UNIVERSITÀ DEGLI STUDI DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

DIPARTIMENTO DI PEDIATRIA
LABORATORIO DI ONCOEMATOLOGIA PEDIATRICA

SCUOLA DI DOTTORATO IN
MEDICINA DELLO SVILUPPO E SCIENZE DELLA PROGRAMMAZIONE
INDIRIZZO: IMMUNOLOGIA ED EMATOONCOLOGIA
XXIII CICLO

Tesi di Dottorato

Role of BCL-2 associated athanogene - 1 (BAG-1) in Acute Myeloid Leukemia (AML): protein with hundred faces

DIRETTORE DELLA SCUOLA: Ch.mo Prof. GIUSEPPE BASSO
SUPERVISORE: Dott.ssa MARTINA PIGAZZI

DOTTORANDO: SANJA AVEIĆ

From
Padova, Italy
MMXI

"If we knew what it was we were doing, it would not be called research, would it?"

-Albert Einstein

List of abbreviations:

AA	Amino acid
AIF	Apoptosis induction factor
ALL	Acute lymphoblastic leukemia
AML	Acute myeloische Leukemia
Apaf	Apoptosis protease activating factor
ATP	Adenosinetriphosphate
BD	BAG domain
Bak	Bcl-2 homologous antagonist/killer
BSA	Bovine serum albumin
BP	Base pair
Bax	Bcl-2 associated X protein
Bcl	B cell lymphoma
BH	Bcl-2 homology
CASPASE	Cysteinyl aspartate protease
CDK	Cyclin dependent kinase
DIABLO	Direct IAP- binding protein with low pI
ERK	Extracellular regulated kinase
FADD	Fas-associated death domain
FMS	Macrophage colony-stimulating factor receptor
DMEM	Dulbecco´s Modified Eagle Medium
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
dNTP	Deoxyribonucleosidetriphosphate
ECL	Enhanced chemiluminescence
FAB	French-American-British
FACS	Fluorescence activated cell sorter
HSC	Heat shock protein (constitutively expressed)
HSP	Heat shock protein (induced after stress)
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
IL-3	Interleukin-3
LAM	Leucemia acuta mieloide
MAPK	Mitogen-activated protein kinase / ERK
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAP/ERK kinase
MDS	Myelodysplastic Syndrome
MLL	Mixed Lineage Leukemia

NFkB	Nuclear factor kappa B
NLS	Nuclear localization signal
OMM	Outer mitochondrial membrane
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidyl inositol-3 kinase
PKC	Protein kinase C
PXXP	Proline rich motif (docking site for interaction with Src homology 3 domains)
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
Smac	Second mitochondria –derived activator of caspases /DIABLO
STAT5	Signal transducer and activator of transcription 5
TNF	Tumor necrosis factor
TRAIL	TNF –related apoptosis inducing ligand
Ub	Ubiquitin
UV	Ultraviolet
WHO	World Health Organization

SUMMARY

Bcl-2 associated AthanoGene-1 (BAG-1) is a multifunctional protein competent to delay cell death by a synergistic action with Bcl-2. BAG-1, as well as Bcl-2, has been reported as deregulated in diverse cancer types. During PhD study, we confirmed BAG-1 protein as over-expressed in a cohort of leukemic cell lines and heterogeneously expressed in patients affected by de novo acute myeloid or lymphoid leukemia. Silencing approach, used for a determination of BAG-1's role in AML, suggested a correlation between BAG-1 down-regulation and decreased expression of certain proteins, which contribution to a proliferative advantage of leukemic cells has already been documented.

BAG-1 expression in leukemic cells was demonstrated to be reciprocally regulated with the expression of BAG-3, gene from the same family which shows functional similarities with BAG-1. In correspondence, major impact on leukemic cell faith was verified by co-silencing both of this genes. The increased cell death, confirmed after co-silencing of leukemic cell lines and primary AML, was sustained by caspase-3 and PARP cleavage and release of cytochrome *c* and Smac/DIABLO. Key effect of BAG-1 and BAG-3 co-silencing was seen at the levels of anti-apoptotic proteins Bcl-2, Mcl-1 and Bcl-X_L, survival protein ERK1/2 and cyclin D1, which all resulted as decreased. Our results indicated that BAG-1 might take an important place in a protection of native Bcl-2 and Mcl-1 proteins from a proteasome derived degradation, since proteins became removed from cell more intensively after transient BAG-1;3 silencing. In consequence, BAG-1, sustained by BAG-3, occurred to be a novel protein able to affect leukemic cells faith when highly expressed, in major part by provoking in these cells a block of apoptosis induction. It is most likely that described role BAG-1 accomplishes either directly or indirectly. In fact, BAG-1 interacts directly with Bcl-2 and most likely with Usp9X, influencing thereby indirectly the expression of Mcl-1, protein of great importance for leukemic blasts survival, and in consequence impairing the apoptosis induction.

