acetyltransferase (HAT) complexes. Usually, the molecule with histone acetyltransferase catalytic activity is associated with adapters and other proteins that target the HAT complex to specific sites in the genome. Following the discovery of the first histone acetyltransferase Gcn5 in *Tetrahymena* (Brownell and Allis 1995; Brownell *et al.* 1996), a number of distinct histone acetyltransferases displaying distinct specificities has been found in eukaryotes, some of them originally identified as transcriptional coactivators. Various histone acetyltransferase complexes differ in preference for specific histone substrates and internal acetylation sites. The activity of the catalytic subunit depends considerably on the context of the other subunits in the complex and although many acetylation events and molecules seem to be conserved throughout eukaryotes, acetylation of a specific lysine residue does not necessarily lead to the same result in yeast, flies and humans. Some of the subunits are, not unfrequently, shared by different histone acetyltransferase complexes; such an example can be yeast catalytic subunit Gcn5 that makes part of four distinct complexes - SAGA, SLIK/SALSA, HAT-A2 (which may however represent only the core enzymatic complex of this HAT group (Sendra *et al.* 2000)) and ADA (Grant *et al.* 1997); in humans, the Gcn5 homologue (hGCN5L) can be found in STAGA (Martinez *et al.* 1998), TFTC (Brand *et al.* 1999), ATAC (Krebs *et al.* 2010) and PCAF histone acetyltransferase complex (Ogryzko *et al.* 1998).

The catalytic activity of histone acetyltransferases consists in transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine side chains within the basic N-terminal tail of the histone. Histone acetylation is a covalent post-translational modification, rapid and precisely controlled.

It is a reversible process, deacetylation is mediated by histone deacetylases (HDAC) which remove acetyl groups from specific lysine residues (Figure 4). HDAC generally function in transcriptional repression.

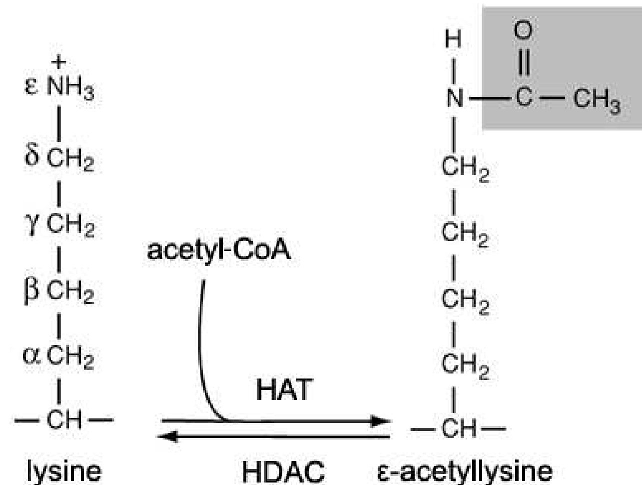


Figure 4: Catalytic function of histone acetyltransferases (HAT) and deacetylases (HDAC). An acetyl group is transferred from acetyl-coenzyme A to the ε-terminal amino group of a specific lysine residue by HAT; the acetyl group can be removed again by HDAC.

Interestingly, lysine acetylation-dependent modulation of protein activity has been found in archaea and eubacteria as well (Bell *et al.* 2002; Starai *et al.* 2002).

Acetylation of histones and resulting activation of transcription seems to be the main biological role of histone acetyltransferase complexes. However, the scope of their function is most probably much broader. Histone acetyltransferases might in addition help to maintain a longer-term particular state of activity of a gene and contribute to the mechanism of epigenetic inheritance (Cavalli and Paro 1999). Indeed, the extent of histone H4 acetylation is the principle of regulation of gene activity that diverges in genomes of different parental origin in a mealybug (Ferraro *et al.* 2001). The acetylation of H4 thus serves as a histone code that can be transmitted to next generations as shown by experiments in *S. pombe* (Ekwall *et al.* 1997).

Histone acetyltransferases have also been shown to participate in DNA repair mechanism (Qin and Parthun 2002). An example could be yeast the histone acetyltransferase Hat1 that is recruited to chromatin sites with double-strand breaks

(Qin and Parthun 2006). Another yeast histone acetyltransferase, Esa1, acetylates histone H4 preferentially near the break sites and is also essential for DNA repair (Bird *et al.* 2002). Possibly, changes in chromatin structure mediated by histone acetyltransferases modulate the accessibility of damaged DNA to the structures involved in DNA repair (Tamburini and Tyler 2005; Kruhlak *et al.* 2006).

Furthermore, there has also been evidence of implication of histone acetyltransferases in the DNA replication. According to a study in yeast, mutation of lysine residues K5, K8 and K12 of histone H4 and subsequent lack of acetylation leads to impaired nucleosome assembly (Ma *et al.* 1998). Histone acetyltransferase HBO1 was shown to bind the origin recognition complex (Iizuka and Stillman 1999) and to recruit Mcm2p, a protein that regulates DNA replication (Burke *et al.* 2001). Replication origins seem to be located in chromatin regions that are globally deacetylated and histone acetylation most probably affects the DNA replication timing (Vogelauer *et al.* 2002).

Nevertheless, the role of HATs and HDACs in transcription regulation does not seem to be unambiguous. Acetylation of histones can be targeted specifically to promoter regions (Kuo *et al.* 1998), but not every gene´s transcription is regulated by HAT-mediated acetylation. The recruitment of a histone acetyltransferase to a specific promoter may be facilitated by specific DNA-binding proteins (Janknecht and Hunter 1996) and different genes usually exhibit differential requirements for distinct histone acetyltransferases (Korzus *et al.* 1998). However, HAT recruitment does not necessarily correlate with activation of transcription since p300/CBP have been found to bind promoters of genes that do not appear to need them for active transcription (Leung *et al.* 2004; Kasper *et al.* 2005). Conversely, under hypoxic conditions, the histone deacetylase HDAC7 translocates to the nucleus and enhances transcription, although this effect may be attributed rather to p300/CBP that is also present in the complex with HDAC7 (Kato *et al.* 2004). Although the transcriptional activation correlates strongly with the acetylated state of many genes, the function of histone acetyltransferases and deacetylases in the regulation of transcription is probably much more complex and some particular details remain uncertain.

4.2.3 Non-histone targets of histone acetyltransferases

Certain histone acetyltransferase complexes acetylate not only histones, but also other, non-histone substrates. Indeed, ε-amino lysine acetylation of non-histone proteins has been recognised as an important regulatory signal in many cellular processes such as transcription (Gu and Roeder 1997; Gu *et al.* 1997; Chen *et al.* 2001b; Yao *et al.* 2001), apoptosis (Fu *et al.* 2004; Ianari *et al.* 2004; Iyer *et al.* 2004a; Sykes *et al.* 2006), cell differentiation (Boyes *et al.* 1998; Miska *et al.* 2001; Mal and Harter 2003; Wong *et al.* 2010) or nuclear transport (Blander *et al.* 2002; Wang *et al.* 2005). Some authors therefore renamed HATs as KATs (lysine or K-acetyltransferases) in 2007 (Allis *et al.* 2007). A significant number of non-histone proteins can be regulated by HAT-mediated acetylation (reviewed in (Glozak *et al.* 2005)), interestingly, the majority of them reside in the mitochondria (Kim *et al.* 2006). Certain acetylation and deacetylation-linked disorders have been associated with diverse human pathologies (for a review see (Close *et al.* 2010)).

An example of a non-histone target of histone acetyltransferases could be the tumour suppressor p53 that is acetylated by p300/CBP (Gu and Roeder 1997; Gu *et al.* 1997; Wang *et al.* 2003; Iyer *et al.* 2004a), PCAF (Sakaguchi *et al.* 1998; Liu *et al.* 1999), hMOF and Tip60 (Sykes *et al.* 2006; Tang *et al.* 2006). Acetylation of p53 is induced by DNA damage (Sakaguchi *et al.* 1998) and this event influences protein stability (Li *et al.* 2002a), increases its sequence-specific DNA binding activity (Gu and Roeder 1997; Liu *et al.* 1999), contributes to the cell arrest in response to DNA damage (Iyer *et al.* 2004a) and affects its function in apoptosis as demonstrated by reduced BAX and PUMA transcription (Sykes *et al.* 2006). Indeed, Tip60-mediated p53 acetylation at lysine 120 possibly contributes to the decision between apoptosis and cell cycle arrest (Tang *et al.* 2006). In agreement with the acetylation-mediated p53 activation, histone deacetylases are involved in negative regulation of p53. HDAC2 has been shown to inhibit p53 binding to target promoters and HDAC2 knockdown leads to the inhibition of proliferation and to cellular senescence (Harms and Chen 2007). Given that the tumour suppressor p53 is inactivated in a wide scale of tumours, histone deacetylases represent a promising target of anti-tumour therapy (Saito *et al.* 1999; Vigushin *et al.* 2001; Butler *et al.* 2002; Finzer *et al.* 2004; Adler *et al.* 2009; Liu *et al.* 2010a).

4.2.4 Histone acetyltransferase types and families

There are two categories of histone acetyltransferases: type A, located in the cell nucleus, and type B, that are cytoplasmic. However, HAT complexes are highly diverse and some of histone acetyltransferases may function in more than one complex and therefore do not have to fit precisely this historic classification. Generally, type A HATs acetylate nucleosomal histones while type B HATs catalyze acetylation of newly synthesised histones prior to their transport to the nucleus. Histone acetyltransferases sharing conserved sequence similarities and catalytic mechanisms are further grouped into families and superfamilies such as GNAT (Gcn5-related N-acetyltransferase) (Neuwald and Landsman 1997), MYST (named after the members MOZ, Ybf2/Sas3, Sas2 and Tip60) or p300/CBP. Although transcription regulation is the main function of these enzymatic complexes, they may be involved also in other cellular processes.

The GNAT superfamily is the largest acetyltransferase family containing over 10,000 members from all kingdoms of life (reviewed in (Vetting *et al.* 2005)). The group includes bacterial aminoglycoside *N*-acetyltransferases mediating resistance to aminoglycoside antibiotics (Shaw *et al.* 1993), archaeal putative acetyltransferase PfGNAT (Biarrotte-Sorin and Mayer 2005) and various eukaryotic N-acetyltransferases of which Gcn5 histone acetyltransferase is the most well-characterised protein.

Histone acetyltransferases from the MYST family share the highly conserved MYST domain that contains an acetyl CoA-binding motif and a zinc finger. Generally they are found in multisubunit complexes rather than as isolated proteins. Among others, human histone acetyltransferase Tip60, its yeast homologue Esa1 or drosophila MOF are members of the MYST family. MYST acetyltransferases are involved in many cellular events including transcription regulation of specific genes, DNA replication or DNA damage response and repair of double-strand breaks. The N-terminal part of the MYST histone acetyltransferase MOZ (monocytic leukaemia zinc finger protein) is commonly found in fusion with the C-terminal part of CBP in acute myeloid leukaemia (Borrow *et al.* 1996), and also other MYST members can play a role in certain human diseases such as cancer (Halkidou *et al.* 2003; Fraga *et al.* 2005; Smith *et al.* 2005), Alzheimer´s disease (Cao and Sudhof 2001) or AIDS (Col *et al.* 2005).

The p300/CBP family in addition to p300 and CBP contains CBP-1 from *C. elegans*, dCBP from drosophila, *Arabidopsis thaliana* CBP-like proteins or other homologous proteins from multicellular animals, plants, but also fungi (Yuan and Giordano 2002; Tang *et al.* 2008). Multiple conserved domains are shared by the protein members of the p300/CBP family such as the CREB-binding domain, the bromodomain, glutamine/proline or cysteine/histidine-rich regions. The p300/CBP family proteins participate in differentiation, proliferation, apoptosis and other cellular processes, acetylate a range of important transcription factors such as p53 and are implicated in certain pathologies including cancer (reviewed in (Giordano and Avantaggiati 1999)).

4.2.5 Yeast histone acetyltransferases

During the past years, many histone acetyltransferases have been isolated from various organisms but a significant portion of work has been done using yeast that is, as a simple eukaryotic model organism, amenable to many distinct genetic techniques. The first histone acetyltransferase, Hat1, was described in yeast in 1995 (Kleff *et al.* 1995) and yeast Gcn5 is probably the best characterised acetyltransferase ever. It is estimated that Gcn5-associated complexes participate in regulation of transcription of at least 10% of the yeast genome (Lee *et al.* 2000), one of the main yeast histone acetyltransferases is also Esa1; Hat1, Elp3, Hpa2, and Sas3 have been shown to have only minor effects on gene regulation (Durant and Pugh 2006). Nevertheless, yeast HATs are to a certain extent redundant and usually one HAT complex can partly compensate for the loss of another (Lee *et al.* 2000). Indeed, only 2 (Taf1 and Esa1) out of 16 currently known* yeast histone acetyltransferase gene knock-outs are inviable (source: *Saccharomyces* Genome Database, http://www.yeastgenome.org).

* genes encoding currently known yeast histone acetyltransferases: Sas2, Sas3, Sas4, Sas5, Spt10, Gcn5, Hpa2, Esa1, Taf1, Elp3, Rtt109, Eaf6, Eaf3, Hat1, Nat4, Nto1 (http://www.yeastgenome.org)

4.2.5.1 The SAGA complex

SAGA is one of the most well-studied HAT complexes and also one of the main transcriptional coactivators in yeast. The acronym SAGA refers to the name of proteins that make part of the complex: Spt-Ada-Gcn5 acetyltransferase. Up to the present, 20 SAGA subunits have been identified (listed in Table 2). In addition to acetylation of histones, SAGA has been also shown to mediate histone H2B deubiquitination (Henry *et al.* 2003; Daniel *et al.* 2004). SAGA is also required for the optimal recruitment of TBP by certain transcription factors to target promoters (Qiu *et al.* 2004).

SAGA has recently been shown to possess a modular structure (Figure 5), being assembled from distinct modules: HAT/Core (histone acetyltransferase catalytic core), DUB (deubiquitinylation module), SA_SPT (SAGA-associated suppressors of Ty) and SA_TAF (SAGA-associated TATA-binding protein-associated factors) (Lee *et al.* 2011). The HAT/Core module carries out the HAT activity of the SAGA complex and is composed of Gcn5, Ada2, Ada3 and Sgf29. The DUB module consists of subunits Ubp8, Sgf11, Sus1 together with Sgf73 that anchors the DUB complex to SAGA (Lee *et al.* 2009). Proteins Ada1, Spt3, Spt7, Spt8 and Spt20 form the SA_SPT module; the SA_TAF module is composed of proteins Taf5, Taf6, Taf9, Taf10 and Taf12; Tra1 could be constituent of the SA_SPT module or represent a single subunit; Chd1 is probably not associated with any of the modules mentioned and its association with the SAGA complex is uncertain (Lee *et al.* 2011).

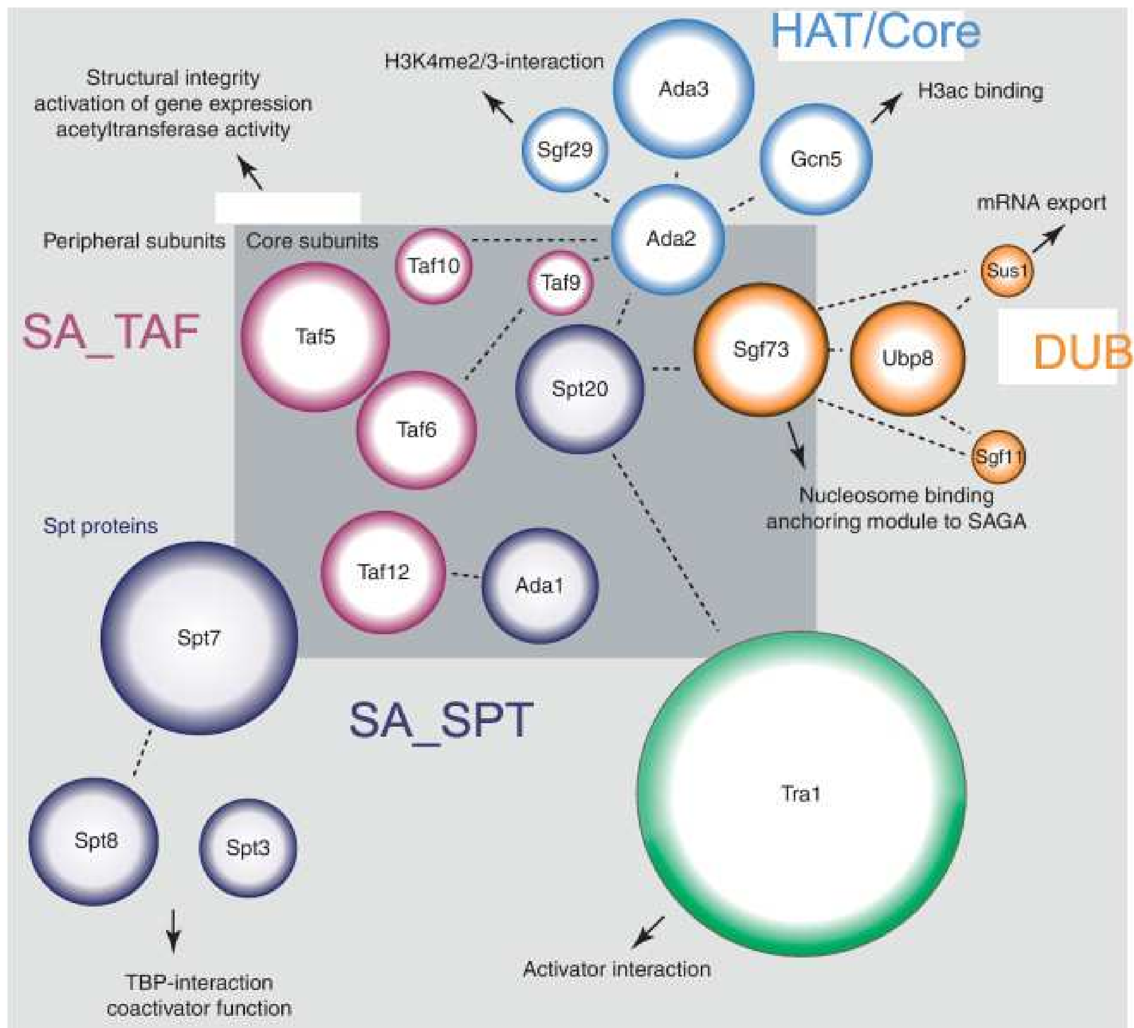


Figure 5: Modular structure of the yeast SAGA complex. Different colours indicate the subunits of different SAGA modules: the HAT/Core module (light blue), DUB module (orange), SA_TAF (violet), SA_SPT (dark blue) and the single subunit Tra1 (green). Chd1 is not represented in this figure; its association with SAGA is still uncertain. The inner box embraces the subunits that are more central to the complex; peripheral subunits are outside. Adopted from (Weake and Workman 2012).

The 51-kDa protein Gcn5 is the catalytic subunit of the SAGA complex mediating acetylation of target histones. It is not essential for the viability of the yeast cells. The crystal structure of the enzyme has been resolved and mutational studies revealed the residues important for its catalytic function as well as the regions binding to histone substrates (Trievel *et al.* 1999). The bromodomain of Gcn5 is important for its function (Marcus *et al.* 1994) and within the bromodomain, Pro371 is the key residue for histone H4 recognition (Pizzitutti *et al.* 2006). Yeast Gcn5 preferentially acetylates free histone H3 at lysine 14 and with somewhat lower efficiency lysines 8 and 16 of histone H4 (Kuo *et al.* 1996). However, in the context of the ADA HAT

complex, H3 is acetylated by Gcn5 on lysine 18 as well and the same catalytic subunit in the SAGA complex acetylates all four lysine residues that are commonly acetylated on H3, i.e. lysine 9, 14, 18, and 23 (Grant *et al.* 1999). Finally, Gcn5 alone is unable to acetylate nucleosomal histones (Grant *et al.* 1997); a role of Gcn5 in regulation of DNA replication has been suggested (Burgess *et al.* 2010; Espinosa *et al.* 2010) and in mammalian cells, GCN5 has been shown to participate in the nucleotide excision pathway (Guo *et al.* 2011).

The molecular function of Sgf29, protein that has also been identified as a SAGA component (Sanders *et al.* 2002) and makes part of the HAT/Core module (Lee *et al.* 2011), is probably to recruit SAGA to target sites and mediate histone H3 acetylation (Bian *et al.* 2011). The rat homologue of Sgf29 as a subunit of the rat STAGA HAT complex seems to be involved in malignant transformation mediated by c-Myc (Kurabe *et al.* 2007).

The SPT genes were originally identified as suppressors of Ty or δ insertions in the promoter regions of the yeast *HIS4* and *LYS2* genes (Simchen *et al.* 1984; Winston *et al.* 1984). Spt7 is a highly negatively charged protein that represents the structural core of SAGA and is required for SAGA complex integrity (Grant *et al.* 1997). It also regulates the levels of Spt20 and Ada1 and binds directly Spt8 (Wu and Winston 2002). The Spt7 protein in SAGA is significantly acetylated at multiple lysine residues (Mischerikow *et al.* 2009) and possibly also ubiquitinated (Saleh *et al.* 1998a). Although both Gcn5 and Spt7 contain a bromodomain, only the Gcn5 bromodomain can bind acetylated nucleosomes (Eberharter *et al.* 1999) and is important for nucleosomal acetylation (Sterner *et al.* 1999).

Spt3 and Spt8 proteins participate in the regulation of TBP binding to the TATA box of certain promoters (Dudley *et al.* 1999; Belotserkovskaya *et al.* 2000; Larschan and Winston 2001; Bhaumik and Green 2002; Sermwittayawong and Tan 2006); loss of these proteins does not affect the SAGA structure significantly (Sterner *et al.* 1999). Spt8 binds directly to TBP and the binding is mediated by the C-terminal WD40 repeats of Spt8 (Sermwittayawong and Tan 2006). A study demonstrated in an cross-linking assay *in vivo* that Spt3 directly contacts TBP as well (Mohibullah and Hahn 2008).

Ada1 is a subunit required for SAGA complex integrity, together with Spt7 and Spt20 (Marcus *et al.* 1996; Sterner *et al.* 1999). Deletion of any of these subunits leads to disruption of the SAGA complex and growth defects (Grant *et al.* 1997).

Ada1, Spt7 and Spt20 are required for SAGA recruitment to SAGA-dependent promoters (Bhaumik and Green 2002). Within SAGA, Ada1 is located in the proximity of the TBP binding site (Sermwittayawong and Tan 2006).

Ada2 and Ada 3 have been found to markedly enhance the ability of Gcn5 to acetylate nucleosomes and to form the catalytic core of the SAGA complex (Candau and Berger 1996; Candau *et al.* 1997; Syntichaki and Thireos 1998; Balasubramanian *et al.* 2002). Gcn5 and Ada3 are bound together by Ada2, forming thus a heterotrimeric complex (Horiuchi *et al.* 1995). Moreover, Ada2 binds telomeric DNA and, probably in the context of the SAGA complex, is involved in transcriptional silencing (Jacobson and Pillus 2009).

Protein Chd1 is a member of the chromodomain family (Chd stands for <u>c</u>hromo domain, an ATPase/<u>h</u>elicase and a <u>D</u>NA binding segment (Delmas *et al.* 1993)) with two chromodomains and other conserved regions (Flanagan *et al.* 2007). Chd1 recognises di- and trimethylated histone H3 through one of the chromodomains (Pray-Grant *et al.* 2005), possesses DNA- and nucleosome-stimulated ATPase activity and probably acts as a nucleosome remodelling factor (Tran *et al.* 2000), but may also be involved in the negative regulation of DNA replication (Biswas *et al.* 2008). The histone acetyltransferase activity of the SAGA complex in the *chd1* deletion strain is compromised (Pray-Grant *et al.* 2005); however, in another study, Chd1 was not co-purified with SAGA (Lee *et al.* 2011).

The essential Tra1 protein is the largest SAGA subunit (Grant *et al.* 1998b; Saleh *et al.* 1998b) that mediates the interaction with acidic activation domains and its loss results in transcription defects and decreased acetylation levels at genes regulated by the transcription activator protein Gcn4 (Brown *et al.* 2001). In addition to SAGA, Tra1 is also present in the NuA4 HAT complex (Allard *et al.* 1999) and in the SLIK/SALSA complex, but was not found to make part of the ADA complex (Grant *et al.* 1998b). The 433-kDa Tra1 protein contains a C-terminal phosphatidylinositol-3-kinase (PI3K) domain that is important for transcriptional regulation (Mutiu *et al.* 2007) and the FAT domain (Malmstrom *et al.* 2007). Interestingly, a *tra1*Δ mutant is viable in *Schizosaccharomyces pombe* and in cells with deleted gene TRA1, SAGA complex assembles but is only recruited to a part of SAGA-dependent promoters (Helmlinger *et al.* 2011). Tra1 is homologous to mammalian TRRAP, which is found in the human STAGA complex and which is almost identical to the PAF400 subunit from the human PCAF complex (Vassilev *et*

al. 1998). Tra1 directly binds transcriptional activators (Bhaumik *et al.* 2004; Fishburn *et al.* 2005; Reeves and Hahn 2005) and possibly regulates the activity of Ada molecules (Hoke *et al.* 2008). Other results point towards the implication of Tra1 in the cellular stress response, membrane sorting/protein trafficking and mitochondrial function (Hoke *et al.* 2008).

The subset of Taf (TATA-binding protein (TBP)-associated factors) proteins (Taf5, Taf6, Taf9, Taf10 and Taf12) (formerly named Taf90, Taf60, Taf17, Taf25, and Taf61/68, respectively (Tora 2002)) are essential proteins shared by SAGA and TFIID (Grant *et al.* 1998a). Together they form the SA_TAF module of SAGA (Lee *et al.* 2011). Deletion of the TAF5, TAF10 and TAF12 gene affects SAGA integrity (Grant *et al.* 1998a; Durso *et al.* 2001; Kirschner *et al.* 2002). Taf6 bears sequence similarity to the histone H4 (Kokubo *et al.* 1994). Taf5 is required for SAGA subunit abundance and overall complex activity (Durso *et al.* 2001) and Taf12 is required for nucleosomal histone acetyltransferase activity (Grant *et al.* 1998a). Taf10 is important for efficient transcription of certain genes (Kirchner *et al.* 2001). Taf6, Taf9 and Taf12 have been demonstrated to be differentially required for TBP binding to specific promoters indicating that various promoters show different degrees of dependance on TBP-associated factors from the SAGA complex (Bhaumik and Green 2002).

Sca7/Sgf73 is, similarly as Spt7, acetylated at multiple lysine residues (Mischerikow *et al.* 2009). It is a homologue of the human ataxin-7 protein which is, in its polyglutamine expanded pathological form, involved in the development of the neurodegenerative disease spinocerebellar ataxia. Interestingly, human ataxin-7 can complement for the loss of yeast Sca7; however, when present in its expanded form, ataxin-7 causes defects of SAGA integrity and histone acetylation function (McMahon *et al.* 2005). The function of Sgf73 in SAGA consists in anchoring Ubp8, Sgf11 and Sus1 subunits into the complex (Lee *et al.* 2009; Bonnet *et al.* 2010).

Sus1, Sgf11 and Ubp8 can form a stable complex that can dissociate from SAGA under high $MgCl_2$ conditions and they constitute a specific functional module mediating histone H2B deubiquitination (Ingvarsdottir *et al.* 2005; Kohler *et al.* 2006) anchored to SAGA via Sgf73 (Lee *et al.* 2009). Removal of the ubiquitin moiety from H2B contributes to the transcriptional activation, probably by means of trimethylation of lysine 4 on H3 (Daniel *et al.* 2004). Ubp8 is the subunit responsible for H2B deubiquitination (Henry *et al.* 2003; Daniel *et al.* 2004); its human counterpart,

USP22, possesses a deubiquitination activity too and can contribute to cellular transformation (Zhang *et al.* 2008b). Sgf11 is a small (11 kDa) SAGA subunit (Powell *et al.* 2004) that also participates in H2B deubiquitination (Ingvarsdottir *et al.* 2005). The N-terminal part of Sgf11 binds Sus1 (Ellisdon *et al.* 2010). Sus1 is required for SAGA-dependent H2B deubiquitination and maintenance of H3 methylation (Kohler *et al.* 2006). Moreover, Sus1 has been associated with nuclear mRNA export (Fischer *et al.* 2004; Rodriguez-Navarro *et al.* 2004; Cuenca-Bono *et al.* 2010; Cuenca-Bono *et al.* 2011).

The three-dimensional model and molecular composition of the complex has been characterised to high resolution (Wu *et al.* 2004) and is schematically represented on Figure 6.

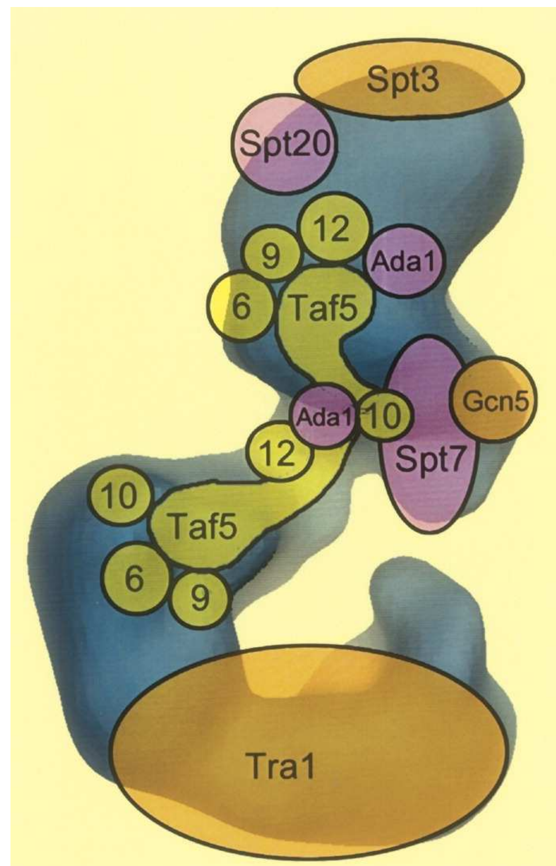


Figure 6: Schematic representation of the localisation of SAGA subunits within the complex. The upper right part of the scheme containing Spt3 (and presumably Spt8) is involved in the interaction of SAGA with TBP (TATA box-binding protein). Subunits Spt7, Ada1 and Spt20 are essential for the complex integrity, Gcn5 is the catalytic subunit. Tra1 interacts with various transcriptional activators. The name of most of the Taf proteins has been shortened to their identification number, e.g. 10 for Taf10 etc. Adopted from (Wu *et al.* 2004).

subunit	size (kDa)	function	human homologue
Gcn5	53	catalytic subunit	PCAF, hGCN5L
Ada1 (Hfi1)	55	complex integrity	STAF42
Ada2 (Swi8)	51	modulation of Gcn5 HAT activity	hAda2, ADA2B
Ada3 (Ngg1)	79	modulation of Gcn5 HAT activity	hAda3, ADA3
Spt3	39	interaction with TBP	hSpt3, SPT3L, SUPT3H
Spt7	152	complex integrity	STAF65γ
Spt8	66	TBP binding	unknown
Spt20 (Ada5)	68	complex integrity	hSPT20
Taf5	90	SAGA activity and abundance of other subunits	TAF_{II}100, PAF65β
Taf6	58	TBP binding at certain promoters	TAF_{II}80, PAF65α
Taf9	17	TBP binding at certain promoters	TAF_{II}32/31
Taf10	23	required for transcription of a subset of genes	TAF_{II}30
Taf12	61	required for nucleosomal HAT activity	TAF_{II}20/15
Tra1	433	interaction with activators	TRRAP, PAF400
Ubp8	54	H2B deubiquitination	USP22
Chd1	168	chromatin remodelling	CHD1
Sus1	11	H2B deubiquitination, H3 methylation	ENY2
Sgf11	11	transcription initiation / regulation	ATXN7L3
Sgf29	29	unknown	STAF36
Sgf73/Sca7	73	important for HAT activity and integrity; anchoring of the deubiquitination subcomplex	ataxin-7

Table 2: Subunits of the yeast SAGA complex and their homologues in human HATs. See the text for detailed description and references of each subunit´s function within the SAGA complex. In the case that two names of a human homologue are indicated, they represent the same protein with alternative names.

SAGA is recruited to the target promoters through interactions with sequence-specific DNA-bound transcriptional activators such as Gal4 (Utley *et al.* 1998; Vignali *et al.* 2000; Bhaumik and Green 2001) and this leads to the subsequent formation of the preinitiation complex and to the translation initiation. The recruitment of SAGA to DNA-bound activators is enhanced by the 19S regulatory particle of the proteasome and this interaction also stimulates SAGA HAT activity (Lee *et al.* 2005). This regulatory mechanism seems to be dependent on ATPases associated with the

19S proteasome particle and in this way the 19S subcomplex contributes to the transcription complex assembly (Malik *et al.* 2009).

Other studies showed that ubiquitination and deubiquitination may be also involved in transcriptional activation (Henry *et al.* 2003; Daniel *et al.* 2004). Given that the SAGA subcomplex Ubp8-Sgf11-Sus1 exhibits the deubiquitination activity and that subsequent ubiquitination and deubiquitination steps occur during transcription (Henry *et al.* 2003), this SAGA catalytic function may be involved in translational regulation as well in addition to histone acetylation.

4.2.5.2 SLIK/SALSA

SLIK (from SAGA-like) is a variant of the SAGA complex occuring during the retrograde response to mitochondrial dysfunction (Pray-Grant *et al.* 2002). The loss of functional mitochondria triggers an intracellular signaling pathway leading to the activation of various stress response genes inducing metabolic changes that compensate for the dysfunction of the electron transport chain. The SLIK histone acetyltransferase complex differs from SAGA by the absence of Spt8, presence of Rtg2 protein and of an altered form of Spt7 and potentially other proteins. However, functions of both SAGA and SLIK seem to be partially overlapping.

The SLIK complex has been also described as SALSA (SAGA altered, Spt8 absent). The loss of Spt8 is presumably due to the fact that Spt8 binds the C-terminal part of Spt7 that is deleted in the truncated form (Sterner *et al.* 2002; Wu and Winston 2002). The Spt7 C-terminal truncation occurs probably early in biosynthesis of Spt7-containing complexes and is partially dependent on the presence of functional ubiquitin protease Ubp8 (Hoke *et al.* 2007). The cleavage site was mapped to the position L1141 (Mischerikow *et al.* 2009) and Pep4 is the protease responsible for the cleavage (Spedale *et al.* 2010). Rtg2 is a subunit required for SLIK integrity since its loss brings about the dissociation of the complex (Pray-Grant *et al.* 2002).

H3 and H2B are histones that are acetylated by the SLIK complex. SLIK preferentially acetylates histone H3 with methylated lysine 4, which is recognised by the chromodomain-containing subunit Chd1 (Pray-Grant *et al.* 2005).

4.2.5.3 ADA

ADA is another Gcn5-containing yeast histone acetyltransferase complex. ADA HAT shares certain subunits with the SAGA complex and its molecular weight is about 0,8 MDa. Gcn5 represents the catalytic subunit (Grant *et al.* 1997) and adapter proteins Ada2 and Ada3 are also present, forming together with Sgf29 the HAT/Core module, shared by SAGA and ADA (Lee *et al.* 2011). Ahc1 is the subunit that maintains the complex integrity and its deletion leads to the complex disintegration (Eberharter *et al.* 1999). Recently, protein Ahc2 has been shown to represent an ADA complex subunit (Lee *et al.* 2011). Importantly, ADA contains none of the Spt proteins and Ada1 or Tra1 does not make part of this complex either (Grant *et al.* 1998b; Eberharter *et al.* 1999; Sterner *et al.* 1999).

Although several subunits are shared by SAGA and ADA, these two complexes doesn´t appear to play the same role in the yeast cell. In contrast to the SAGA complex, ADA does not interact with the acidic activators Gcn4 and VP16 (Utley *et al.* 1998). The lysine acetylation pattern is also different to that of SAGA and unlike ADA, deletion of the Gcn5 bromodomain only reduced the nucleosomal acetylation by SAGA (Grant *et al.* 1999). Due to the absence of the Spt proteins, ADA probably does not bind TBP. A hypothesis suggested that ADA represents a subcomplex of SAGA (Grant *et al.* 1998c), but nowadays, ADA has been recognised as a distinct HAT complex with specific functions and activities (Eberharter *et al.* 1999).

4.2.6 Mammalian histone acetyltransferases

In humans, about a hundred of transcriptional coactivators and corepressors together with about 2000 transcription factors ensure a well-regulated and tissue-specific transcriptional control (Messina *et al.* 2004). Histone acetyltransferase complexes are generally very well conserved from yeast to higher eukaryotes and therefore we can often find mammalian counterparts of yeast HAT complexes. p300/CBP, PCAF and STAGA are the best characterised mammalian histone acetyltransferase complexes. Similarly to yeast, some of the mammalian HAT complexes seem to have redundant and overlapping functions but this probably only reflects the complexity and combinatorial potential of the involvement of transcriptional regulators in mammalian transcription control.

Taking into account the significancy of histone acetylation for the transcriptional regulation, histone acetyltansferase activity is particularly important for gene expression in embryonic development. In general, histone acetyltransferase and deacetylase inhibitors interfere with normal embryogenesis. Histone acetylation also seems to be involved in the development of cancer and other diseases since the role of various histone acetyltransferase complexes in certain human disorders and pathologies has been suggested (Ionov *et al.* 2004; Iyer *et al.* 2004b; Kurabe *et al.* 2007; Zhang *et al.* 2008a).

4.2.6.1 p300/CBP

p300 and CBP are two distinct mammalian acetyltransferases which share a high degree of similarity (Arany *et al.* 1994) and also their roles are largely interchangeable; however, in certain physiological processes, they execute different and unique functions. Therefore, they are referred to as p300/CBP. p300 was first described as a nuclear protein binding the adenovirus E1A oncoprotein (Eckner *et al.* 1994) and CBP was originally found to bind CREB (cAMP response element-binding protein) phosphorylated by proteinkinase A (Chrivia *et al.* 1993).

p300/CBP serve as transcriptional coactivator, not binding directly to DNA but acting in cooperation with DNA-bound proteins at specific promoters. There are many transcriptional factors interacting with p300/CBP, including c-Jun (Bannister *et al.* 1995), c-Fos (Bannister and Kouzarides 1995), c-Myb (Dai *et al.* 1996; Oelgeschlager *et al.* 1996), p53 (Lill *et al.* 1997), YY1 (Lee *et al.* 1995; Austen *et al.* 1997), Stat2 (Bhattacharya *et al.* 1996), CREB (Chrivia *et al.* 1993) or TBP (Dallas *et al.* 1997). The total number of p300/CBP interaction partners in mammalian cells most probably exceeds 400 (for a review see (Bedford *et al.* 2010)).

p300/CBP contain a bromodomain, three cysteine/histidine-rich (CH) domains and N- and C-terminal transactivation domains (see Figure 7). The transactivation domains can interact with transcriptional activators and other components of the preinitiation complex and transactivate transcription at target promoters. The central acetyltransferase domain is crucial for the catalytic function and is evolutionary well conserved also in plant orthologs of p300/CBP (Bordoli *et al.* 2001). In addition to the acetyltransferase activity, p300/CBP posess intrinsic ubiquitin ligase activity

(Grossman *et al.* 2003; Linares *et al.* 2007) that seems to be exclusively cytoplasmic (Shi *et al.* 2009).

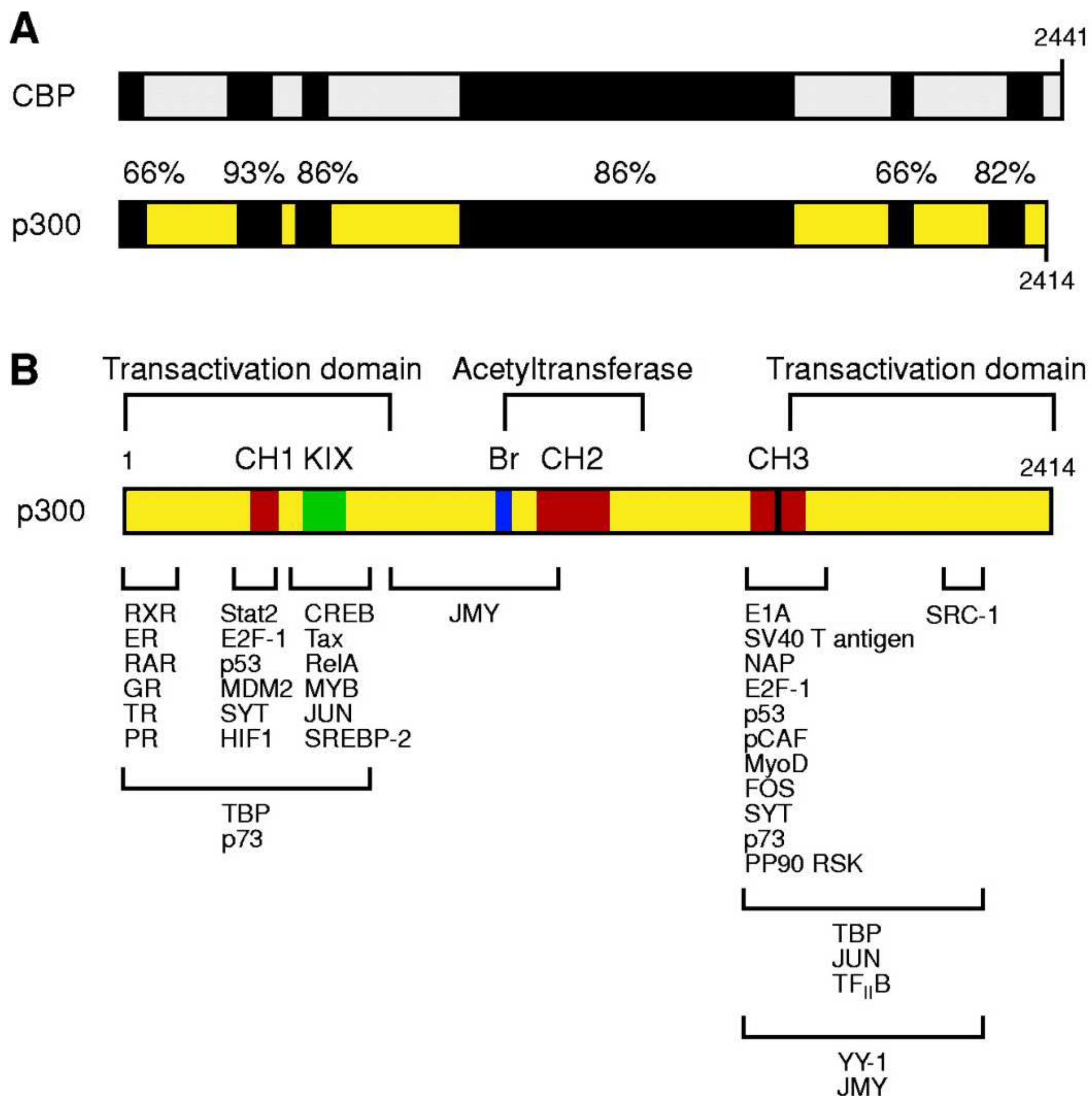


Figure 7: (A) Schematic comparison of CBP and p300 protein sequences. Percents indicate the amino acid identity in certain regions of both molecules.
(B) Schematic structure of p300 showing functional domains and cellular interacting partners. N- and C-terminal transactivation domain and the central acetyltransferase domain together with corresponding interacting transcription factors are indicated. Adapted from (Chan and La Thangue 2001).