Finally, BAG-1 and BAG-3 seem to be new molecules capable to sustain AML. Therefore, identification of their molecular targets, such as Bcl-2, Mcl-1 and Usp9X, significant for leukemia development, gives a meaning to the novel pathways which could be of importance for paediatric AML development and opens a possibility for the future studies for a new therapeutic targets.

SOMMARIO

Bcl-2 associated AthanoGene-1 (BAG-1) è una proteina multifunzionale competente per ritardare la morte cellulare mediante un'azione sinergica principalmente con Bcl-2. BAG-1 così come Bcl-2, è stato segnalato essere molto spesso deregolato in diversi tipi di cancro. Durante il dottorato di ricerca, abbiamo trovato che la proteina BAG-1 è sovra-espressa in una coorte di linee cellulari leucemiche e si esprime in modo eterogeneo in pazienti affetti da leucemia acuta mieloide o linfoide all'esordio. L'approccio del silenziamento genico è stato utilizzato per determinare il ruolo che BAG-1 svolge principalmente nelle leucemie acute mieloidi (LAM), permettendoci di scoprire che l'indotta sotto-espressione di BAG-1 comporta una ridotta espressione di alcune proteine molto importanti per conferire un vantaggio proliferativo alle cellule tumorali.

L'espressione di BAG-1 nelle cellule leucemiche è stata dimostrata essere inversamente proporzionale all'espressione di BAG-3, gene della stessa famiglia genica con funzione altamente simile a quella di BAG-1, e questo fenomeno di compensazione genica è stato dimostrato svolgere un ruolo di forte impatto sulla sopravivenza delle cellule leucemiche soprattutto se entrambi i geni venivano silenziati insieme. Un aumento della morte cellulare, confermata dopo il co-silenziamento di BAG-1;3 in linee leucemiche e in colture primarie di LAM è stata osservata, così come l'attivazione delle proteine caspasi-3 e PARP e il rilascio delle molecole pro-apoptotiche citocromo *c* e Smac/DIABLO. L'elemento chiave del co-silenziamento di BAG-1 e BAG-3 è stato individuato essere soprattutto a livello di repressione dell'espressione proteica di elementi anti-apoptotici, quali Bcl-2, Mcl-1 e Bcl-X_L, così come di alcuni regolatori della proliferazione come ERK1/2 e ciclina D1. La nostra ipotesi è che BAG-1 possa rivestire un ruolo importante nella protezione della degradazione di alcune di queste proteine che normalmente vengono degradate via proteasoma. Infatti, dato che queste proteine risultavano diminuite dopo il silenzia

mento transitorio di BAG-1;3, la mancanza di questi fattori noti per regolare il turnover proteico, dunque supportava il loro contributo alla aumentata degradazione proteica oservata. E' molto probabile che il ruolo di BAG-1/-3 avvenga direttamente o indirettamente. Infatti, BAG-1 è noto interagire direttamente con particolari fattori, e noi abbiamo dimostrato che così avviene per Bcl-2, fenomeno già descritto in altri tessuti, e con una proteina di recente interesse la Usp9X, mai prima identificata tra i target diretti di BAG-1. L'abbassamento di BAG-1;3, molto probabilmente tramite Usp9X, ha influenzato

l’espressione di Mcl-1, un altro fattore importante nell’apoptosi delle LAM, dimostrando che silenziamento era in grado di influenzare le cellule leucemiche provocando un blocco di induzione dell’apoptosi.

Infine, BAG-1 e BAG-3 si candidano a nuove molecole con un potenziale ruolo nel mantenimento delle LAM. L’identificazione dei loro principali targets molecolari, quali Bcl-2, Mcl-1 e Usp9X, affetti particolarmente da BAG-1, portano novità sul ruolo di nuovi pathway che potrebbero essere considerati nelle LAM pediatriche e per futuri sviluppi di targets terapeutici.

History Of Leukemia

The term "leukemia" takes its origin from the Greek language. The terminology is a combination of words "leukos", meaning clear or white, and "haima or emia" which refers to blood. History reflecting times around the 4th or 5th century Before Christ (BC) confers a discover of the cancer upon the Greeks.[1]

Many centuries later in the year of 1845, blood cancer, named leukemia, was described in Edinburgh.[1] This finding of John Hughes Benett was followed by the observations of various doctors which all characterized leukemia as a disease of uncontrolled multiplication of anomalous white blood cells. Through the year of 1913, diverse classes of leukemia, including chronic lymphocytic, chronic myelogenous, acute lymphocytic, myeloblastic or monocytic, and erythroleukemia, were desribed. During those times, the chances for survival of a patient with leukemia were unacceptably low. A note of success in terms of decoding a cure for leukemia began much later, within the decade of the 1970s. Arsenic was used as the first form of treatment for leukemia. Although history reveals evidence of Hippocrates (460-370 BC) and the Greeks utilizing this remedy, it was finally in 1865, in Germany, that Arsenic was found to be effective in treating leukemia.[2] Interestingly, the recommendations of this cure could be also found in the Hindu scriptures, ancient Ramayana, of India. With the passage of time, radiation and chemotherapy became two powerful arms against leukemia.

The greatest improvement in the treatment of leukemia came during 20th century with discovery of DNA and, in following, advent of bone marrow transfusion.[1] Today, bone marrow transfusion stands apart as the most successful cure for leukemia. With the progression in research related to the treatment of leukemia, in the last few decades the rate of survival has been risen radically to around 70 %.[3-5]

However, the battle is keep on going since there are still 30 % of patients for which the cure should be discovered. The following work gives its contribution in the fight against leukemia in children.

CHAPTER I:

GENERAL INTRODUCTION:

HAEMATOPOIESIS AND LEUKEMOGENESIS

---

1. INTRODUCTION

1.1. HAEMATOPOIESIS

Continuous process by which the production of all types of blood cells (red cells, white cells or leukocytes and the platelets) occurs is known as haematopoiesis. In developing embryos, blood formation begins in aggregates of blood cells in the yolk sac, called blood islands.[6] As development progresses, blood formation arises in the spleen, liver and lymph nodes, giving further to bone marrow the task of forming the most of the blood cells for the entire organism. In the human adult, the bone marrow produces all of the red blood cells, 60–70 % of the white cells (*i.e.* the granulocytes) and all of the platelets. However, maturation, activation and some proliferation of the lymphoid cells occurs in secondary lymphoid organs (spleen, thymus and lymph nodes), comprising 20–30 % of the white cells.[7] The reticuloendothelial tissues of the spleen, liver and lymph nodes, produce also monocytes (4–8 % of the white cells). In children, haematopoiesis occurs in the marrow of the long bones such as the femur and tibia, while in adults, it occurs mainly in the pelvis, cranium, vertebrae and sternum.

Haematopoiesis involves a remarkable self-regulated system which is comprised of a complex interplay between the intrinsic genetic processes of blood cells and their environment (*Figure 1*).[8] This interplay keeps the balance between the fundamental cellular processes and determines whether multipotential, long-lived haematopoietic stem cells (HSCs)[9], with self-renewal capability, lymphoid and myeloid progenitors and short-lived mature blood cells originated from the HSCs, remain quiescent, proliferate, differentiate, or undergo apoptosis.[10-12]