Recent results confirmed the essential role of p300 in human keratinocyte differentiation and showed that p300 but not CBP knockdown leads to decreased protein p53 stability (Wong *et al.* 2010). Other study demonstrates that the rate of protein p53 turnover depends on the ubiquitin ligase activity mediated by CBP (Shi

et al. 2009). Both p300 and CBP thus apparently contribute to the tightly balanced p53 level and activity and these results underlie the hypothesis of involvement of p300/CBP in cancer. p300/CBP appear to be mutated in colon cells with microsatellite instability (Ionov *et al.* 2004) and in other disorders including leukaemia, breast cancer or pancreatic cancer (Borrow *et al.* 1996; Muraoka *et al.* 1996; Gayther *et al.* 2000), although the mutations are not very common (Bryan *et al.* 2002; Ozdag *et al.* 2002). Given that p300/CBP interact with a large number of cellular transcription factors including known oncoproteins (c-Jun, c-Fos), tumour suppressors (p53, p73, E2F-1) or transforming viral proteins (E1A, large T antigen), they constitute an important node in the protein interaction network and most probably can indeed influence transcription during the key steps of cancer progression. Although p300/CBP mutations do not appear to play a significant role (Ozdag *et al.* 2002), any dysbalance in the p300/CBP regulation or function may have an impact on cellular homeostasis and may result in malignant transformation.

4.2.6.2 PCAF (p300/CBP-associated factor)

Originally identified as the factor competing with E1A for binding to p300/CBP (Yang *et al.* 1996), mammalian PCAF displays striking similarity to the yeast acetyltransferase Gcn5 and also to human hGCN5L. It is found in a multisubunit protein complex containing more than 20 associated polypeptides (Ogryzko *et al.* 1998). The known subunits of the PCAF complex are $TAF_{II}31$, $hTAF_{II}30$, $hTAF_{II}20$ and $hTAF_{II}15$, PAF65α that contains histone H4-like regions and PAF65β, hSPT3, hADA2 and PAF400 as the largest subunit (Ogryzko *et al.* 1998; Vassilev *et al.* 1998). Most of the PCAF complex subunits represent mammalian counterparts of the components of the yeast SAGA complex. The PCAF HAT can be found within the SAGA complex in humans (Krebs *et al.* 2010) and is associated with actin in human cells (Obrdlik *et al.* 2008).

Unlike hGCN5L, the gene encoding PCAF is not essential for viability (Schiltz and Nakatani 2000). Studies on knockout mice showed that both genes are regulated independently and they play distinct roles in embryogenesis (Yamauchi *et al.* 2000). In addition to histone acetylation, PCAF acetylates various non-histone proteins including p53 (Sakaguchi *et al.* 1998; Liu *et al.* 1999), poly-ADP-ribose polymerase-2 (Haenni *et al.* 2008), c-Abl (di Bari *et al.* 2006), cyclin-dependent kinase 2 (Mateo *et*

al. 2009b) or β-catenin (Ge *et al.* 2009). Certain results point towards the novel function of PCAF in cell cycle regulation through acetylation of cyclin A (Mateo *et al.* 2009a). PCAF itself is methylated *in vivo* (Masatsugu and Yamamoto 2009).

4.2.6.3 STAGA

STAGA is a human transcriptional coactivator complex with Gcn5 as the catalytic subunit. The STAGA complex is highly related to the yeast SAGA complex and certain STAGA subunits share significant homology with their yeast counterparts in SAGA. In this way, hSPT3 (also known as SPT3L or SUPT3H), hADA2, hADA3, hGCN5L, TAF5, TAF6, TAF9, TAF10, TAF12, STAF65γ (similar to yeast Spt7), STAF42 (similar to yeast Ada1), STAF36 (homologous to yeast Sgf29), ataxin-7 (homologue of yeast Sgf73), USP22 (homologue of Ubp8), ENY2 (Sus1 homologue), ATXN7L3 (homologue of Sgf11) or TRRAP (homologue of Tra1) can be found in the STAGA complex (Candau *et al.* 1996; Yu *et al.* 1998; Martinez *et al.* 2001; Helmlinger *et al.* 2004; Kurabe *et al.* 2007; Zhao *et al.* 2008); the exception is yeast Spt8, having no known homologue in the human complex (Martinez *et al.* 2001; Sterner *et al.* 2002). More recently, hSPT20, a human homologue of yeast Spt20, was identified and it was shown that, similarly to its yeast counterpart, this protein is required for the STAGA complex integrity (Nagy *et al.* 2009).

STAGA preferentially acetylates nucleosomal histone H3 and in addition to the histone acetylation catalytic function, the complex can be involved in DNA repair or mRNA splicing as well (Martinez *et al.* 2001). STAF65γ, the homologue of Spt7 in SAGA, is required for the STAGA complex integrity (Liu *et al.* 2008). Analogously to SAGA, the deubiquitination activity of the STAGA subcomplex ATXN7L3-USP22-ENY2 (human homologues of Sgf11, Ubp8 and Sus1, respectively) specifically removes the ubiquitin moiety from histones H2A and H2B and facilitates transcription (Zhang *et al.* 2008a; Zhao *et al.* 2008). Novel results indicate that USP22 influences the turnover of TRF1 protein and consequently affects telomere stability (Atanassov *et al.* 2009) and this finding could illuminate the results of the in-depth microarray analysis putting USP22 on the list of 11 genes associated with poor prognosis of cancer patients (Glinsky *et al.* 2005).

As it was already mentioned, ataxin-7 that makes part of the STAGA complex (Helmlinger *et al.* 2004) can be found in the polyglutamine expanded form that

contributes to the spinocerebellar ataxia. Taking into account that the STAGA complex loses its nucleosomal HAT function after the incorporation of the expanded form of ataxin-7, it can be speculated that altered HAT function as a consequence of polyglutamine expansion of ataxin-7 may be involved in the pathogenesis of spinocerebellar ataxia or other neurodegenerative diseases (Palhan *et al.* 2005).

One of the transcription factors that functions through association with STAGA is c-Myc. STAGA subunits TRRAP, GCN5L and STAF65γ have been shown to interact with c-Myc at target promoters (McMahon *et al.* 1998; McMahon *et al.* 2000; Liu *et al.* 2008). This interaction is mediated by the coactivator complex Mediator and the STAGA complex contributes to the transcriptional activation by acetylating chromatin histones and probably also by facilitating the recruitment of TFIID and RNA polymerase II (Liu *et al.* 2008). Interestingly, due to the evolutionary conservation of the histone acetyltransferase complexes among eukaryotes, both SAGA and Ada complex have been shown to interact with c-Myc in the yeast cells and activate transcription at certain promoters (Flinn *et al.* 2002).

4.2.6.4 ATAC

The ATAC complex is specific for higher eukaryotes and is not present in yeast cells; it emerged from SAGA and differs in the presence of the ADA2A protein variant while SAGA (or its human homologue STAGA) contains ADA2B (Guelman *et al.* 2006; Spedale *et al.* 2012). ATAC harbours two histone acetyltransferases: ATAC2 that also maintains the integrity of STAGA (Guelman *et al.* 2009) and either GCN5 or PCAF in a mutually exclusive manner (Nagy *et al.* 2010). Furthermore, ATAC is composed of other proteins: ADA3, STAF36, WDR5, POLE3/CHRAC17, POLE4, TAK1/MAP3K7, MBIP, the YEATS2-NC2β histone fold module that interacts with TBP and other cofactors (Wang *et al.* 2008). ATAC acetylates histone H3 and H4 (Suganuma *et al.* 2008; Nagy *et al.* 2010) and has been shown to localise to the mititic spindle and to control the cell cycle progression through cyclin A acetylation (Orpinell *et al.* 2010).

4.2.7 Association and interaction of human histone acetyltransferases with the tumour suppressor p53

As it was already mentioned, the tumour suppressor p53 belongs to the non-histone protein targets of various human histone acetyltransferase complexes; indeed, p53 has been found to be physically associated with various histone acetyltransferase complexes (Iizuka and Stillman 1999; Berger 2002; Coutts and La Thangue 2005). As a sequence-specific transcription factor, p53 is recruited to the promoters of target genes to activate transcription after various stimuli such as DNA damage or cellular stress; however, p53 can also act as a transcriptional repressor (Ho *et al.* 2005; Spurgers *et al.* 2006; Jurvansuu *et al.* 2007).

Similarly as in the case of c-Myc interaction with histone acetyltransferases, the chromatin remodelling complexes are likely to participate in the transcriptional activation of certain promoters by p53 as well. Consistently with this, Xiao *et al.* showed that the region of DNA protected by p53 binding appears to be larger than expected (Xiao *et al.* 1998) and this observation could result from the simultaneous binding of a larger protein complex, possibly modifying the chromatin structure and facilitation transcription.

Lill *et al.* (1997) proved that p300/CBP and p53 form a stable DNA-binding complex and that p300/CBP act as a transcriptional adapter for p53. The binding of p300 to p53 is dependent on the consensus p53 target DNA sequence (Dornan *et al.* 2003b), occurs through the proline repeat domain at the N-terminus of p53 and is crucial for p53 acetylation (Dornan *et al.* 2003a). Furthermore, association of p300 with p53 can protect the tumour suppressor against the binding of other interacting proteins, e.g. Mdm2 (Teufel *et al.* 2007).

According to a study by Gamper and Roeder (2008), STAGA directly binds the tumour suppressor p53 both *in vitro* and *in vivo* and this interaction is induced by UV irradiation (Gamper and Roeder 2008). The STAGA subunits found in this study to be directly interacting with p53 are GCN5L, ADA2b and hTAF9. hADA3 association with p53 has been reported previously (Wang *et al.* 2001b).

The biological effects attributed to the association of p53 with histone acetyltransferase complexes are not unambiguous. Some authors describe the activation of transcription resulting from the recruitment of histone acetyltransferases to certain promoters by p53 (Barlev *et al.* 2001; Espinosa and Emerson 2001). For

example, the transactivation of the *mdm2* promoter by p53 requires TRRAP, a component shared by several mammalian histone acetyltransferase complexes; this effect correlates with an increased level of histone acetylation, acetylation of p53 is, however, not required for *mdm2* transcription (Ard *et al.* 2002). In contrast, Barlev *et al.* observed a reduction of p53 transcription activation function in its deacetylated state (Barlev *et al.* 2001). According to the latter study, p53 acetylation promotes recruitment of histone acetyltransferase complexes (as shown by the presence of TRRAP and CBP at the p21 promoter) and is crucial for p53 function *in vivo*. This is consistent with the statement of Wang *et al*. that the interaction of p53 with hADA3 is required for full p53 activity (Wang *et al.* 2001b) and with the results of another study showing that Tip60 HAT is important for activation of p21 expression by p53 as well as for p53 stability (Legube *et al.* 2004). Another study confirmed the p300 histone acetyltransferase activity at the p21 promoter (Espinosa and Emerson 2001) indicating that the requirements for specific coactivator complexes at the same promoter may differ.

In general, it seems that modulation of chromatin structure by histone acetyltransferases associated with p53 is important for p53 transcriptional activation function. Nucleosomal acetylation of the p53 target promoter regions possibly keeps the chromatin relaxed and enhances the binding of the transcriptional machinery complexes. However, p53 interaction with histone acetyltransferases at various promoters may represent a mechanism that differs in a gene-specific manner or according to the current condition of the cell.

Interaction of p53 with histone deacetylase HDAC-1 that is probably mediated by the transcriptional corepressor mSin3a and leads to the transcriptional repression has been also reported (Murphy *et al.* 1999). Therefore, the association with histone deacetylases and resulting repression of transcription could provide a compensatory mechanism for the transcriptional activation via histone acetylation in order to achieve appropriate level of transcriptional activity.

4.3 Acute lymphoblastic leukaemia

4.3.1 The disease

4.3.1.1 Disease manifestation, treatment and outcome

Acute lymphoblastic leukaemia (ALL) is a malignant neoplasm characterised by excess of immature lymphoid cells, lymphoblasts, that proliferate uncontrollably and can infiltrate virtually any organ and tissue in the body. Consequently, normal haematopoiesis is suppressed and patients experience anaemia, fatigue, frequent and repeated infections, increased or unexpected bleeding. Generally, a multi-drug treatment regimen is required immediately.

In the first phase of ALL treatment (called „induction therapy“), the aim is to achieve remission. A remission is defined as the absence of the signs of the disease, elimination of leukaemic cells from the blood and the presence of less than 5% leukaemic cells in the bone marrow. Chemotherapy is the first choice of treatment and combinations of drugs used in this first phase include prednisone, vincristine, L-asparaginase, anthracycline or cyclophosphamide. The second phase of treatment is the „consolidation therapy“. In order to further reduce the number of leukaemic cells, patients are treated with chemotherapeutics (that can also be administered into the cerebrospinal fluid) and bone marrow transplantation may be used. However, residual leukaemic cells still can be present in ALL patients and therefore „maintenance therapy“ follows the first two phases of the treatment with low-dose chemotherapy for 2-3 years. Although most patients achieve remission, a relapse can occur and the disease returns. For certain patients after the relapse radiotherapy followed by the bone marrow transplantation may be effective.

Most commonly, ALL affects children aged 2-5 years and another peak of incidence is observed after 50 years of age. The clinical outcome is different in adult and childhood ALL in spite of similar gene expression profiles (Kuchinskaya *et al.* 2005) - treatment of adults is generally less successful. Fortunately, the rate of success in the treatment of paediatric ALL has reached 85 % (Starý 2010) in comparison to 30-50% in adults (Tjonnfjord *et al.* 2007).

The cause of ALL in humans is unclear, but most probably multiple factors such as inherited genetic predispositions, DNA damage by chemicals, radiation or viral infection may play a role in leukaemogenesis.

4.3.1.2 Classification of ALL

ALL is a heterogeneous disease and that means that morphologic, cytogenetic, immunologic, biochemical and molecular genetic characterisation of malignant cells is needed in order to establish the diagnosis and adjust the medication and intensity of therapy. ALL can be classified into subtypes according to the origin of lymphoblasts, to the cell morphology or to immunophenotype.

4.3.1.2.1 The FAB classification

The French-American-British (FAB) classification scheme (Bennett *et al.* 1976) divided ALL into three subtypes (L1, L2, L3) according to the lymphoblast morphology. This system has largely been replaced with more accurate classification.

4.3.1.2.2 Classification according to immunophenotype

This system takes into account the type of lymphocyte giving rise to the leukaemic cells and its degree of differentiation or maturation.

80-85% cases of ALL are of B lineage and these can be further divided into four subtypes: early pre-B ALL, common precursor B ALL, pre-B ALL and mature B cell ALL (Burkitt leukaemia). These subtypes are defined using surface or cytoplasmic markers-e.g. CD10, CD19, CD22, CD79a, surface immunoglobulins or cytoplasmic immunoglobulin μ.

T lineage ALL accounts for approximately 15-20% cases. T-cell markers studied in T-ALL are e.g. CD1a, CD2, CD3 (cytoplasmic in immature thymocytes), CD4 or CD8. T-ALL subtypes are pro-T, pre-T ALL, cortical T ALL and mature T ALL.

4.3.1.2.3 Cytogenetic alterations involved in ALL

Abnormalities in chromosome number or structure are often found in ALL patients. Hyperdiploidy with more than 50 chromosomes is mostly present in paediatric ALL and generally predicts favorable outcome. On the contrary, hypodiploidy (less than 44 chromosomes) is associated with poor prognosis.

One of the most commonly found chromosomal translocations is t(12;21)(p13;q22) resulting in the *TEL-AML1* (also known as *ETV6-RUNX1*) gene fusion. Another translocation, t(4;11)(q21;q23) giving rise to the fusion of *MLL* and *AF4* genes, is a hallmark chromosomal aberration in infant ALL with poor prognosis. The so-called „Philadelphia“ translocation t(9;22)(q34;q11), leading to the *BCR-ABL1* fusion, generates a chimeric tyrosine kinase oncogene that is mostly present in adult ALL cases. This abnormality generally predicts worse response to the induction therapy.

Although there is a clear evidence of involvement of certain fusion genes in pathogenesis of ALL, in approximately 10% of paediatric and 30% of adult ALL cases no chromosomal abnormalities are detected by currently known methods (Kebriaei *et al.* 2003).

4.3.2 *HOX* genes and their role in human haematopoietic disorders

4.3.2.1 The essential *HOX* genes

The evolutionary well-conserved *HOX* genes form a subgroup of the homeobox gene superfamily and encode transcription factors acting as key regulators of development. Spatiotemporally controlled expression of these genes in a developing embryo is essential for the correct morphogenesis of the anterioposterior axis. *HOX* genes contain a DNA sequence motif called the homeobox that encodes a 60-aa DNA-binding domain referred to as the homeodomain. The sequence and three-dimensional structure of the homeodomain is highly conserved and so are the Hox proteins. Their functional conservation has been demonstrated by an experiment in which a chicken Hox protein can complemented for the function of its knocked-out ortholog in *Drosophila* (Lutz *et al.* 1996).

In mammals, 39 *HOX* genes are arranged into four clusters (A, B, C and D) that map to four different chromosomes and appear to be a result of a duplication over evolutionary time. The corresponding *HOX* genes between the clusters (e.g. A4, B4, C4, D4 called paralogs) are more related than adjacent genes within one cluster (e.g. A3, A4, A5). The synergism of mutations of paralogous *HOX* genes in contrast to the relatively mild effect of mutations in each paralogous gene alone indicates the

probability of functional redundancy among certain *HOX* genes (Favier *et al.* 1996; Fromental-Ramain *et al.* 1996).

The position of *HOX* genes within the cluster roughly corresponds to the order in which they are expressed during the development and also reflects the localisation of their gene product along the body axis of the embryo. In general, earlier genes are located at the 3´end are active in the anterior part of the embryo and genes from the 5´end pattern the posterior of the embryo at later time points (Dressler and Gruss 1989; Izpisua-Belmonte *et al.* 1991; Gaunt and Strachan 1996) (Figure 8).

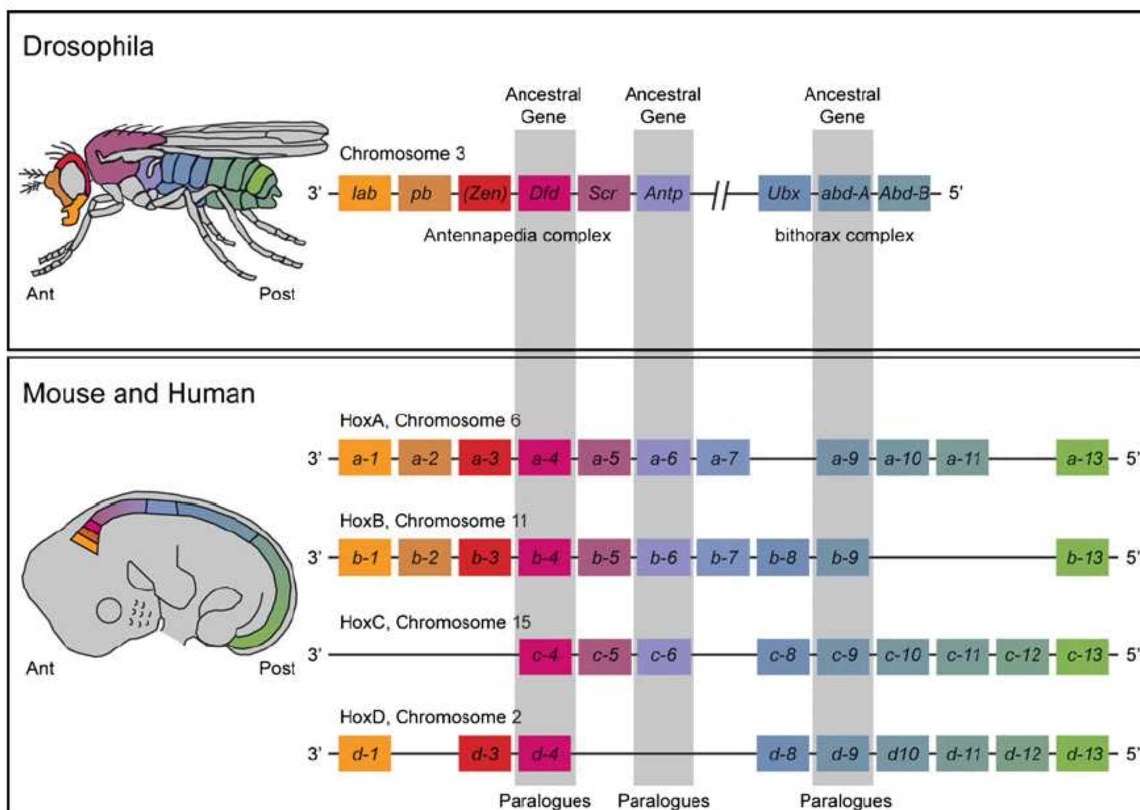


Figure 8: Human and mouse *HOX* genes with their phylogenetic counterparts in *Drosophila.* In humans, the *HOXA-D* clusters are located on chromosomes 7, 17, 12 and 2, respectively (adopted from (Pang and Thompson 2011)).

4.3.2.2 *HOX* genes in human congenital malformations and haematopoietic disorders

Despite their crucial role in the development of the embryo, mutations of human *HOX* genes usually do not cause severe developmental defects as they do in *Drosophila*. This may be explained by the above-mentioned functional redundancy

of *HOX* genes in vertebrates. Certain mutations in *HOX* genes have been described yet in humans, being the cause of congenital malformations synpolydactyly (*HOXA13*) or the hand-foot-genital syndrome (*HOXD13*) (Muragaki *et al.* 1996; Mortlock and Innis 1997).

Furthermore, especially genes from the *HOXA* and *HOXB* cluster are important in normal haematopoiesis. Typically, the expression of *HOX* genes gradually decreases in the course of haematopoiesis, with a high level of HOX expression in haematopoietic stem cells and undetectable expression in differentiated cells (Magli *et al.* 1991; Sauvageau *et al.* 1994). Deregulated or aberrant expression of certain *HOX* and non-*HOX* homeotic genes have thus been associated with various haematopoietic malignancies. For example, overexpression of *HOXA10* in murine bone marrow cells disturbs differentiation of myeloid and B-lymphoid progenitors and leads to acute myeloid leukaemia (AML) (Thorsteinsdottir *et al.* 1997). Another study provides evidence that the t(4;11) translocation is associated with increased expression of *HOXA9* and a non-*HOX* gene *MEIS1* (Rozovskaia *et al.* 2001). In T-ALL with the *MLL* gene rearrangement, increased levels of *HOXA9, HOXA10* and *HOXC6* have been detected (Ferrando *et al.* 2003). Importantly, in two consecutive studies, Drabkin *et al.* showed that HOX gene espression patterns may be relevant for AML leukaemia patients clinical outcome, where low overall *HOX* and especially *HOXA9* gene expression predicted better prognosis (Drabkin *et al.* 2002; Andreeff *et al.* 2008)

In addition to this, chromosomal translocations involving homeotic genes are implicated in the development of leukaemia. *HOXA9* or *HOXD13* gene fusion with the *NUP98* nucleoporin gene result in a rare case of AML. Fusion of the homeotic gene *PBX1* to the transcription factor E2A can cause pre- and pro-B cell ALL (Nourse *et al.* 1990; Kamps and Baltimore 1993).

4.3.3 IL-1 and human haematopoietic disorders

It is now generally accepted that inflammatory processes underlie a vast variety of human diseases and exacerbate the progress of both immune and non-immune diseases. It is therefore not surprising that due to its numerous proinflammatory activities, interleukin-1 appears to be involved in the development of leukaemia.

Indeed, IL-1 has been reported to be spontaneously produced from B cells of chronic lymphocytic leukaemia (Pistoia *et al.* 1986), T-cell leukaemia cells (Shirakawa *et al.* 1989), myeloma cells (Yamamoto *et al.* 1989) or cells from patients with AML (Griffin *et al.* 1987; van der Schoot *et al.* 1989).

According to the literature sources, IL-1 has been studied mostly in the relation to AML. IL-1α and IL-1β may contribute to AML cell proliferation (Cozzolino *et al.* 1989) most probably because they stimulate endothelial cells to produce GM-CSF and G-CSF, colony stimulating factors important for the growth of AML cells (Griffin *et al.* 1987). The spontaneous IL-1 production from myeloid cells, as well as the AML cell proliferation, can be reduced by addition of IL-1Ra or soluble IL-1 receptors (Rambaldi *et al.* 1991; Estrov *et al.* 1992). AML cells producing higher IL-1β levels are also more resistant to apoptotic cell death (Turzanski *et al.* 2004).

However, certain studies also exist that evaluate the role of IL-1 in ALL. Similarly to AML, IL-1 has been demonstrated to act as an autocrine growth factor for ALL cells of B-lineage (Mori *et al.* 1994). Soluble IL-1 receptors at lower concentrations increase the proliferative effect of IL-1 on ALL blasts while at higher concentrations, they bring about its inhibition (Carter *et al.* 1994). IL-1 also stimulates the proliferation of leukaemic cells in the testis of pediatric ALL patients and most probably contributes to testicular relapse; this effect of IL-1 appears to be suppressed by Leydig cells (von Euler 2002). A recent study revealed the increased susceptibility to septic shock in children with ALL harbouring certain polymorphisms of the gene *IL1RN* encoding IL-1Ra (Zapata-Tarres *et al.* 2013).

5 MATERIALS AND METHODS

5.1 Yeast strains

For the IL-1α expression, plasmid stability analysis and co-localisation experiments, I was using the standard *Saccharomyces cerevisiae* W303-1a strain (ATCC 208352; obtained from Beate Schwer). For the co-immunoprecipitation experiments addressing the association of the IL-1α precursor with histone acetyltransferase subunits, I was using the *Saccharomyces cerevisiae* BY4741 strain (derived from S288C; ATCC 201388; obtained from Zuzana Storchová) (Brachmann *et al.* 1998). This strain had been used for the construction of the TAP-tagged yeast strain collection where the histidine auxotrophy was used for the selection of the TAP fusion gene-containing transformants (Ghaemmaghami *et al.* 2003). All the yeast strains used throughout this study are summarised in Table 3.

***S. cerevisiae* strain**	**Genotype**	**Reference/Source**
W303-1a	MATa*; ade2-1; can1-100; his3-11,15; leu 2-3,112; trp1-1; ura3-1*	ATCC 208352
TAP/Gcn5	MATa*; leu2-0; met15-0; ura3-0; GCN5-TAP*::*HIS3*	(Ghaemmaghami *et al.* 2003)
TAP/Spt7	MATa*; leu2-0; met15-0; ura3-0; SPT7-TAP*::*HIS3*	(Ghaemmaghami *et al.* 2003)
TAP/Spt8	MATa*; leu2-0; met15-0; ura3-0; SPT8-TAP*::*HIS3*	(Ghaemmaghami *et al.* 2003)
TAP/Ada1	MATa*; leu2-0; met15-0; ura3-0; ADA1-TAP*::*HIS3*	(Ghaemmaghami *et al.* 2003)
TAP/Ada2	MATa*; leu2-0; met15-0; ura3-0; ADA2-TAP*::*HIS3*	(Ghaemmaghami *et al.* 2003)
TAP/Ada3	MATa*; leu2-0; met15-0; ura3-0; ADA3-TAP*::*HIS3*	(Ghaemmaghami *et al.* 2003)
TAP/Ahc1	MATa*; leu2-0; met15-0; ura3-0; AHC1-TAP*::*HIS3*	(Ghaemmaghami *et al.* 2003)
TAP/Ahc1,*gcn5Δ*	MATa*; leu2-0; met15-0; ura3-0; AHC1-TAP*::*HIS3; gcn5Δ*::*kanMX*	B. Zámostná
TAP/Spt8,*gcn5Δ*	MATa*; leu2-0; met15-0; ura3-0; SPT8-TAP*::*HIS3; gcn5Δ*::*kanMX*	B. Zámostná
TAP/Spt7,*gcn5Δ*	MATa*; leu2-0; met15-0; ura3-0; SPT7-TAP*::*HIS3; gcn5Δ*::*kanMX*	B. Zámostná
TAP/Gcn5,*gcn5Δ*	MATa*; leu2-0; met15-0; ura3-0; GCN5-TAP*::*HIS3; gcn5Δ*::*kanMX*	B. Zámostná
TAP/Gcn5,*ahc1Δ*	MATa*; leu2-0; met15-0; ura3-0; GCN5-TAP*::*HIS3; ahc1Δ*::*kanMX*	B. Zámostná
TAP/Spt8,*ahc1Δ*	MATa*; leu2-0; met15-0; ura3-0; SPT8-TAP*::*HIS3; ahc1Δ*::*kanMX*	B. Zámostná
TAP/Spt7,*ahc1Δ*	MATa*; leu2-0; met15-0; ura3-0; SPT7-TAP*::*HIS3; ahc1Δ*::*kanMX*	B. Zámostná
TAP/Ahc1,*ahc1Δ*	MATa*; leu2-0; met15-0; ura3-0; AHC1-TAP*::*HIS3; ahc1Δ*::*kanMX*	B. Zámostná

TAP/Spt8,*spt7Δ*	MATa*; leu2-0; met15-0; ura3-0; SPT8-TAP*::*HIS3; spt7Δ*::*kanMX*	B. Zámostná
TAP/Ahc1,*spt7Δ*	MATa*; leu2-0; met15-0; ura3-0; AHC1-TAP*::*HIS3; spt7Δ*:: *kanMX*	B. Zámostná
TAP/Spt7,*spt7Δ*	MATa*; leu2-0; met15-0; ura3-0; SPT7-TAP*::*HIS3; spt7Δ*:: *kanMX*	B. Zámostná
TAP/Gcn5,*ahc2Δ*	MATa*; met15-0; ura3-0; GCN5-TAP*::*HIS3; ahc2Δ*::*LEU2*	V. Vopálenský
TAP/Spt8,*ahc2Δ*	MATa*; met15-0; ura3-0; GCN5-TAP*::*HIS3; ahc2Δ*::*LEU2*	V. Vopálenský
TAP/Spt7,*ahc2Δ*	MATa*; met15-0; ura3-0; GCN5-TAP*::*HIS3; ahc2Δ*::*LEU2*	V. Vopálenský

Table 3: Yeast strains used in this study.

5.1.1 Culturing and handling yeast strains

All cultivations were done at 28 ℃. I used liquid growth medium YPD and YPD agar plates for growing the non-transformed strains. For selection of strains transformed with recombinant plasmids I used SD (synthetic drop-out) selective medium lacking an appropriate nutrition factor (amino acid or base) depending on the auxotrophy marker specific for each plasmid. For selection of yeast strains harbouring the *kanMX* gene disruption cassette, YPD agar plates containing G418 antibiotic (500 µg/ml) were used. Cells were grown in a shaker at 200 rpm in the liquid growth medium, agar plates were kept in a temperature-controlled incubator. Yeast strains were stored at -70 ℃ in 12.5% glycerol for a long-term storage.

5.1.2 Cultivation media

SD: 1.74 g Yeast Nitrogen Base; 5 g $(NH_4)_2SO_4$; 50 ml 20% glucose; ddH_2O up to 1 l. The SD medium was supplemented with adenine, uracil, leucine, histidine, methionine, lysine, tryptophan (30 mg/l)

SD selective medium: SD medium lacking one or more appropriate nutrients

SD gal: SD medium (lacking one or more amino acids or bases if needed) where glucose was replaced with galactose

YPD: 0,5% yeast autolysate; 1% peptone; 2% glucose

YPD gal: YPD medium where glucose was replaced with galactose

5.1.3 Agar plates

SD: SD medium (lacking one or more amino acids or bases if needed), 2% agar

SD gal: SD medium (lacking one or more amino acids or bases if needed) where glucose was replaced with galactose, 2% agar

YPD: YPD medium, 2% agar (G418 500 µg/ml for G418 plates)

5.2 Bacterial strains

E. coli XL-1 Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10(*Tet^{r}*)*] (Stratagene)

5.2.1 Culturing and handling the bacterial strains

All cultivations were done at 37 ℃. For growing th e bacterial cells for large-scale plasmid DNA isolation, I was using 2xTY liquid growth medium containing an appropriate antibiotic
(ampicilline 100 µg/ml or kanamycin 50 µg/ml). For the selection of colonies harbouring a recombinant plasmid I was using 2xTY agar plates with selective antibiotic.

5.2.2 Cultivation media

2xTY: 1.6% peptone; 1% yeast autolysate; 0.5% NaCl; adjust to pH 7.0 with NaOH; antibiotics in selective media (ampicilline 100 µg/ml or kanamycin 50µg/ml)

5.2.3 Agar plates

2xTY: 2xTY medium, 2% agar; antibiotics (ampicilline 100 µg/ml or kanamycin 50 µg/ml)

5.3 Mammalian cell lines

NIH 3T3 (mouse embryonic fibroblasts, transformed) (ATCC number CRL-1658)
Mrc-5 (human lung fibroblasts, non-transformed) (ATCC number CCL-171)
Saos-2 (human osteosarcoma, p53-deficient) (ATCC number HTB-85)
A375 (human malignant melanoma) (ATCC number CRL-161)
H1299-R273H (human non-small-cell lung carcinoma, stably transfected with pCMV-neo-Bam-p53R273H, expressing mutant p53 not binding to DNA)
K562 (human chronic myelogenous leukaemia; positive for the *BCR/ABL* fusion) (ATCC number CCL-243)
MV4;11 (human acute myelomonocytic leukaemia with t(4;11)) (ATCC number CRL-9591)
NB4 (human acute promyelocytic leukaemia with t(15;17))
RS4;11 (human acute biphenotypic leukaemia with t(4;11)) (ATCC number CRL-1873)

5.3.1 Culturing and handling the mammalian cells

Cells were cultivated in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics/antimycotics (penicillin 100 iu/ml, streptomycin 100 mg/ml) and 2 mM L-glutamine (medium and all suplements were from Gibco) in 5% CO_2 and a humidified atmosphere at 37 ℃. The only exc eption was the RS4;11 cell line that was maintained in the RPMI medium supplemented with

10% fetal calf serum, antibiotics/antimycotics (penicillin 100 iu/ml, streptomycin 100 mg/ml) and 2mM L-glutamine. Confluent adhesive cells were detached by trypsin-EDTA solution (Gibco) and split into 6-cm dishes at 1:3-1:5 depending on the cell line. Non-adhesive cells were split between new bottles according to the requirements of each cell line. For a long-term storage, cells were stored in the DMEM medium containing 20% fetal calf serum and 10% DMSO in liquid nitrogen.

5.4 Recombinant plasmids used throughout this study

For the expression of the IL-1α variants both in yeast and in mammalian cells, I was using various recombinant plasmids that are summarised in Table 4. Together with the comercially available plasmids from Clontech, Ingenius and Invitrogen and plasmids created by myself I also used vectors constructed by Ladislav Burýšek, Jayne Stommel (Stommel *et al.* 1999) and Johannes H. Hegemann (Gueldener *et al.* 1996; Gueldener *et al.* 2002). The expression plasmid pEGFP-C1/IL-1αNTP was constructed by Denisa Dolečková, an undergraduate student from our laboratory, under my supervision.

name	usage	origin
pYX133	protein expression studies in yeast	Ingenius
pYX133N/S IL-1αFull	IL-1α precursor expression in yeast	B. Zámostná
pYX133N/X IL-1αFull	IL-1α precursor expression in yeast	B. Zámostná
pYX212	protein expression studies in yeast	Ingenius
pYX212N/S IL-1αFull	IL-1α precursor expression in yeast	B. Zámostná
pYX212N/X IL-1αFull	IL-1α precursor expression in yeast	B. Zámostná
pYX212N/S IL-1αMat	mature IL-1α expression in yeast	B. Zámostná
pYX212N/X IL-1αMat	mature IL-1α expression in yeast	B. Zámostná
pEGFP-C1	fluorescence microscopy in mammalian cells	Clontech
pEGFP-C1/IL-1αFull	fluorescence microscopy in mammalian cells	B. Zámostná
pEGFP-C1/IL-1αMat	fluorescence microscopy in mammalian cells	B. Zámostná
pEGFP-C1/IL-1αNTP	fluorescence microscopy in mammalian cells	D. Dolečková
pcDNA4C/TO/Myc-His	protein expression studies in mammalian cells	Invitrogen
pcDNA4/TO/FLAGMycHis-IL-1αFull	IL-1α precursor expression in mammalian cells	L. Burýšek
pcDNA4/TO/FLAGMycHis-IL-1αMat	mature IL-1α expression in mammalian cells	L. Burýšek
pcDNA4/TO/FLAGMycHis-IL-1αNTP	IL-1αNTP expression in mammalian cells	L. Burýšek
pDsRED2-C1	fluorescence microscopy in mammalian cells	Clontech
pDsRED2-C1/IL-1αFull	fluorescence microscopy in mammalian cells	B. Zámostná
p53/EGFP	fluorescence microscopy in mammalian cells	J. Stommel
pUG6	gene disruption in yeast	J.H.Hegemann
pUG36	fluorescence microscopy in yeast	J.H.Hegemann
pUG36/IL-1αFull	fluorescence microscopy in yeast	B. Zámostná
pUG36/ IL-1αMat	fluorescence microscopy in yeast	B. Zámostná

Table 4: Recombinant plasmids used throughout this study.

5.5 Protocols

5.5.1 Polymerase chain reaction (PCR)

In all PCR reactions, 10µM primers and 10µM deoxyribonucleotide triphosphates (dNTPs; 10µM dATP, dCTP, dGTP and dTTP in water) were used. Precise quantities of all reagents, temperatures and incubation times are mentioned in each protocol separately. I was using the Biorad C1000™ Thermal cycler or Eppendorf Mastercycler epgradient S apparatus.

5.5.2 Co-immunoprecipitation from yeast cells using a non-denaturing lysis buffer

I was using co-immunoprecipitation for the confirmation of protein-protein interaction *in vivo*. Two proteins tagged with two different epitopes were co-expressed in yeast strains, the cell lysate was incubated with the antibody specific to one of the proteins together with protein G agarose. The agarose-immobilised protein-antibody complexes were resolved by SDS-PAGE and the interacting proteins were detected by western blotting using the antibody recognising the epitope on these proteins.

Cells overexpressing IL-1α proteins fused to the FLAG tag were grown in 50-200 ml (depending on the abundance of the protein studied) of the selective SD liquid growth medium in a shaker at 200 rpm in 28 ℃ until they reached OD_{600}=0.8-1.2. I centrifuged them for 10 min at 5000 g and kept on ice from now. I washed them with 1 ml of ddH_2O (4 ℃) and centrifuged again. To the cell pellet I added 3 volumes of ice-cold nondenaturing lysis buffer supplemented with 1 mM PMSF and protease inhibitor cocktail (Roche) added immediately before use and at least 1 volume of glass beads (0.45 mm). In a cold room (4 ℃), I shaked the cells by vortexing vigorously at maximum speed for five 30-sec periods, keeping the tubes on ice for 30 sec between the periods. I removed the lysate to a clean tube, washed the beads by 1 volume of ice-cold nondenaturing lysis buffer with PMSF and protease inhibitors, vortexed for 5 sec and combined both supernatants. I cleared the lysate by centrifugation at 20000 g and 4 ℃ for 20 min, k ept aside 40 µl of the cell lysate as input sample fot western blotting and incubated the rest of the lysate with 15 µl of protein G agarose slurry (Exalpha) for 1 h at 4 ℃ on wheel. I centrifuged the tubes at 2000 g for 20 sec and transferred the lysate to a new tube. I added 25 µl of protein G agarose slurry and 1 µg of the mouse monoclonal anti-FLAG antibody (Sigma) and incubated overnight on a wheel at 4 ℃. Next day I washed the protein G agarose slurry containing bound proteins 3x for 15 min by ice-cold nondenaturing lysis buffer, discarded the supernatant and finally added 20-25 µl of 2x SLB and incubated at 100 ℃ for 5 min.

Nondenaturing lysis buffer: 1% Triton X-100; 50 mM Tris-Cl, pH 7.5; 300 mM NaCl; 5 mM EDTA; 0.02% sodium azide
2x SLB: 6x SLB (5.5.11) diluted 3x in water

5.5.3 Large-scale co-immunoprecipitation from yeast cells for protein identification using mass spectrometry

This protocol is a large-scale variant of the protocol „Co-immunoprecipitation from yeast cells using a non-denaturing lysis buffer". I used 1 liter of yeast cell culture per sample. After clarification, the lysate (final volume cca 20 ml) was incubated with 40 µl of protein G agarose slurry and 1.5 µl of the mouse monoclonal anti-FLAG antibody (Sigma) overnight on a wheel at 4 °C. Next day I washed the protein G agarose slurry containing bound proteins 5x for 20 min by ice-cold nondenaturing lysis buffer, added 20 µl of 3x SLB and incubated at 100 °C for 5 min.

3x SLB: 6x SLB (5.5.11) diluted 2x in water

5.5.4 Co-immunoprecipitation from mammalian cells

I was using this protocol to study protein-protein interactions in mammalian cells. Transiently transfected cells growing in 6-cm culture dishes were washed with PBS and 1 ml of IP lysis buffer supplemented with protease inhibitor cocktail (Roche) was added. Cells were scraped into an eppendorf tube, incubated on ice for 30 min and vortexed repeatedly. Samples were spinned for 10 min at 13 000 g in 4 °C and supernatants were transferred into new tubes and kept on ice. 100 µl of Buffer B supplemented with protease inhibitor cocktail (Roche) and 100 µM DTT was added to the pellet, vortexed and incubated on ice for 15 min. After a 10 min centrifugation at 13 000 g in 4 °C, the Buffer B supernatant was added to the lysate obtained with the IP lysis buffer. I added 20 µl of protein G agarose slurry to the lysate and incubated for 1 h at 4 °C on wheel. I centrifuged the tubes at 2000 g for 20 sec and transferred the lysate to a new tube. I added 30 µl of protein G agarose slurry and 1 µg of the mouse monoclonal anti-FLAG antibody (Sigma) or 2 µg of the mouse monoclonal anti-p53 antibody and incubated overnight on a wheel at 4 °C. Next day I washed the protein G agarose slurry containing bound proteins 3x for 15 min by ice-cold nondenaturing lysis buffer (the same I used for the co-immunoprecipitation from yeast), centrifuged at 2000 g for 20 sec and added 30 µl of 2x SLB and incubated at 100 °C for 5 min.

IP lysis buffer: 50 mM Tris-Cl, pH 7.6; 150 mM NaCl; 1.5 mM $MgCl_2$; 1 mM EDTA; 0.5% NP40; 10% glycerol
Buffer B: 0.02 M HEPES; 25% glycerol; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA

5.5.5 Fluorescence microscopy of yeast cells

I was using this protocol to study the subcellular localisation of the yGFP-labeled IL-1α proteins in *S. cerevisiae*. Cells bearing recombinant plasmids derived from pUG36 encoding IL-1α proteins were grown in a selective growth medium lacking methionine and uracil on a shaker until they reached OD_{600}=0.8. Cells were sedimented by centrifugation at 3000 g for 5 min and resuspended in an eppendorf tube in 1 ml of growth medium. 100 µl of formaldehyde was added to the tubes and samples were incubated for 1 h in room temperature. Fixed cells were washed by 0.5 ml of KPO_4/sorbitol and resuspended in 300 µl of KPO_4/sorbitol. 2 µl of the cell suspension was mixed with 2 µl of mounting medium+DAPI and samples were observed under the fluorescence microscope.

KPO_4/sorbitol: 60 ml 2M sorbitol, 10 ml 1M potassium phosphate solution, 30 ml water
Potassium phosphate solution 1M: 83.4 ml 1M K_2HPO_4, 16.6 ml 1M KH_2PO_4
mounting medium+DAPI: 100 mg p-phenylenediamine in 10 ml PBS; 90 ml glycerol; 2.25 µl fresh DAPI solution (1mg/ml in water).

5.5.6 Fluorescence microscopy of mammalian cells

Adherent cells growing on round microscope coverglasses transfected with recombinant plasmids encoding fluorescent protein fusions of the IL-1α proteins were fixed by 3% PFA for 30 min in room temperature. After washing 3x with PBS, the solution of DAPI (0.1 µg/ml) was added to the cells and incubated for 5 min in room temperature. Coverglasses were washed by PBS three times and then in water before the mounting medium was added and cells were observed under the fluorescence microscope.

3% PFA: 3% paraformaldehyde in PBS
Mounting medium: 70% glycerol in water

5.5.7 Drop test

For the yeast phenotype characterisation and verification of the nutritional requirements of the yeast clones I was using this drop test. Monocolonies of selected yeast clones were resuspended in 100 µl of sterile water and diluted 10x, 5 µl of the cell suspension was seeded on agar plates made from SD medium lacking an amino acid (histidine, lysine, leucine, tryptophan, methionine), a base (uracil, adenine), lacking all components mentioned (SD-) or including all of them (SD+). Cells were incubated in an incubator up to five days and the growth of yeast colonies was observed and recorded daily.

5.5.8 Gene disruption in yeast strains

In order to study the interactions of human IL-1α with yeast histone acetyltransferase complexes and to study these interactions in yeast strains with deleted SAGA and ADA complex subunits, I used the *kanMX* gene disruption cassette encoded by plasmid pUG6 (Gueldener *et al.* 1996). This cassette contains a kan^r marker which confers resistance to the antibiotic G418. The resistance marker is flanked by two 34 bp *loxP* sequences. The disruption cassette is produced by PCR using primers complementary to the sequence of pUG6 as well as to the genomic regions flanking the gene to be deleted; these free ends serve for the cassette integration into the yeast genome by homologous recombination (see Figure 9). After transformation of yeast strains with the PCR product, I selected the transformants on YPD agar plates containing G418 (500 µg/ml).

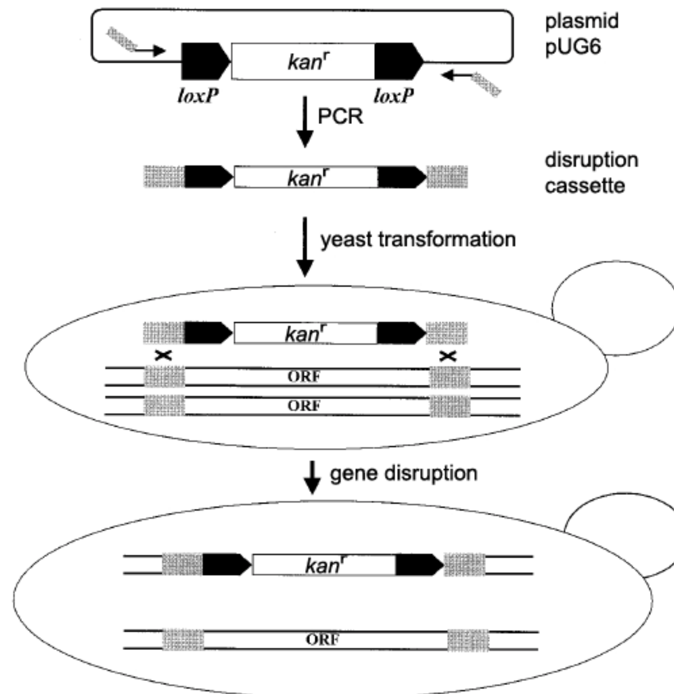


Figure 9: Gene disruption in yeast using the kanMX gene disruption cassette (Gueldener *et al.* 1996).

5.5.8.1 PCR of the kanMX gene disruption cassette

I amplified the *kanMX* gene disruption cassette from the plasmid pUG6 using following primer sets designed for the disruption of *GCN5*, *SPT7* and *AHC1* genes (Table 5).

PCR reaction scheme for the amplification of the *kanMX* gene disruption cassette for *GCN5* gene disruption

template (pUG6 stock diluted 500x)	1.5 µl
dNTPs	0.5 µl
primer Gcn5Fwd	1 µl
primer Gcn5Rev	1 µl
Taq polymerase + 1% Pfu	0.5 µl
10x PCR buffer	2.5 µl
water	18 µl

94 ºC	2 min	
94 ºC	40 sec	25x
49 ºC	30 sec	
68 ºC	90 sec	
68 ℃	5 min	

This reaction scheme is the same as for the disruption of *SPT7* and *AHC1* except for a different primer set.

name	sequence 5´ - 3´
Gcn5Kan5	GGCAGCAAAAAATGCGTCTTTCTTCCCTCGTCTGTTGTTTTATGTAG GGCCGTACGCTGCAGGTCGACAACC
Gcn5Kan3	TGAAAGGAAAAGTAGTAAAATAACCTCAATTGATCACATCGTCTCGC CGTCGACTCACTATAGGGAGACCG
Gcn5Fwd	AGAAGGCACGTAAATAATCTTAAACACTTATGGGCAGCAAAAAATGC GTCTTTCTTCCCCGTACGCTGCAGGTCGACAACC
Gcn5Rev	GAAGCTGCAGAAAGTCCAGAAGAAGCGGATGTTGAAATGTCATAAA TATAGTTACACGACTCACTATAGGGAGACCG
Spt7Fwd	GCTCTTATCTTTGAGAGAAACAACGGTAGTACTTAAAGGCTACGAAT AAGCCAATTTCCGTACGCTGCAGGTCGACAACC
Spt7Rev	CAGATCCAAGTAATGAAAAGTGAATGTACACTCGTAAGTCTTCAATT ATGTTAGATGTACGACTCACTATAGGGAGACCG
Ahc1Kan5	ATACAATCCACTTTTTCTTCCAAAGCGACAAATTAGGTCAGAAAACCA ACCGTACGCTGCAGGTCGACAACC
Ahc1Kan3	ATAGCCGTAGGAAGAGGGAAAAACGAACAGGGAAGAAGCAGGAAG AGCAGACGACTCACTATAGGGAGACCG
Ahc2-delALL-F	CGCTTACAAGATCAGTACTAGCGTGTACCAGTCTATTTAAACTGGAC GCATAGGCCACTAGTGGATCTG
Ahc2-delALL-R	ATATATATAAATATATGTTTAAAAATGTCCATAGCCGCTATTTGACAG CTGAAGCTTCGTACGC

Table 5: Primer sets used for amplification of the *kanMX* gene disruption cassette designed to disrupt *GCN5*, *SPT7* and *AHC1*, respectively. In the case of the *GCN5* gene disruption, I used first the Gcn5Kan5/Gcn5Kan3 primer set. Since the *GCN5* gene disruption in the TAP/Gcn5 yeast strain performed using the *kanMX* cassette amplified with this primer pair seemed to be unsuccessful (see Results part .6.2.5), I designed another primer set Gcn5Fwd/Gcn5Rev that was used for all the *GCN5* disruptions described in this study.

The correct integration of the *kanMX* gene disruption cassette was checked by PCR screening. I used primers complementary to the kanamycin resistance gene (KanR1, KanR2) and a second primer set composed of one primer annealing within the kanamycin resistance gene (KanV2F) and the other complementary to a sequence upstream or downstream of the gene to disrupt (Gcn5VerR, Spt7VerR or Ahc1VerR; see Figure 10 and Table 6).

Figure 10: Schematic representation of the *kanMX* gene disruption cassette integrated in the genome and the primer sets used for the PCR screening of transformed yeast cells. Primers for the amplification of the kanamycin resistance gene (red) and for the verification of the correct *kanMX* cassette position in the genome (yellow) are shown.

name	sequence 5´ - 3´
KanR1	ATGGGTAAGGAAAAGACTCACG
KanR2	TTAGAAAAACTCATCGAGCATCA
KanV2F	GTTGTATTGATGTTGGACGAGTCGG
Gcn5VerR	CAGAACCATCTGGGACATTTTACCGT
Spt7VerR	CTAACTTGTCGAGGGAGCGTCAACG
Ahc1VerR	GGCGAGGGCGATACTGAAGTCATTT

Table 6: Primers used for the verification of correct integration of the *kanMX* gene disruption cassette into the yeast genome.

5.5.8.2 PCR screening of the strains transformed with the *kanMX* gene disruption cassette

A- screening for the presence of the kanMX gene disruption casette

template (gDNA diluted 50x)	1 µl
dNTPs	0.5 µl
primer KanR1	0.5 µl
primer KanR2	0.5 µl
Taq polymerase	0.5 µl
10x PCR buffer	2 µl
water	15 µl

95 ºC	2 min	
95 ºC	40 sec	30x
50 ºC	1 min	30x
72 ºC	1 min	30x
72 ºC	5 min	

B- screening for the correct integration of the kanMX gene disruption casette

template (gDNA diluted 50x)	1 µl
dNTPs	0.5 µl
primer KanV2F	0.5 µl
primer 2	0.5 µl
Taq polymerase + 1% Pfu	0.5 µl
10x PCR buffer	2 µl
water	15 µl

95 ºC	2 min	
95 ºC	40 sec	30x
50 ºC	1 min	30x
72 ºC	1 min	30x
72 ºC	5 min	

Primer 2 stands for either Gcn5VerR, Spt7VerR or Ahc1VerR. The primer annealing temperature was 50 ºC for Gcn5VerR/KanV2F set, 50 ºC with 90 sec elongation time for Spt7VerR/KanV2F set and 65 ºC for Ahc1VerR/KanV2F primer set.

5.5.9 High-yield transformation of yeast cells

I was using this protocol for a high-efficiency transformation of TAP fusion yeast strains with the *kanMX* gene disruption cassette. Cells were inoculated into the appropriate growth medium and cultured overnight, next day inoculated into a pre-warmed YPD growth medium at OD_{600}=0.1 and incubated in a shaker at 28 ºC. After 5 hours the cells were pelleted by centrifugation at 3000 g for 5 min, washed by 25ml of sterile water and resuspended in 1 ml of sterile water. 100 µl samples were used for each transformation. Cells were centrifuged and water was removed, 360 µl of the transformation mix (240 µl of 50% PEG, 36 µl of 1M lithium acetate (LiAc), 50 µl

of ssDNA, 34µl of *kanMX* cassette PCR product) was added to each tube. Cells were resuspended by vortex mixing vigorously. I incubated the cells in 42 ℃ for 40-50 min and centrifuged at top speed for 30 sec. The transformation mix was removed and cells were seeded on YPD agar plates containing G418 (500 µg/ml).

5.5.10 LiAc transformation of yeast cells

This transformation method is a modified protocol according to (Gietz and Woods 2002).
Cells were grown for 1-2 days on appropriate agar plates in the incubator. At the day of the transformation, a quantity of yeast corresponding to a bigger pinhead was transferred into 1 ml of 100 mM LiAc in a 1.5 ml eppendorf tube and resuspended. Cells were pelleted by centrifugation at 14000 g for 15 sec and supernatant was aspirated. To the cell pellet I added 240 µl of 50% PEG, 36 µl of 1M LiAc, 50 µl of ssDNA (kept on ice) and 25 µl of plasmid DNA resuspended in sterile water (1:5), respectively. I resuspended the cells by vortexing, incubated at 42 ℃ for 20 min and in room temperature overnight. Next day I pelleted the cells by centrifugation at 5000 g for 1 min, resuspended in sterile water and seeded in three different quantities on appropriate selective agar plates. The plates were incubated for 2-6 days in the incubator.

5.5.11 Yeast cell lysate for western blotting

In order to check quickly the expression of a particular protein in a *S. cerevisiae* strain, I followed this procedure: 1-5 ml of the overnight yeast culture was pelleted by centrifugation, 100-500 µl of 1xSLB and 200 mg of glass beads (0.45mm) was added and cells were resuspended quickly. The tubes were transferred immediately to a 95 ℃ dry heating block for 5-10 min. Subsequently the tubes were vortexed for 2-4 min and again incubated in 95 ℃ for 5-10 min. 5-20 µl of the sample was loaded onto the gel.

6x SLB: 7 ml 4x Tris-HCl pH 6.8; 5 ml glycerol; 1 ml β-mercaptoethanol; 1 g SDS; 5 mg bromophenol blue

5.5.12 Mammalian cell lysate for western blotting

Rapid lysis was used for a quick control of protein expression in mammalian cells. 1x SLB (50-500 µl depending on the cell growth surface) was added directly to the adherent cells, cells were scraped to an eppendorf tube and sonicated for 20 sec. The samples were incubated in 100 ℃ for 5 min.

1x SLB: 6x SLB (5.5.11) diluted 6x in water

5.5.13 SDS-PAGE protein electrophoresis

The sodium dodecyl sulfate polyacrylamide gel electrophoresis was routinely used for the separation of proteins. I was using the following protocol: for the separating 14% polyacrylamide gel (2 gels) I prepared a mixture containing 7 ml 30% acrylamide/0.8% bisacrylamide; 3.75 l 4x Tris-HCl/SDS pH 8.8; 4.25 ml ddH_2O; 50 µl ammonium persulfate and 10 µl TEMED. I poured the solution between two glasses

and overlayed with 1x Tris-HCl/SDS pH 8.8. After 20-30 min of polymeration I prepared the loading gel composed of 650 µl 30% acrylamide/0.8% bisacrylamide; 1.25 ml 4x Tris-HCl/SDS pH 6.8; 3.05 ml ddH_2O; 25 µl ammonium persulfate and 5 µl TEMED. I placed the combs and allowed the gel to polymerate for 15-20 min. After loading the samples I runned the electrophoresis for 1-2 h at 150 V in 1x SDS-PAGE buffer.

4x Tris-HCl/SDS pH 8.8: 91 g Tris; 2g SDS; adjust pH with HCl; ddH_2O up to 500 ml
4x Tris-HCl/SDS pH 6.8: 6.05 g Tris; 0.4 g SDS; adjust pH with HCl; ddH_2O up to 100 ml
5x SDS-PAGE buffer: 15.1 g Tris; 72 g glycine; 5 g SDS; ddH_2O up to 1 l

5.5.14 Western blotting

To detect specific proteins separated by SDS-PAGE using antibodies I was using western blotting. After SDS-PAGE electrophoresis I incubated the gel in the blotting buffer for 15 min, I prepared a „sandwich" composed of filter paper, nitrocellulose membrane and the gel and run the blotting on a semidry blotting apparatus (The Panther™ Semidry Electroblotter, OWL) at 2 mA/cm^2. The membrane was blocked in the milk solution for 1 h and incubated with the primary antibody in an appropriate concentration overnight in 4 ℃. Next day the membr ane was washed 3x15 min in TBS/Tween, blocked again in the milk solution and incubated with the secondary antibody for 2 h in 4 ℃. Then it was washed 3x15 m in in TBS/Tween.
In the case that unstained protein ladder was used, I incubated the membrane in Ponceau S solution for 2 min and washed in water. I marked the ladder bands by pencil.
If needed, the membrane was stripped using the stripping solution heated to 50 ℃. I incubated the membrane in this solution for 2x30 min, washed it in distilled water and TBS/Tween, blocked in the milk solution and reprobed with another primary and secondary antibody.

Blotting buffer: 200 ml methanol; 2.9 g glycine; 5,8 g Tris; 0.37 g SDS; ddH_2O up to 1l
TBS/Tween: 50 mM Tris; 150 mM NaCl; 0.5% Tween-20
Milk solution: 5% Sunar baby (Heinz) in 1x TBS/Tween
Ponceau S solution: 0.1% Ponceau S; 5% acetic acid
Stripping solution: 5 ml 4x Tris-HCl/SDS pH 6.8; 0.8 g SDS, 300 µl β-mercaptoethanol; ddH_2O up to 40 ml

5.5.15 Chemiluminescence detection

The signal of horseradish peroxidase conjugated to the secondary antibody used in western blotting experiments was detected using luminol and p-coumaric acid. I mixed 200 µl of 0.25M luminol, 88 µl of 0.1M p-coumaric acid and 12 µl of 30% hydrogen peroxide solution in 40 ml of 0.1M Tris pH 8.8. The membrane was immersed in this solution and incubated 1 min, then placed in a film cassette together with a sheet of autoradiography film. After 1-45 min (depending on the signal intensity) the film was incubated in developer for 2-5 min, washed with water and incubated in fixer for 2-10 min.

5.5.16 Silver staining of polyacrylamide gels

To visualise protein bands on polyacrylamide gels after SDS-PAGE with high sensitivity I was using this silver staining protocol. The gel was first incubated in 50% methanol and 10% acetic acid for 30 min, then washed with 5% methanol and 7% acetic acid for 30 min and fixed with 5% glutaraldehyde solution in water for 30-45 min. After washing for 3x15 min and 3x10 min in ddH_2O I incubated the gel in DTT solution (5mg/l) for 20 min and then in 0.1% $AgNO_3$ solution for 20 min. I washed the gel with ddH_2O for 1 min and incubated it in 3% Na_2CO_3 with formaldehyde (100 µl 30% formaldehyde solution for 100 ml of the Na_2CO_3 solution). To stop the staining reaction I added 5 ml of 2.3M citric acid, incubated for 5 min and washed 3x-5x with 250 ml of ddH_2O.

5.5.17 Agarose electrophoresis of DNA

Agarose (concentration vary according to the character of the DNA samples) was resuspended in 1x TAE buffer and heated until it melted completely. Then it was cooled to approximately 50 ℃, poured to the gel ca sting tray and EtBr was added (final concentration 0.175 µg/ml). A comb was placed and the gel was allowed to cool at room temperature.

50x TAE: 242 g Tris; 57.1 ml acetic acid; 100 ml 0.5 M EDTA-NaOH pH 8; water up to 1 l

5.5.18 Plasmid minipreparation from *E. coli*

For a quick isolation of plasmids from bacterial cells I was using this simple protocol. Cells growing in an area of approx. $4cm^2$ were scraped from the selective agar plate and transferred into an eppendorf tube containing 400 µl of STET buffer, 5 µl of 5% lysozyme was added and tubes were incubated in 95 °C for 2 min. Samples were centrifuged for 10 min at 13000 g and pellet was carefully removed using a toothpick. To the supernatant 0.4 volume of isopropanol was added and tubes were incubated in -20 ℃ for 1 h. Precipitated DNA was sedimented by centrifugation for 15 min at 13000 g, washed by 1 ml of 70% ethanol and centrifuged again for 15 min at 13000 g. Pellets were allowed to dry at the room temperature and they were resuspended in 30 µl of TE buffer. Samples were analyzed by agarose electrophoresis.

STET buffer: 10% saccharose; 50 mM Tris-HCl pH 8; 50 mM EDTA-NaOH pH 8; 1% Triton X-100
TE buffer: 10 mM Tris-HCl pH 8; 1 mM EDTA-NaOH pH 8

5.5.19 Plasmid isolation from *E.coli*

This is the protocol for a large-scale plasmid isolation from bacterial cells. 50 ml of cell culture cultivated overnight was centrifuged for 10 min at 5000 g, the pellet was resuspended in 2 ml of TEG solution and 4 ml of cell lysis solution was added, the tubed were inverted a few times carefully and cells were allowed to lyse for 5 min at room temperature. Afterwards 3 ml of 3M KAc was added and the tubes were incubated for 20 min on ice. The cell lysate was centrifuged for 30 min at 4000 g and

4 ℃, the supernatant was filtered and 0.7 volume of isopropanol was added. Tubes were incubated 10 min in 4 ℃ and centrifuged for 30 min at 4000 g and 4 ℃. The sediment was washed with 3 ml of 70% ethanol and allowed to dry at the room temperature. DNA was resuspended in 400 µl TE buffer, 3 µl of RNase A solution was added and tubes were incubated for 1 h at 37 ℃. Samples were extracted by 400 µl of phenol equilibred to pH 8, 400 µl of phenol/chloroform/isoamylalcohol (25:24:1) (repeated until a protein layer was visible in the tubes) and 400 µl of chloroform/isoamylalcohol (24:1). To the water phase 0.1 volume of 3M NaAc and 2.5 volumes of 96% ethanol was added and tubes were incubated at -20 ℃ overnight. Next day DNA was sedimented by centrifugation for 10 min at top speed, pellet was washed by 70% ethanol and allowed to dry at the room temperature. Plasmid DNA was resuspended in 300 µl of TE buffer.

TEG: 25 mM Tris-HCl pH 8; 10 mM EDTA-NaOH pH 8
Cell lysis solution: 1% SDS; 0.2 M NaOH
TE buffer: 10 mM Tris-HCl pH 8; 1 mM EDTA-NaOH pH 8
RNase A solution: Ribonuclease A 110 mg/ml; 0.1 mM Tris-HCl pH 7.5; 0.045 mM NaCl

5.5.20 Rapid yeast genomic DNA isolation

For PCR screening of yeast colonies growing on YPD agar plates containing G418 after transformation with the *kanMX* disruption cassette, I was using this rapid genomic DNA isolation protocol. Single colonies were plated on YPD agar plates and after 1-2 days yeast cells growing in an area of approx. 4cm^2 were scraped and transferred into an eppendorf tube containing 200 µl of lysis buffer. The tubes were placed in a -80 ℃ freezer for 15 min, then incubated in a 95 ℃ dry heating block for 2 minutes to thaw quickly. The process was repeated once, and the tubes were vortexed vigorously for 30 seconds. 200 µl of fenol/chloroform (1:1) was added and the tubes were vortexed for 2 minutes and then centrifuged 3 minutes at room temperature at 14000 g. The aqueous layer was transferred to a tube containing 400 µl of ice-cold 96% ethanol. The samples were allowed to precipitate 1 hour at 4 ℃ and then centrifuged 5 minutes at room temperature at 14000 g. Supernatants were aspirated and DNA pellets were washed with 0.5 ml of 70% ethanol and allowed to air dry at room temperature. I resuspended DNA in 30 µl of TE buffer and diluted 50x-100x to use as a template for my PCR screening reactions.

Lysis buffer: 2% Triton X-100; 1% SDS; 100 mM NaCl; 10 mM Tris-HCl (pH 8.0); 1 mM EDTA (pH 8.0)

5.5.21 Restriction cleavage

All restriction enzymes and corresponding buffers used were from Fermentas.

5.5.22 Transformation of *E. coli* by electroporation

For electroporation I was using the *E. coli* XL-1 blue competent cells.
In a 2-mm electroporation cuvette, 40 µl of cell suspension wes mixed with DNA (ligation mixture or plasmid) and after 1 min incubation on ice an electric pulse was applied (electroporation instrument settings: capacitance 25 µF; 2.5 kV; 200 Ω). 1 ml

of 2xTY non-selective growth medium was added immediately to the cell suspension and the tube was incubated for 1 h in 37 °C in a shaker. Cells were plated on appropriate selective plates in at least three different densities.

5.5.23 Transient transfection of mammalian cells using Turbofect transfection reagent (Fermentas)

I transfected the cells according to the manufacturer´s instructions.

5.5.24 Transient transfection of mammalian cells using FuGENE 6 transfection reagent (Roche)

The transient transfection was performed according to the manufacturer´s instructions. I used 2 µg of the plasmid DNA and 5 µl of the FuGENE reagent per well in the 6-well plate and analyzed the expression after 24 hours.

5.5.25 Transient transfection of mammalian cells using ExGen transfection reagent (Fermentas)

The transient transfection was performed according to the manufacturer´s instructions.

5.5.26 Transient transfection of mammalian cells using the Amaxa Nucleofector device (Lonza)

I was using this device for transient transfection of the NIH 3T3 cells together with the Amaxa Cell Line Nucleofector kit R.
2×10^6 cells were trypsinised and transfected according to the manufacturer´s instructions using the programme U-030 (NIH 3T3).

5.5.27 Antibodies used throughout this study

mouse monoclonal anti-FLAG antibody (Sigma-Aldrich)
mouse monoclonal anti-HA antibody (Sigma-Aldrich)
rabbit anti-calmodulin binding peptide (CBP) antibody (ICL Labs)
mouse monoclonal anti-p53 antibody (Chemicon)
rabbit anti-p53 antibody CM-1 (kindly provided by Bořivoj Vojtěšek)
goat anti-mouse antibody, HRP-conjugated (Santa Cruz Biotechnology)
swine anti-rabbit antibody, HRP-conjugated (Sevapharm)

5.5.28 Construction of expression plasmids used in this study

5.5.28.1 Construction of pUG36/IL-1αFull and pUG36/IL-1αMat

I was using these plasmids for yeast fluorescence microscopy experiments.

PCR of the IL-1α precursor (Full) cDNA
I amplified cDNA encoding the full-length IL-1α precursor from the pcDNA4/TO/FLAGMycHis-IL-1α Full plasmid using the following primer set:

name	sequence 5´ - 3´
5´BglII	CGACGTAGATCTATGGCCAAAGTTCCAGACAT
3´SalI	CTTACTGTCGACCTACGCCTGGTTTTCCAGTA

PCR reaction scheme

template (plasmid diluted 100x)	2 µl
dNTPs	0.5 µl
primer 5´BglII	0.6 µl
primer 3´SalI	0.6 µl
Taq polymerase + 1% Pfu	0.5 µl
10x PCR buffer	2 µl
water	13.8 µl

95 ºC	2 min	
95 ºC	40 sec	25x
68,6 ºC	30 sec	25x
72 ºC	1 min	25x
72 ℃	7 min	

Restriction cleavage of the PCR product

PCR product	10 µl
restriction buffer O^+	2 µl
BamHI	1 µl
XhoI	1 µl
water	6 µl

incubated at 37 ℃ for 2 h 30 min

Restriction cleavage of the plasmid encoding mature IL-1α (Mat)

plasmid pcDNA4/TO/FLAGMycHis-IL-1αMat	5 µl
restriction buffer Y^+Tango	4 µl
BamHI	1 µl
XhoI	1 µl
water	9 µl

incubated at 37 ℃ for 3 h

Restriction cleavage of the plasmid pUG36

plasmid pUG36	7 µl
restriction buffer Y^+Tango	4 µl
BamHI	1 µl
XhoI	1 µl
water	7 µl

incubated at 37 ℃ for 2 h

All restriction cleavage mixtures were loaded onto a 0.5% agarose gel and separated by electrophoresis.

Isolation from the TAE agarose gel

I isolated the bands corresponding to the cleaved plasmid pUG36, IL-1αFull and IL-1αMat using the Geneclean II kit (Bio101) according to the manufacturer´s instructions.

Ligation

The vector and the insert (both Full and Mat) were ligated using the T4 DNA ligase

cleaved plasmid	2 µl
insert	3 µl
T4 DNA ligase	1 µl
ligation buffer	1 µl
water	3 µl

incubated at 16 ℃ for 10 hours

3 µl of each ligation mixture was used for transformation of *E. coli* by electroporation (see the protocol above).

Verification of the constructed recombinant plasmids
I isolated plasmid DNA from selected bacterial clones following the protocol „Plasmid minipreparation from *E. coli*“ and cleaved the isolated plasmids by a specific combination of restriction endonucleases (Full: EcoRI, XbaI; Mat: XhoI, XbaI). I checked the presence of the insert by agarose electrophoresis.

5.5.28.2 Construction of pYX212N/S IL-1αMat and pYX212N/X IL-1αMat

I used the plasmid pYX212 encoding mature IL-1α for yeast co-immunoprecipitation experiments. I constructed two variants of this plasmid - pYX212N/S IL-1αMat and pYX212N/X IL-1αMat - that differ only in the presence of the HA tag in the first plasmid (N/S). However, I only used pYX212N/X IL-1αMat throughout this study.

Restriction cleavage of the plasmid encoding mature IL-1α (Mat)

plasmid pcDNA4/TO/FLAGMycHis-IL-1αMat	7 µl
restriction buffer Y^{+}Tango	4 µl
NcoI	0.5 µl
XhoI	0.5 µl
water	8 µl

incubated at 37 ℃ for 3 h

Restriction cleavage of the plasmid pYX212 by NcoI and SalI (for the construction of pYX212N/S IL-1αMat)

plasmid pYX212	8 µl
restriction buffer Y^+Tango	4 µl
SalI	0.5 µl
XhoI	0.5 µl
water	7 µl

incubated at 37 °C for 3 h

Restriction cleavage of the plasmid pYX212 by NcoI and XhoI (for the construction of pYX212N/X IL-1αMat)

plasmid pYX212	8 µl
restriction buffer Y^+Tango	4 µl
NcoI	0.5 µl
XhoI	0.5 µl
water	7 µl

incubated at 37 °C for 3 h

Isolation from the TAE agarose gel

I isolated the appropriate bands using the Geneclean II kit (Bio101) according to the manufacturer´s instructions.

Ligation

The vector and the insert were ligated using the T4 DNA ligase

cleaved plasmid (N/S or N/X)	1.5 µl
cleaved insert	4 µl
T4 DNA ligase	1 µl
ligation buffer	1 µl
water	2.5 µl

incubated in room temperature for 1.5 h and in 16 °C for 10 hours

2.5 µl of both ligation mixtures (N/S and N/X variants) was used for transformation of *E. coli* by electroporation (see the protocol above).

Verification of the constructed recombinant plasmids

I isolated the plasmid DNA from selected *E. coli* clones and verified the presence of the insert by restriction cleavage with BamHI and XhoI (for the plasmid pYX212N/S IL-1αMat) or NcoI and XhoI (for the plasmid pYX212N/X IL-1αMat) restriction endonucleases.

5.5.28.3 Construction of pDsRED2-C1/IL-1αFull

I amplified cDNA encoding the full-length IL-1α precursor from the pcDNA4/TO/FLAGMycHis-IL-1α plasmid using the following primer set:

name	sequence 5´ - 3´
5´BglII	CGACGTAGATCTATGGCCAAAGTTCCAGACAT
3´SalI	CTTACTGTCGACCTACGCCTGGTTTTCCAGTA

PCR reaction scheme

template (plasmid diluted 100x)	1 µl
dNTPs	0.5 µl
primer 5´BglII	0.5 µl
primer 3´SalI	0.5 µl
Taq polymerase + 1% Pfu	0.5 µl
10x PCR buffer	2 µl
water	15 µl

95 ºC	1 min	
95 ºC	40 sec	25x
74 ºC	30 sec	25x
72 ºC	1 min	25x

The PCR product was loaded onto a 0.5% agarose gel and separated by electrophoresis.

Isolation from the TAE agarose gel
I isolated the band using the Geneclean II kit (Bio101) according to the manufacturer´s instructions.

Restriction cleavage of the PCR product isolated from the gel

PCR product	10 µl
restriction buffer Y^{+}Tango	4 µl
BglII	1 µl
SalI	1 µl
water	4 µl

incubated at 37 °C for 1 h 30 min

Restriction cleavage of pDsRED2-C1

plasmid pDsRED2-C1	12 µl
restriction buffer Y^{+}Tango	4 µl
BglII	1 µl
SalI	1 µl
water	2 µl

incubated at 37 °C for 2 h

Both the vector and the insert were separated on 0.5% agarose gel and isolated from the gel using the Geneclean II kit.

Ligation

The vector and the insert were ligated using the T4 DNA ligase

cleaved plasmid	4 µl
cleaved PCR product	4 µl
T4 DNA ligase	1 µl
ligation buffer	1 µl

incubated in room temperature for 2 h and in 16 ℃ for 10 hours

3 µl of the ligation mixture was used for transformation of *E. coli* by electroporation (see the protocol above).

Verification of the constructed recombinant plasmid
I isolated the plasmid DNA from selected *E. coli* clones and verified the presence of the insert by PCR using 5´BglII and 3´SalI primers (see the sequence above). The plasmid from one verified clone was isolated from bacteria using QIAprep Spin Miniprep Kit (Qiagen) and the insert region was sequenced (BigDye® Terminator v3.1 Cycle Sequencing Kit by Applied Biosystems).

PCR for sequencing of pDsRED2-C1/IL-1αFull

name	sequence 5´ - 3´
DsRedSeqPrimer	AGCTGGACATCACCTCCCACAACG

plasmid	1 µl
DsRed sequencing primer	0.5 µl
reaction mix	4 µl
5x sequencing buffer	2 µl
water	12.5 µl

96 ºC	1 min	
96 ºC	30 sec	24x
58 ºC	1 min	24x
60 ºC	4 min	24x

5.5.28.4 Construction of pEGFP-C1/ IL-1αMat

This plasmid was used for the fluorescence microscopy experiments in mammalian cells.

Restriction cleavage of the plasmid encoding mature IL-1α (Mat)

plasmid cDNA4/TO/FLAGMycHis-IL-1α Mat	14 µl
restriction buffer Y^{+}Tango	4 µl
BamHI	1 µl
XhoI	1 µl
water	2 µl

incubated at 37 ℃ for 3 h

Restriction cleavage of pEGFP-C1

plasmid pEGFP-C1	6 µl
restriction buffer Y^{+}Tango	4 µl
BamHI	1 µl
XhoI	1 µl
water	8 µl

incubated at 37 °C for 2 h

Both reaction mixtures were loaded onto a 0.5% agarose gel and separated by electrophoresis.

Isolation from the TAE agarose gel
Both DNA fragments were isolated from the TAE agarose gel using the Geneclean II kit (Bio101) according to the manufacturer´s instructions.

Ligation
The vector and the insert were ligated using the T4 DNA ligase

plasmid	2 µl
insert	4 µl
T4 DNA ligase	1 µl
ligation buffer	1 µl
water	2 µl

incubated at 16 °C for 10 hours

2 µl of the ligation mixture was used for transformation of *E. coli* by electroporation (see the protocol above).

Verification of the constructed recombinant plasmid
I isolated plasmid DNA from selected bacterial clones following the protocol „Plasmid minipreparation from *E. coli*" and verified the presence of the insert by PCR (primers EGFP-C_Seq and 3´SalI). I isolated the plasmid from a selected clone with QIAprep Spin Miniprep Kit (Qiagen) and used it for sequencing (BigDye® Terminator v3.1 Cycle Sequencing Kit by Applied Biosystems).

PCR for sequencing of pEGFP-C1/ IL-1αMat

name	**sequence 5´ - 3´**
EGFP-C_Seq	CATGGTCCTGCTGGAGTTCGT

plasmid	0.5 µl
EGFP-C_Seq primer	0.5 µl
reaction mix	4 µl
5x sequencing buffer	2 µl
water	13 µl

96 °C	1 min	
96 °C	30 sec	} 24x
58 °C	30 sec	
60 °C	4 min	

5.5.28.5 Construction of pEGFP-C1/ IL-1αNTP

This plasmid construct was prepared by Denisa Dolečková during her diploma thesis experimental work. It encodes IL-1αNTP fused to EGFP.

5.5.28.6 Plasmids pYX133N/S IL-1αFull, pYX133N/X IL-1αFull, pYX212N/S IL-1αFull and pYX212N/X IL-1αFull

These constructs encoding the IL-1α precursor were prepared by myself and described in my MSc. diploma thesis (Vicenova 2005).

5.5.28.7 Plasmids pcDNA4/TO/FLAGMycHis-IL-1αFull, Mat and NTP

These plasmids were prepared previously by Ladislav Burýšek.

5.5.29 Samples used for the *HOX* expression analysis

In this part of my work, peripheral blood from healthy volunteer donors was used for the analysis of mature primary lymphocytes. Sorted bone marrow populations from paediatric patients without evidence of malignancy were used as non-malignant counterparts of leukemic cells. Thymic T cells from paediatric patients undergoing heart surgery were used to analyze primary T-cell precursors. Diagnostic samples of 61 paediatric ALL patients were used for the analysis of malignant lymphoid precursors. The patient samples were categorised according to their immunophenotypic (T-ALL, B-cell precursor-ALL), prognostic (prednisone good responders, prednisone poor responders), and genotypic (BCR/ABL, MLL/AF4, TEL/AML1, hyperdiploid ALL; ALL without gene rearrangement) characteristics. Approximately $1–10\times10^6$ bone marrow or peripheral blood cells was used to isolate RNA according to the protocol „RNA isolation from mononuclear leukocytes" (5.5.30). The quality of total RNA was examined by the RNA 6000 Nano chip kit (Agilent Technologies). The age of the patients ranged from 1 month to 13 years. An informed consent was obtained from all subjects or their guardians.

5.5.30 RNA isolation from mononuclear leukocytes

This is the modified RNA isolation protocol by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Cells pelleted by centrifugation and frozen at -80 °C were placed on ice and 500 µl ITG/β-mercaptoethanol was added, tubes were incubated 5 min on ice. 50 µl of 3M sodium acetate solution (pH 4) was added together with 455 µl of acidic phenol pre-warmed at 65 °C and 130 µl chloroform/isoamylalcohol (24:1) solution. Samples were mixed by vortexing for 2 min, incubated on ice for 2-3 min and then centrifuged at 4 °C and maximum speed for 15 min. The aqueous phase was collected into a new tube and 1 volume of ice-cold isopropanol was added. Samples were incubated for 30 min at -20 °C, then centrifuged at 4 °C and maximum speed for 10 min, the pellet was washed with 1ml

of ice-cold 75% ethanol, centrifuged at 4 ℃ and maximum speed for 5 min. All ethanol was removed and RNA pellet was air-dryed for 15 min at room temperature, resolved in sterile water (50 µl for 10 million cells used) for 10 min at 55 ℃ and stored at -40 ℃.

ITG/β-mercaptoethanol: 4M guanidine isothiocyanate solution, 25mM sodium citrate, 0.5% sodium lauryl sarcosinate, adjust pH to 7.0 and add β-mercaptoethanol 7.2 µl/1 ml ITG

5.5.31 Preparation of cDNA by reverse transcription

I used RNA isolated from all the diagnostic samples by the protocol „RNA isolation from mononuclear leukocytes (5.5.30) and performed the on-column treatment of RNA by DNase I using the RNase-Free DNase Set (Qiagen). I reverse-transcribed 11 µl of RNA into cDNA using the iScript cDNA Synthesis Kit (Bio-rad) and diluted 20 µl of cDNA obtained three times in sterile water. Both kits were used according to the instructions of the manufacturer.