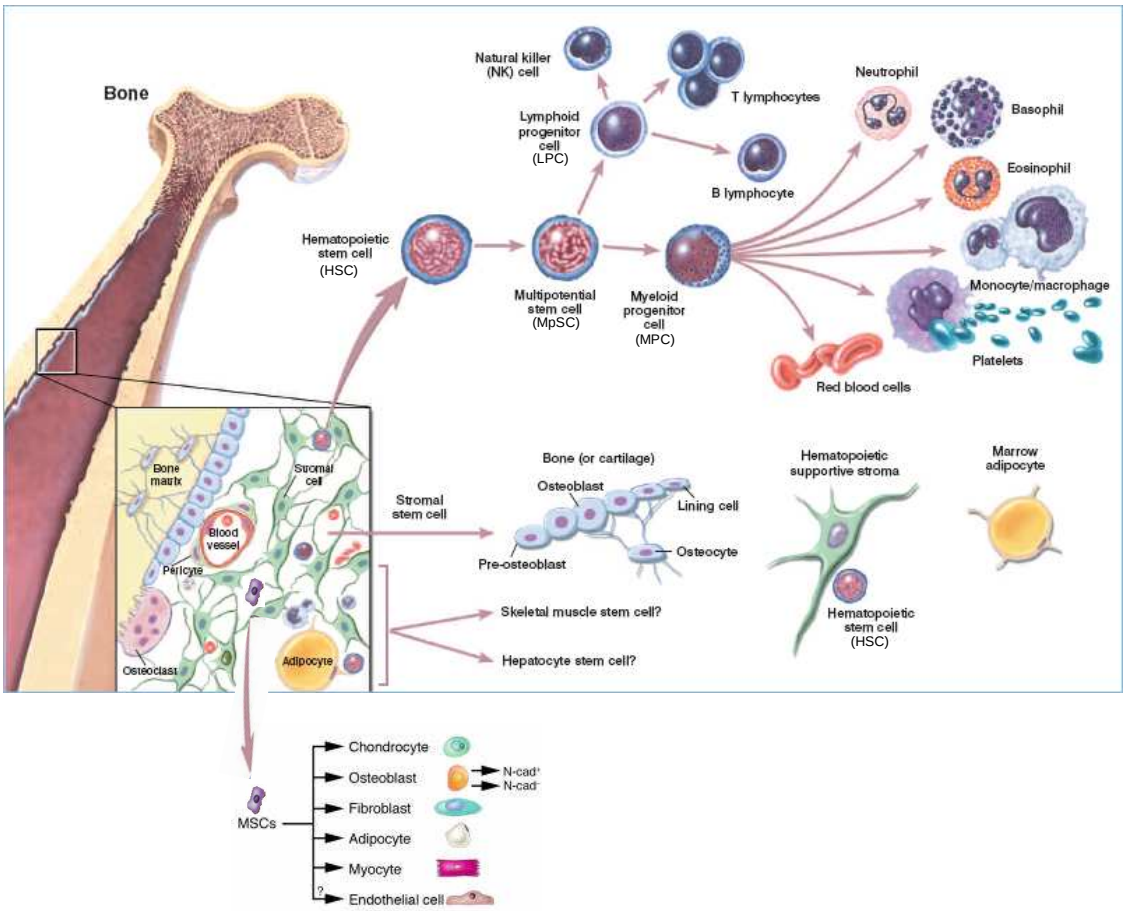


Figure 1. Haematopoiesis of bone cells and marrow stromal cells. In the bone marrow Haematopoietic Stem Cells (HSCs) reside either next to osteoblasts on the endosteal niche or adjacent to endothelial cells of sinusoidal vessels. Upon each division, one daughter cell leaves the bone to proliferate and differentiate into various lineages. HSCs and their progeny are surrounded by stromal cells derived from Mesenchimal Stem Cells (MSCs), which also reside in the bone cavity. MSCs give rise to chondrocytes, osteoblasts, fibroblasts, adipocytes, endothelium, and myocytes. Osteoblasts (expressing M-CSF, RANKL, and OPG and producing IL-7) may regulate lymphoid (expressing IL-7R) and osteoclast (derived from monocytes, expressing the M-CSF ligand c-Fms and RANK) cell development. Abbreviations: MpSC - Multipotent (Progenitor) stem cell; MPC - Myeloid Progenitor Cell; LPC - Lymphoid Progenitor Cell. N-cad – N cadherin.(modified from Regenerative Medicine. Department of Health and Human Services. Chapter 2: Bone Marrow (Hematopoietic) Stem Cells. 2006. © Terese Winslow assisted by Lydia Kibiuk and Caitlin Duckwall, 2001).

Under normal conditions, the majority of HSCs and many progenitors are quiescent, locked in the G0 phase of the cell cycle (*BOX I*), but since a permanent demand for a new haematopoietic cells is put upon them, some of the mature progenitors continue to proliferate and produce more mature offspring. [13] In the event of a stress, such as bleeding, infection or allergic reactions, stored pools of cells in the bone marrow or cells adherent to the endothelium can be quickly released into the circulation in order to localize to the site of "stress alarm".[14] In addition, quiescent progenitors and HSCs are stimulated by a variety of

growth factors (cytokines*; BOX II*) to proliferate and differentiate into mature white cells, red cells or platelets (*Figure 2*).[15-18]

Haematopoietic growth factors

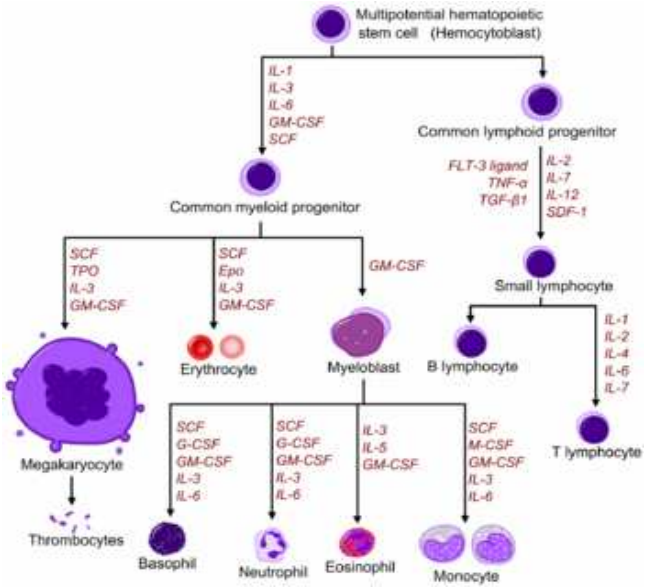


Figure 2. Simplified scheme of the different blood cells development from haematopoietic stem cell to mature cells and some of the most important cytokines that determine which type of blood cell will be created.
Abbreviations: SCF = Stem Cell Factor, Tpo = Thrombopoietin, IL = Interleukin, GM-CSF = Granulocyte Macrophage-Colony Stimulating Factor, Epo = Erythropoietin, M-CSF = Macrophage-Colony Stimulating Factor, G-CSF = Granulocyte-Colony Stimulating Factor, SDF-1 = Stromal cell-Derived Factor-1, FLT-3 ligand = FMS-like tyrosine kinase 3 ligand, TNF-a = Tumour Necrosis Factor – alpha, TGFβ = Transforming Growth Factor beta. (taken from Molecular cell biology. Lodish H, 5th edition; © 2000, W. H. Freeman and Company).

When the bleeding, infection, or other underlying stress, comes to an end and the demand for blood cells returns to normal, the anti-apoptotic and proliferative processes wind down and blood cells swing back to their storage sites, returning the kinetic of haematopoiesis to its basal level. During the lifespan of an individual this process repeats itself innumerable times and is seen in an amplified form after chemotherapy or bone marrow transplantation, two approaches used for a treatment of blood cell's cancer or leukemia.

1.1-a. BOX I: THE CELL CYCLE: A UNIVERSAL CELLULAR DIVISION PROGRAM (By David Secko)

In the 17th century, Robert Hooke peered through a microscope at a slice of cork and discovered that it was composed of cells. Centuries later, Rudolf Virchow set forth the cell theory, in which he proposed that the cell was the basic unit of all living organisms.[19] We now know that this theory is true; cells make up all living things, whether they are plants, animals, or microorganisms. This realization produces a reflective concept on the continuity of life. There is only one way to make more cells—by the division of those that already exist. All animals come from animals, all plants from plants, and all cells from cells. Nature has produced an orderly sequence for the cell to accomplish this feat. In the simplest sense, a cell duplicates its contents and then divides in two. The cycle of duplication and division is known as the cell cycle and is schematically presented in Figure 3..