5.5.32 Quantitative real-time PCR

I performed the quantitative real-time PCR analysis using an iCycler iQ system (Bio-rad). Primer sequences are summarised in Table 7. PCR reactions were performed with 2.5 pmol of each primer and 1 µl of (diluted) cDNA template, in a final volume of 25 µl, using iQ™SYBR Green Supermix (Bio-rad) following the manufacturer's recommended protocol. I always used a 'master mix' containing all components, except the cDNA template, that was aliquoted to each reaction tube. After the initial denaturation at 95 ℃ for 5 min, 50 cycles of 95 ℃ for 15 sec and 65 ℃ (60 ℃ for *HOXA4*, *HOXB7*, *HOXB8* and *CDX2*; 68 ℃ for CDX1) for 1 min were carried out. Fluorescence was detected at each annealing step. Melting curve analysis was automatically performed at the end of each run by measuring change in SYBR Green fluorescence within 80 cycles with temperature increasing by 0.5 ℃ each cycle starting at 55 ℃. All reactions were run in duplicate, in the case of an unclear result third run was performed. The qRT-PCR analysis of *ABL1*, a housekeeping gene commonly used in analyses of the haematopoietic system, was used to evaluate the quantity and quality of cDNA.

name	sequence 5´ - 3´
HOXA1fwd	ACCCCTCGGACCATAGGATTAC
HOXA1rev	AAGGCGCACTGAAGTTCTGTG
HOXA2fwd	ACATCTTTTCCCCCTGTCGC
HOXA2rev	TTCAGGCTGGGAATGGTCTG
HOXA3fwd	TGCTTTGTGTTTTGTCGAGACTC
HOXA3rev	CAACCCTACCCCTGCCAAC
HOXA4fwd	GCTCTGTTTGTCTGAGCGCC
HOXA4rev	AATTGGAGGATCGCATCTTGG
HOXA5fwd	AGATCTACCCCTGGATGCGC
HOXA5rev	CCTTCTCCAGCTCCAGGGTC

HOXA6fwd	CGAGCAGCAGTACAAACCCGACAGCA
HOXA6rev	CGCCCATGGCTCCCATACACAGCAC
HOXA7fwd	ATCACTCTACCTCGTAAAACCGACAC
HOXA7rev	ACATAATACGAAGAACTCATAATTTTGACC
HOXA9fwd	GAGTGGAGCGCGCATGAAG
HOXA9rev	GGTGACTGTCCCACGCTTGAC
HOXA10fwd	GAGAGCAGCAAAGCCTCGC
HOXA10rev	CCAGTGTCTGGTGCTTCGTG
HOXA11fwd	TGGATTTTGATGAGCGTGGTC
HOXA11rev	GGGCAGGTTGGAGGAGTAGG
HOXA13fwd	GAACGGCCAAATGTACTGCC
HOXA13rev	GTGGCGTATTCCCGTTCAAG
HOXB1fwd	CAATCAGAAGGAGACGGAGGC
HOXB1rev	GATCAGCATAGGCCGGTGC
HOXB2fwd	TCTCCCCTAGCCTACAGGGTTC
HOXB2rev	AAACTGCAGGTCGATGGCAC
HOXB3fwd	CCGTGTAGAGATGGCCAACC
HOXB3rev	CAATCCCTTGGCCTTCTGG
HOXB4fwd	AACTCAAACTATGTCGACCCCAAG
HOXB4rev	TACCCGGGCGAGTGGTC
HOXB5fwd	GTTCCTGCTGCCTGGGTAGG
HOXB5rev	GCCCTCTAGCCAGACCCATG
HOXB6fwd	AGGACAAGAGCGTGTTCGGC
HOXB6rev	GGCCCAAAGGAGGAACTGTTG
HOXB7fwd	CCAGCCTCAAGTTCGGTTTTC
HOXB7rev	CCCGAACCCGCTCCATAG
HOXB8fwd	TTTAATCCCTATCTGACTCGTAAGCG
HOXB8rev	GCTCGCATTTGCTGCTGG
HOXB9fwd	AAAAAGCGCTGTCCCTACACC
HOXB9rev	AGGAGTCTGGCCACTTCGTG
HOXB13fwd	TGACTCCCTGTTGCCTGTGG
HOXB13rev	GGCGTCAGGAGGGTGCTG
CDX1fwd	CAGCCGTTACATCACAATCCG
CDX1rev	GGCTGTTGCTGCTGCTGTTTC
CDX2fwd	CACTACAGTCGCTACATCACCATCCG
CDX2rev	GATTTTCCTCTCCTTTGCTCTGCGG
ABLfwd	TTCAGCGGCCAGTAGCATCTGACTT
ABLrev	TGTGATTATAGCCTAAGACCCGGAGCTTTT

Table 7: Primer sets used for the *HOX* gene expression analysis. The *ABL* primer set was designed in the CLIP laboratory Prague, the *HOXA6* primer set was designed by myself, others were described previously (Drabkin *et al.* 2002).

6 RESULTS

6.1 PART I: Study of the function of IL-1α in mammalian cells

The aim of this part of my work was to study the function of human IL-1α precursor, mature IL-1α and the N-terminal part of IL-1α in mammalian cells. I focused exclusively on the role of IL-1α in the cell nucleus and more precisely I was particularly interested in the putative interaction of the IL-1α precursor with the tumour suppressor p53. I was also interested by the possibility to modulate the subcellular localisation of IL-1αNTP in fusion with the enhanced green fluorescent protein (EGFP), that appeared to be unequally distributed among the cell nucleus and cytoplasm.

I was using various mammalian cell lines throughout this study. NIH 3T3 represents a mouse embryonic fibroblast cell line that is relatively easy to transfect and to cultivate; hence, I used these cells in most experiments. For the characterisation of subcellular localisation of the IL-1α proteins I also employed human osteosarcoma Saos-2 cells and Mrc-5 cells, human lung fibroblasts that are not transformed. To study the nuclear interaction of the IL-1α precursor with the tumour suppressor protein p53, I also used human melanoma A375 cells, where p53 can be induced and stabilized with roscovitine, and human non-small-cell lung carcinoma line H1299-R273H, expressing a mutated variant of p53.

6.1.1 Overexpression of the IL-1α precursor, mature IL-1α and IL-1αNTP in mammalian cells

Overexpression of proteins is one of the possible ways to examine their function. I employed transient transfection of selected mammalian cell lines in order to study the IL-1α function in mammalian cells. For the experiment confirming the expression of the IL-1α precursor, mature IL-1α and IL-1αNTP in mammalian cells, I used the NIH 3T3 mouse embryonic fibroblast cell line and recombinant plasmids derived from pcDNA4C/TO/Myc-His (see Table 4 in Materials and methods) encoding these three IL-1α variants. I transiently transfected the cells with the appropriate

plasmids using the FuGENE transfection reagent (5.5.24) and after a 24-hour cultivation I checked the expression of the recombinant proteins in the cell lysate by western blotting (Figure 11).

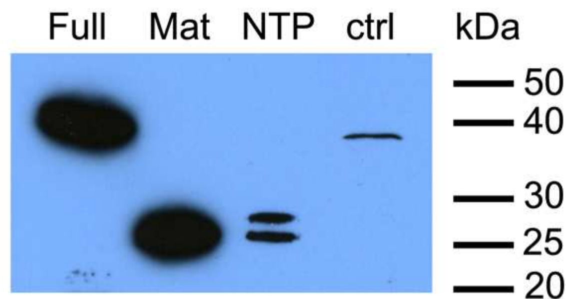


Figure 11: Overexpression of the IL-1α proteins in the mammalian cell line NIH 3T3. Western blotting was performed with the anti-FLAG antibody recognising the FLAG tag at the N terminus of the proteins studied. The proteins appear bigger in size due to their fusion to the FLAG, Myc and His tag. The two bands in the IL-1α sample may result from posttranslational modifications that can occur at the N-terminal part of IL-1α. The band in the control (ctrl) sample is unspecific. Primary antibody: anti-FLAG; secondary antibody: goat anti-mouse.

6.1.2 Subcellular localisation of the IL-1α precursor, mature IL-1α and IL-1αNTP in mammalian cells studied by fluorescence microscopy

Due to the presence of a nuclear localisation sequence within the N-terminal part of the IL-1α precursor, both the IL-1α precursor and IL-1αNTP should be localised in the cell nucleus in contrast to mature IL-1α that lacks the N-terminal part and remains in the cytoplasm. In order to study the subcellular localisation of the IL-1α precursor, mature IL-1α and IL-1αNTP in mammalian cells, I created a set of plasmids encoding fluorescence protein fusions of the IL-1α proteins (see Table 4). These plasmids enabled me to produce an EGFP fusion of the IL-1α precursor and mature IL-1α as well as the DsRED2 variant of the IL-1α precursor. Furthermore, the plasmid construct encoding IL-1αNTP fused to EGFP was prepared by our undergraduate student Denisa Dolečková under my supervision. The cells were transiently transfected with the recombinant plasmids encoding the fluorescent protein IL-1α fusions using the ExGen transfection reagent (5.5.25) and after 24-48 h the cells were fixed and analyzed by fluorescence microscopy (5.5.6).

The precursor of IL-1α fused to DsRED2 or EGFP was located exclusively in the cell nucleus in both NIH 3T3 and Saos-2 cells (Figure 12). In contrast, the subcellular

localisation of mature IL-1α/EGFP fusion protein was cytoplasmic, although a small portion of the mature IL-1α seemed to be localised in the cell nucleus as well (Figure 13). Similarly, cells transiently transfected with the emply plasmid pEGFP-C1 appeared to contain EGFP in their nucleus (Figure 15). This phenomenon was not due to the DAPI staining and resulting emission spectra overlap since the cells unstained with DAPI appeared to contain IL-1α Mat or EGFP in the nucleus as well (Figure 13B). However, when compared to the nuclear localisation of IL-1αNTP/EGFP (Figure 14), which is mediated by NLS, the difference in the amount of protein in the nucleus can be clearly seen.

Interestingly, IL-1αNTP was located both in the nucleus and in the cytoplasm. Our pilot experiments indicated that the localisation of IL-1αNTP varied according to the cell density (addressed in Results part 6.1.5).

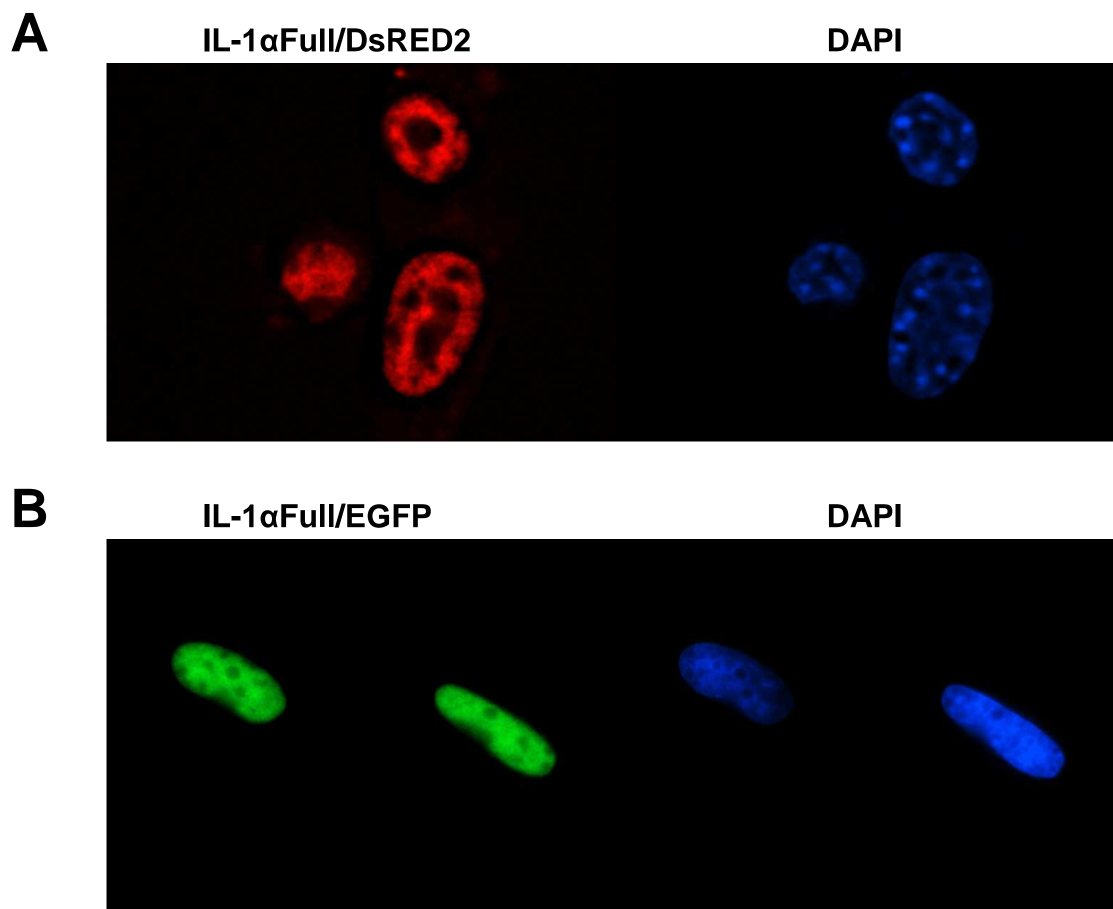


Figure 12: (A) Subcellular localisation of the IL-1α precursor fused to the red fluorescent protein DsRED2 in NIH 3T3 cells. (B) Subcellular localisation of the IL-1α precursor fused to the green fluorescent protein EGFP in Saos-2 cells. In both cell lines used, the IL-1α precursor was located exclusively in the cell nucleus. (×600)

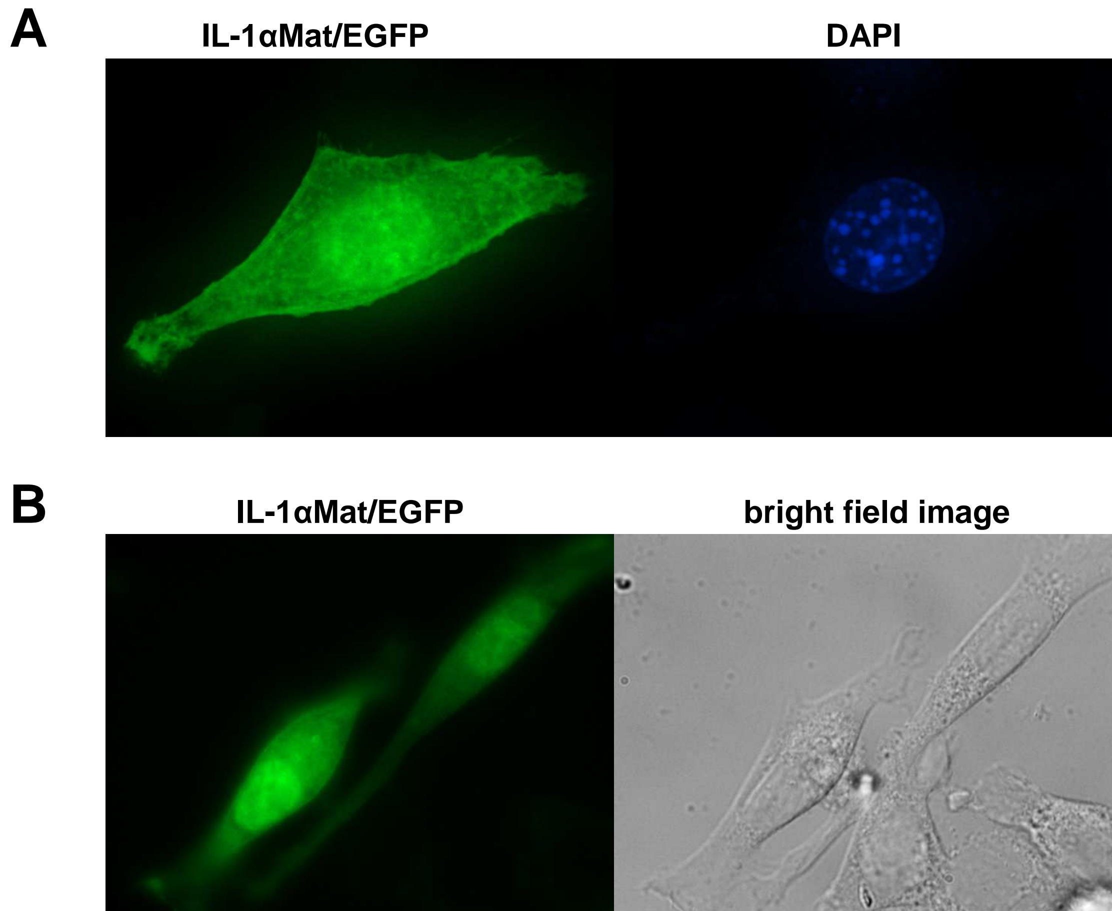


Figure 13: (A) Subcellular localisation of mature IL-1α fused to EGFP in NIH 3T3 cells stained with DAPI. (B) Subcellular localisation of mature IL-1α fused to EGFP in NIH 3T3 cells without DAPI staining. The localisation of mature IL-1α was cytoplasmic although a part of IL-1α Mat appears to be located in the cell nucleus; this occurs even in the cells unstained with DAPI (B). (×600)

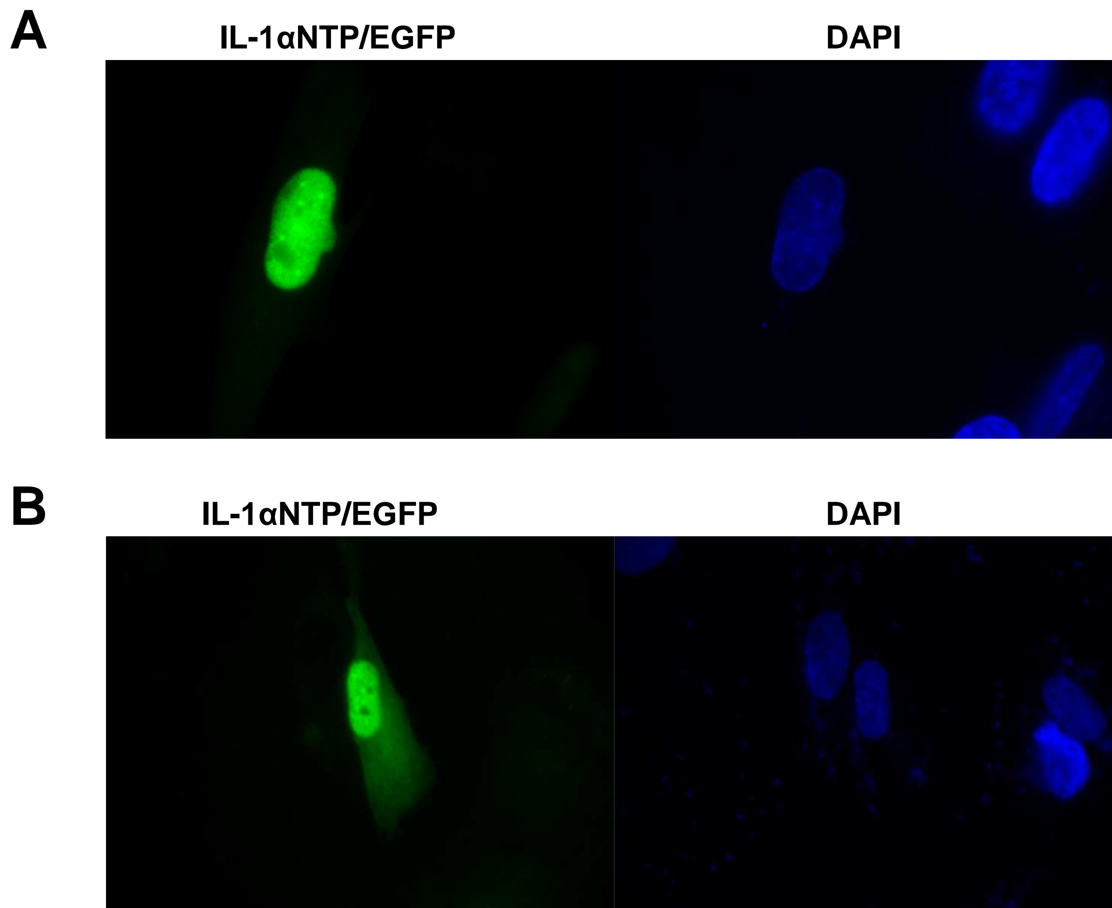


Figure 14: Subcellular localisation of IL-1αNTP fused to EGFP in Mrc-5 cells. IL-1αNTP was located both in the nucleus and in the cytoplasm. In certain cells, only nuclear localisation was observed (A) while other cells contained a part of IL-1αNTP in the cytoplasm as well (B). (×600)

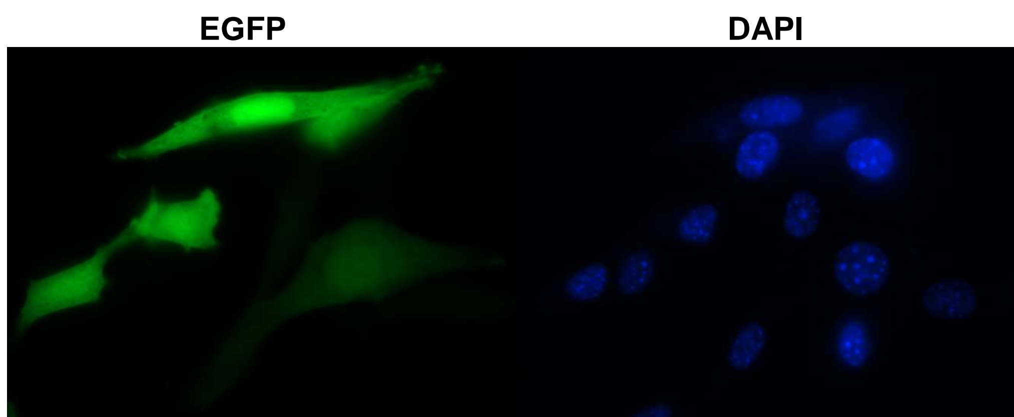


Figure 15: Subcellular localisation of EGFP in the NIH 3T3 cells. Control cells were transfected with the empty plasmid pEGFP-C1. In certain cells, EGFP seems to be partly localised in the cell nucleus probably due to the small EGFP protein size. Similar results were obtained using the plasmid pDsRED2-C1 (encoding the red fluorescent protein DsRED2) for transient transfection. (×600)

6.1.3 Co-localisation of the IL-1α precursor with protein p53 in NIH 3T3 cells

In our laboratory, we have previously analyzed the involvement of the IL-1α precursor in virus-induced apoptosis and we were particularly interested in determining the role of p53 in this process (Vicenova 2005). Our pilot experiments indicated that p53 could co-operate with the IL-1α precursor in activating the apoptotic program in response to viral infection. Since p53 represents a „hub protein" accomodating interactions with many other protein binding partners, there is a possibility that p53 interacts with IL-1α either directly or, more probably, through another p53-associated molecule. Therefore we intended to investigate the putative interaction of the IL-1α precursor with p53 in the current study.

Preliminary co-immunoprecipitation experiments performed by Miroslava Burýšková indicated that the IL-1α precursor co-immunoprecipitates with the tumour suppressor p53. In order to study the interactions of the IL-1α precursor with p53, I co-transfected the NIH 3T3 cells with plasmids encoding DsRED2 fusion of the IL-1α precursor and EGFP-fused p53 using the Amaxa Nucleofector device. I fixed the cells with 3% paraformaldehyde and analyzed them by fluorescence microscopy.

The results indicated that both proteins showed similar distribution patterns in the cell nucleus (Figure 16) and therefore it is possible that both proteins co-localise in the same macromolecular complex. The EGFP-fused p53 was also present in the cytoplasm in the majority of cells while the IL-1α precursor was localised exclusively in the cell nucleus.

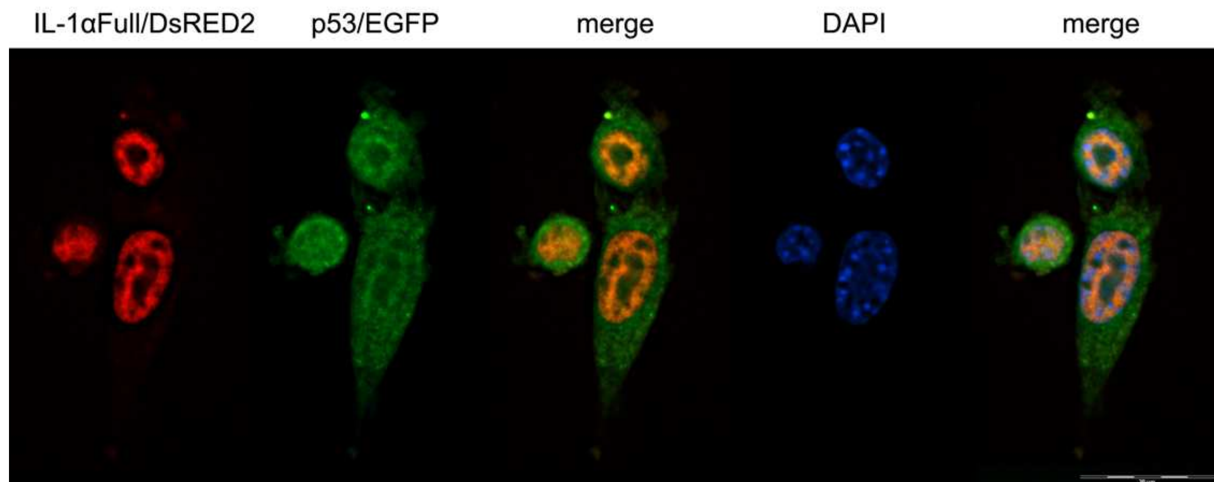


Figure 16: Co-localisation of the IL-1α precursor with p53 in the nucleus of the NIH 3T3 cells. Co-transfection of the NIH 3T3 cells with the fluorescent protein fusion variant of both proteins and subsequent analysis with fluorescence microscopy revealed the co-localisation of the IL-1α precursor with p53 in the nucleus. (×600)

6.1.4 Analysis of the interaction of the IL-1α precursor with p53 by co-immunoprecipitation

Since the co-localisation experiments did not exclude the possibility of an intranuclear interaction between the IL-1α precursor and the tumour suppressor p53, I decided to analyze the physical association of these two proteins by co-immunoprecipitation. This technique is widely used to identify either direct or indirect protein-protein interactions. I performed these experiments according to the protocol „Co-immunoprecipitation from mammalian cells“ (Materials and Methods part 5.5.4).

In this experiment I was using the IL-1α precursor produced from the plasmid pcDNA4/TO/FLAGMycHis-IL-1αFull as a bait. In order to avoid problems with co-transfection by two different plasmids encoding the IL-1α precursor and p53, I intended to upregulate the p53 synthesis and increase its nuclear fraction. Therefore I decided to use the human melanoma cell line A375 and treatment with roscovitine. Roscovitine is a purine derivative that act as a CDK inhibitor and activates and stabilises nuclear p53 in tumour cells (Lu *et al.* 2001). The effect of roscovitine on p53 induction in A375 cells is greatly enhanced by UV irradiation (Bořivoj Vojtěšek, personal communication) that I verified by western blotting (Figure 17). In parallel I was using the human non-small-cell lung carcinoma line H1299-

R273H that is stably transfected with pCMV-neo-Bam-p53R273H, expressing mutant p53 that does not bind to target DNA. In this cell line p53 is produced in large quantities and does not need to be induced with UV and roscovitine.

To study the physical association of the IL-1α precursor and the tumour suppressor p53, I transiently transfected both cell lines with the expression plasmid pcDNA4/TO/FLAGMycHis-IL-1αFull using the transfection reagent Turbofect (5.5.23). 24 h after transfection I irradiated the A375 cell line with 2 mJ/cm^2 UV and added fresh growth medium containing 20 µM roscovitine. H1299-R273H cells remained untreated. After 2 h I lysed the cells of both lines and performed immunoprecipitation of the IL-1α precursor using anti-FLAG antibody. To detect the co-precipitated proteins, I performed western blotting and probed the membrane with anti-p53 CM-1 primary antibody. I detected a faint band corresponding to p53 co-immunoprecipitated together with the IL-1α precursor from A375 cells; no signal was detected from cells H1299-R273H (Figure 18).

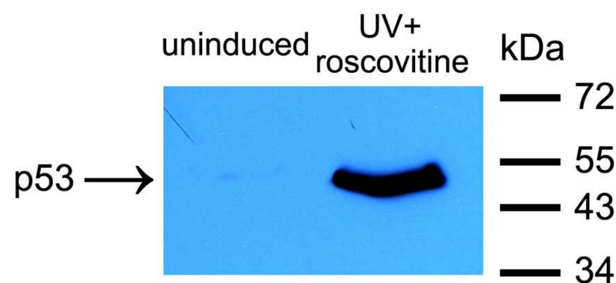


Figure 17: Induction of p53 by UV irradiation and roscovitine treatment in A375 cells. Cells were irradiated with 2 mJ/cm^2 UV and treated with 20 µM roscovitine. After 2 h the cells were lysed and western blotting was performed. Primary antibody: anti-p53 (mouse monoclonal); secondary antibody: goat anti-mouse.

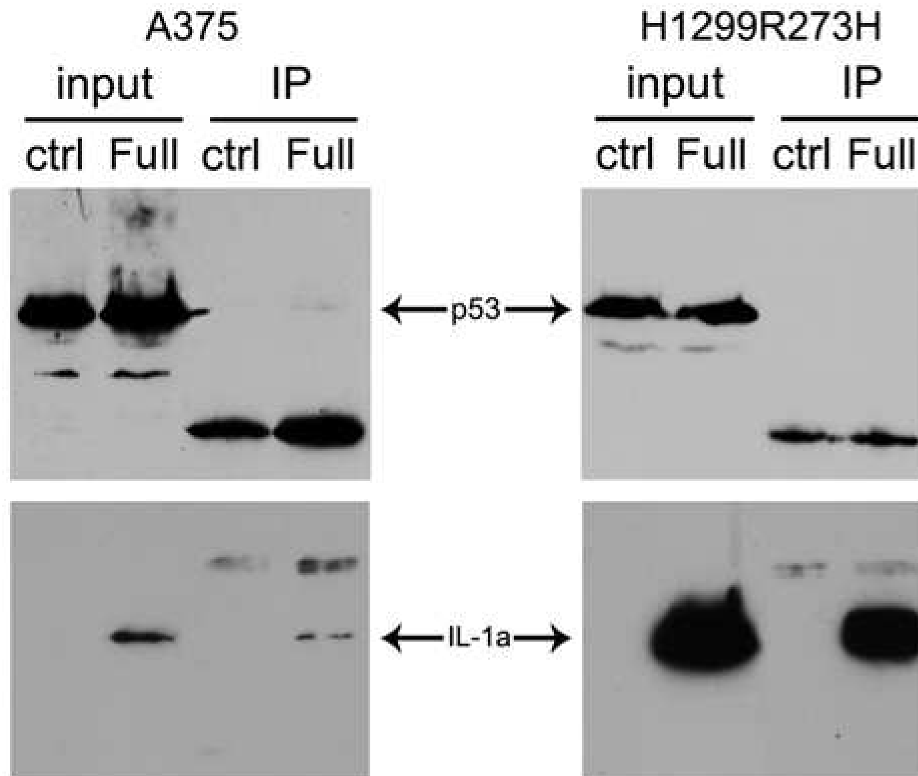


Figure 18: Co-immunoprecipitation of the IL-1α precursor and protein p53 from A375 and H1299-R273H cells. In A375 cells, p53 was induced using UV and roscovitine, H1299-R273H express larger amounts of p53 that, however, bears a point mutation preventing the protein from binding to target DNA. Cells were transiently transfected with pcDNA4/TO/FLAGMycHis-IL-1αFull (Full), control cells (ctrl) remained untransfected. A faint band indicates that p53 can be co-immunoprecipitated together with the IL-1α precursor from A375 cells, but not from H1299-R273H cells. p53 was detected with the CM-1 anti-p53 antibody, the expression and immunoprecipitation of the IL-1α precursor was confirmed with the anti-Flag antibody. Secondary antibodies: swine anti-rabbit and goat anti-mouse, respectively.

6.1.5 Modulation of IL-1αNTP subcellular localisation in Mrc-5 cells

In this part of my work, I focused on the study of subcellular localisation of IL-1αNTP fused to EGFP. Interestingly, in Mrc-5 cells transiently transfected with the plasmid pEGFP-C1/IL-1αNTP, I always observed two types of IL-1αNTP/EGFP subcellular localisation under the fluorescence microscope: the „nuclear“ and the „diffuse“ localisation (as it can be seen in Figure 19), one of which each time prevailed. I intended to investigate whether the subcellular localisation of IL-1αNTP/EGFP can be shifted towards one prevailing type of localisation after

cultivation under different conditions or not. Therefore I performed a set of experiments aimed at the modulation of IL-1αNTP/EGFP localisation using various agents or culturing conditions:

a) different cell confluency (50% vs. 100%) (6.1.5.1)
b) conditioned medium vs. fresh medium (6.1.5.2)
c) extracellular IL-1α in growth medium (6.1.5.3)

All experiments were performed in the Mrc-5 cells. Cells were transiently transfected with plasmid pEGFP-C1/IL-1αNTP, cultivated under different growth conditions according to the scheme above and observed under a fluorescence microscope.

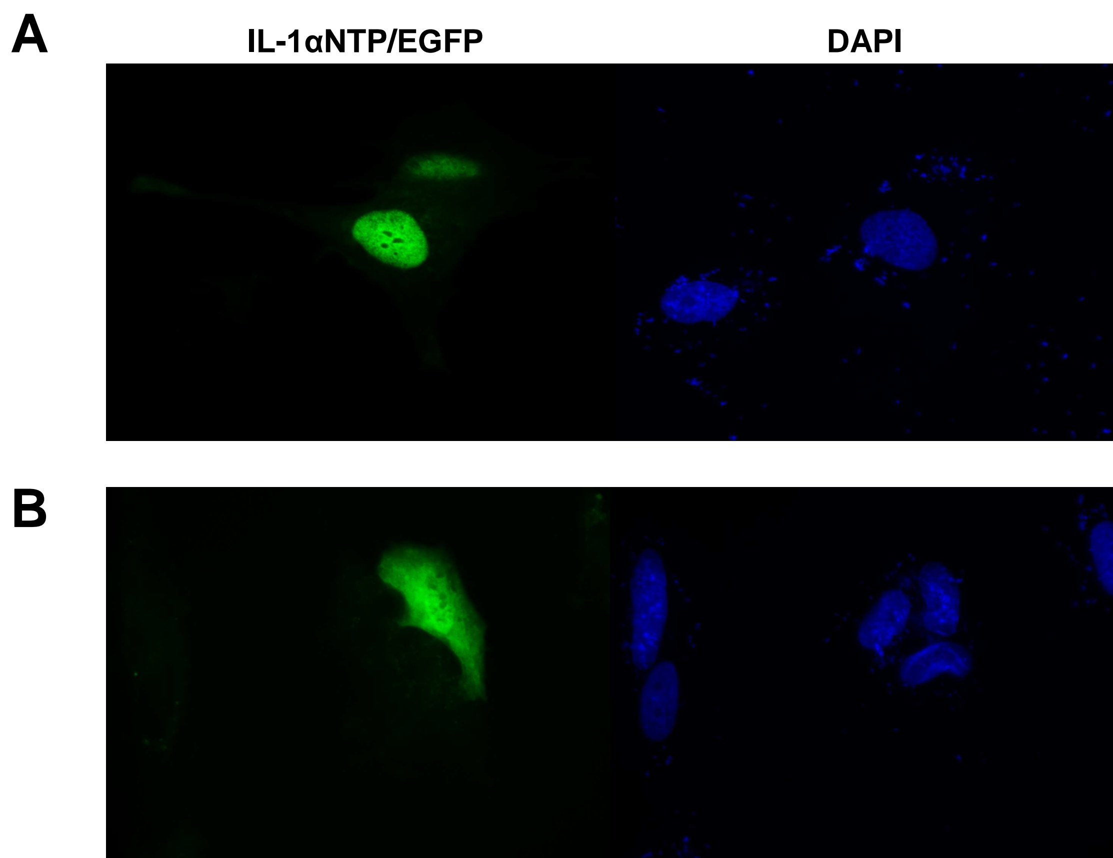


Figure 19: The two types of IL-1αNTP/EGFP subcellular localisation observed. (A) The nuclear and **(B)** the diffuse localisation of IL-1αNTP can be distinguished. (×600)

6.1.5.1 Modulation of the IL-1αNTP subcellular localisation by cell confluency

In this experiment Mrc-5 cells of different confluency (approximately 50% and 100%) were used to study the influence of cell confluency on the IL-1αNTP/EGFP subcellular localisation. A total number of four experiments was performed by Denisa Dolečková during her diploma thesis (Dolečková 2011) experimental work under my supervision. In 100%-confluent cells, nuclear localisation of IL-1αNTP/EGFP prevailed (Table 8).

Confluency	**Experiment number**	**1**		**2**		**3**		**4**	
	Localisation	**N. of cells**	**%**	**N. of cells**	**%**	**N. of cells**	**%**	**N. of cells**	**%**
100%	Nuclear	19	**65,52**	69	**65,71**	83	**80,58**	78	**50,65**
	Diffuse	10	**34,48**	36	**34,29**	20	**19,42**	76	**49,35**
	Total cell n.	29		105		103		154	
50%	Nuclear	12	**34,29**	31	**36,05**	4	**6,67**	13	**19,70**
	Diffuse	23	**65,71**	55	**63,95**	56	**93,33**	53	**80,30**
	Total cell n.	35		86		60		66	

Table 8: Modulation of IL-1αNTP/EGFP subcellular localisation by cell confluency. In 100%-confluent cells, nuclear localisation of IL-1αNTP/EGFP prevailed in contrast to 50%-confluent cells where prevailing localisation of IL-1αNTP/EGFP was diffuse. Performed by Denisa Dolečková under my supervision.

6.1.5.2 Modulation of the IL-1αNTP subcellular localisation using conditioned medium

Since I observed a change in the subcellular localisation of IL-1αNTP/EGFP in the cell cultures of higher densities, a hypothesis emerged that the shift towards the nuclear localisation of IL-1αNTP/EGFP may be caused by a substance which is secreted by the cells and diffuses along the growth medium. Therefore, in this experiment, I intended to investigate the effect of the conditioned medium on the subcellular localisation of IL-1αNTP/EGFP. Conditioned medium is a growth medium collected from cultured cells after several days of culturing. It is enriched with many growth factors and other substences produced and secreted by the cells cultivated in this medium. In this experiment, the conditioned medium was collected from Mrc-5

cells after one week of culturing. Cells were washed twice with a fresh medium and incubated in the conditioned medium for 2 hours. In the control samples, I used a fresh D-MEM medium. The confluency of the cellular culture varied from approximately 60% to 75%.

The first experiment was performed by Denisa Dolečková during her diploma thesis experimental work. According to the result of this experiment, the subcellular localisation of IL-1αNTP/EGFP can be shifted towards the nuclear localisation in the cells growing in the conditioned medium. I repeated this experiment and found that the portion of cells with nuclear localisation of IL-1αNTP/EGFP was only slightly larger in cells treated with conditioned medium than in control cells. In the third experiment I obtained the results showing that in cells grown in the conditioned medium, nuclear localisation of IL-1αNTP/EGFP prevails (Table 9).

Medium	**Experiment number**	**1**		**2**		**3**	
	Localisation	**N. of cells**	**%**	**N. of cells**	**%**	**N. of cells**	**%**
Conditioned medium	Nuclear	71	**83,53**	50	**29,8**	39	**57,4**
	Diffuse	14	**16,47**	118	**70,2**	29	**42,6**
	Total cell n.	85		168		68	
Fresh medium	Nuclear	8	**9,76**	23	**20,5**	26	**36**
	Diffuse	74	**90,24**	89	**79,5**	46	**64**
	Total cell n.	82		112		72	

Table 9: Modulation of the IL-1αNTP/EGFP subcellular localisation by conditioned medium. In the first experiment, the subcellular localisation of IL-1αNTP/EGFP is shifted towards the nuclear localisation in cells grown in the conditioned medium. However, **in the second experiment**, the portion of cells with nuclear localisation of IL-1αNTP/EGFP is only slightly larger in cells treated with conditioned medium than in control cells. **In the third experiment** I obtained the results showing that in cells grown in the conditioned medium, nucelar localisation of IL-1αNTP/EGFP prevails. Experiment number 1 was performed by Denisa Dolečková.

6.1.5.3 Modulation of the IL-1αNTP subcellular localisation using extracellular IL-1α in medium

The experiment using the conditioned medium showed that the modulation of the subcellular localisation of IL-1αNTP/EGFP may be mediated by a substance

secreted by the cells into the growth medium. In this experiment, I tried to add recombinant IL-1α (Peprotech) into the growth medium. I used the concentration of 15 ng/ml, that has been used for cell stimulation with IL-1 previously (Scheel *et al.* 2006; Jura *et al.* 2008). First I washed the cells with the fresh growth medium twice, I added the growth medium enriched with IL-1α and incubated the cells for 2 h. In the cell culture treated with recombinant IL-1α, the nuclear localisation of IL-1αNTP/EGFP was slightly or significantly more frequent than in untreated cells (Figure 20 and Table 10).