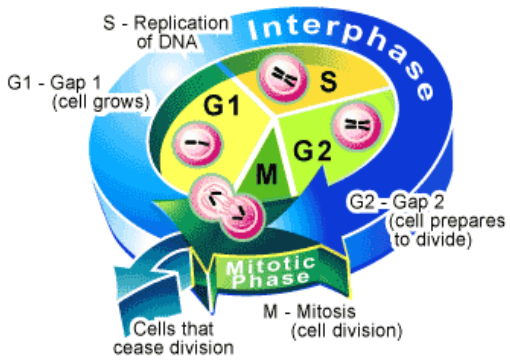


Figure 3. The scheme of the cell cycle. (taken from The science creative quartery, Secko D, 2003)

The goal of the Cell Cycle is to produce two genetically identical cells from one precursor cell.[20] Details of the cell cycle vary from organism to organism and may occur at different times in an organism's life. Nevertheless, certain aspects are universal.[21,22] This is because every cell must accomplish its most fundamental task — copy and pass-on its genetic information to the next generation of cells. This requires the replication of the DNA in each chromosome, followed by the accurate separation of the chromosomes into the daughter cells, so that each cell receives a copy of the entire genome. Once accomplished, the cell will physically divide to produce two identical daughter cells. Since the cell is dividing also its contents in two, under most circumstances, it will also double the mass (including duplicating their cellular machinery). This helps cell to avoid the problem of getting smaller after each division.

The entire cell cycle will take different amounts of time to complete, depending of the organism. One of the more rapidly dividing mammalian cells has a cycle that takes about 24 hours.[23]

However, every cell will pass through the following phases of the cell cycle before division is completed :

- *G1 = growth and preparation of the chromosomes for replication;*
- *S = synthesis of DNA and duplication of the centrosome;*
- *G2 = preparation for the next phase*
- *M = mitosis.*

The passage of a cell through the cell cycle is controlled by the cell cycle control - proteins which can be found in the cytoplasm. Two main protein groups are Cyclins and Cyclin – dependent Kinases (CDKs):

Cyclins	*CDKs*
G1 cyclins (D cyclins)	*G1 CDK (Cdk4)*
S-phase cyclins (cyclins E and A)	*S-phase CDK (Cdk2)*
mitotic cyclins (B cyclins)	*M-phase CDK (Cdk1)*

Within the cell the level of Cyclins rise and fall with the stages of the cell cycle, while CDKs retain their levels fairly stable. However, each CDK must bind the appropriate cyclin (whose levels fluctuate) in order to be activated. The three check-points of the cell cycle progression are placed at the end of the G1 and G2 phases and within M phase.[24]

Resting (G0 phase): The term "post-mitotic" is sometimes used to refer to both, quiescent and senescent cells. Non-proliferative cells in multicellular eukaryotes generally enter the quiescent G0 state from G1 and may remain quiescent for long periods of time, possibly indefinitely (as is often the case for neurons). This is very common for cells that are fully differentiated. Cellular senescence is a state that occurs in response to DNA damage or degradation that would make a cell's progeny non-viable; it is often a biochemical alternative to the self-destruction of such a damaged cell by apoptosis.

1.1-b. BOX II: Cytokines in Haematopoiesis:

Production of the red and white blood cell is regulated with high precision in healthy human. When infection occurs, the production of granulocytes is rapidly increased. Both parameters of these cells, proliferation and self-renewal, depend on stem cell factor (SCF) presence. Proliferation and maturation of the cells that enter the blood from the marrow is regulated by glycoprotein growth factors which can cause cells in one or more committed cell lines to proliferate and mature.[25] *Another factors that stimulate the production of committed stem cells are called colony-stimulating factors (CSFs) and include granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF) and macrophage CSF (M-CSF). All these factors stimulate granulocyte formation and are active on either progenitor cells or end product cells. Erythropoietin is required for a myeloid progenitor cell to become an erythrocyte. On the other hand, presence of thrombopoietin makes myeloid progenitor cells to differentiate into megakaryocytes (thrombocyte-forming cells).*[26]

1.2. LEUKEMIA

Leukemia is a cancer of blood progenitor cells of either lymphoid (which form mature B cells or T cells), or myeloid (which yield neutrophils, monocytes, or eosinophils) origin, that show an expanded proliferative capacities in the bone marrow (*Figure 4*). A fundamental problem that appeared during the research of a different cancer types, including leukemia, was to identify the exact cell type capable to initiate and, in following, sustain the growth of the neoplastic clone once it has appeared. During the past decade, the most accepted theory supports the existence of the rare subset of so-called "cancer stem cells" (CSCs), which have tumor-initiating capacities within neoplastic tissue and are responsible for its maintenance.[27,28] In the case of leukemia, these cells are named as Leukemic Stem Cells (LSCs).[29,30] The results that strongly supported "CSCs theory" derives from the haematological studies in mice, where a fundamental differences between normal blood cells and LSCs biology were described (*BOX III*).