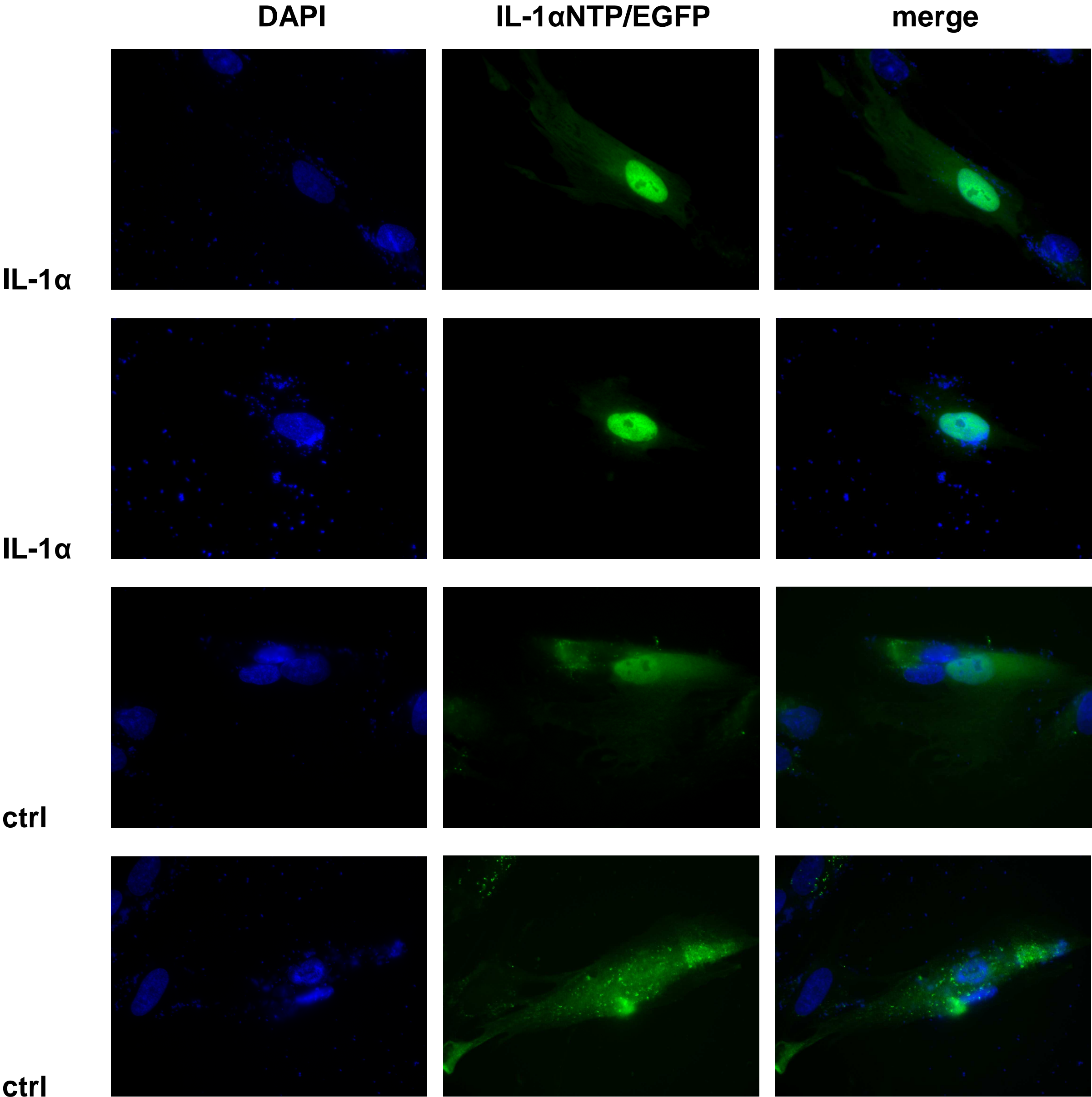


Figure 20: Modulation of IL-1αNTP/EGFP subcellular localisation in Mrc-5 cells using recombinant IL-1α. In the cells treated with recombinant IL-1α, the nuclear localisation of IL-1αNTP/EGFP was slightly or significantly more frequent than in untreated cells. (×600)

Treatment	Experiment number	1		2		3	
	Localisation	Number of cells	%	Number of cells	%	Number of cells	%
IL-1α-treated	Nuclear	67	**64,5**	**48**	**27,7**	**57**	**36,3**
	Diffuse	37	**35,5**	**125**	**72,3**	**100**	**63,7**
	Total n. of cells	104		**173**		**157**	
untreated	Nuclear	28	**28,6**	**23**	**20,5**	**53**	**29,1**
	Diffuse	70	**71,4**	**89**	**79,5**	**129**	**70,9**
	Total n. of cells	98		112		182	

Table 10: Modulation of the IL-1αNTP/EGFP subcellular localisation by extracellular IL-1α. In the cells treated with recombinant IL-1α, the nuclear localisation of IL-1αNTP/EGFP was slightly or significantly more frequent than in untreated cells.

Results of the experiments studying the subcellular localisation of IL-1αNTP/EGFP suggest that it can be modulated using various agents or under different culturing conditions. When cultivated in higher densities, with 15 ng/ml recombinant IL-1α or using conditioned medium, Mrc-5 cells transiently transfected with plasmids encoding IL-1αNTP/EGFP exhibit the nuclear localisation of the fluorescence fusion protein slightly or significantly more frequently than control cells.

6.2 PART II: Study of the IL-1α function in the model organism *Saccharomyces cerevisiae*

Yeast as a simple eukaryotic organism can often serve as a model for studying the function, interactions and various other features of the proteins specific for higher eukaryotes. It was actually in yeast that the interaction of IL-1α with the histone acetyltransferase complexes was discovered (Buryskova *et al.* 2004). Due to the evolutionary conservation of the HAT complexes throughout eukaryotes, the results gained in the experiments with the yeast cells may help to elucidate the interactions of IL-1α with mammalian HAT complexes and to focus on specific mammalian HAT subunits homologous to their yeast counterparts. Therefore we decided to further characterise the reported interaction of IL-1α with the histone acetyltransferase complexes in the yeast cells and to take advantage of the potential of molecular genetic techniques that are easier to employ in yeast than in mammalian cells.

In this part of my work, I first examined certain aspects of the heterologous expression of IL-1α in the yeast cells such as the expression plasmid stability, subcellular localisation and possibility of immunoprecipitation. Then I studied the interactions of IL-1α with the yeast SAGA HAT complex that is well conserved accross all eukaryotes and currently serves as an archetype of the HAT complexes. I was using co-immunoprecipitation of the IL-1α precursor with selected subunits of the SAGA complex and also of another yeast HAT complex, ADA. I demonstrate here that yeast proved to be an excellent model for the study of the IL-1α interaction with the HAT complexes and show that the IL-1α precursor physically interacts with the HAT/Core module of SAGA complex.

6.2.1 Heterologous expression of the IL-1α precursor, mature IL-1α and IL-1αNTP in *Saccharomyces cerevisiae*

For the expression of human IL-1α proteins in a yeast host system I was using the *S. cerevisiae* W303-1A strain that has been widely used in our laboratory. I inserted human cDNA sequences coding for the IL-1α precursor (Full) and mature IL-1α (Mat) to the yeast expression plasmid pYX212 that allow transcription of the gene from the TPI promoter. I constructed two variants of Full and Mat differing in the

in the presence of the epitope: both N-terminal FLAG and C-terminal HA (influenza virus haemagglutinin) tag (briefly „N/S“) or N-terminal FLAG tag only („N/X“) (see Table 4 in Materials and Methods), but I preferentially used the N/X variant of Full and only the N/X variant of Mat in subsequent experiments. I performed transformation of the yeast cells according to the protocol „LiAc transformation of yeast cells“ (5.5.10). Expression of both IL-1α forms in yeast was confirmed by western blotting using anti-HA and anti-FLAG antibody (Figure 21).

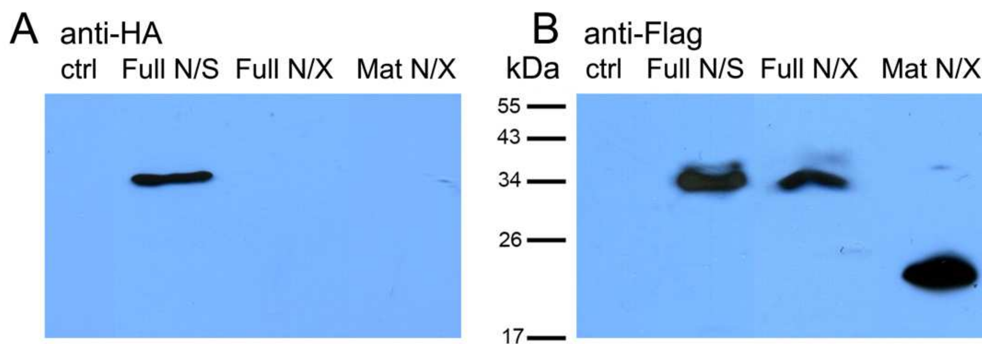


Figure 21: Heterologous expression of the IL-1α proteins in *Saccharomyces cerevisiae.* The IL-1α precursor (Full) and mature IL-1α (Mat) are expressed from the plasmid pYX212. **(A) HA tagged IL-1α precursor.** Western blotting was performed with the anti-HA antibody recognising the HA tag at the C-terminus of the IL-1α precursor (Full N/S). **(B) FLAG-tagged IL-1α precursor and mature IL-1α.** Western blotting was performed with the anti-FLAG antibody recognising the FLAG tag at the N terminus of all IL-1α variants. Secondary antibody: goat anti-mouse.

6.2.2 Effect of the presence of the functional IL-1α precursor gene on plasmid stability in yeast

During the longer-term cultivation and passaging of the W303 strains overexpressing the IL-1α precursor, I observed a marked decrease of the protein expression in the course of time and also a decreased viability of yeast strains, harbouring the expression plasmids coding for the IL-1α precursor, after prolonged storage on agar plates. This phenomenon could have reflected a toxic effect resulting from the IL-1α precursor overexpression; it seemed that the plasmids encoding the IL-1α precursor are eliminated by the host cells more rapidly than the control empty plasmids. Therefore I decided to perform a growth experiment focusing on the influence of the functional IL-1α precursor gene on plasmid stability in the yeast cells.

For this experiment I used two recombinant expression plasmids that carry the IL-1α precursor gene under the control of the inducible GAL1 promoter. pYX133N/S IL-1αFull (hereafter referred to as „N/S“) encodes the IL-1α precursor with C-terminal HA tag and N-terminal FLAG tag whereas IL-1α precursor expressed from pYX133N/X IL-1αFull („N/X“) lacks the HA tag. In this way I was also able to observe the possible effect of the presence of the HA tag. Control strains were bearing empty plasmid pYX133.

I performed this experiment directly after the transformation of the W303-1a strain with the IL-1α-encoding plasmids according to the protocol „LiAc transformation of yeast cells“ (5.5.10). I plated the cells onto the SD agar plate without tryptophan and randomly selected three colonies from each strain (labeled 133-1, 2, 3; N/S-1, 2, 3; N/X-1, 2, 3). I checked the auxotrophic requirements of selected transformants, inoculated the nine selected strains into the liquid SD gal medium lacking tryptophan and next day I re-inoculated them into the non-selective liquid SD gal medium at OD_{600}=0,05. At this time (t=0), first samples were collected (labeled t0) and plated onto non-selective SD plates. When the liquid cultures reached the exponential growth phase (OD_{600}=0,9-1,2), I collected the samples again (labeled t1) and re-inoculated the cell culture into the non-selective SD gal liquid medium at OD_{600}=0,05. Following this scheme I collected also the samples t2-t4 and plated them onto non-selective SD plates; the time period t0-t4 corresponded approximately to 16 yeast generations (Table 11). Subsequently, I picked randomly 50 colonies per strain and sample growing on the non-selective plates and tested them for tryptophan requirement indicating the presence or absence of the recombinant plasmid.

The exact number of colonies capable of growth on the selective agar plates is shown in Table 12, Table 13 shows the percentage of colonies grown and Graph 1 depicts the percentage of cells growing on the selective plates after the incubation in non-selective conditions relative to the total number of colonies tested. The experiment has shown that the plasmids encoding the IL-1α precursor are removed from the cells more rapidly than the empty vectors and has confirmed the role of the functional IL-1α precursor gene on plasmid stability in yeast. It also seemed that the C-terminal HA tag possibly could slightly contribute to the IL-1α-mediated plasmid instability as can be seen from the Graph 1, although this observation may not be significant due to the relatively small number of colonies tested.

time	133-1	133-2	133-3	N/S-1	N/S-2	N/S-3	N/X-1	N/X-2	N/X-3
t0	0	0	0	0	0	0	0	0	0
t1	3,61	3,93	3,97	3,76	3,75	3,8	3,99	3,87	3,69
t2	7,76	8,18	7,99	8,16	8,3	8,4	8,29	8,25	7,83
t3	11,8	12,12	11,96	12,28	12,36	12,31	12,1	12,08	11,77
t4	15,68	16,12	15,72	16,4	16,33	16,02	15,76	15,73	15,46

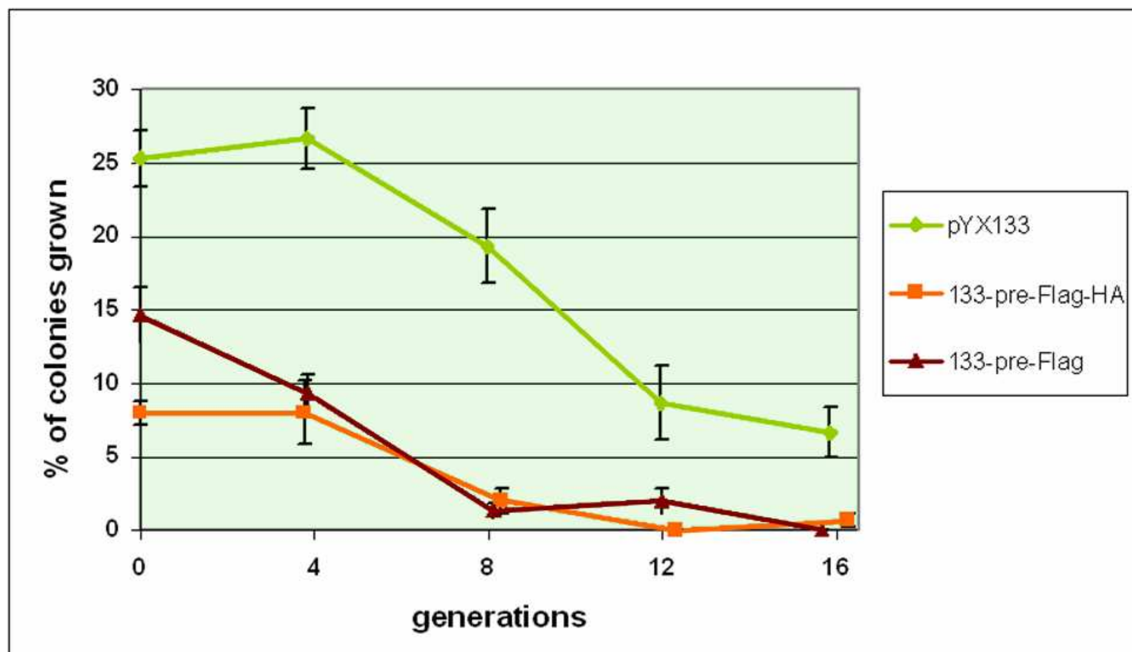
Table 11: Number of generations reached by each strain at the time point indicated. See the text for details.

time	133-1	133-2	133-3	N/S-1	N/S-2	N/S-3	N/X-1	N/X-2	N/X-3
t0	14	14	10	3	5	4	6	10	6
t1	11	13	16	5	6	1	6	3	5
t2	9	13	7	2	0	1	1	0	1
t3	7	5	1	0	0	0	2	0	1
t4	1	5	4	0	0	1	0	0	0

Table 12: Number of colonies grown on selective plates after the incubation in non-selective conditions. 50 colonies were tested in total. See the text for details.

time	133-1	133-2	133-3	N/S-1	N/S-2	N/S-3	N/X-1	N/X-2	N/X-3
t_0	28	28	20	6	10	8	12	20	12
t_1	22	26	32	10	12	2	12	6	10
t_2	18	26	14	4	0	2	2	0	2
t_3	14	10	2	0	0	0	4	0	2
t_4	2	10	8	0	0	2	0	0	0

Table 13: Percentage of colonies growing on selective plates after the incubation in non-selective conditions. See the text for details.



Graph 1: Percentage of cells growing on selective plates after the incubation in non-selective conditions relative to the total number of colonies tested. Obviously, the strains harbouring the functional IL-1α precursor gene (N/S and N/X) get rid of the corresponding plasmids more rapidly than the control strain carrying the empty plasmid pYX133.

6.2.3 Subcellular localisation of the IL-1α precursor and mature IL-1alpha in yeast

Given that the IL-1α precursor contains a nuclear localisation sequence in its N-terminal domain, both the full-length precursor and IL-1αNTP are commonly found in the nucleus of various mammalian cells while mature IL-1α resides in the cytoplasm. In order to study the nuclear function of the IL-1α precursor in the cells of *Saccharomyces cerevisiae*, I intended to examine whether the IL-1α proteins in yeast show a similar subcellular localisation as in mammalian cells.

To study the subcellular localisation of the IL-1α precursor and IL-1αMat in the yeast cells, I used fluorescence microscopy of the yeast-enhanced GFP (yGFP)-labeled IL-1α fusion proteins. The IL-1α precursor gene as well as the coding sequence of the mature IL-1α were inserted into the yeast expression vector pUG36 (Materials and Methods part 5.5.28.1). The constructs were introduced to the *S. cerevisiae* W303-1A strain. After fixation and DAPI staining of the nuclei I observed the cells under the fluorescence microscope (part 5.5.5).

The experiment confirmed the nuclear localisation of the IL-1α precursor in yeast cells since the yGFP fluorescence co-localised with DAPI-stained nuclei in this sample (Figure 22). In contrast, the localisation of mature IL-1α was cytoplasmic, as well as the localisation of yGFP in control cells bearing the empty vector pUG36. Therefore I concluded that the subcellular localisation of the IL-1α proteins corresponds to the locatisation in the mammalian cells and that and the nuclear trafficking of the IL-1α precursor appears to be conserved in the yeast cells.

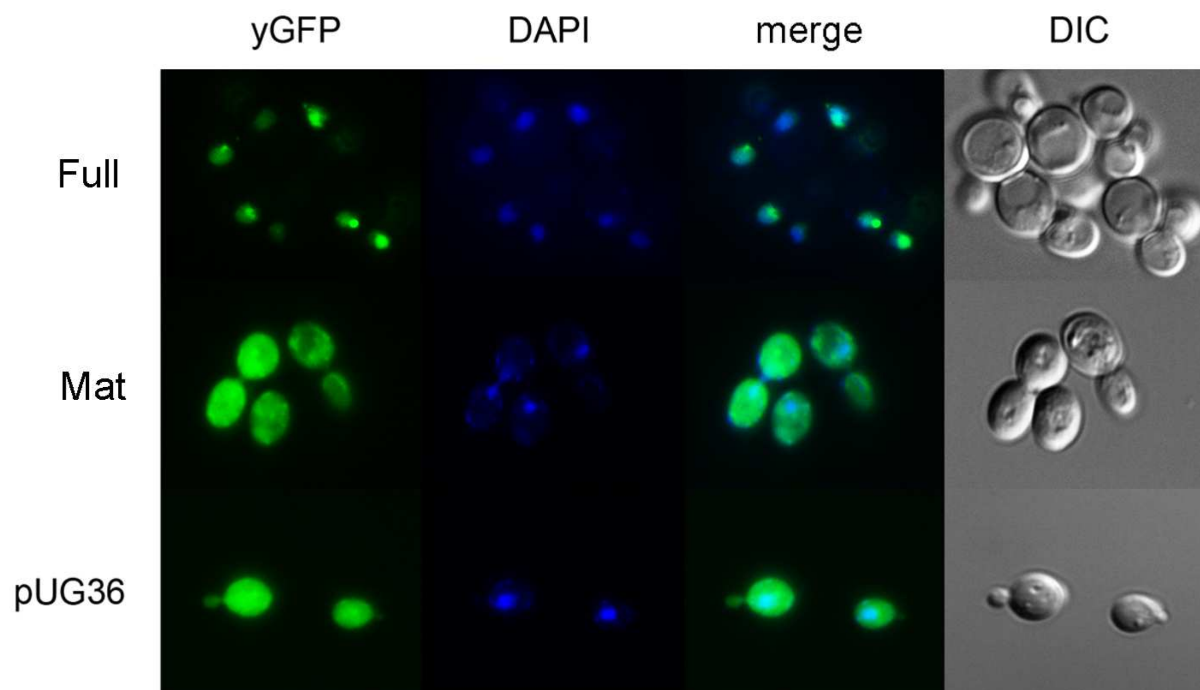


Figure 22: Subcellular localisation of the IL-1α precursor and mature IL-1α in *Saccharomyces cerevisiae.* The IL-1α precursor (Full) is located exclusively in the nucleus of the yeast cells as shown by the co-localisation with the DAPI-stained nuclei in contrast to the observed cytoplasmic localisation of mature IL-1α (Mat). Control cells (pUG36) carry the empty vector pUG36. (×600)

6.2.4 Identification of SAGA HAT subunits binding the IL-1α precursor in the yeast cells

Previous studies of human IL-1α in the yeast two-hybrid system revealed the transcription transactivation potential of IL-1αNTP, but not mature IL-1α, fused to the Gal4p DNA-binding domain (Gal4BD), that required an intact SAGA histone acetyltransferase complex (Buryskova *et al.* 2004). Due to interference with host

transcription, IL-1αNTP/Gal4BD exhibited a toxic phenotype in *S. cerevisiae*. This toxicity was completely abrogated in strains harbouring *GCN5*, *ADA2*, *ADA3* and *SPT7* gene deletions but not in the strain containing a deletion of the *AHC1* gene encoding the Ahc1 protein being the structural subunit of the ADA complex. The results suggested that IL-1αNTP interacts with the SAGA complex, but not with the ADA complex in the yeast cells. In the current work I intended to elucidate the interactions between the IL-1α precursor and the yeast SAGA complex in more detail through immunoprecipitation using IL-1αFull as a bait for binding various SAGA and ADA subunits. IL-1αMat was used as a control because I verified previously that it does not enter the yeast nucleus and therefore should not interact with nuclear histone acetyltransferases.

In order to study the interaction of the IL-1α precursor with SAGA, I needed first to optimise the conditions of co-immunoprecipitation of yeast HAT complexes via interleukin-1α from the yeast cells. I chose N-terminal tagging of the interleukin-1α proteins by the FLAG epitope and vectors based on yeast expression multicopy plasmid pYX212, which contains the strong constitutive triose phosphate isomerase promoter. I separately introduced the recombinant plasmids encoding the IL-1α precursor (pYX212N/X IL-1αFull) and mature IL-1α (pYX212N/X IL-1αMat) into the yeast BY4741 strain harbouring TAP-tagged SAGA subunit Ada1 (Ghaemmaghami *et al.* 2003) and performed co-immunoprecipitation of the IL-1α proteins with TAP/Ada1 using the anti-FLAG antibody recognising the FLAG tag at the N-terminus of IL-1α and protein G-agarose (see Materials and methods part 5.5.4). The possibility to immunoprecipitate both proteins from a yeast cell lysate with low background was verified by western blotting (Figure 23).

Subsequently, I performed, according to the same protocol, a set of co-immunoprecipitations from yeast BY4741 strains bearing various SAGA and ADA histone acetyltransferase subunits labeled with the TAP tag at the C terminus (Ghaemmaghami *et al.* 2003) and harbouring pYX212-derived IL-1α expression plasmids. Due to a limited number of TAP-fusion strains that were available to me I was only able to examine the interaction of IL-1α with yeast histone acetyltransferase complexes containing TAP-tagged Gcn5, Ada2, Ada3 (found both in the SAGA and ADA complex HAT core), Spt7, Spt8, Ada1 (specific for SAGA) and Ahc1 (specific for ADA). To detect these TAP-fused HAT subunits by western blotting, I used a single antibody raised against the calmodulin-binding peptide (CBP)

that makes part of the TAP tag. This approach enabled me to identify at least a part of proteins contained in the complex binding IL-1α in the yeast cells.

Results of my co-immunoprecipitation experiments revealed that all of the studied HAT subunits were present in the complex of yeast proteins binding the IL-1α precursor (Figure 24). In contrast, none of these subunits was found to bind mature IL-1α although western blotting confirmed the expression of the studied proteins (Figure 25A) as well as the succesful immunoprecipitation of mature IL-1α (Figure 25B).

The binding of the Ahc1 protein to IL-1α was surprising for me since previously published results indicated that IL-1α doesn´t functionally interact with the ADA complex (Buryskova *et al.* 2004). However, based on the Ahc1 interaction with the IL-1α precursor, a new hypothesis emerged that in addition to SAGA, IL-1α may interact with the ADA complex as well. The only known protein complex shared among SAGA and ADA is the HAT/Core module.

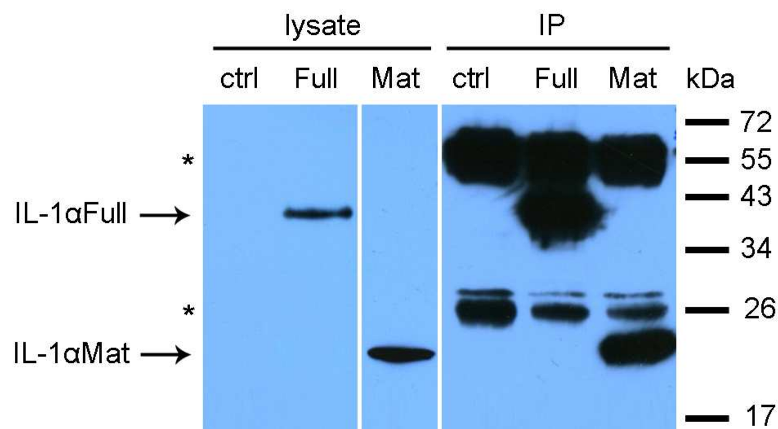


Figure 23: Immunoprecipitation of the IL-1α precursor and mature IL-1α from the yeast TAP/Ada1 strain lysate. Detection of IL-1αFull (from TAP/Gcn5,*ahc1Δ*) and IL-1αMat (from TAP/Ahc1 strain) in the yeast cell lysate is also shown; immunoprecipitation was performed using the anti-FLAG antibody. Asterisks indicate the bands corresponding to the heavy and light chains of the anti-FLAG antibody. Molecular size marker positions are shown at the right. Primary antibody: mouse anti-FLAG; secondary antibody: goat anti-mouse.

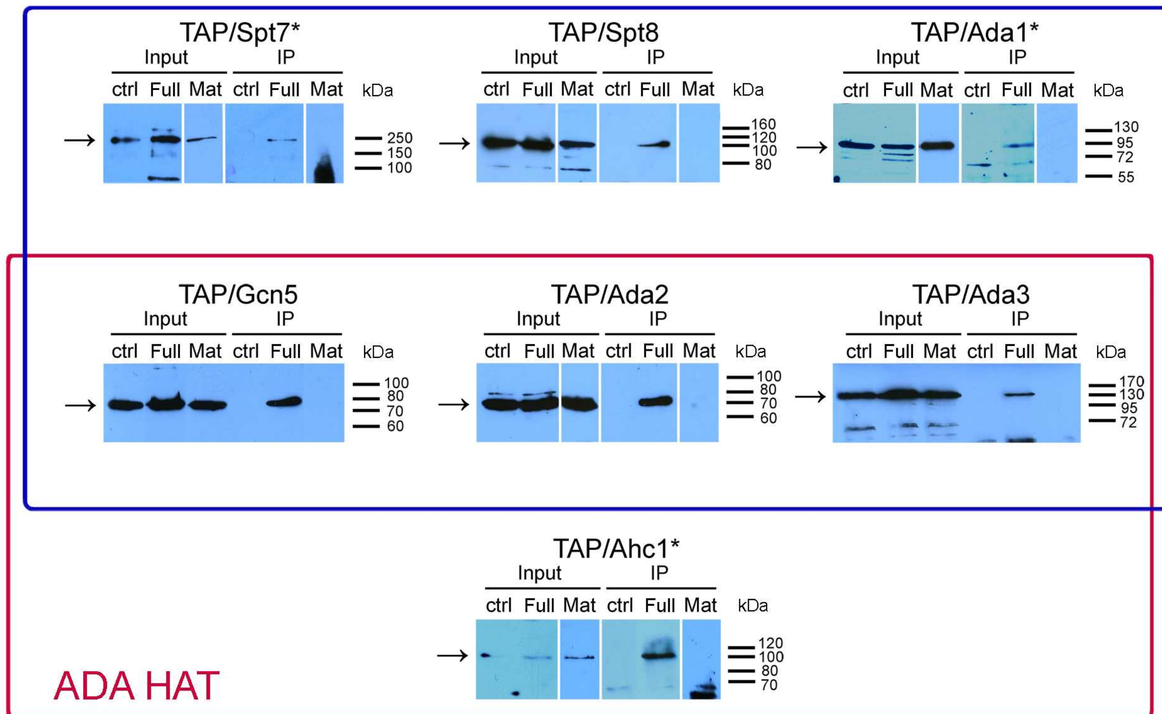


Figure 24: SAGA and ADA HAT subunits identified as a part of the IL-1α precursor-binding complex in *S. cerevisiae.* Western blotting was performed with anti-CBP antibody recognising the TAP tag at the C terminus of the HAT subunits. All of the studied proteins co-immunoprecipitated with the IL-1α precursor (Full). In contrast, mature IL-1α (Mat) doesn´t bind any of these proteins. Control cells (ctrl) carry the empty plasmid pYX212. The asterisk indicates the subunit required for the particular HAT complex integrity. Secondary antibody: swine anti-rabbit.

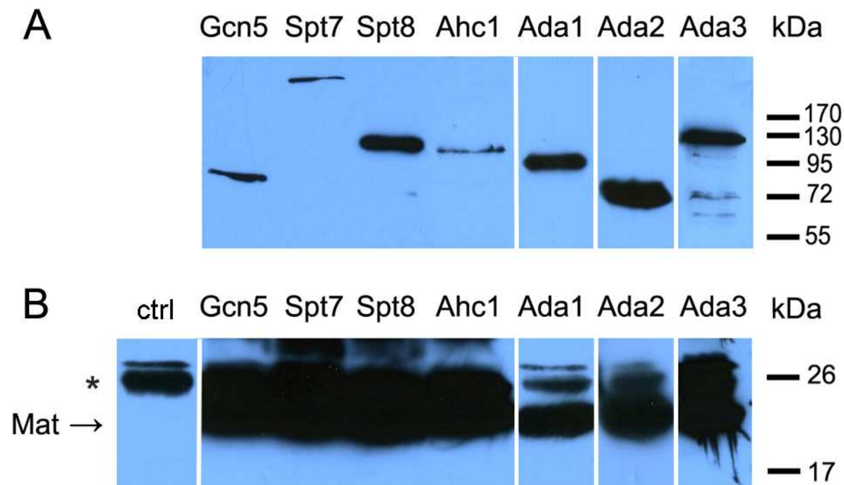


Figure 25: (A) Expression of the TAP-fused HAT subunits in corresponding yeast cell lysates used as input for the mature IL-1α immunoprecipitation. The expression of all subunits was confirmed by western blotting performed with anti-CBP antibody recognising the TAP tag at the C terminus of the HAT subunits. Secondary antibody: swine anti-rabbit. **(B) Mature IL-1α immunoprecipitated from the lysates of the *S. cerevisiae* strains expressing corresponding TAP-fused HAT subunits.** Western blotting was performed with anti-FLAG antibody recognising the FLAG tag at the N terminus of mature IL-1α (Mat). Asterisk indicates the band corresponding to the light chain of the anti-FLAG antibody. Positions of molecular size markers are shown at right. Secondary antibody: goat anti-mouse.

6.2.5 Analysis of the IL-1α-binding protein complex in yeast strains with gene deletions of selected HAT subunits

In order to refine my results and to get more inside the interactions of IL-1α with the yeast histone acetyltransferase complexes, I performed a series of gene disruptions resulting in a loss of expression of the studied HAT subunits. I replaced the genes *GCN5* (catalytic subunit of both the SAGA and ADA complexes), *SPT7* (maintains the integrity of SAGA) and *AHC1* (a unique ADA component essential for the integrity of ADA) with the *kanMX* gene disruption cassette (Gueldener *et al.* 1996).

I designed the primers containing the sequences complementary to the *kanMX* disruption cassette as well as to the sequences flanking the genomic region containing the gene to disrupt. I performed PCR amplification of the *kanMX* cassette and used the PCR product for the transformation of yeast BY4741 strains with various TAP-fused SAGA and ADA histone acetyltransferase subunits. Cells in which

the PCR product integrated into the genome gained resistance to the G418 antibiotic. I selected the transformants on agar plates containing G418 (500 μg/ml), isolated genomic DNA from selected clones and verified the correct *kanMX* cassette position in the genome by PCR screening (Figure 26). Strains in which the target gene was replaced by the *kanMX* cassette were used for transformation with cDNA coding for the IL-1α precursor in pYX212 or with empty pYX212 as a control.

In this way I succesfully created 10 new deletion strains: TAP/Gcn5,*ahc1*Δ, TAP/Spt8,*gcn5*Δ, TAP/Spt8,*spt7*Δ, TAP/Spt8,*ahc1*Δ, TAP/Ahc1,*gcn5*Δ, TAP/Ahc1,*spt7*Δ, TAP/Ahc1,*ahc1*Δ, TAP/Spt7,*gcn5*Δ, TAP/Spt7,*spt7*Δ and TAP/Spt7,*ahc1*Δ.

Furthermore, the gene *AHC2* (a recently identified specific subunit of the ADA complex) was deleted using the Leu2 gene disruption cassette (J. H. Hegemann). The *AHC2* gene deletion in the strains TAP/Gcn5, TAP/Spt7 and TAP/Spt8 were performed by Václav Vopálenský and kindly provided to me.

During my experimental work, I was not succesful in an attempt to delete the *SPT7* gene from the TAP/Gcn5 strain. From the total number of 156 colonies tested after the transformation with the *kanMX* cassette amplified using primers Spt7Fwd and Spt7Rev, none was found to bear the *SPT7* deletion. I also intended to disrupt the *SPT7* gene from TAP/Ada2 and TAP/Ada3 strains, but no positives were found among 74 and 72 colonies tested, respectively.

For an unknown reason I was also unable to delete the *GCN5* gene from the TAP/Gcn5 strain by the *kanMX* cassette amplified using primers Gcn5Kan5 and Gcn5Kan3. Although the PCR screening showed that the *kanMX* cassette integrated to the right place (Figure 26) and the drop test also indicated the deletion of TAP/*GCN5* fusion gene (Figure 28A), repeated western blotting experiments showed that the TAP fusion *GCN5* gene product was still translated and bound the IL-1α precursor as shown by co-immunoprecipitation (Figure 28B). Therefore I designed a new primer set for the *GCN5* disruption (Gcn5Fwd and Gcn5Rev), but got the same results as before. Figure 29 shows the position of *GCN5* on *S. cerevisiae* chromosome VII (996 874-998 193); the oligonucleotides Gcn5Fwd and Gcn5Rev enclose the chromosomal region 996 218-998 407. Since this was a persistent problem, I excluded the TAP/Gcn5,*gcn5*Δ deletion strain from the analysis.

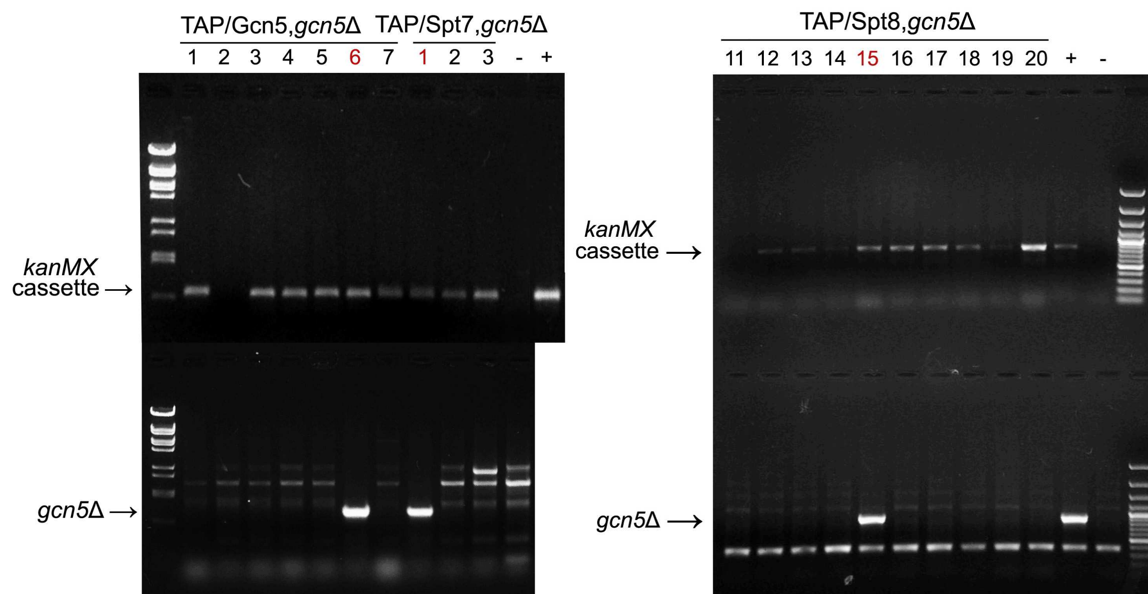


Figure 26: PCR verification of the *GCN5* gene disruption by the *kanMX* gene disruption cassette. An example of a PCR screening experiment showing a succesful *GCN5* gene deletion in three TAP fusion strains. Upper part shows the presence of the *kanMX* cassette (primers KanR1 and KanR2), bands in the lower part indicate the integration of the *kanMX* cassette in place of *CGN5* gene (primers KanV2F and Gcn5VerR; see Materials and Methods part 5.5.8.1). For TAP/Spt8,*gcn5*Δ (on the right), one of the previously identified *gcn5*Δ strain served as a positive control; positive control was not available yet for the verification of *GCN5* gene disruption in TAP/Gcn5 and TAP/Spt7 strains.

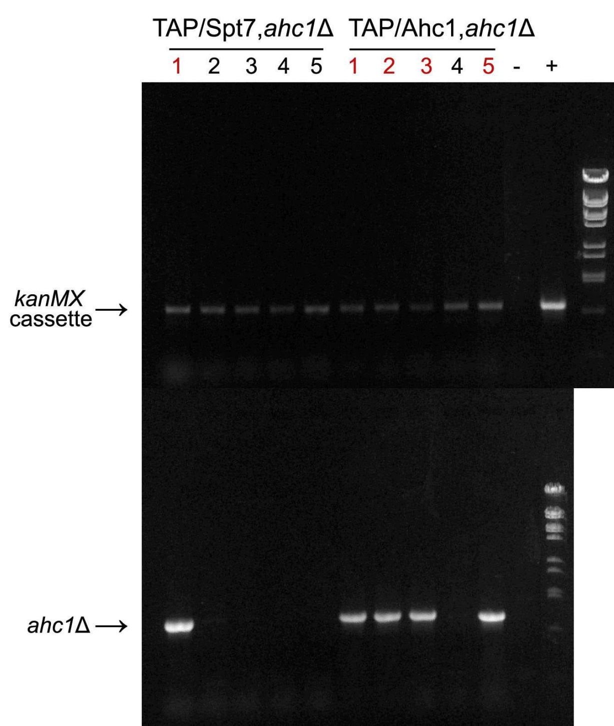
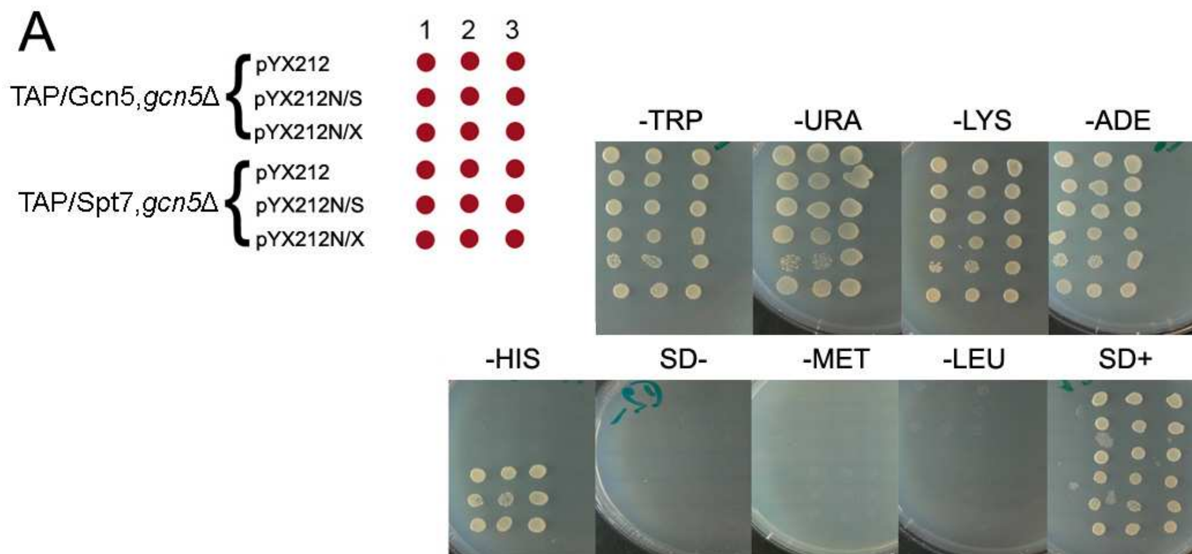


Figure 27: PCR verification of the *AHC1* gene disruption by the *kanMX* gene disruption cassette. PCR screening shows *AHC1* gene deletion in two TAP fusion strains. The upper part shows the presence of the *kanMX* cassette (primers KanR1 and KanR2), the band in the lower part indicates the integration of the *kanMX* cassette in place of *AHC1* gene (primers KanV2F and Ahc1VerR) Positive control was only available for the *kanMX* cassette.



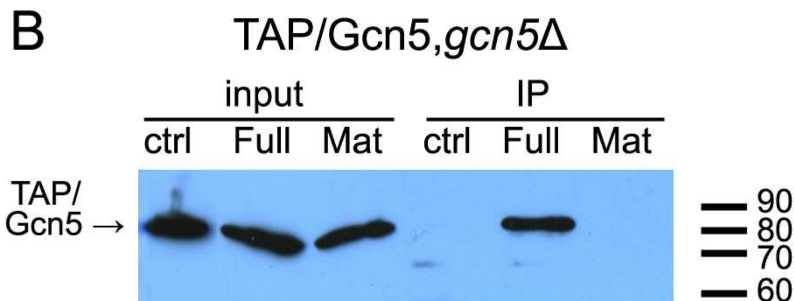


Figure 28: Unsuccessful *GCN5* gene disruption in the TAP/Gcn5 strain. In spite of the PCR screening result (see Figure 26 confirming the deletion of *GCN5* in the TAP/Gcn5 strain, the TAP-fusion Gcn5 protein is still expressed in the deletion strain. **(A) Drop test showing the nutritional requirements of the TAP/Gcn5 and TAP/Spt7 strains with *GCN5* gene deletion overexpressing the IL-1α precursor.** The TAP/Gcn5,*gcn5*Δ and TAP/Spt7,*gcn5*Δ strains bearing the plasmids pYX212, pYX212N/S IL-1αFull (pYX212N/S) or pYX212N/X IL-1αFull (pYX212N/X) were tested for growth on the SD agar plates lacking tryptophan (-TRP), uracil (-URA), lysine (-LYS), adenine (-ADE), histidine (-HIS), methionine (-MET) or leucine (-LEU); SD+ represents the SD agar plate; SD- represents the SD agar plate lacking all of the amino acids/bases mentioned. Note the difference between the TAP/Gcn5 and TAP/Spt7 strains on the SD agar plate without histidine. Since histidine was the marker that had been previously used for selection of the TAP fusion strains (Ghaemmaghami *et al.* 2003), no growth of TAP/Gcn5,*gcn5*Δ on the plates lacking histidine should point to the successful deletion of TAP/*GCN5* fusion gene. Other auxotrophies correspond to the strain genotype; growth on SD plate without uracil is the consequence of the transfection with pYX212 plasmid variants. Three independent yeast monocolonies have been tested. **(B) Western blotting experiment confirming that the TAP/*GCN5* gene product is still present in the cell lysates of the TAP/Gcn5 strain with deleted *GCN5* and binds the IL-1α precursor but not mature IL-1α as shown by co-immunoprecipitation.** The specific binding to the IL-1α precursor indicates that this band does not represent a protein recognised by the antibody due to an unspecific interaction. Primary antibody: anti-CBP; secondary antibody: swine anti-rabbit.

Strains TAP/Spt7,*spt7*Δ and TAP/Ahc1,*ahc1*Δ appeared to have lost the *SPT7* and *AHC1* gene, respectively, since I was unable to detect the expression of both Spt7 and Ahc1 proteins neither in the corresponding cell lysate nor by co-immunoprecipitation using the IL-1α precursor as a bait (Figure 30).

As I expected, *GCN5* gene deletion did not abolish the interaction of IL-1α precursor with either of the histone acetyltransferase complex as it is clearly visible from the co-immunoprecipitation experiments in the *gcn5*Δ yeast strains producing either Ahc1-TAP or Spt8-TAP proteins (Figure 31A). This result shows that the IL-1α precursor does not interact directly with this catalytic subunit of the HAT/Core module of SAGA and ADA. Furthermore, *AHC1* knock-out did not affect the interaction of SAGA subunits Gcn5 and Spt8 with IL-1α (Figure 31B). Disruption of *SPT7* gene, encoding a subunit that is important for the maintenance of the integrity of the SAGA complex, disturbed the binding of Spt8 to IL-1α (Figure 31C). Surprisingly, Ahc1 protein was still found to interact with IL-1α in yeast cells with a disintegrated SAGA complex (Figure 31C). This result is consistent with my previous finding that IL-1α possibly interacts with the HAT/Core module of both yeast histone acetyltransferase complexes SAGA and ADA (6.2.3).

Surprisingly, I was only rarely able to detect the Spt7-TAP protein in a complex with the IL-1α precursor from the TAP/Spt7,*ahc1*Δ yeast strain (Figure 31B). If obtained, the signal was weak and the success rate of detectable co-immunoprecipitation of Spt7-TAP with the IL-1α precursor in the *ahc1*Δ strain was 1 in 4 cases. Since Ahc1 and Spt7 proteins have not been found together in one HAT complex so far, this result could possibly reveal a novel or alternative model of ADA function in the SAGA complex assembly where the binding of Spt7 and Ahc1 is mutually exclusive. This hypothesis is further supported by the confirmation that the disruption of the *AHC1* gene does not significantly affect intracellular level of the Spt7 protein (Figure 32).

Results obtained in this part of my experimental work suggest that the IL-1α-binding site should comprise Ada2, Ada3 and Sgf29. Binding of Gcn5, the fourth currently known subunit of the HAT/Core module of SAGA and ADA, to the IL-1α precursor has been excluded by the positive co-immunoprecipitation experiment from the *gcn5*Δ strain (Figure 31A).

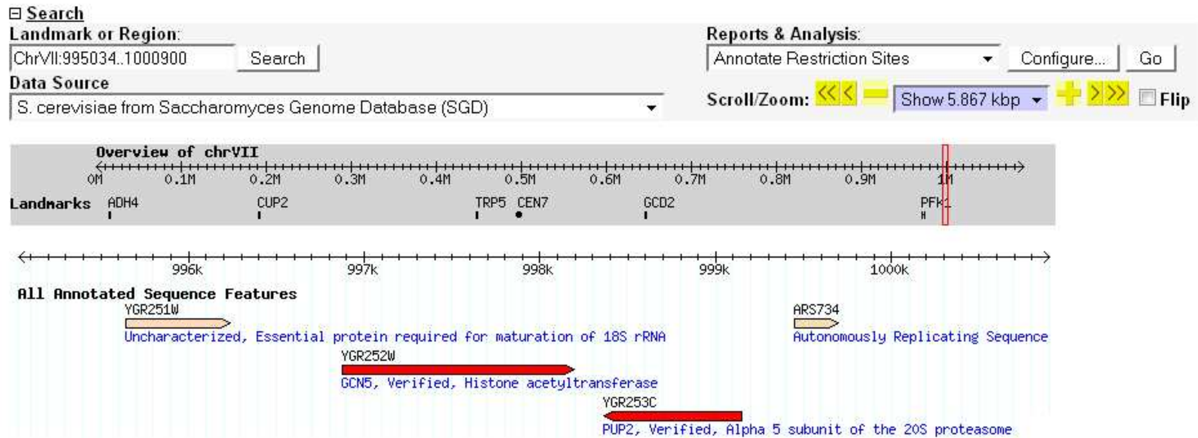


Figure 29: Position of the *GCN5* gene on *S. cerevisiae* chromosome VII. Adapted from the *Saccharomyces* genome database (http://www.yeastgenome.org/)

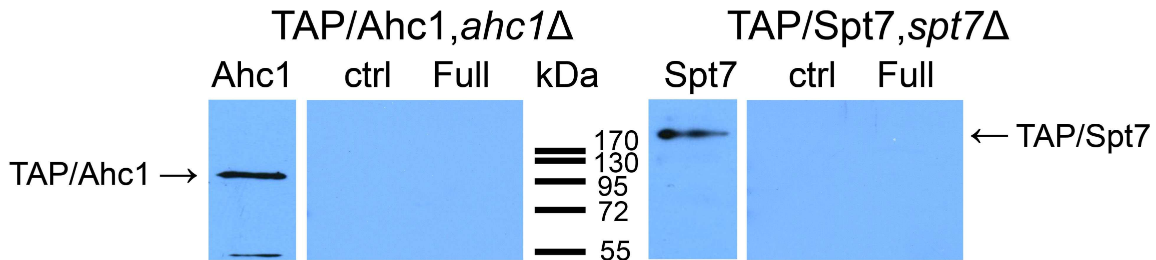


Figure 30: Verification of the successful deletion of the *AHC1* and *SPT7* genes. The TAP/ Ahc1,*ahc1*Δ and TAP/Spt7,*spt7*Δ strains do not produce the TAP-tagged Ahc1 and Spt7 proteins, respectively. To increase the detection sensitivity, co-immunoprecipitation with the IL-1α precursor (Full) from the disruption strain lysates was performed using the anti-FLAG antibody; negative control (ctrl) represents the co-immunoprecipitation from the cells bearing the empty plasmid pYX212. For the cell lysates, 200 ml of cell culture was used that should be enough for the production of a sufficient and detectable amount of both proteins if they were expressed. To show the protein size, for Ahc1, the lysate obtained from the TAP/Ahc1,*spt7*Δ strain bearing pYX212N/X IL-1αFull (Ahc1) was used; for Spt7, the lysate from the TAP/Spt7 strain bearing pYX212 (Spt7) was used. Primary antibody: anti-CBP; secondary antibody: swine anti-rabbit.

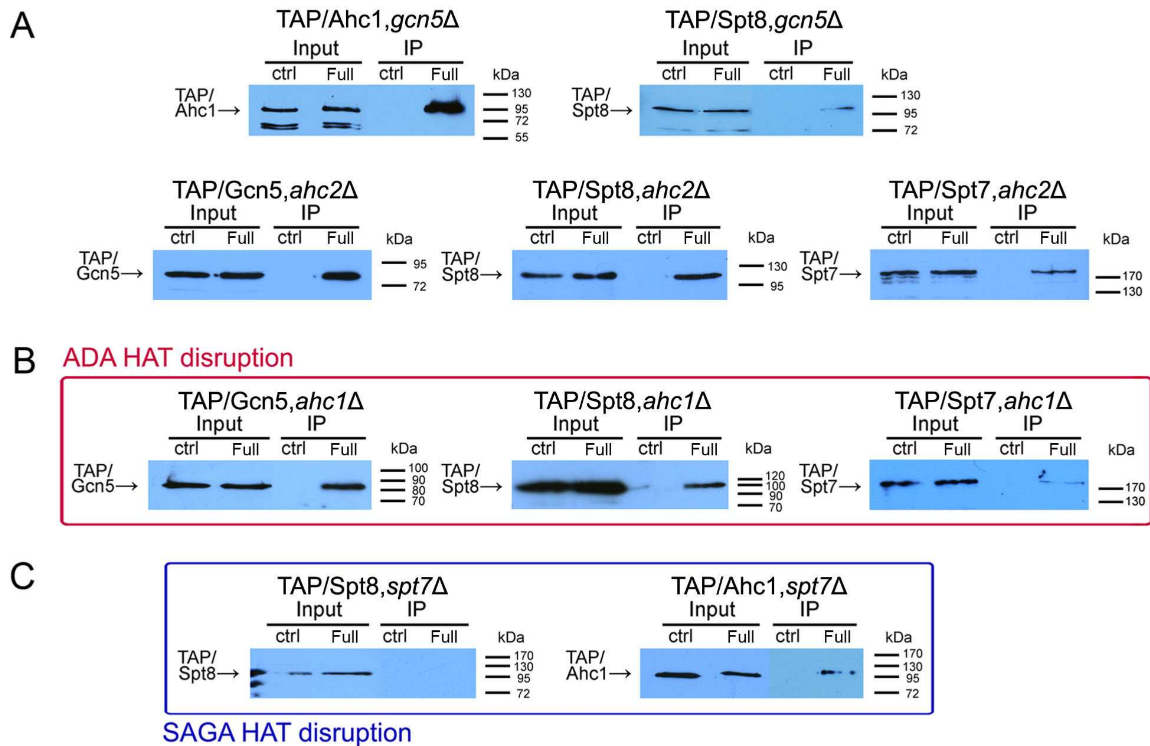


Figure 31: Analysis of the IL-1α precursor binding to the selected SAGA and ADA HAT subunits in various HAT disruption *S. cerevisiae* strains. Co-immunoprecipitation was performed from the corresponding yeast lysates using the anti-FLAG antibody recognising the FLAG tag at the N terminus of the IL-1α precursor; western blotting was performed with anti-CBP antibody binding the TAP tag at the C terminus of the HAT subunits. **(A)** As expected, since Gcn5 is not required for SAGA or ADA complex integrity, its deletion has no influence on Ahc1 or Spt8 binding to the IL-1α precursor (Full). Deletion of the *AHC2* gene doesn't impair co-immunoprecipitation of the IL-1α precursor with Gcn5, Spt8 and Spt7. **(B)** Similarly, disruption of the ADA HAT complex did not affect the binding of Gcn5 and Spt8 to IL-1α. However, the interaction between Spt7 and the IL-1α precursor was significantly weakened. The binding of Spt7 to the IL-1α precursor was only rarely detected. **(C)** The disintegration of the SAGA complex abolished the interaction Spt8 with IL-1α, but had no effect on Ahc1 binding to the IL-1α precursor. Control cells (ctrl) carry the empty plasmid pYX212. Secondary antibody: swine anti-rabbit.

Figure 32: Disruption of the *AHC1* gene does not significantly affect the intracellular level of the Spt7 protein. Similar number of cells of TAP/Spt7 and TAP/Spt7,*ahc1*Δ yeast strains bearing the plasmid pYX212N/X IL-1αFull (Full) or the empty plasmid pYX212 (ctrl) was lysed and analyzed by western blotting. Both strains appear to express similar amount of Spt7. Primary antibody: anti-CBP; secondary antibody: swine anti-rabbit.

6.2.6 Mass spectrometry identification of the IL-1α-binding proteins

In an effort to shed more light on the interaction of the IL-1α precursor with yeast HAT complexes using a different approach I performed an experiment aimed at the identification of the IL-1α-binding proteins by mass spectrometry (MALDI-TOF). For this analysis, the TAP/Spt8,*spt7*Δ and TAP/Spt7,*ahc1*Δ strains were chosen. These strains lack the SAGA and ADA complex, respectively, and enable me to study both IL-1α-binding protein complexes separately.

For this experiment, a large-scale variant of the co-immunoprecipitation from the yeast cells was used (5.5.3). 20 µl of each sample (corresponding to 0,5 liter of the cell culture) was loaded onto the polyacrylamide gel and separated using SDS-PAGE. The part of the gel containing proteins that are approximately 35-200 kDa in size was cut into 20 tiny bands (1 mm of thickness). Samples were sent to the mass spectrometry laboratory for analysis. The rest of both samples was loaded onto another polyacrylamide gel and stained with silver (Figure 33).

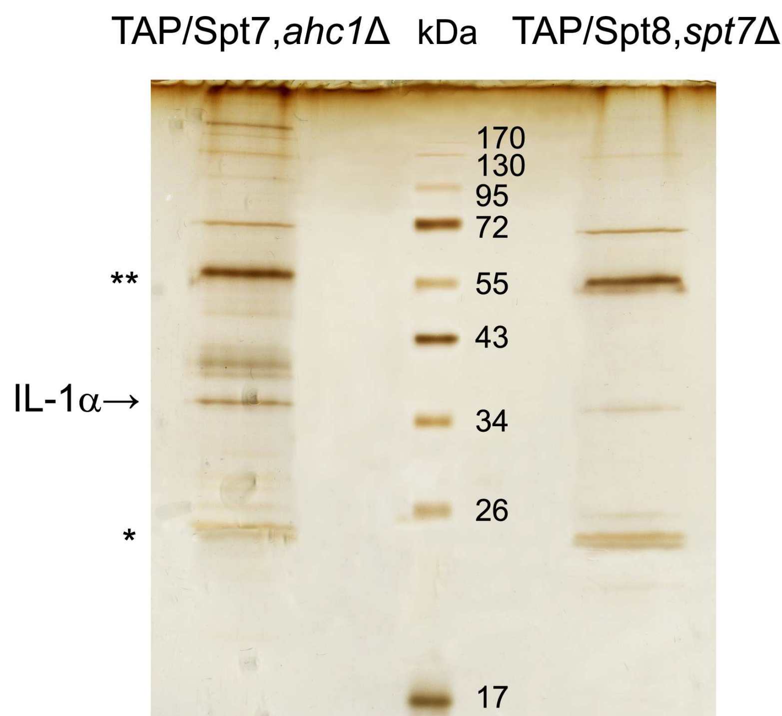


Figure 33: Co-immunoprecipitation from yeast TAP/Spt8,*spt7*Δ and TAP/Spt7,*ahc1*Δ strains. SDS-PAGE gel stained with silver. Single and double asterisks indicate bands corresponding to the light and heavy chain of the anti-FLAG antibody, respectively. The band corresponding to the IL-1α precursor is also indicated (IL-1α).

Unfortunately, only one protein has been identified in the TAP/Spt7,*ahc1*Δ sample, Ssa2 (YLL024C). This HSP70 family member may, however, represent a molecule binding the IL-1α precursor due to an unspecific interaction because its interactions either with IL-1α or with histone aetyltransferases have not been described yet in the literature.

Although the attempt to identify the proteins binding to the IL-1α precursor in yeast cells by mass spectrometry failed, my results suggest that IL-1α precursor binds to the HAT/Core module consisting of Ada2, Ada3, Gcn5 and Sgf29 being the only polypeptides shared by both SAGA and ADA complexes (Figure 24). Furthermore, my co-immunoprecipitation experiments in TAP/Ahc1,*gcn5*Δ and TAP/Spt8,*gcn5*Δ strains further excluded Gcn5 from the candidates for the IL-1α binding. Results of Part II of this thesis therefore show that the IL-1α-binding site should be formed by Ada2, Ada3, probably Sgf29 and perhaps some other proteins making part of the HAT/Core module.

6.3 PART III: Study of the *HOX* gene expression in subgroups of paediatric ALL

HOX gene expression is not only crucial during the embryonic development, but also at later developmental stages since dysregulated *HOX* expression is often considered to be the cause of certain human diseases including various types of leukaemia. Although childhood ALL has been one of the great recent success stories in therapy, there are still too many unknowns concerning the intensity of treatment and accuracy of prognosis especially in patients with no characteristic chromosomal abnormalities. One of the possibilities to improve our understanding of the disease resides in searching new prognostic and diagnostic markers. *HOX* genes are candidates for studies of their association with ALL progress and prognosis since they are often dysregulated in ALL, their overexpression may obviously be leukaemogenic and in certain subtypes of childhood AML, *HOX* gene expression patterns appears to be predictive of outcome (Drabkin *et al.* 2002).

In this part of my work, I was interested in studying the *HOX* gene expression in different genotypic and phenotypic subgroups of childhood ALL. I examined RNA samples of 61 patients categorised according to their phenotypic (T-ALL - 10 samples, B-cell precursor ALL), prognostic (PGR; prednisone good responders - 9 samples, PPR; prednisone poor responders - 9 samples; all with normal karyotype), and genotypic (BCR/ABL - 6 samples, MLL/AF4 - 9 samples, TEL/AML1 - 9 samples, hyperdiploid ALL - 8 samples, ALL without a known gene rearrangement - 18 samples) characteristics. I also analyzed the *HOX* gene expression in 4 samples of immature myeloid progenitors (IMPs; defined as CD34-positive, CD33-positive, and CD19-negative), 4 samples of B-lymphocyte progenitors (BLPs; defined as CD34-positive and CD19-positive), 4 samples of B-lymphocyte precursors (pre-Bs; defined as CD19-positive, CD10-positive, and CD34-negative), 4 samples of mature B lymphocytes (matBs; defined as CD34-negative, CD19-positive, CD20-positive, and CD10-negative), 2 samples of cortical T-cells (corTs; defined as CD3-positive, CD4-positive, and CD8-positive) and 1 sample of mature T lymphocytes (matTs; defined as CD3-positive and CD45-positive). These cells were gained from healthy donors, sorted using the FACS Aria III sorter and represent the physiological counterparts of the malignant cells from patients with leukaemia.

The level of expression of *HOX* genes in all the samples was assessed by quantitative real-time polymerase chain reaction (qRT-PCR; 5.5.32) using SYBR Green incorporation. As a reference gene, *ABL1* was used.

6.3.1 Optimising of the qRT-PCR conditions

Prior to working with the patient samples I needed to test all the *HOX* primers (Table 7) using various human leukaemic cell lines that were available in the laboratory, i.e. K562 (chronic myelogenous leukaemia), MV4;11 (acute myelomonocytic leukaemia), NB4 (acute promyelocytic leukaemia) and RS4;11 (acute biphenotypic leukaemia). I isolated RNA from the cells (5.5.30), performed reverse transcription into cDNA (5.5.31) and run qRT-PCR using cDNA from the above-mentioned cell lines for each *HOX* primer set. Thus I assessed the intensity of transcription of the *HOX* genes in these cell lines, determined the cell line suitable for the calibration curve construction in each gene system as well as the optimal annealing temperature for each primer set (Table 14).

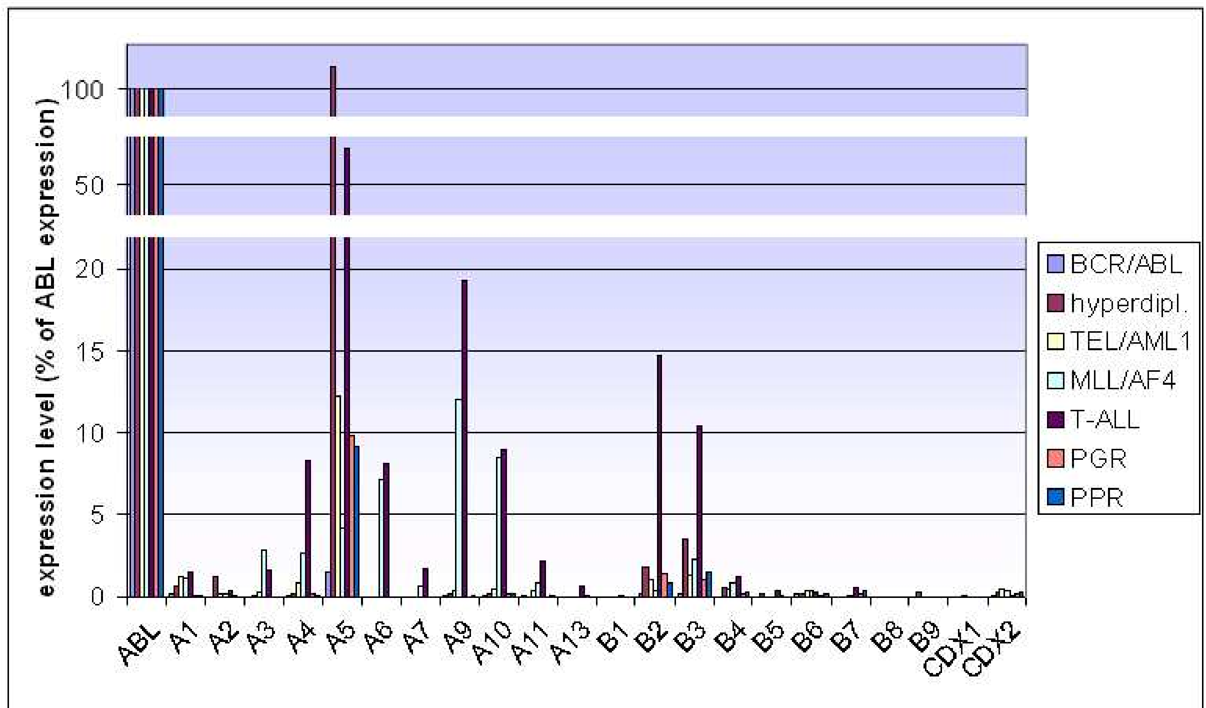
gene system	line for calibration curve	annealing temperature
HOXA1	NB4	65 ℃
HOXA2	NB4	65 ℃
HOXA3	RS4;11	60 ℃
HOXA4	NB4	65 ℃
HOXA5	K562	65 ℃
HOXA6	RS4;11	65 ℃
HOXA7	RS4;11	65 ℃
HOXA9	RS4;11	65 ℃
HOXA10	MV4;11	65 ℃
HOXA11	MV4;11	65 ℃
HOXA13	NB4	65 ℃
HOXB1	NB4	65 ℃
HOXB2	K562	65 ℃
HOXB3	K562	65 ℃
HOXB4	K562	65 ℃
HOXB5	K562	65 ℃
HOXB6	MV4;11	60 ℃
HOXB7	K562	60 ℃
HOXB8	K562	65 ℃
HOXB9	K562	65 ℃
HOXB13	MV4;11	68 ℃
CDX1	RS4;11	60 ℃
CDX2	NB4	65 ℃
ABL1	NB4	60 ℃

Table 14: Cell lines used for the calibration curve construction and optimal annealing temperature for each *HOX* primer set and *ABL1*.

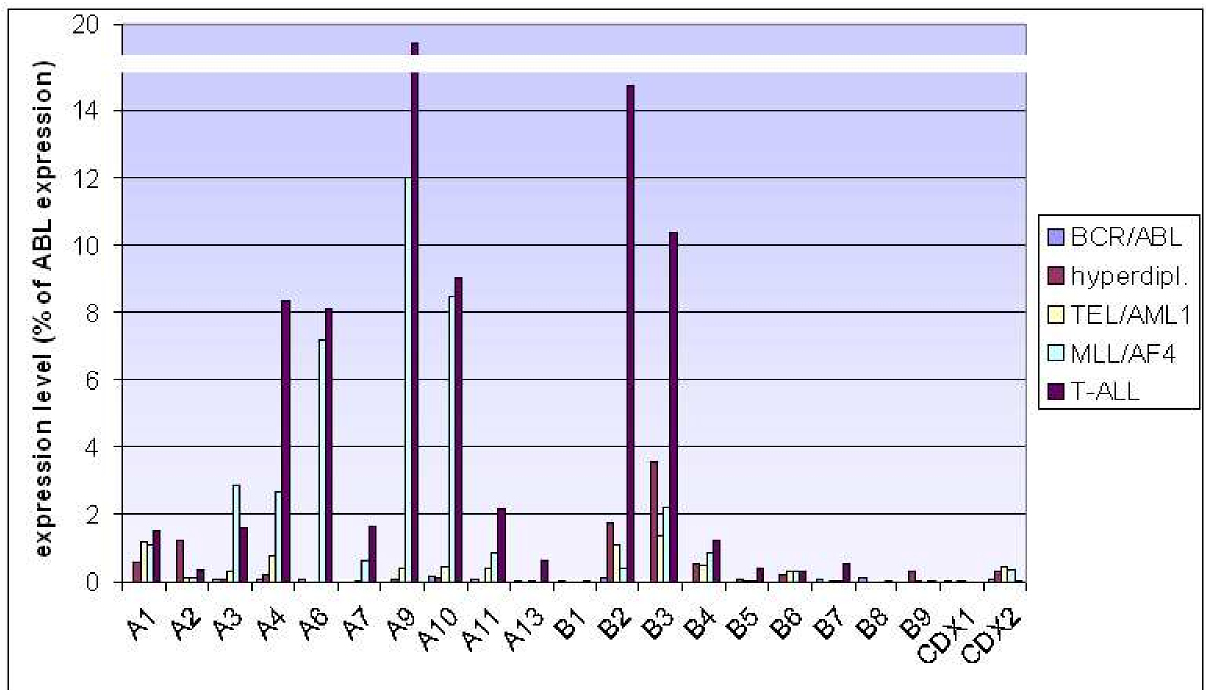
6.3.2 qRT-PCR analysis of *HOX* gene expression in ALL patient and sorted cell population samples

After optimising all the *HOX* gene qRT-PCR systems, I performed the analysis of *HOX* gene expression in all the samples, i.e. 61 patient samples and 19 sorted cell populations. *ABL1* was used as the reference gene and all *HOX* gene expression data were normalised to the quantity of *ABL1* expression. All reactions were run in duplicate and the arithmetical mean value of both Ct („cycles to threshold") values was calculated. In the case that only one Ct value was measured within the two duplicates, it was used instead of the mean value. Mean Ct values from the *HOX* gene expression analysis in the ALL patients are shown in Table 15, 16, 17 and 18. Expression data from the *HOX* gene analysis in sorted cell populations are shown in Table 19 and 20. „0" designates samples where no expression was detected, samples where the amplified product did not correspond to the expected product according to the melting curve analysis (the case of *HOXB13* that I had to exclude from the analysis) or where the fluorescence measurement was influenced by the presence of primer dimers (the case of *CDX2*) are designated „-". N/A significates that the sample was not analyzed for the particular *HOX* gene expression. Using the Bio-Rad iQ5 Optical System Software, the SQ („starting quantity") of cDNA in each sample was determined.The mean SQ values in each sample analyzed were sebsequently divided by the mean SQ value of *ABL1* expression to take account of the concentration and quality od cDNA (data not shown due to their volume).

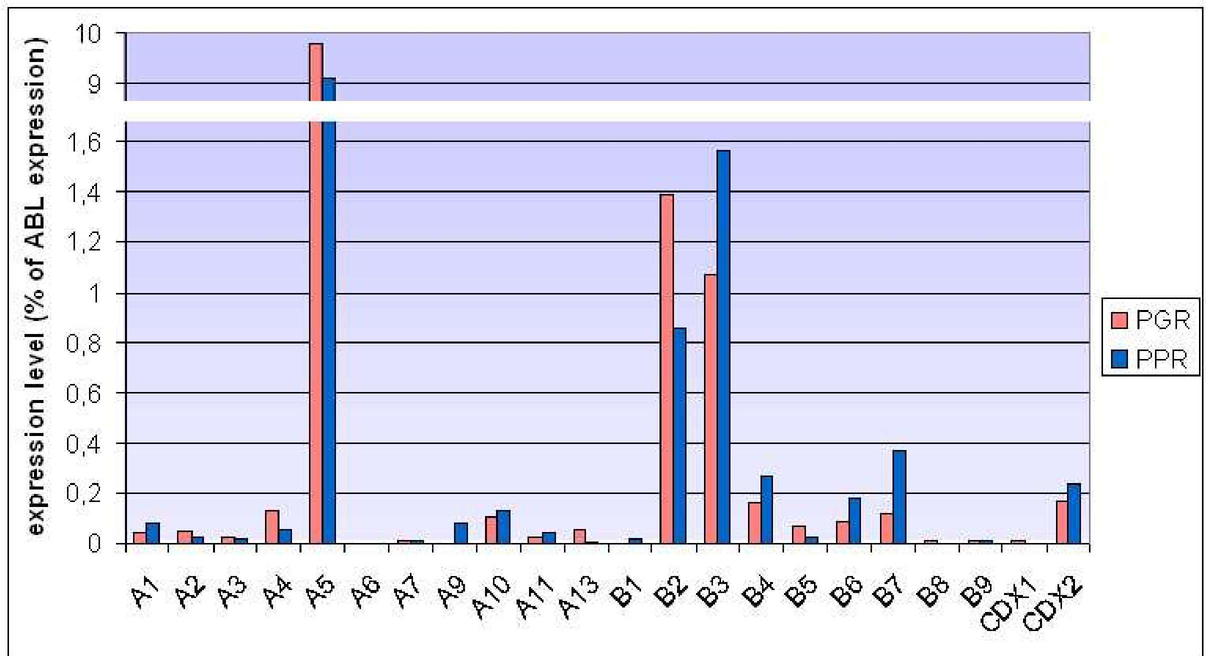
Results of the *HOX* gene expression analysis in ALL patients is illustrated by Graph 2 and Graph 3. Overall, patients with the *MLL/AF4* rearrangement and patients with T-ALL showed the highest *HOX* gene expression. *HOXA5* was expressed strongly in patients with hyperdiploid karyotype. *CDX1* was almost not expressed in the samples studied, *HOXB8* expression was very low. Not many differences were found in the *HOX* gene expression among the PGR and PPR group except for a higher expression of *HOXB2* in the PGR group and a more intense transcription of *HOXB3* and *HOXB7* in the PPR group (Graph 4).



Graph 2: Expression of *HOX* genes in the defined groups of patients with ALL. *ABL1* gene expression is also shown.

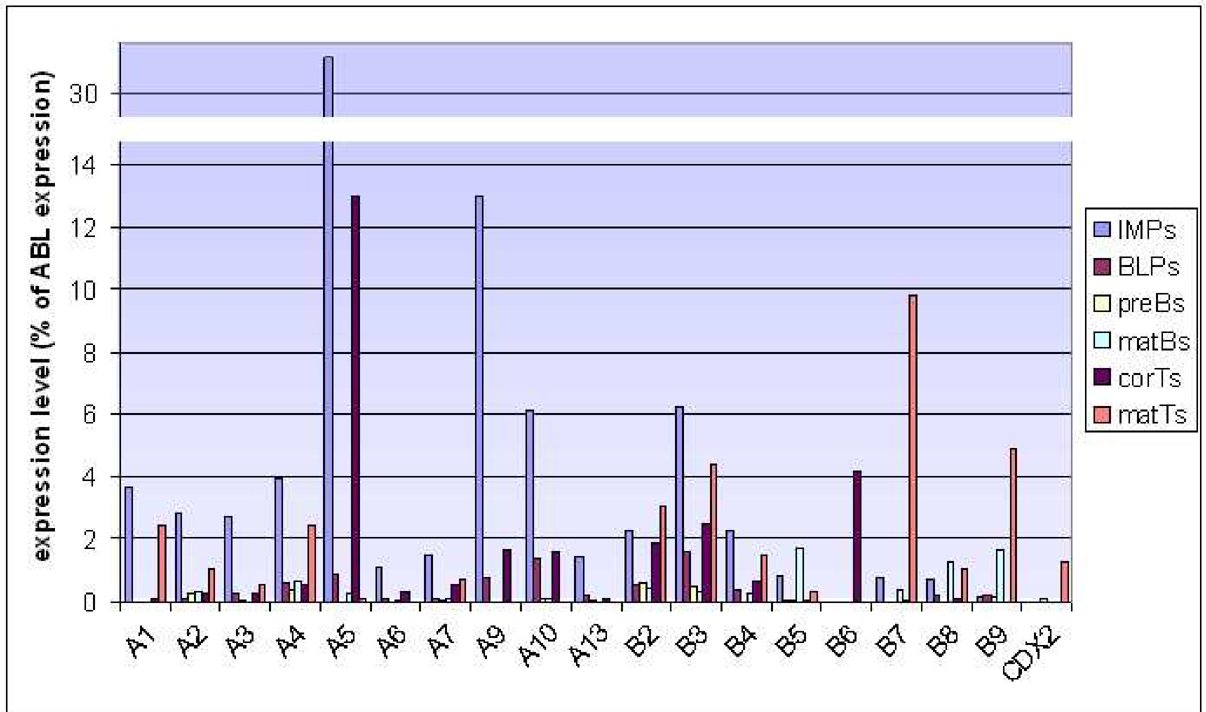


Graph 3: Expression of *HOX* genes in defined genotypic and phenotypic groups of patients with ALL. Corresponds to Graph2 but *HOXA5* and *ABL1* expression is not shown here.



Graph 4: *HOX* gene expression in prednisone good responders (PGR) and prednisone poor responders (PPR).

Results of the analysis of sorted cell populations (Graph 5) showed that the *HOX* gene expression was generally higher and more *HOX* genes were transcribed in the sorted population of immature myeloid progenitors than in later developmental stages of B-lymphocytes, where especially most of the genes from the *HOXA* cluster were silenced. Among these genes, *HOXA5* expression in IMPs was particularly high. Among the *HOXB* cluster, only *HOXB2*, *HOXB3* and *HOXB4* were expressed in the B-lineage precursors. In contrast, both cortical and mature T lymphocytes showed the expression of a wide range of *HOX* genes. Cortical T-cells showed high expression of *HOXA5*; *HOXB7, HOXB9* and *HOXB3* genes were transcribed extensively in mature T-cells. Interestingly, *CDX2* was expressed only in mature T lymphocytes and *HOXB6* was transcribed solely in cortical T lymphocytes. *HOXB2* and *HOXB3* were expressed across all the developmental stages studied.



Graph 5: *HOX* gene expression in sorted B- and T-cell populations. The expression of HOX genes in immature myeloid progenitors (IMPs), B-lymphocyte progenitors (BLPs), B-lymphocyte precursors (pre-Bs), mature B lymphocytes (matBs), cortical T-cells (corTs) and mature T lymphocytes (matTs) was analyzed.

patient	ALL group	*ABL1*	*HOXA1*	*HOXA2*	*HOXA3*	*HOXA4*	*HOXA5*	*HOXA6*	*HOXA7*	*HOXA9*	*HOXA10*	*HOXA11*	*HOXA13*
212	**BCR/ABL**	22,995	39,915	17,43	17,73	32,42	31,15	34,785	35,605	30,115	29,97	-	0
429	**BCR/ABL**	22,8	0	0	17,635	35,255	33,47	18,015	17,815	32,08	33,505	-	0
739	**BCR/ABL**	23,735	0	37,28	34,57	32,495	31,05	34,125	34,97	29,985	30,245	35,57	0
861	**BCR/ABL**	23,04	17,54	16,8	32,455	31,175	30,785	34,255	34,465	32,01	32,455	34,84	0
1029	**BCR/ABL**	23,555	-	34,335	16,96	32,835	30,06	33,125	35,8	31,7	32,23	-	0
1092	**BCR/ABL**	22,15	40,32	34,195	32,14	31,415	27,095	30,035	31,31	26,76	27,875	35,59	0
815	**hyperdiploid**	26,46	0	-	17,645	0	17,33	-	0	0	17,88	0	0
863	**hyperdiploid**	26,41	0	34,295	17,575	0	33,375	34,855	0	31,8	17,61	-	0
874	**hyperdiploid**	31,985	0	37,97	0	0	31,345	0	0	28,88	0	0	0
890	**hyperdiploid**	25,895	34,99	0	34,23	32,77	30,98	33,345	18,73	28,88	32,19	-	17,025
920	**hyperdiploid**	27,53	21,985	17,455	35,42	32,88	32,34	35,24	0	32,055	34,115	0	0
1058	**hyperdiploid**	24,815	21,485	35,775	17,04	33,255	31,715	34,265	18,525	31,585	32,65	-	0
1074	**hyperdiploid**	24,97	43,035	0	0	33,45	30,32	34,615	35,98	30,675	31,255	-	0
1091	**hyperdiploid**	24,025	43,67	17,14	34,615	31,64	30,255	33,985	35,785	28,995	30,99	-	0
995	**TEL/AML1**	25,575	17,45	34,87	16,59	31,4	0	34,985	34,525	31,655	31,55	0	0
1020	**TEL/AML1**	23,99	35,785	33,6	32,86	33,36	31,105	34,15	36,725	30,37	31,9	33,955	0
1040	**TEL/AML1**	25,295	41,895	34,005	30,57	27,09	31,33	35,11	36,535	30,675	31,83	-	17,005
1046	**TEL/AML1**	27,93	0	21,475	36,345	35,62	33,01	35,385	36,995	33,68	33,685	-	0
1115	**TEL/AML1**	23,915	36,48	32,885	34,155	30,37	30,49	33,555	34,19	30,78	30,59	0	31,355
1121	**TEL/AML1**	26,35	20,9	17,51	31,485	30,98	28,855	31,44	34,46	28,14	28,685	33,6	17,925
1139	**TEL/AML1**	27,245	21,49	33,485	17,76	18,065	16,82	0	0	0	18,755	0	0
1155	**TEL/AML1**	27,525	37,23	35,04	33,935	-	16,485	17,68	17,97	0	17,835	0	24,095
1190	**TEL/AML1**	26,66	44,93	34,18	17,225	32,375	32,895	16,56	18,715	29,965	34,36	0	17
132	**MLL/AF4**	25,36	43,45	33,96	32,685	29,975	30,985	32,345	35,16	23,92	24,65	33,76	17,09
193	**MLL/AF4**	25,775	45,51	17,41	39,44	33,72	33,185	35,725	37,785	30,86	31,395	0	0
252	**MLL/AF4**	23,47	0	37,41	34,335	32,765	32,77	35,15	35,485	33	32,135	0	0
571	**MLL/AF4**	24,39	39,745	30,1	23,24	22,825	20,815	22,02	24,165	20,25	21,23	30,155	31,175
587	**MLL/AF4**	23,72	37,345	34,14	33,585	31,52	29,03	32,595	34,825	28,595	28,65	34,555	0
765	**MLL/AF4**	25,21	33,865	32,155	33,79	27,99	28,78	35,07	36,335	30,55	31,68	34,065	33,93
923	**MLL/AF4**	25,51	34,91	34,805	16,78	29,125	29,975	34,785	35,255	27,68	28,885	17,815	16,81
1178	**MLL/AF4**	27,195	0	20,88	0	-	19,775	0	0	16,57	17,655	-	23,305
481	**MLL/AF4**	22,37	41,79	0	34,46	-	32,57	36,08	33,90	33,64	31,80	N/A	32,57
524	**MLL/AF4**	23,56	37,69	32,87	34,68	33,05	34,35	34,75	34,98	24,30	22,65	N/A	31,67

Table 15: Expression (Ct value) of the *HOXA* gene cluster and *ABL1* in ALL patients from the BCR/ABL, hyperdiploid ALL, TEL/AML1 and MLL/AF4 group. See the text for details.

patient	ALL group	*ABL1*	*HOXA1*	*HOXA2*	*HOXA3*	*HOXA4*	*HOXA5*	*HOXA6*	*HOXA7*	*HOXA9*	*HOXA10*	*HOXA11*	*HOXA13*
855	**T-ALL**	26,3	26,3	21,995	34,77	32,71	31,005	29,67	32,94	33,88	31,965	29,385	-
857	**T-ALL**	25,025	25,025	42,225	0	35,015	33,18	31,795	36,325	35,445	34,12	31,065	37,45
975	**T-ALL**	26,005	26,005	42,725	33,79	32,33	28,275	26,985	32,18	31,66	29,3	26,655	18,15
976	**T-ALL**	23,83	23,83	39,37	-	32,835	31,865	30,495	34,5	33,65	33,14	29,43	18,185
998	**T-ALL**	25,825	25,825	41,955	32,655	31,085	29,93	29,9	30,995	29,23	28,69	25,395	36,39
1037	**T-ALL**	26,155	26,155	37,015	32,48	32,08	30,035	28,685	34,67	32,005	31,595	31,155	34,395
1061	**T-ALL**	27,705	27,705	39,115	0	32,625	32,41	29,265	33,635	34,925	32,675	30,465	0
1095	**T-ALL**	24,105	24,105	39,485	33,16	33,395	30,965	28,455	32,99	33,255	31,83	29,74	0
1109	**T-ALL**	28,22	28,22	45,17	32,185	28,405	26,64	24,29	25,455	26,95	25,84	24,695	0
1111	**T-ALL**	25,04	25,04	33,53	31,31	28,58	22,11	23,095	28,56	30,955	32,44	16,91	28,62
944	**PGR**	25,755	25,755	42,475	17,85	34,18	31,47	31,77	34,12	35,34	33,365	30,835	0
980	**PGR**	24,7	24,7	43,205	-	35,21	32,75	30,53	34,7	37,27	32,875	31,53	18,225
996	**PGR**	25,025	25,025	22,845	16,575	0	34,44	32,655	0	36,28	35,315	32,29	0
1010	**PGR**	23,98	23,98	44,1	34,505	35,43	30,79	29,395	33,26	34,565	32,355	29,865	0
1021	**PGR**	25,49	25,49	0	17,23	35,34	32,44	31,45	35,165	35,22	34,265	31,975	0
1049	**PGR**	24,635	24,635	21,99	40,67	0	34,765	29,465	35,78	19,22	36,995	33,19	0
1080	**PGR**	25,405	25,405	0	17,24	16,94	33,36	30,955	34,66	37,42	33,29	31,815	0
1104	**PGR**	25,425	25,425	0	17,8	34,675	29,925	31,185	34,78	34,97	34,93	31,555	36,63
1114	**PGR**	23,855	23,855	46,335	17,97	34,69	33,76	32,03	35,465	35,015	34,01	31,55	-
458	**PPR**	25,505	25,505	23,865	0	0	33,46	16,02	17,245	35,785	33,795	32,075	18,12
459	**PPR**	24,855	24,855	42,915	33,765	33,175	32,24	30,045	16,85	35,15	31,335	28,8	17,99
483	**PPR**	24,295	24,295	45,96	0	0	32,53	32,77	0	17,625	34,225	30,63	-
906	**PPR**	24,675	24,675	42,54	40,305	17,175	32,245	31,2	35,455	36,655	35,51	32,44	42,73
910	**PPR**	24,535	24,535	43,985	17,42	35,4	33,815	30,36	35,58	36,275	33,91	31,01	0
1032	**PPR**	23,745	23,745	41,62	0	0	32,565	30,345	33,905	18,015	33,615	34,275	23,89
1090	**PPR**	25,42	25,42	19,555	34,585	34,31	32,62	30,77	33,12	36,095	32,7	31,56	-
1093	**PPR**	24,3	24,3	44,85	46,75	17,44	33,605	30,555	34,29	36,535	34,085	31,17	18,185
1129	**PPR**	25,505	25,505	0	0	0	34,99	16,935	-	18,67	0	34,25	-