Leukemia originates from a single cell that has undergone clonal expansion caused by some of the genetic changes that have been shown as crucial for the aggressive behavior of leukemic cells.[31-33] However, it is well accepted the notion that the tumor development represents a multistep, progressive conversation of normal cells into cancer cells and, hence, haematopoietic cells into leukemic cells.

Beside the presumed cell origin (myeloid or lymphoid), leukemia can be further classified according to its clinical course as chronic or acute. In acute leukemia, a rapid increase, progression and accumulation of the immature blood cells cause them to spill over into blood stream and often into other organs. This type is commonly found in children and requires immediate treatment. Chronic leukemia, which likely originate in primitive stem cells, are characterized by protracted, subacute, phase and commonly affects elder people.

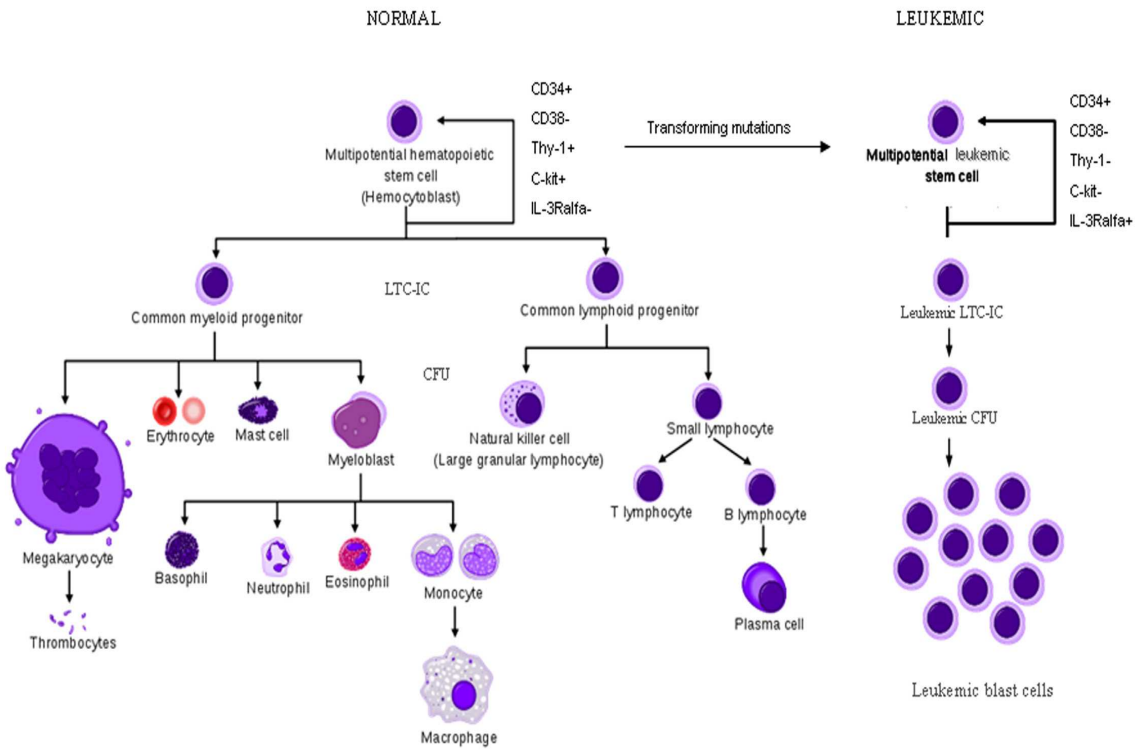


Figure 4. Simplified schematic illustration of the normal and leukemic human haematopoietic hierarchies. Human haematopoietic cells are organized in a hierarchy that is sustained by a small population of self-renewing haematopoietic stem cells (HSCs). HSCs give rise to progressively more lineage-restricted, differentiated progenitors with reduced self-renewal capacity, which in turn produce functionally mature blood cells. Disruption of pathways regulating self-renewal and differentiation through the acquisition of transforming mutations generates leukemic stem cells (LSCs), capable of sustaining growth of the leukemic clone in vivo. LSCs possess an altered differentiation program, as demonstrated by aberrant expression of some cell-surface markers, and give rise to an aberrant developmental hierarchy that retains aspects of its normal counterpart. Abbreviations: LTC-ICs = long-term culture-initiating cells; CFU = colony-forming units. (modified from Hematopoietic Stem Cells: A Long History in Brief; 2009, by Rowland T)