Table 16: Expression (Ct value) of the *HOXA* gene cluster and *ABL1* in ALL patients from the T-ALL, prednisone good responders (PGR) and prednisone poor responders (PPR) group. See the text for details.

patient	ALL group	*HOXB1*	*HOXB2*	*HOXB3*	*HOXB4*	*HOXB5*	*HOXB6*	*HOXB7*	*HOXB8*	*HOXB9*	*HOXB13*	*CDX1*	*CDX2*
212	**BCR/ABL**	-	27,21	30,51	33,265	33,755	0	34,2	0	0	-	0	30,64
429	**BCR/ABL**	34,75	28,875	31,6	35,025	46,99	0	0	0	17,14	-	0	30,4
739	**BCR/ABL**	-	27,125	31,26	35,75	34,575	35,185	18,83	0	0	-	0	30,09
861	**BCR/ABL**	33,18	27,76	29,675	33,14	31,17	31,705	34,9	34,5	34,88	-	0	29,795
1029	**BCR/ABL**	34,15	28,795	30,97	35,155	33,625	34,545	0	0	0	-	17,095	31,155
1092	**BCR/ABL**	40,14	24,68	27,935	30,795	33,54	33,98	34,385	34,89	0	-	0	30,44
815	**hyperdiploid**	37,99	30,765	33,06	0	-	34,94	0	0	0	-	0	-
863	**hyperdiploid**	43,26	29,52	31,69	36,32	33,42	0	0	0	0	-	0	30,565
874	**hyperdiploid**	39,85	30,74	31,39	35,545	-	0	0	0	17,425	-	0	-
890	**hyperdiploid**	41,06	28,48	31,355	35,245	33,91	17,54	0	0	0	-	0	29,5
920	**hyperdiploid**	-	28,01	32,335	18,165	34,08	0	18,27	0	34,99	-	0	31,56
1058	**hyperdiploid**	39,01	28,495	32,075	34,77	32,965	0	0	0	17,71	-	0	31,12
1074	**hyperdiploid**	36,73	27,43	30,98	32,96	33,96	17,025	37,94	0	17,05	-	0	31,11
1091	**hyperdiploid**	-	27,545	31,48	34,545	33,67	17,51	17,605	0	36,655	-	0	30,205
995	**TEL/AML1**	37,31	26,975	29,14	32,06	36,45	0	17,875	0	0	-	18,305	31,255
1020	**TEL/AML1**	-	27,575	31,395	35,115	34,5	0	0	17,54	32,55	-	0	31,72
1040	**TEL/AML1**	-	27,1	28,005	29,925	33,245	0	0	0	17,16	-	0	31,705
1046	**TEL/AML1**	40,68	29,255	31,155	33,985	34,23	17,515	0	0	0	-	17,895	31,87
1115	**TEL/AML1**	33,1	25,285	28,27	31,635	32,585	34,92	0	17,495	33,965	-	0	29,87
1121	**TEL/AML1**	-	26,3	30,33	33,475	34,22	34,78	21,46	0	0	-	0	-
1139	**TEL/AML1**	19,835	29,685	31,16	34	0	17,84	17,69	0	17,645	-	0	-
1155	**TEL/AML1**	38,615	30,26	32,095	41,285	0	0	0	0	17,575	-	0	-
1190	**TEL/AML1**	-	30,83	28,84	30,925	34,78	0	0	0	0	-	0	28,445
132	**MLL/AF4**	34,75	26,8	27,34	29,755	34,14	17,485	37,005	34,63	34,965	-	0	30,3
193	**MLL/AF4**	-	30,21	31,895	29,735	37,475	17,005	37,22	0	20,935	-	0	31,505
252	**MLL/AF4**	37,75	29,115	29,73	31,08	-	0	0	0	0	-	18,725	30,36
571	**MLL/AF4**	37,58	27,26	29,155	31,415	33,405	34,855	19,43	0	35,135	-	0	28,63
587	**MLL/AF4**	-	26,795	26,565	29,945	31,77	33,53	17,955	0	0	-	0	28,095
765	**MLL/AF4**	34,39	27,69	28,02	29,935	34,615	17,34	34,54	0	0	-	0	29,4
923	**MLL/AF4**	-	27,765	27,34	29,925	32,73	17,295	33,44	0	17,6	-	0	29,115
1178	**MLL/AF4**	37,705	33,805	30,94	32,82	35	0	17,975	0	0	-	0	31,595
481	**MLL/AF4**	N/A	29,04	26,02	27,23	34,55	33,65	28,04	0	0	N/A	N/A	31,46
524	**MLL/AF4**	N/A	27,98	26,65	27,03	32,59	0	36,07	34,27	34,55	N/A	N/A	30,87

Table 17: Expression (Ct value) of the *HOXB* gene cluster, *CDX1* and *CDX2* in ALL patients from the BCR/ABL, hyperdiploid ALL, TEL/AML1 and MLL/AF4 group. See the text for details.

patient	ALL group	*HOXB1*	*HOXB2*	*HOXB3*	*HOXB4*	*HOXB5*	*HOXB6*	*HOXB7*	*HOXB8*	*HOXB9*	*HOXB13*	*CDX1*	*CDX2*
855	**T-ALL**	-	24,095	25,68	27,985	32,24	16,72	35,44	42,93	16,96	-	0	-
857	**T-ALL**	34,075	23,895	25,28	28,135	34,29	0	31,625	0	34,865	-	0	-
975	**T-ALL**	33,01	23,415	28,365	30,79	27,7	34,09	33,495	0	32,365	-	0	32,53
976	**T-ALL**	40,23	25,56	26,71	29,34	32,395	33,345	33,11	0	17,44	-	0	31,75
998	**T-ALL**	-	31,015	30,78	35,63	34,06	-	18,11	0	0	-	0	-
1037	**T-ALL**	32,62	22,57	25,845	29,27	31,24	0	31,64	32,85	32,65	-	0	31,7
1061	**T-ALL**	36,88	28,455	29,2	31,855	34,81	0	18,08	0	0	-	0	-
1095	**T-ALL**	-	20,555	25,025	28,2	31,705	17,135	29,29	0	31,62	-	0	32,12
1109	**T-ALL**	-	34,2	-	18,71	-	0	0	0	0	-	0	-
1111	**T-ALL**	-	28,585	28,385	31,36	16,465	-	37,785	0	0	-	18,42	31,8
944	**PGR**	38,16	27	30,69	33,43	33,57	0	18,67	34,56	33,54	-	0	31,3
980	**PGR**	-	29,615	31,49	34,995	32,73	34,63	0	0	32,1	-	17,455	31,41
996	**PGR**	-	28,71	31,04	32,1	34,22	17,325	36,38	0	33,475	-	0	31,47
1010	**PGR**	-	24,92	31,21	37,215	31,3	0	31,7	0	33,26	-	0	31,195
1021	**PGR**	-	27,225	31,71	33,395	31,755	0	35,585	0	38,18	-	0	31,2
1049	**PGR**	-	27,21	28,02	30,855	38,945	0	32,645	0	17,31	-	0	-
1080	**PGR**	-	27,565	27,705	30,58	33,965	0	35,83	0	40,635	-	0	31,91
1104	**PGR**	-	26,96	29,4	31,925	34,26	35,52	35,19	0	0	-	0	31,665
1114	**PGR**	-	28,47	29,135	31,675	32,185	0	18,38	0	0	-	0	30,79
458	**PPR**	35,795	29,13	27,235	29,83	35,065	0	18,31	0	17,32	-	0	30,885
459	**PPR**	34,865	25,44	30,795	38,86	31,92	16,41	36,05	0	33,95	-	0	30,31
483	**PPR**	37,64	27,65	31,335	29,87	33,83	17,03	38,33	0	34,755	-	0	30,615
906	**PPR**	-	28,435	-	0	32,325	17,24	32,825	0	31,955	-	0	30,35
910	**PPR**	39,18	28,89	26,475	28,655	16,15	39,42	29,17	0	17,355	-	0	30,155
1032	**PPR**	-	28,085	30,74	34,655	17,5	-	36,505	0	0	-	0	30,325
1090	**PPR**	35,22	27,88	30,88	33,775	34,205	0	0	0	0	-	0	32,29
1093	**PPR**	34,7	27,945	31,6	36,895	33,145	0	0	-	17,39	-	0	29,92
1129	**PPR**	36,89	31,525	31,76	36,03	36,01	0	0	0	0	-	0	31,765

Table 18: Expression (Ct value) of the *HOXB* gene cluster, *CDX1* and *CDX2* in ALL patients from the T-ALL, prednisone good responders (PGR) and prednisone poor responders (PPR) group. See the text for details.

number	cell type	*ABL1*	*HOXA1*	*HOXA2*	*HOXA3*	*HOXA4*	*HOXA5*	*HOXA6*	*HOXA7*	*HOXA9*	*HOXA10*	*HOXA11*	*HOXA13*
B5	**IMPs**	30,35	-	34,97	33,45	31,68	33,01	33,20	32,71	30,48	29,38	0	-
H13	**IMPs**	28,44	-	0	-	0	30,95	31,39	31,12	30,76	29,26	32,10	-
S1	**IMPs**	29,89	39,24	32,83	0	0	0	32,65	33,79	30,68	30,98	31,92	39,24
D8	**IMPs**	30,42	44,54	33,51	32,41	32,44	0	-	33,61	30,44	29,88	45,60	44,54
G3	**BLPs**	30,36	0	0	36,26	0	-	35,34	16,34	34,07	31,89	0	0
K7	**BLPs**	33,27	0	0	0	-	0	0	37,17	34,94	34,38	34,36	0
H10	**BLPs**	30,18	0	37,65	35,22	34,68	35,23	34,88	35,68	34,73	33,20	45,67	0
D7	**BLPs**	31,65	0	0	0	34,67	35,04	0	0	0	32,58	0	0
S2	**pre-Bs**	30,91	0	45,73	38,32	34,36	0	0	0	0	33,97	0	0
K6	**pre-Bs**	29,81	0	34,55	0	0	0	0	0	0	35,46	0	0
O2	**pre-Bs**	31,41	0	0	0	0	0	0	35,88	0	0	0	0
D6	**pre-Bs**	31,93	0	0	0	0	0	0	0	0	0	40,71	0
S3	**mature Bs**	32,26	0	38,48	0	34,63	36,15	35,72	36,11	0	34,27	0	0
B14	**mature Bs**	32,32	0	0	0	34,46	0	0	0	0	36,08	0	0
S+B18	**mature Bs**	30,50	0	44,03	0	44,23	0	0	36,94	0	0	0	0
D5	**mature Bs**	32,59	0	34,95	0	0	0	0	0	0	0	0	0
T1	**cortical Ts**	24,42	39,69	31,32	31,50	29,05	29,40	29,67	28,47	28,11	26,88	30,54	39,69
T2	**cortical Ts**	24,90	40,81	31,75	32,05	29,66	30,43	30,25	28,88	27,95	27,23	44,54	40,81
S+B19	**mature Ts**	29,80	40,05	34,26	34,80	32,85	41,12	0	32,57	-	0	0	40,05

Table 19: Expression (Ct value) of the *HOXA* genes and *ABL1* in sorted cell populations of immature myeloid progenitors (IMPs), B-lymphocyte progenitors (BLPs), B-lymphocyte precursors (pre-Bs), mature B-lymphocytes, cortical T lymphocytes and mature T lymphocytes. See the text for details.

number	cell type	HOXB2	HOXB3	HOXB4	HOXB5	HOXB6	HOXB7	HOXB8	HOXB9	CDX2
B5	**IMPs**	24,095	25,68	27,985	32,24	16,72	35,44	42,93	16,96	0
H13	**IMPs**	23,895	25,28	28,135	34,29	0	31,625	0	34,865	0
S1	**IMPs**	23,415	28,365	30,79	27,7	34,09	33,495	0	32,365	0
D8	**IMPs**	25,56	26,71	29,34	32,395	33,345	33,11	0	17,44	0
G3	**BLPs**	31,015	30,78	35,63	34,06	-	18,11	0	0	0
K7	**BLPs**	22,57	25,845	29,27	31,24	0	31,64	32,85	32,65	0
H10	**BLPs**	28,455	29,2	31,855	34,81	0	18,08	0	0	0
D7	**BLPs**	20,555	25,025	28,2	31,705	17,135	29,29	0	31,62	0
S2	**pre-Bs**	34,2	-	18,71	-	0	0	0	0	0
K6	**pre-Bs**	28,585	28,385	31,36	16,465	-	37,785	0	0	18,42
O2	**pre-Bs**	27	30,69	33,43	33,57	0	18,67	34,56	33,54	0
D6	**pre-Bs**	29,615	31,49	34,995	32,73	34,63	0	0	32,1	17,455
S3	**mature Bs**	28,71	31,04	32,1	34,22	17,325	36,38	0	33,475	0
B14	**mature Bs**	24,92	31,21	37,215	31,3	0	31,7	0	33,26	0
S+B18	**mature Bs**	27,225	31,71	33,395	31,755	0	35,585	0	38,18	0
D5	**mature Bs**	27,21	28,02	30,855	38,945	0	32,645	0	17,31	0
T1	**cortical Ts**	27,565	27,705	30,58	33,965	0	35,83	0	40,635	0
T2	**cortical Ts**	26,96	29,4	31,925	34,26	35,52	35,19	0	0	0
S+B19	**mature Ts**	28,47	29,135	31,675	32,185	0	18,38	0	0	0

Table 20: Expression (Ct value) of the *HOXB* genes and *CDX2* in sorted cell populations of immature myeloid progenitors (IMPs), B-lymphocyte progenitors (BLPs), B-lymphocyte precursors (pre-Bs), mature B-lymphocytes, cortical T lymphocytes and mature T lymphocytes. See the text for details.

6.3.3 Statistical analysis of the *HOX* gene expression data from the ALL patients

The expression data of the patient samples were subjected to the statistical analysis. The most significantly differentially expressed genes among the defined groups of ALL patients were identified by the Kruskal-Wallis test, statistical values were then subtracted with the Bonferroni correction and *HOX* gene expression among different groups was compared by the Wilcoxon signed-rank non-parametric test in order to determine expression of which *HOX* genes is specifically associated with certain ALL patient group (performed by Roman Krejčí).

According to the results of the Kruskal-Wallis test, *HOXA7* (P=0.000045), *HOXA3* (P=0.000098), *HOXB3* (P=0.00015), *HOXA4* (P=0.000619), and *HOXB4* (P=0.001925) were differentially expressed among the studied groups of ALL patients. The comparison of couples of groups using the Wilcoxon signed-rank test showed that *HOXA3*, *HOXA4* and *HOXB3* expression distinguished T-ALL from hyperdiploidy or ALL without a known gene rearrangement (i.e. the PGR group together with PPR). In hyperdiploid ALL, *HOXA7* expression was significantly lower when compared to the levels in all other groups (i.e., *P*=0.0361 vs. TEL/AML1; *P*=0.0273 vs. MLL/AF4; *P*=0.0232 vs. T-ALL). Furthermore, *HOXB7* expression was specifically lower in patients with *TEL/AML1*-positive ALL (*P*=0.0048 vs. T-ALL; *P*=0.0110 vs. the *MLL/AF4*-positive group) and *CDX2* was downregulated in patients with the *BCR/ABL* gene fusion (*P*=0.0011 vs. hyperdiploid ALL; *P*=0.0065 vs. TEL/AML1; *P*=0.0367 vs. MLL/AF4). These differences in *HOX* gene expression may potentially be associated with the specific genetic background of these subgroups.

7 DISCUSSION

Over the last several years, increasing attention has been directed towards the intracellularly localised interleukin-1α. In addition to the „classic“ cytokine function mediating the communication among the cells from the immune system, it has become evident that IL-1α exerts a specific role inside various types of cells and especially in the nucleus. This study aimed to shed more light on the role of the IL-1α precursor in the cell nucleus emphasising its interactions with the transcription factor p53 and histone acetyltransferase complexes, two distinct regulatory components of eukaryotic transcription. The idea is based on previously published works confirming the involvement of both IL-1α precursor and IL-1αNTP in the transcription process via interaction with nuclear histone acetyltransferase complexes (Buryskova *et al.* 2004), surface receptor-independent activation of transcription factors (Werman *et al.* 2004) and interaction with necdin (Hu *et al.* 2003) that has been found to be widely implicated in transcription (Taniura *et al.* 1999; Matsumoto *et al.* 2001; Kuwako *et al.* 2004; Kaul *et al.* 2009).

Besides of the experiments studying IL-1α in its natural milieu in the mammalian cells (Part I), I was using yeast *Saccharomyces cerevisiae* as a simple eukaryotic model organism (Part II). Here I intended to extend our previous results that had revealed a functional (genetic) interaction of IL-1α and yeast histone acetyltransferase complexes (Buryskova *et al.* 2004). The yeast model enabled me to use efficient genetic techniques such as gene disruption and *in vivo* protein tagging to analyze the physical association of IL-1α with distinct subunits of the yeast HATs SAGA and ADA. My data refined our previous results and precised the interaction of the IL-1α precursor with the SAGA complex to the HAT/Core module. Results obtained during this part of research also allowed us to propose a new model of the SAGA complex assembly. Last but not least, we defined a new domain at the C-terminus of the Snf1/AMPK kinase and hypothesized its function. This part of my work was recently published (Zamostna *et al.* 2012).

We expect the results obtained from the yeast model to be useful in subsequent experiments addressing the role of the IL-1α interaction with histone acetyltransferases in mammalian cells. Given that yeast and mammalian HAT complexes are to a large extent homologous to each other, I suppose that the

interaction of IL-1α with histone acetyltransferases is most probably conserved in both eukaryotic systems and would be crucial for elucidating the nuclear role of IL-1α in higher eukaryotes including humans.

From 2007 to 2010 I was involved in a project examining the role of *HOX* gene expression in childhood acute lymphoblastic leukaemia. This project was conducted in cooperation with the Childhood Leukaemia Investigation Prague (CLIP) laboratory at the 2nd Faculty of Medicine, Charles University in Prague. While there have been reports of dysregulation of *HOX* gene expression in AML and the study by Drabkin *et al.* (2002) indeed showed that elevated overall transcription of *HOX* genes predicts worse clinical outcome, there was no quantitative analysis of *HOX* gene expression in genotypically- and immunophenotypically-defined subgroups of paediatric ALL to date. Results that I gained during this four-year project are summarised in Part III of this thesis. Although the objectives of this part of my work may seem to stand a little bit aside from the main topic of this thesis, both themes may indeed be related since there have been evidences in the literature of the IL-1α stimulation of leukaemic cells and of various proinflammatory cytokines contributing to the pathogenesis of leukaemia (part 4.3.3). The data I obtained in this part broaden our understanding of the *HOX* genes and their expression in childhood ALL and were published (Starkova *et al.* 2010).

7.1 Overexpression and subcellular localisation of the IL-1α proteins in mammalian cells

To study the IL-1α proteins in mammalian cells, I was using transient transfection with the appropriate recombinant plasmids derived from pcDNA4, pEGFP and pDsRED2 (see Table 4 in Materials and Methods). However, during the experimental work, I was facing the problem of low transfection efficiency and low levels of the IL-1α protein overproduction in mammalian cells. The use of electroporation, nucleofection (Amaxa) or transfection reagents FuGENE (Roche) and ExGen (Fermentas) resulted in transfection efficiency rarely higher than 10%. Although relatively high transfection efficiency was achieved with EGFP alone (30-60%), IL-1α expression never reached similar levels. I assign this phenomenon to a possible toxic effect of IL-1α overexpression in mammalian cells. A similar behaviour was observed in my experiments with yeast cells that eliminated plasmids

encoding the IL-1α precursor significantly more rapidly than the corresponding empty vectors (6.2.2). In my opinion, the protein expression toxicity may arise out of the presumable interference of IL-1α with host transcription.

Because I was interested in studying the nuclear function of IL-1α, I subsequently checked he subcellular localisation of all of the IL-1α variants fused to EGFP. Due to the presence of the nuclear localisation signal, both the IL-1α precursor and IL-1αNTP were located in the cell nucleus. In contrast, the subcellular localisation of mature IL-1α was cytoplasmic. A small portion of the EGFP-fused mature IL-1α seemed to be localised in the cell nucleus (Figure 13). This is possible due to the small size of IL-1αMat/EGFP (approx. 44 kDa) so the fusion protein can freely diffuse through the nuclear pores. According to a study addressing the diffusion limit set by the size of the nuclear pore, even a protein larger than 60 kDa can pass through the nuclear pore without a functional NLS (Wang and Brattain 2007). The same phenomenon may explain the apparent presence of EGFP in the nucleus (Figure 15); indeed, the EGFP translocation to the nucleus has been reported in multiple studies (Seibel *et al.* 2007; Dross *et al.* 2009). However, in my experiments, the amount of protein in the nucleus is significantly higher in the case that intranuclear transport is driven by NLS (IL-1α precursor and IL-1αNTP; Figure 12 and 14).

It is also important to say that I never observed the proteolytic cleavage of the IL-1α precursor by endogenous calpain in the cell lines I used. This would be manifested on the western blotting as a band corresponding to IL-1αNTP bearing the FLAG tag on its N-terminus. The calpain cleavage site lies between amino acids 112 (R) and 113 (S) of the IL-1α precursor. Both μ-calpain and m-calpain, two „classical“ calpain forms, have been shown to cleave the IL-1α precursor (Kobayashi *et al.* 1990; Carruth *et al.* 1991; Sultana *et al.* 2003). Rise in intracellular calcium level is absolutely required for the processing (Kobayashi *et al.* 1990) as well as phosphorylation of certain serine residues of IL-1α (Watanabe and Kobayashi 1995). Both calpain forms are ubiquitously expressed in mammalian cells, but processing by calpain does not usually occur in cells producing the IL-1α precursor *in vitro* (Watanabe and Kobayashi 1994), possibly as a consequence of the association of IL-1α with the intracellular IL-1 receptor type II that protects intracellular IL-1α from calpain cleavage (Zheng *et al.* 2013).

7.2 Evidence and significance of the putative interaction of IL-1α with p53

The hypothesis of the possible interaction of the tumour supressor p53 with IL-1α in the cell nucleus is based on our previous results that showed that p53 co-immunoprecipitates with the IL-1α precursor in Mrc-5 cells (Miroslava Burýšková, personal communication). Moreover, my co-localisation experiment showed that the IL-1α precursor labeled with DsRED2 and p53/EGFP co-expressed in the mouse NIH 3T3 cell line exhibited similar intranuclear distribution patterns and it is therefore possible that they co-localise (Figure 16). This result indicates that a putative physical association of these two molecules may occur in the nucleus of mammalian cells.

However, I failed to confirm these promising results in subsequent co-immunoprecipitation experiments aimed at the detection of a physical interaction of the IL-1α precursor and p53/EGFP co-expressed in mammalian cells. The EGFP fusion p53 protein was used because of the overlap of the 53-kDa p53 band with the 55-kDa band corresponding to the heavy chain of the anti-p53 mouse monoclonal antibody on western blotting. Neither using different transient transfection methods (electroporation, nucleofection, FuGENE, ExGen) nor diferent cell lines (Mrc-5, NIH 3T3, Saos-2) leaded to the successful co-immunoprecipitation. In my opinion, the overall low transfection efficiency did not produce sufficient protein levels. Possibly, the co-expression with p53 also decreases the levels of IL-1a protein within the cell (M. Pospíšek, unpublished observation). IL-1a co-expressed with p53 may be targeted for degradation, but this issue remains to be addressed in further experiments.

Therefore I changed the experimental setup and used endogenous p53, expression and nuclear localisation of which was induced by UV irradiation and roscovitine treatment in A375 cells, as a bait. In this experimental setup the use of the fluorescent precursor of IL-1α was not necessary since the heavy chain of a novel p53 antibody (CM-1) was not recognised by the secondary antibody used. Following this scheme, a faint band was detected in the sample of the co-immunoprecipitation of transiently transfected IL-1α with endogenous p53 from the A375 cells (Figure 18).

Importantly, no IL-1α and p53 co-immunoprecipitation was observed from the H1299-R273H cell lysate. The H1299-R273H cells produce high quantities of protein

p53 that, however, bears a point mutation (arginine to histidine at amino acid 273) in the region contacting DNA. In consequence, it possesses different biological properties than wild-type p53, e.g. almost exclusively cytosolic subcellular localisation in HCT 116 cells (Morselli *et al.* 2008), modulation of drug resistance through downregulation of procaspase-3 (Wong *et al.* 2007), increased tumorigenicity (Dittmer *et al.* 1993) and decreased (although still functional) sequence-specific DNA binding (Cho *et al.* 1994). The absence of IL-1α in the co-immunoprecipitation sample from the H1299-R273H cells therefore suggests that p53 is possibly tethered into the same site on the chromatin as the IL-1α precursor. Indeed, IL-1α was found to be associated with chromatin (Cohen *et al.* 2010).

In the future, the hypothesis of the putative interaction of IL-1α with p53 needs to be examined by further experiments involved other techniques such as the Förster resonance energy transfer (FRET). FRET represent a method that is widely used to study the interaction between two proteins; if the proteins are in close proximity, a non-radiative energy transfer occurs between the two fluorescent dyes fused to both examined proteins. Although DsRED2 and GFP can possibly be used for FRET analysis (Erickson *et al.* 2003), these two fluorophores do not represent an optimal setup for FRET due to the fact that the excitation spectra for GFP and DsRED2 overlap significantly. Furthermore, although DsRED2 should represent an improved form of the DsRED1 protein, some unspecific aggregation can still occur since the aromatic residues involved in these interactions probably remained unchanged (Yarbrough *et al.* 2001). As an efficient FRET partner for GFP, the red fluorescent protein mRFP1 could be used (Peter *et al.* 2005) that is monomeric and its emission is negligible when excited at wavelengths optimal for GFP (Campbell *et al.* 2002); construction of the IL-1α fusion with mRFP1 and subsequent FRET analysis of the interaction of IL-1α with p53 is planned in the future.

p53 is one of the „hub proteins“ representing important nodes in the protein-protein interaction network in higher eukaryotes. Although the physical association of p53 with IL-1α has not been reported so far, in addition to the chromatin fiber, both molecules could possibly be interconnected by a third protein, e.g. by the histone acetyltransferase p300. Given that the physical interaction of p300 with p53 has been described (Lill *et al.* 1997; Dornan *et al.* 2003a), as well as the interaction between p300 and IL-1α (Buryskova *et al.* 2004), it could be possible that IL-1α association with p53 may be mediated by the acetyltransferase p300. p300 has been reported

to contain multiple binding sites for p53 and certain of these allow the interaction of p53 with other high-affinity p53 N-terminal binding proteins (Teufel *et al.* 2007). p53 can thus bridge p300 to other proteins and this may be the reason of the co-precipitation of p53 together with IL-1α.

p300 represents a key molecule regulating transcription and possibly other cellular processes through chromatin remodelling and acetylation of non-histone proteins. Regarding the large number of the protein interaction partners of p300 (Bedford *et al.* 2010), it is evident that p300 is able to form a „scaffold“ providing interaction surfaces for many other cellular proteins. IL-1α has been shown to make part of a protein complex bound to p300 (Buryskova *et al.* 2004). An intriguing hypothesis therefore arises that nuclear IL-1α could in a certain way influence the p300 acetyltransferase function and serve as a transcriptional coactivator modulating the transcriptional activity of selected (possibly proinflammatory) (Werman *et al.* 2004) genes via histone acetyltransferase-catalyzed chromatin remodelling. It would be also interesting to observe p53 acetylation status sequentially after the interaction with IL-1α with the histone acetyltransferases since p53 represents one of the non-histone target molecules of the p300-mediated acetylation (Gu and Roeder 1997; Wang *et al.* 2003).

The IL-1α-binding protein necdin has also been shown to interact with p53 (Taniura *et al.* 1999) and to regulate p53 acetylation and consequently apoptosis in neuronal cells (Hasegawa and Yoshikawa 2008). It is tempting to speculate that elucidation of the molecular mechanism of the possible involvement of IL-1α in modulation of the p53 transcriptional activity could have an impact on understanding the role of p53 in the regulation of cellular events including apoptosis or malignant transformation.

7.3 Modulation of the IL-1αNTP subcellular localisation

The N-terminal domain of the IL-1α precursor harbours a functional nuclear localisation signal KVLKKRRL between residues 79-86 (Wessendorf *et al.* 1993) and a study employing fluorescent IL-1αNTP protein version confirmed that IL-1αNTP is localised in the cell nucleus (Pollock *et al.* 2003). During the fluorescence microscopy analysis of the IL-1αNTP/EGFP fusion protein expressed in human Mrc-5

cells I remarked that IL-1αNTP/EGFP was localised both in the nucleus and in the cytoplasm (Figure 14). The subcellular localisation of IL-1αNTP/EGFP appeared to be variable according to the cell density with more cells with the nuclear IL-1αNTP/EGFP in cell cultures of higher density.

Results of the experiments studying the subcellular localisation of IL-1αNTP/EGFP performed by me and Denisa Dolečková under my supervision showed that it can be shifted towards the nuclear localisation when cultivated in higher densities (6.1.5.1). We thus sought to identify the mechanism of this effect and found that IL-1αNTP/EGFP exhibits the nuclear localisation more frequently in cells cultivated in the conditioned growth medium (6.1.5.2). Denisa next performed a time-scale experiment studying the change in the subcellular localisation of IL-1αNTP/EGFP within one cell in the 25 min interval demonstrated that the nuclear relocalisation occurs within 2,5 h after the addition of conditioned medium and remains stable until the end of the experiment (cca 8 h) (Dolečková 2011). It appears that certain mediators or growth factors secreted by the cells to the growth medium presumably mediate the change in the subcellular localisation of IL-1αNTP/EGFP.

Interestingly, the effect of higher cell culture density on the subcellular localisation of IL-1αNTP/EGFP was opposite to that observed by Luheshi *et al.* with the IL-1α precursor. In the BV-2 microglial cell line, the nuclear localisation of the IL-1α precursor was inhibited at higher cell densities and was predominantly cytosolic as shown by immunostaining. The authors assumed that the subcellular distribution of the IL-1α precursor was mediated by cell contact (Luheshi *et al.* 2009a).

The transport of IL-1αNTP into the cell nucleus is most probably mediated by HAX-1 (Kawaguchi *et al.* 2006). Until present, various biological functions have been attributed to the nuclearly localised IL-1αNTP, including malignant transformation (Stevenson *et al.* 1997), induction of apoptosis in malignant cells (Pollock *et al.* 2003) or transcriptional activation in cooperation with histone acetyltransferases (Buryskova *et al.* 2004). It was not possible to observe the malignant transformation of Mrc-5 cells by IL-1αNTP in my experiments since this is a long process that would require the generation of stable transfectants. Importantly, even after exhaustive attempts, I have never not observe apoptosis in cells transiently transfected with IL-1αNTP (Figure 34). The apoptotic behaviour of malignant cells that was described by Lovett and colleagues may be

a consequence of the specific transfection procedure. While I used ordinary recombinant vectors and commercially available transfection reagents, Lovett *et al.* used retroviral vectors pseudotyped with the VSV_G coat protein that could have mimicked viral infection, interacted with cellular defence mechanisms and triggered apoptotic pathways (Ladislav Burýšek, personal communication).

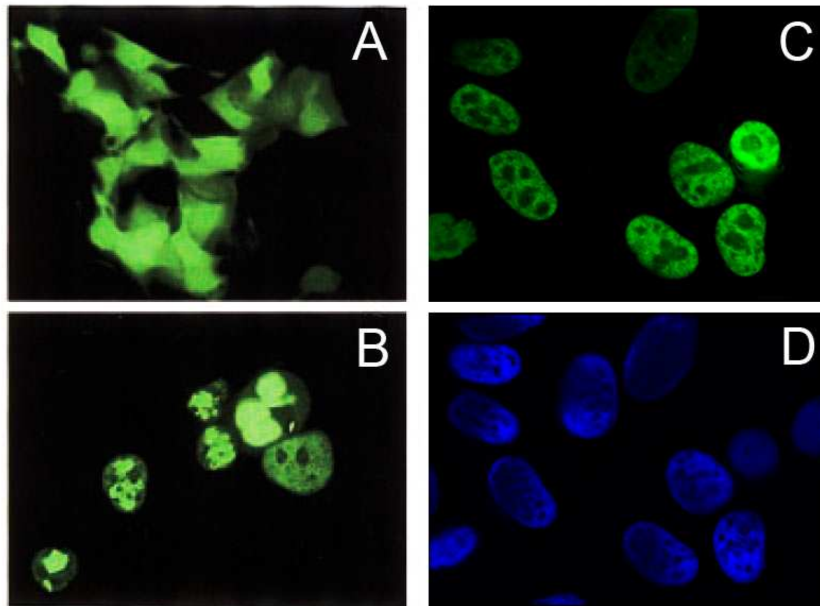


Figure 34: Different results of a similar experiment performed in two laboratories. In David H. Lovett´s lab (images **A** and **B**) (Pollock *et al.* 2003), human 293 tsa cells producing EGFP exhibit unaltered morphology **(A)** while overexpression of IL-1αNTP/EGFP triggered apoptosis in these cells **(B)**. However, IL-1αNTP/EGFP production in human A375 melanoma cells in my experiments **(C)** did not lead to any apoptotic changes. The nuclei of EGFP-positive cells appear to be intact; DAPI staining is also shown **(D)**. (×600)

One of the mediators that promoted the IL-1αNTP/EGFP relocalisation towards the nucleus was extracellular IL-1α in the growth medium (6.1.5.2). Mrc-5 cells exhibit fibroblast morphology and IL-1RI is expressed on their surface since extracellular IL-1 was found to induce IL-6 in these cells (Economides *et al.* 2003). Generally, treatment of cells with IL-1 stimulates transcription of many genes (4.1.4). The relocalisation of IL-1αNTP into the cell nucleus under various stimuli such as high cell density or stimulation with extracellular mediators could be thus connected with transcription and IL-1αNTP in cooperation with histone acetyltransferases (Buryskova *et al.* 2004) may contribute to the modulation of gene expression.

7.4 Heterologous expression of the IL-1α proteins in the yeast cells, the possible toxic effect of the IL-1α production in yeast and subcellular localisation of the IL-1α isoforms

With the intention to facilitate the experimental work with the mammalian cell-specific protein interleukin-1α, I was using yeast *Saccharomyces cerevisiae* as a simple eukaryotic model suitable to study the nuclear function of this molecule. Given that the essential pathways and mechanisms of cellular machinery are conserved here, heterologous expression of proteins in yeast allows the functional analysis of the selected protein in combination with a wide range of molecular and genetic techniques, ease of cultivation and the well-defined genetic background.

Importantly, yeast has played a crucial role in contributing to our understanding of the nuclear role of IL-1α. The finding that the N-terminal part of IL-1α transactivates transcription in yeast cells when fused to the Gal4 DNA binding domain (Gal4BD) (Buryskova *et al.* 2004) was a key step in elucidating the possible function of IL-1α in the cell nucleus. In a yeast assay originally used for the characterisation of the transactivation function of the bacterial σ54-Gal4 fusion protein (Chen *et al.* 2001a), the functional interaction of IL-1α with yeast histone acetyltransferase complexes has been revealed (Buryskova *et al.* 2004). Yeast has been a potent tool for investigation of this higher eukaryote-specific protein and results obtained from the yeast experiments have inspired a wealth of further research of IL-1α nuclear function (Hu *et al.* 2003; Pollock *et al.* 2003; Werman *et al.* 2004).

After the construction of the recombinant IL-1α precursor-encoding plasmids, I introduced these plasmids into the W303-1a strain and confirmed the succesful expression of IL-1α by western blotting (Figure 21). However, the initial experiments with the IL-1α production in yeast indicated that the expression of the IL-1α precursor is gradually attenuated after several passaging steps. Yeast strains producing the IL-1α precursor also showed slightly decreased viability after prolonged storage on agar plates. According to the previous results obtained in our lab, expression of IL-1NTP fused to Gal4BD produces a toxic effect on yeast cells (Buryskova *et al.* 2004), therefore I intended to investigate the putative toxicity of the whole IL-1α precursor expression in yeast. In order to address this issue, I performed an experiment

to determine the stability of plasmids encoding the IL-1α precursor in the course of yeast cell cultivation in non-selective conditions (6.2.2).

The results of this experiment showed that the plasmids bearing a functional IL-1α precursor are removed from the cells more rapidly than the corresponding empty vectors and this supported our hypothesis of interference of IL-1α with yeast growth and viability. This analysis has been described in my MSc. diploma thesis before (Vicenova 2005), but I consider it important for further experiments presented in this work. In my opinion, intracellular production of the IL-1α precursor disturbs some important intracellular pathways or structures in the yeast cell. Very probably, the production of the IL-1α precursor affects the transcriptional system. The interaction of IL-1α with yeast histone acetyltransferases could result in deregulation of certain target genes and thus influence yeast cell fitness.

Regarding the possible effect of the haemagglutinin epitope on the C-terminus of the IL-1α precursor, I can not rule out the contribution of this tag to the observed plasmid instability although the significance of this observation remains speculative. However, the influence of the HA epitope tagging on protein function has already been described in an unrelated field as it was proved that epitope and chimeric tags have a significant effect on human gonadotropin-releasing hormone receptor localisation and function (Brothers *et al.* 2003). Similarly, a toxic effect of the FLAG tag has been reported in a study of polyglutamine expansion proteins in yeast (Duennwald *et al.* 2006). In my experiment comparing two strains producing the IL-1α precursor labeled with both FLAG and HA tag („N/S"; bearing pYX212N/S IL-1αFull) or with the FLAG tag only („N/X"; bearing pYX212N/X IL-1αFull), I observed, in the case of the „N/S" variant, only a slight reduction in the number of colonies growing on selective plates after incubation in non-selective conditions, there was no dramatic difference. Though I do not attach great significance to the possibility that HA tag would contribute to this effect, I was preferentially using the IL-1α variants lacking this epitope in subsequent experiments.

Prior to further experiments addressing the study of the nuclear signaling of the IL-1α precursor in the yeast cells, I decided to determine the subcellular localisation of both IL-1α isoforms with the intention to examine whether the IL-1α proteins show in yeast a similar subcellular localisation as in mammalian cells. The fluorescence microscopy of the yeast-enhanced GFP (yGFP) fusion IL-1α proteins revealed that, similarly to the mammalian cells, the IL-1α precursor is located in the

yeast cell nucleus. This was not a surprising result given that the IL-1α precursor contains the nuclear localisation sequence in its N-terminal part and that the nuclear transport mechanisms are generally very well conserved among eukaryotes (Malik *et al.* 1997; Quan *et al.* 2008; Nguyen Ba *et al.* 2009). In contrast to the IL-1α precursor, mature IL-1α lacking the NLS was localised in the cytoplasm. The subcellular localisation of the IL-1α isoforms therefore corresponds to the situation in the mammalian cells. This finding was an important prerequisite for studying the nuclear function of the IL-1α precursor in the yeast model and the results also indicated that cytoplasmic mature IL-1α can serve as a negative control in further experiments studying nuclear histone acetyltransferases.

7.5 Physical interaction of the IL-1α precursor with yeast histone acetyltransferase complexes

Previous experiments conducted in our laboratory aimed at the screening of IL-1α interactions in the two-hybrid system showed that the expression of Gal4BD/IL-1αNTP can transactivate transcription of the Gal4 promoter *per se*, without the presence of any interaction partner (Buryskova *et al.* 2004). Furthermore, overexpression of the Gal4BD/IL-1αNTP fusion protein in yeast resulted in the growth-inhibitory effect that was markedly dependent on the presence of an intact SAGA complex. The Gal4BD/IL-1αNTP-mediated growth inhibition was partly or completely relieved in the yeast strains with a deletion of an adaptor (Ada2, Ada3), enzymatic (Gcn5) or structural (Spt7) subunit of the SAGA complex. Interestingly, deletion of the *AHC1* gene leading to the disintegration of the ADA complex had no effect and failed to rescue the inhibition of growth and to transactivate transcription of the Gal4 promoter (Buryskova *et al.* 2004).

According to these results, I assumed that IL-1α expressed in the yeast cells interacts with the yeast histone acetyltransferase complex SAGA, but not with the ADA HAT complex. When browsing the yeast TAP-fusion strain collection (Ghaemmaghami *et al.* 2003) to choose the strains for my experiments, I considered using the TAP/Ahc1 strain as a negative control to the remaining TAP-fused HAT subunits-expressing strains that were available to me, e.g. TAP/Gcn5, TAP/Spt7, TAP/Spt8, TAP/Ada1, TAP/Ada2 and TAP/Ada3. Out of these HAT subunits, only

Ahc1 is ADA-specific and should not be present in the SAGA complex (Eberharter *et al.* 1999).

Results of my co-immunoprecipitation experiments surprisingly indicated that all of the studied HAT subunits can be found in the complex of yeast proteins binding IL-1α. Obviously, only the IL-1α precursor conferred the interaction with the TAP-fused SAGA and ADA subunits (Figure 24). Although I was able to immunoprecipitate mature IL-1α from the yeast lysates as well (that I confirmed by western blotting; Figure 25), none of the histone acetyltransferase subunits were found to bind IL-1αMat. The most probable explanation of this phenomenon is that the IL-1α precursor interact with HAT complexes via the N-terminal domain (IL-1αNTP) that is not present in the mature protein. This has been shown previously in GST pull-down experiments in mammalian cells (Buryskova *et al.* 2004). Moreover, unlike IL-1αMat, the IL-1α precursor can translocate to the nucleus due to the nuclear localisation sequence. Although cytoplasmic histone acetyltransferase complexes also exist in yeast such as Hat1 (Parthun *et al.* 1996), this is not the case of SAGA and ADA histone acetyltransferase complexes that, according to the literature available, have not been found in the cytoplasm so far. Therefore, the interaction with the yeast histone acetyltransferase complexes is also dependent on the nuclear localisation of IL-1α.

7.6 Role of the N-terminal domain in the binding of the IL-1α precursor to the HAT complexes and its similarity to the C-terminal regulatory domain of the Snf1 kinase

Unfortunately, due to cloning difficulties and problems with protein expression and stability, I was not able to characterise the interaction of particular histone acetyltransferase subunits with the N-terminal part of IL-1α as I intended. This part of the project was carried out by Josef Novák, a student from our laboratory, under my supervision. However, we did not obtain enough data from the co-immunoprecipitations using IL-1αNTP as a bait, mainly due to the instability of the protein in the yeast cells (Novák 2012).

However, Josef Novák contributed significantly to the possible explanation of the mechanism of the IL-1α N-terminal domain binding to the yeast SAGA complex. In order to find a structure similar to that of IL-1αNTP in *S. cerevisiae*, he

employed the Robetta server (Kim *et al.* 2004) to obtain *ab initio* models of the IL-1αNTP 3-D structure and used five of the structures obtained to perform a structure similarity search using the Dali server at the Institute of Biotechnology, University of Helsinki (Holm and Rosenstrom 2010). The best-scored and only meaningful hit returned by one of the IL-1αNTP structure predictions was the C-terminal regulatory domain of the Snf1 kinase alpha subunit. IL-1αNTP and the Snf1 C-terminal regulatory domain exhibit a high degree of their structural similarity in 3-D (Figure 35 A and B); therefore we named this domain Interleukin-1αNTP-Like domain (INL domain). Moreover, the INL domain appears to be highly conserved among yeast and rat (Figure 35 C).

Snf1 is a member of the AMP-activated protein kinase (AMPK) family and has been shown to functionally and physically interact with certain subunits of the SAGA complex (Gcn5, Sgf73, Spt3, Spt8 and Ubp8) and with Ahc1, an identifying member of the ADA complex (Liu *et al.* 2005; Collins *et al.* 2007; Wilson *et al.* 2011). Mutant strains *snf1*Δ and *snf1-108,* truncated after the 108th codon of Snf1, show growth defects on agar plates containing 3-Amino-1,2,4-triazole (3-AT; a competitive inhibitor of His3) (Liu *et al.* 2005). Interestingly, according to Josef´s drop test results, expression of the IL-1α precursor slightly suppressed this growth defect compared to the strains producing mature IL-1α or strains containing an empty control plasmid only. The highest suppression of the 3-AT toxicity was observed in the yeast strain *snf1-108* (Liu *et al.* 2005) growing on agar plates containing 10 mM 3-AT and glycerol as a carbon source; this probably reflects the fact that Snf1 kinase is, besides other stimuli, activated during amino acid and generally nitrogen starvation (Orlova *et al.* 2006; Orlova *et al.* 2010) and after the shift from glucose to glycerol media, during which the relocation of Snf1 to the nucleus was also reported (Vincent *et al.* 2001). The results of Josef´s drop test not only support his structural model of IL-1αNTP but also, because of the known role of Snf1 and SAGA complex in the regulation of transcription initiation at *HIS3* promoter (Liu *et al.* 2005; Liu *et al.* 2010b), point to the possibility of a competition between the IL-1α precursor and Snf1/AMPK for the same binding sites in HAT complexes (Zamostna *et al.* 2012). Moreover, in our previuos study, p300, being a direct AMPK target in mammalian cells (Hardie 2007; Towler and Hardie 2007), was subjected to deletional mapping in human HEK293 cells in order to determine the region responsible for p300- and Gal4BD/IL-1αNTP-dependent transactivation of the Gal4 reporter system. One of the two most active

p300 mutants studied (Δ143-957) lacked a large portion of the N-terminal region but the N-terminal 142 amino acid residues (Buryskova *et al.* 2004) containing also an AMPK phosphorylation site at Ser89 (Zhang *et al.* 2011). This result further supports our hypothesis of competitive binding of IL-1α precursor and Snf1/AMPK to HAT complexes and extends it into the realm of mammals.

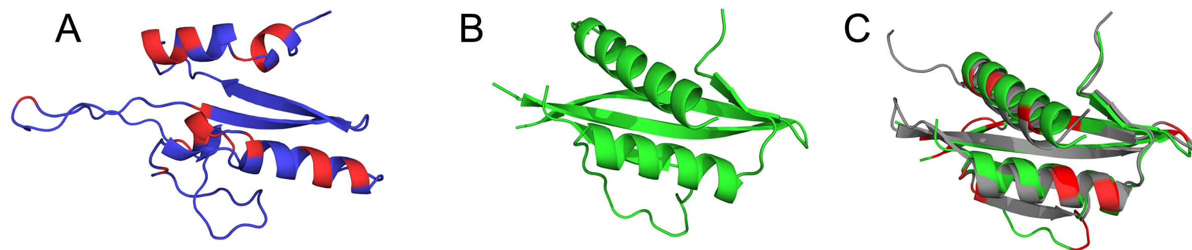


Figure 35. The structure of IL-1αNTP resembles the C-terminal portion of the catalytical subunit of the eukaryotic AMP-activated protein kinase. (A) Prediction of the 3-D structure of the first N-terminal 112 amino acid residues of the IL-1α precursor (IL-1αNTP). Acidic amino acid residues are depicted in red. (B) The 3-D structure of the INL domain of the yeast Snf1 protein kinase (PDB ID: 3T4N). (C) A superimposition of the INL domains of yeast Snf1 (green, PDB ID: 3T4N) and rat AMP-activated protein kinase (grey, PDB ID: 2V92). Acidic amino acid residues are depicted in red. Performed by Josef Novák (Zamostna *et al.* 2012).

7.7 Determining the specific SAGA and ADA HAT subunits forming the possible IL-1α precursor binding site

Although I intended to identify the SAGA subunit that directly binds to the IL-1α precursor by a mass spectrometry analysis (6.2.6), I was not successful due to technical constraints and difficulties of the method. One of the reasons of failure probably lies in the unsufficient amount of cells used for my co-immunoprecipitation experiment. While I only used 1 litre of cell culture (half of which corresponded to the sample loaded on SDS-PAGE), others often use much higher quantities of yeast culture to purify histone acetyltransferase complexes such as 20 litres (Grant *et al.* 1997), 60 litres (Sterner *et al.* 2002) or even 90 litres (Eberharter *et al.* 1999). Protein Ssa2 that has been identified by MALDI-TOF as an IL-1α-binding protein in the TAP/Spt7,*ahc1*Δ sample can be, however, considered as a contamination.

It is a HSP70 family member that is found in the cytoplasm, vacuolar membrane and cell wall (Mager and Ferreira 1993; Lopez-Ribot and Chaffin 1996; Satyanarayana *et al.* 2000). Its interaction with the nuclearly localised IL-1α precursor should therefore be purely coincidental. Interestingly, yeast Ssa2 has 344 unique interaction partners according to the Biogrid database (thebiogrid.org) and appears to represent a highly promiscuous molecule.

Although the attempt to identify the specific proteins binding to the IL-1α precursor in yeast cells by mass spectrometry failed, my data suggest that the IL-1α precursor binds to the HAT/Core module consisting of Ada2, Ada3, Gcn5 and Sgf29 being the only polypeptides shared by both SAGA and ADA complexes (Figure 24) (Grant *et al.* 1997; Lee *et al.* 2011). Furthermore, my co-immunoprecipitation experiments in TAP/Ahc1,*gcn5*Δ and TAP/Spt8,*gcn5*Δ strains excluded Gcn5 from the candidates for the direct IL-1α binding. The IL-1α-binding site should be therefore formed by Ada2, Ada3, Sgf29 and perhaps some other proteins making part of the HAT/Core module.

Unexpectedly, my co-immunoprecipitation experiments revealed that not only the subunits of SAGA but also Ahc1, that does not make part of the SAGA complex (Eberharter *et al.* 1999), can bind IL-1α in yeast cells (Figure 24). This could be the evidence that, contrarily to previous findings (Buryskova *et al.* 2004), the IL-1α precursor may associate also with the ADA complex, but it could also point towards a novel and unexpected role of Ahc1 in *S. cerevisiae*.

7.8 A novel model of the SAGA complex assembly

Multiple evidences suggest that the ADA complex is not merely a SAGA subcomplex as suggested by Grant *et al.* (1998c), but that it represents a distinct HAT complex in yeast (Eberharter *et al.* 1999). This complex possesses enzymatic activity since ADA is able to stimulate transcription in an acetyl-CoA-dependent manner *in vitro* (Steger *et al.* 1998). However, ADA does not interact with acidic transcription coactivators VP16 or Gal4 neither with adenovirus E1A (Utley *et al.* 1998; Shuen *et al.* 2002) though it contains Ada2p that has been shown to interact with these molecules (Barlev *et al.* 1995). Since ADA contains none of the Spt proteins, that are responsible for the interaction with TBP, it probably needs SAGA

for proper TBP binding to target promoters (Grant *et al.* 1998c). Altogether, ADA complex appears to be only partially active in comparison to SAGA and functionally distinct. Ahc1 is believed to be the core structural subunit of ADA deletion of which affects ADA integrity (Eberharter *et al.* 1999).

While no functional interaction of human IL-1α with the yeast ADA complex has been proved (Buryskova *et al.* 2004), my experiments showed that Ahc1 can be co-immunoprecipitated together with IL-1α from yeast cell lysates (Figure 24). The binding of Ahc1, protein that has not been found in SAGA but represents the core structural subunit of ADA, to IL-1α was one of the unexpected results of this study. Furthermore, the analysis of the interaction of IL-1α with the HAT complexes in strains harbouring a disruption of structural subunits showed that Ahc1 can still bind the IL-1α precursor in the strain lacking the functional SAGA complex (Figure 31), its binding is therefore independent on the integrity of SAGA. Since Ahc1 was found to be a subunit specific for the ADA complex that does not co-immunoprecipitate with certain specific SAGA components (Eberharter *et al.* 1999), the association of IL-1α with ADA could be an intriguing idea.

Surprisingly, I was only rarely able to co-immunoprecipitate the Spt7-TAP protein with the IL-1α precursor from the TAP/Spt7,*ahc1*Δ yeast strain - I succeeded in one case from four only and the signal was weak. I however verified that the *AHC1* gene deletion does not reduce Spt7 protein levels. Similarly, IL-1α precursor immunoprecipitation from the yeast cell lysate was confirmed by western blotting (Figure 36). Neither unsufficient amount of Spt7 nor unsuccessful IL-1α precursor immunoprecipitation could therefore have influenced the result.

Spt7 is the core SAGA subunit, maintaining the complex integrity, and is present even in the absence of Spt3, Spt8, Spt20, Gcn5 and Ada1 (Wu and Winston 2002). In the absence of Spt7, the SAGA HAT complex is disrupted, and this disruption severely affects transcriptional activation (Grant *et al.* 1997; Belotserkovskaya *et al.* 2000). Therefore, there is no obvious reason why Spt7 should be absent from a protein complex co-immunoprecipitated with IL-1α precursor in the *ahc1*Δ strain unless ADA is the complex responsible.

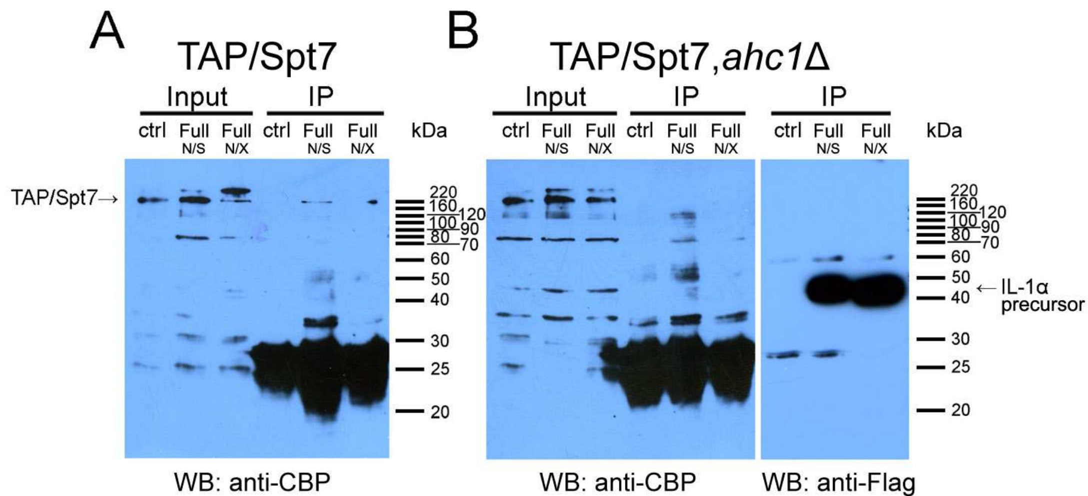


Figure 36: Co-immunoprecipitation of Spt7-TAP with the IL-1α precursor from the TAP/Spt7 and TAP/Spt7,*ahc1*Δ strains. (A) Strain TAP/Spt7; Spt7-TAP co-precipitated with both FLAG-tagged IL-1α precursor (N/X) and FLAG/HA-tagged IL-1α-precursor (N/S). Primary antibody: anti-CBP, secondary antibody: swine anti-rabbit. **(B)** Strain TAP/Spt7,*ahc1*Δ; no Spt7-TAP could be co-precipitated neither with FLAG-tagged IL-1α precursor nor with FLAG/HA-tagged IL-1α precursor from the TAP/Spt7,*ahc1*Δ strain in this experiment. After several rounds of experiments I was able to obtain a weak signal of Spt7-TAP in lysates from TAP/Spt7,*ahc1*Δ in one of four experiments in average (see Figure 31). As it is clearly seen from input lines of all experiments, the disruption of the *AHC1* gene does not significantly affect the intracellular levels of the Spt7 protein. Primary antibody: anti-CBP, secondary antibody: swine anti-rabbit. Staining with anti-FLAG antibody in the last panel confirmed successful immunoprecipitation of the IL-1α precursor from the TAP/Spt7,*ahc1*Δ lysates used in the experiment; primary antibody: mouse anti-FLAG, secondary antibody: goat anti-mouse.

After careful evaluation of the results of my co-immunoprecipitations, as well as of other studies published in the literature,we hypothesized a novel model of SAGA complex assembly and indicate a possible role of the ADA complex in this process. According to this model, Ahc1 functions as an exchange factor that is not exclusively required for but facilitates the association of Spt7 and perhaps other factors with the ADA HAT complex. As a result, a fully functional SAGA complex, that is capable of interacting with various general and non-canonical transcriptional co-activators and accessory proteins such as the IL-1α precursor and Snf1/AMPK, is formed. ADA thus would, at least for some of the cellular regulatory loops, not represent a *bona fide* HAT complex but rather an intermediate and/or reserve protein complex that is associated with non-canonical co-activators and other accessory proteins that may be necessary for the proper assembly of the SAGA complex with all of its co-activators. This is further supported by the observation of Lee *et al.* (2011) who assigned a novel function to the Ahc2 protein, suggesting it as a loader of Ahc1 to

the ADA complex. Important and supportive of our model is that they were able to co-precipitate neither SAGA nor ADA complexes from the *ahc2Δ* yeast strain using TAP-tagged Ada2 as a bait. In that experiment, only the HAT/Core containing Ada2, Ada3, Gcn5 and Sgf29 could be obtained by the co-precipitation. However, our data show that deletion of the *AHC2* gene does not affect the co-precipitation of the IL-1α precursor with Gcn5, Spt8 and Spt7 (Figure 31). We hypothesize that these differences could be caused by different yeast strain background and that our and Lee's observations, if combined, rather point to the importance of Ahc1 for the SAGA complex completion.

It should be noted that the purification of the SAGA complex has also been reported from the *ahc1*Δ strain (Eberharter *et al.* 1999). However, the proposed model does not exclude ADA-independent SAGA assembly but rather suggests that some co-activators or accessory proteins may be brought to the SAGA complex only via the ADA complex, which thus may also exhibit a regulatory function in the control of gene expression (Figure 37).

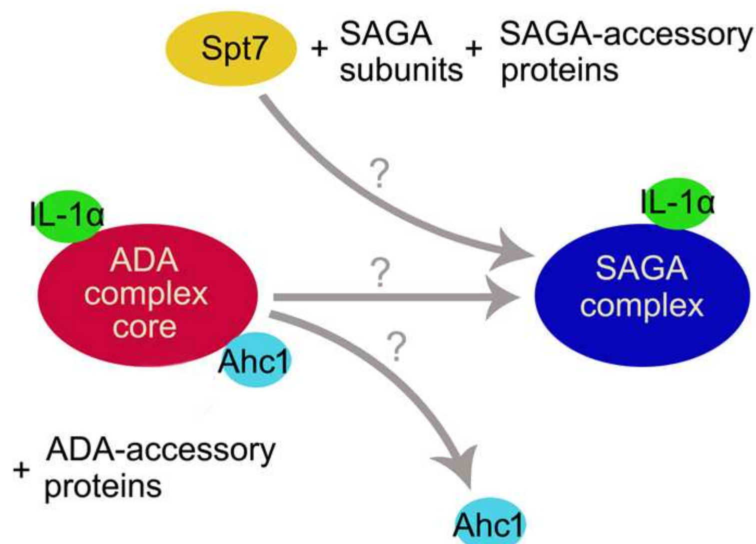


Figure 37: A model suggesting a mutually exclusive role of Ahc1 and Spt7 in the SAGA complex assembly. Co-immunoprecipitation experiments showed that the IL-1α precursor binds to the HAT/Core of both the ADA and SAGA complexes. In the TAP/Spt7,*ahc1*Δ strain, only rarely weak co-precipitation of Spt7-TAP and IL-1α precursor was observed. Ahc1 could thus operate as an exchange factor that facilitates Spt7 binding to a silent or partly functional ADA HAT, bringing various non-canonical co-activators and accessory proteins (e.g.,IL-1α or Snf1) and providing the resulting complex with DNA binding abilities and thus giving rise to a fully functional SAGA complex. ADA might therefore, at least from the point of IL-1α function, not represent a *bona fide* HAT complex, but rather an intermediate protein complex that is however necessary for the assembly and proper function of the SAGA HAT complex in broader sense.

Last but not least, it should be noted that the subunit composition of yeast histone acetyltransferase complexes may vary, as is the case of the SAGA and SALSA HAT complexes (Belotserkovskaya *et al.* 2000; Sterner *et al.* 2002). As far as protein-protein interactions are concerned, the estimated number of subunits of the SAGA complex is at least 20 and the number of protein-protein interactions of core SAGA subunits and SAGA-associated molecules could result in hundreds of possibilities of binding. For example, according to the *Saccharomyces* genome database (http://www.yeastgenome.org), only Ada2 itself confers more than 50 physical interactions with various non-SAGA proteins. Moreover, especially the ADA complex has not been studied sufficiently in my opinion since the literature references addressing the ADA subunit composition can mostly be found before 1999 (Grant *et al.* 1997; Grant *et al.* 1998b; Eberharter *et al.* 1999; Sterner *et al.* 1999; Lee *et al.* 2011). Variable subunits may participate in the assembly of yeast histone acetyl transferase complexes, making them difficult to define precisely as far as the subunit composition is concerned. Likewise, the precise physiological role of the histone acetyltransferase complexes both in yeast and in humans still remains largely ununderstood.

7.9 The role of *HOX* gene expression in paediatric ALL

The aim of this project was to assess the expression of selected *HOX* genes in genotypically and phenotypically defined subgroups of human acute lymphoblastic leukaemia. The *HOX* expression analysis was carried out in cooperation with Harry A. Drabkin who had previously performed an analogous study in chromosomally defined subgroups of acute myeloid leukaemia (Drabkin *et al.* 2002). This is the reason of using the same *HOX* primer sets for the qRT-PCR quantification as described in this article, except for *HOXA6*, that was created by myself. Out of the 23 *HOX* gene systems listed in Table 14, only the *HOXA5, HOXA6, HOXA9, HOXA10, HOXA13, HOXB6, HOXB13, CDX1* and *CDX2* primers anneal to different exons of the *HOX* gene concerned. Human *HOX* genes are small and contain only two exons one of which is represented by the extremely conserved homeodomain, *HOX* primer designing is therefore challenging. qRT-PCR performed using primers

annealing within one exon, however, could be influenced by the presence of genomic DNA (gDNA) in the sample. Because of this, DNase I treatment to eliminate the gDNA contamination was needed. During the qRT-PCR optimising phase (6.3.1), I routinely included control reactions without reverse transcriptase into the experimental protocols in order to control the quality of DNase I treatment and found that no residual gDNA is present in the DNase I-treated samples.

Our study was the first analysis of this extent that studied the expression of 23 *HOX* genes in paediatric ALL. Before that, only the roles of individual *HOX* genes in ALL were examined. For example, high levels of *CDX2* expression have been correlated with adverse prognosis in paediatric ALL (Riedt *et al.* 2009). Importantly, the leukaemogenic potential of many *HOX* genes was proven (Bach *et al.* 2010).

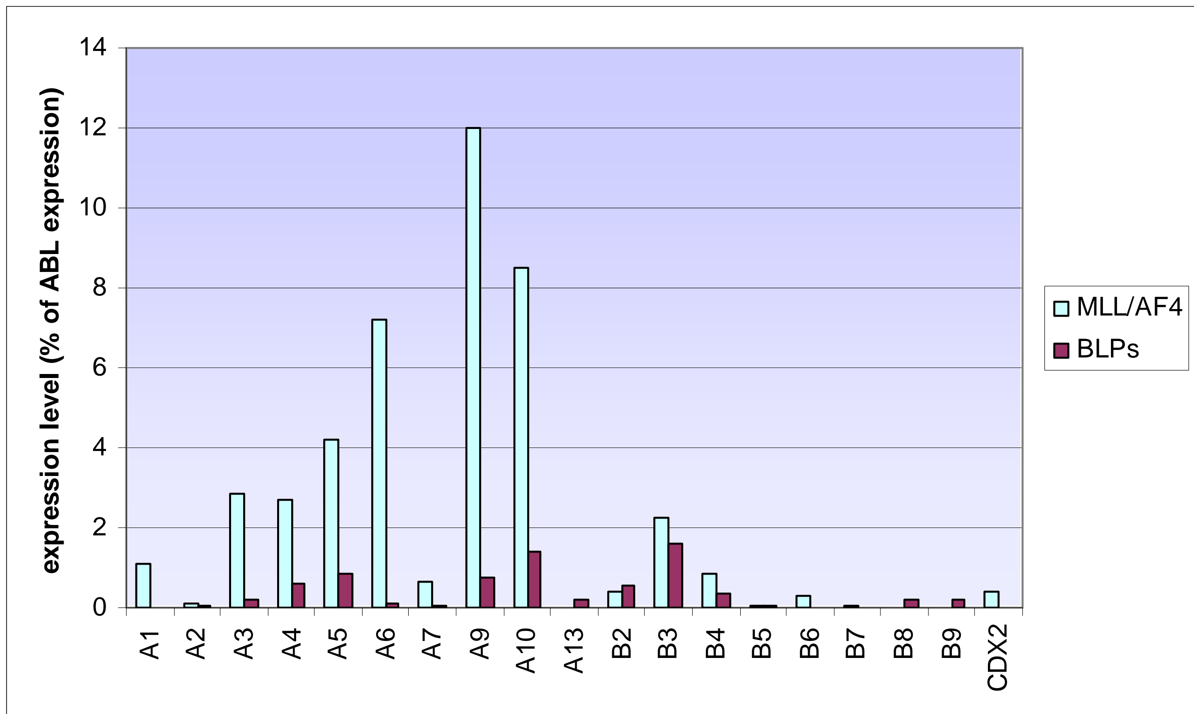
As it can be seen from Graph 2 and Graph 3, the highest overall levels of *HOX* gene expression were determined in the T-ALL subgroup. My qRT-PCR analysis showed that *HOXA4*, *HOXA6*, *HOXA9*, *HOXA10* and *HOXA11*, but also *HOXB2* and *HOXB3*, were extensively transcribed in patients with T-ALL. T-cell acute lymphoblastic leukaemias represent approximately 10-15% of paediatric ALL cases and according to Taghon *et al.* (2003), especially *HOXA7*, *HOXA9*, *HOXA10*, *HOXB3* and *HOXB4* gene expression is important in T-cell development. The *HOXA* cluster genes involved in thymic differentiation pathways, i.e. *HOXA5, HOXA9, HOXA10* and *HOXA11*, may also participate in oncogenic processes (Soulier *et al.* 2005). Aberrant expression of *HOXA9* and *HOXA10* has been shown to contribute to the deregulation of ID2 transcription factor, that may be a crucial step in thymocytic transformation (Nagel *et al.* 2010). Furthermore, *HOXA10* and *HOXA11* can be upregulated in consequence of a cryptic inv(7)(p15q34) that brings the strong *TCRβ* enhancer within the *HOXA* locus (Speleman *et al.* 2005). The frequency of this inversion in ALL patients is not known, but was found in approximately 20% of T-ALL patients in the study by Speleman *et al.* and it is therefore possible that some of our patients may also be affected.

T lymphocytes develop in the thymus through a well-defined order of differentiation steps, during which *HOXA7*, *HOXA9*, *HOXA10* transcription is gradually attenuated (Taghon *et al.* 2003). My expression analysis of these *HOX* genes in sorted T-cell populations was in agreement with this observation only in the case of *HOXA9* and *HOXA10*; I did not observe the attenuation of *HOXA7* in the T-cell maturation process. However, due to technical difficulties, I only evaluated the

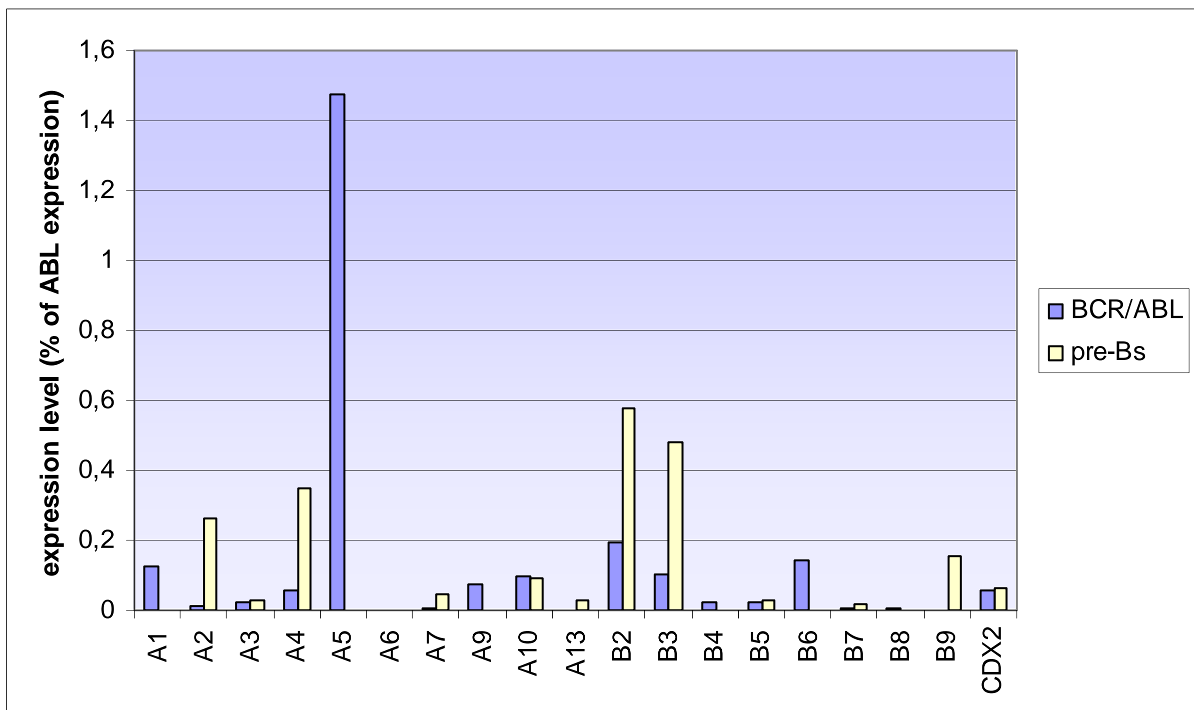
HOX gene expression in two samples of cortical T lymphocytes and one sample of mature T-cells, that is, of course, largely insufficient to draw a conclusion regarding this question.

According to my analysis, the pattern of *HOX* gene expression in patients with T-ALL, notably from the *HOXA* cluster, was similar as in the *MLL/AF4*-positive subgroup. ALL harbouring this fusion gene represents an aggresive, high-risk type of childhood leukaemia and have a particularly poor prognosis. The *MLL* gene encodes a DNA-binding protein that methylates histone H3 on lysine 4 and activates transcription of multiple *HOX* genes (Milne *et al.* 2002). Rearrangements of the *MLL* gene occur in a variety of leukaemia types and translocations with more than 50 different genes have been described until now. However, the leukaemic *MLL* gene fusions encode proteins that have lost the methyltransferase activity. The leukaemogenic potential of *MLL* gene fusions resides in the amino-terminal part of the MLL protein, that is conserved in the MLL fusions. The *MLL* gene rearrangement has been found to correspond with higher expression levels of *HOXA5*, *HOXA7*, *HOXA9*, *HOXA10* and *MEIS1* (Rozovskaia *et al.* 2001; Ferrando *et al.* 2003; Zangrando *et al.* 2009). Indeed, MLL/AF4 suppression using short interfering RNAs decreases the expression of *HOXA7*, *HOXA9* and *MEIS1* (Thomas *et al.* 2005). My data showed high expression of *HOXA3*, *HOXA4*, *HOXA6*, *HOXA9* and *HOXA10* in the MLL/AF4 group, out of which *HOXA9* expression level was the most prominent, that is in agreement with the study by Ferrando *et al.* (2003).

The maturation stages of all the B-lineage ALL subtypes examined in this study ranged between BLPs and pre-B cells, with *MLL*-rearranged leukaemia being the least mature and *BCR/ABL*-positive leukaemia being the most mature. In comparison to their closest possible physiological counterparts, leukaemic cells display an aberrant expression pattern of many *HOX* genes as it can be seen from Graph 6 and Graph 7. These graphs compare the average *HOX* gene expression levels in the MLL/AF4 and BCR/ABL group to the BLPs and pre-B group, respectively. Particularly *HOXA3*, *HOXA4*, *HOXA5*, *HOXA6*, *HOXA9* and *HOXA10* are upregulated in patients with the *MLL/AF4* gene fusion; in the *BCR/ABL*-positive group, *HOXA1*, *HOXA5* and *HOXB6* expression is higher than in the physiological counterparts while *HOXA2*, *HOXA4*, *HOXB2*, *HOXB3* and *HOXB9* are downregulated.



Graph 6: Comparison of *HOX* gene expression in the MLL/AF4 group and in sorted population of B-lymphocyte progenitors (BLPs) as their physiological counterparts. Difference in *HOX* gene expression among these groups demonstrate an aberrant profile in leukaemic cells.



Graph 7: Comparison of *HOX* gene expression in the BCR/ABL group and in sorted population of B-lymphocyte precursors (pre-Bs) as their physiological counterparts. Difference in *HOX* gene expression among these groups demonstrate an aberrant profile in leukaemic cells.

The study also showed the difference in *HOX* gene expression among the cells of B-cell lineage and T-cells, where only B-cells showed the attenuation of the *HOXA* cluster at later developmental stages (6.3.2 and Graph 5). T-cells retain the expression of a variety of *HOX* genes during maturation, indeed, under normal physiological conditions, expression of *HOXB* gene cluster has been found to be associated with differentiation and T-cell activation (Care *et al.* 1994). The contrast between the *HOX* transcription in their physiological counterparts may explain the difference in *HOX* expression levels between T-ALL and B-cell precursor ALL. While T-ALL patients show high overall *HOX* gene expression, transcription of *HOX* genes in cells gained from the *BCR/ABL*-positive patients (maturation stage of which corresponds to B-lymphocyte precursors) are very low.

One of the aims of my analysis was to investigate whether a distinctive *HOX* gene expression pattern in ALL can be predictive of prognosis. This has been previously reported in AML patients (Drabkin *et al.* 2002). However, the study of *HOX* gene expression in patients, divided into two groups according to the response to prednisone therapy, did not reveal any significant difference in *HOX* expression levels (Graph 4). Although the prednisone response is an important predictor of treatment outcome in ALL (Dordelmann *et al.* 1999), it showed little predictive force throughout my analysis, evidenced by the observation that four of the patients in the PGR group experienced disease relapse compared to none in the PPR group.

Neither the hypothesis of a distinctive *HOX* gene RNA expression pattern in immunophenotypically and genotypically defined ALL subgroups has been proven true. The statistical analysis performed by Roman Krejčí revealed that certain *HOX* genes, i.e. *HOXA3, HOXA4*, *HOXA7, HOXB3* and *HOXB4,* were differentially expressed among the studied groups of ALL patients. Furthermore, the cross-comparison of *HOX* gene expression in couples of patient groups characterised particular *HOX* genes expressed in specific genotypic subgroups. According to the Wilcoxon signed-rank test, *HOXA3*, *HOXA4* and *HOXB3* expression distinguished T-ALL from hyperdiploidy or ALL without a known gene rearrangement (i.e. the PGR group together with PPR).

In hyperdiploid ALL, *HOXA7* expression was significantly lower when compared to the levels in the MLL/AF4, TEL/AML1 and T-ALL group. *HOXA7* has been previously found to contribute to the *MLL*-associated leukaemogenesis (Ayton and Cleary 2003). It can be upregulated by MLL-fusion proteins (Zeisig *et al.* 2004) and

although it is probably not required for transformation, its knock-down affects leukaemogenicity in mice (So *et al.* 2004; Orlovsky *et al.* 2011). However, a contradictory result has been reported from the mouse leukaemia model where a MLL fusion protein MLL/AF9 downregulated, rather than activated, the *HOXA7* gene expression (Joh *et al.* 1999).

Furthermore, *HOXB7* expression was lower in patients with *TEL/AML1*-positive ALL and *CDX2* was found to be downregulated in the BCR/ABL group. *HOXB7* overexpression promotes cellular proliferation (Care *et al.* 1999) and is associated with a variety of tumours and poor prognosis (Wu *et al.* 2006; Liao *et al.* 2011; Storti *et al.* 2011; Nguyen Kovochich *et al.* 2013). Lower expression of *HOXB7* can thus be correlated with an overall good prognosis of ALL patients harbouring the *TEL/AML1* gene fusion. The expression of *CDX2* is aberrant in most cases of ALL and AML (Scholl *et al.* 2007; Thoene *et al.* 2009) and there has been evidence that certain *HOX* genes serve as direct transcriptional targets of Cdx2 (Taylor *et al.* 1997; Chawengsaksophak *et al.* 2004; Tabaries *et al.* 2005). Downregulated *CDX2* expression may therefore explain the overall low *HOX* gene transcription in *BCR/ABL*-positive patients shown in my experiments. However, the observation of low levels of *CDX2* in paediatric patients with the *BCR/ABL* gene fusion is in contrast with a study that reported higher *CDX2* expression from adult *BCR/ABL*-positive ALL patients (Thoene *et al.* 2009).

The marked differences in *HOX* gene expression among the leukaemic cels and their closest physiological counterparts (i.e. MLL/AF4 and B-lymphocyte progenitors; BCR/ABL and B-lymphocyte precursors) suggests that the *HOX* expression patterns do not reflect well the developmental stages of B-lymphocytes. More likely, a new *HOX* gene expression pattern is established in the cells subsequent to their transformation. The group of *HOX* genes deregulated during the transformation may include *HOXA1* and *HOXB6*, expression of which has not been detected in normal cells, and possibly other *HOX* genes such as *HOXA3*, *HOXA4*, *HOXA5*, *HOXA9* or *HOXA10*, that appear to be markedly deregulated in leukaemic cells (Graph 6 and 7).

8 CONCLUSIONS

The present study has contributed to our understanding of the role of the IL-1α precursor in the cell nucleus and has uncovered certain aspects of the IL-1α interactions with distinct components of the cellular transcription machinery. It has also analyzed the expression of *HOX* genes in genotypically and phenotypically defined subgroups of paediatric ALL and the results of this analysis suggested that a novel *HOX* gene gene expression pattern is established in the cells subsequent to malignant transformation.

Based on the experimental observations, following conclusions can be drawn:

1/ The subcellular distribution of the IL-1α proteins has been studied both in yeast and mammalian cells and is analogous in both eukaryotic cell models

2/ The subcellular localisation of IL-1αNTP/EGFP in human cells can be modulated by the cell culture density, use of the conditioned medium or addition of recombinant IL-1α into the growth medium

3/ When introduced to the yeast cells, human IL-1α precursor gene affects the stability of the recombinant plasmid bearing this gene; IL-1α overexpression possibly has a toxic effect on yeast cells

4/ The IL-1α precursor physically associates with yeast histone acetyltransferase complexes; the protein complex(es) binding the IL-1α precursor in yeast comprise Gcn5, Spt7, Spt8, Ada1, Ada2, Ada3 and surprisingly also Ahc1 protein while none of these HAT subunits binds mature IL-1α

5/ Part of the HAT/Core module, composed of Ada2, Ada3, Sgf29 and perhaps some other proteins, is the site of the IL-1α precursor binding to the yeast SAGA complex. The binding of the IL-1α precursor to yeast HAT complexes can be explained by the structural similarity of IL-1αNTP with the C-terminal regulatory domain of yeast Snf1

6/ A novel model of the SAGA complex assembly has been suggested in which ADA represents an intermediate subcomplex and Ahc1 facilitates the binding of Spt7 to this partly functional HAT complex

7/ The IL-1α precursor and tumour suppressor p53 show similar distribution patterns, possibly co-localise within the same molecular complex in the nucleus and can be co-immunoprecipitated from mammalian cells

8/ Aberrant *HOX* gene expression patterns identified in the leukaemic cells of paediatric patients subdivided into genotypically and immnophenotypically defined subgroups, i.e. ALL with *BCR/ABL*, *TEL/AML1* and *MLL/AF4* gene rearrangements, hyperdiploid ALL and ALL without a known gene rearrangement, compared to the *HOX* expression in their closest physiological counterparts suggest the establishment of a novel *HOX* expression pattern susbequent to malignant transformation

9 LIST OF PUBLICATIONS

9.1 Publications

Zamostna B, Novak J, Vopalensky V, Masek T, Burysek L, Pospisek M.: N-terminal domain of nuclear IL-1α shows structural similarity to the C-terminal domain of Snf1 and binds to the HAT/Core module of the SAGA complex. PLoS ONE 2012;7(8):e41801

Starkova J*, **Zamostna B***, Mejstrikova E, Krejci R, Drabkin H, Trka J.: HOX Gene Expression in Phenotypic and Genotypic Subgroups and Low HOXA Gene Expression as an Adverse Prognostic Factor in Pediatric ALL. Pediatric Blood & Cancer 2010, 55(6):1072-82
*Authors contributed equally

Vicenová B, Vopálenský V, Burýšek L, Pospíšek M.: Emerging role of interleukin-1 in cardiovascular diseases. Physiological Research 2009, 58(4):481-498

Vopálenský V, Masek T, Horváth O, **Vicenová B**, Mokrejs M, Pospísek M.: Firefly luciferase gene contains a cryptic promoter. RNA 2008 14(9):1720-1729

Zámostná B, Vopálenský V, Burýšková M, Novák J, Burýšek L, Pospíšek M: Evidence of the nuclear interaction of the tumour suppressor p53 and the intracellular cytokine interleukin-1alpha. Manuscript.

9.2 Conferences

Lectures:

Zamostna-Vicenova Blanka, Novak Josef, Burysek Ladislav, Pospisek Martin: Yeast as a model for investigation of the human interleukin-1alpha nuclear signaling. SGM Autumn Conference, University of Nottingham, UK (2010)

Vicenová Blanka, Novák Josef, Pospíšek Martin: Studium interakce interleukinu-1 alfa a histonacetyltransferáz v kvasinkách. XVIII. konference mladých mikrobiologů Tomáškovy dny 2009, Brno, Czech Republic (2009)

Vicenová Blanka, Burýšková Miroslava, Burýšek Ladislav, Pospíšek Martin: Studium jaderné funkce interleukinu-1 alfa v kvasinkách. 24. Kongres Československé společnosti mikrobiologické, Liberec, Czech Republic (2007)

Posters:

Starkova Julia, **Vicenova Blanka**, Krejci Roman, Drabkin Harry A., Trka Jan: HOX Genes Expression Pattern Is Related to Phenotypical and Genotypical Subgroups but Not to Treatment Response in Pediatric ALL. 51th ASH Annual Meeting and Exposition, San Francisco, USA (2009)

Vicenova Blanka, Buryskova Miroslava, Burysek Ladislav, Novak Josef, Pospisek Martin: Dissecting interactions between interleukin-1alpha and histone acetyltransferase complexes by *in vivo* protein-tagging and immunoprecipitation techniques. Tri-Society Annual Conference of the International-Cytokine-Society/International-Society-of-Interferon-and-Cytokine-Research/Society-of-Leukocyte-Biology, Cellular and Cytokine Interactions in Health and Disease, Lisbon, Portugal (2009)

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11 APPENDIX - Selected publications

Zamostna B, Novak J, Vopalensky V, Masek T, Burysek L, Pospisek M.: **N-terminal domain of nuclear IL-1α shows structural similarity to the C-terminal domain of Snf1 and binds to the HAT/Core module of the SAGA complex.**
PLoS ONE 2012;7(8):e41801

Starkova J, **Zamostna B**, Mejstrikova E, Krejci R, Drabkin H, Trka J.: **HOX Gene Expression in Phenotypic and Genotypic Subgroups and Low HOXA Gene Expression as an Adverse Prognostic Factor in Pediatric ALL.**

Pediatric Blood & Cancer 2010, 55(6):1072-82

Vicenová B, Vopálenský V, Burýšek L, Pospíšek M.:

Emerging role of interleukin-1 in cardiovascular diseases.

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Zámostná B, Vopálenský V, Burýšková M, Novák J, Burýšek L, Pospíšek M:

E**vidence of the nuclear interaction of the tumour suppressor p53 and the intracellular cytokine interleukin-1alpha.**

Manuscript.