1.2-a. Box III. Fundamental lessons in stem cell biology (Studies in murine haematopoiesis)

The modern era of haematopoiesis and stem cell research was founded by early studies in mice. Using marker chromosomes to distinguish donor from host, investigators demonstrated that bone marrow (BM) contains cells capable of reconstituting all the haematopoietic tissues of irradiated recipient animals. However, the crucial question remained as to whether a single stem cell could reconstitute all blood lineages, or whether there were stem cells for each lineage. In 1961, Till and McCulloch demonstrated the formation of multilineage colonies in the spleen following injection of BM into irradiated mice. Through the use of radiation-induced chromosomal markers, it was shown that each spleen colony arose from a single cell, termed the spleen colony-forming unit (CFU-S), conclusively establishing the existence of multipotent stem cells. The low frequency of CFU-S (1 in 104 BM cells) implied that the haematopoietic system is organized as a hierarchy, with mature, differentiated cells being produced from a smaller number of more immature precursors. Spleen colonies contained CFU-S and were able to give rise to new colonies in secondary recipients, demonstrating that CFU-S are capable of self-renewal. This key property, together with their capacity for multilineage differentiation and extensive proliferation, suggested that CFU-S could be considered as a class of stem cells. Later evidence, including their inability to differentiate into lymphocytes, showed that CFU-S are distinct from true pluripotent repopulating haematopoietic stem cells (HSCs), and that the latter can only be assayed by their ability to stably reconstitute the haematopoietic system of recipient animals. The subsequent development of efficient retroviral gene transfer techniques to uniquely mark HSCs, enabling clonal tracking of the progeny of individual stem cells, as well as more refined methods for purification of stem cells, enabled elucidation of the surface phenotype of self-renewing HSCs. In addition, these approaches led to the recognition of functional heterogeneity within the stem cell compartment, the basis of which was later shown to be the existence of distinct classes of stem cells with differing functional capacities.

Early progress in the identification and characterization of human HSCs was hampered by the lack of transplantation assays that would allow functional testing of candidate stem cell populations in vivo. The development of quantitative xenotransplantation assays using immune-deficient mouse recipients (SCID = severe combined immune-deficient and NOD/SCID = non-obese diabetic/ SCID) to detect primitive human haematopoietic cells with in vivo repopulating ability (SCID-repopulating cells, SRCs) thus represented a significant advance in the field of human haematopoiesis research. Studies to characterize SRCs have shown them to be very primitive cells possessing properties attributed to HSCs, including multipotentiality, high proliferative capacity and the ability to self-renew. Recent improvements to gene transfer

techniques that enable efficient marking of primitive human haematopoietic cells, combined with strategies to purify SRCs, have allowed detailed analysis of the clonal behavior of human HSCs and pointed to the existence of distinct classes of SRC with variable proliferative and self-renewal potentials. It is becoming clear that the human stem cell compartment, like its murine counterpart, is heterogeneous and comprises cell populations with varying capacities for differentiation, proliferation and self-renewal.

(taken from- Cancer stem cells: lessons from leukemia Jean C.Y. Wang and John E. Dick TRENDS in Cell Biology).

1.2.1. BIOLOGY OF CHILDHOOD LEUKEMIA

Leukemia is the most common malignancy during childhood. In industrialized countries, it accounts for 30 % of all cancers diagnosed in children under 15 years of age. Around the year of 2000, the average incidence for this age group in the European region was 46.7 cases per million per year, with a slightly lower level in eastern than in western European countries. The incidence of leukemia is also higher among more industrialized nations and among people of higher socioeconomic status. European population-based cancer registries show an average increase in the incidence of childhood leukemia of 0.7 % per year between 1970 and 1999.[34] The increase of childhood leukemia in modern times may be lifestyle-related. In developed countries, families are usually smaller and hygiene has been improved, so infants are no longer exposed to infections at an early age.[1] The immune systems of children exposed at later ages, without having confronted microbes earlier, may not respond that well. These children may have increased risk of developing leukemia, because their surrounding environment is least like the environment humans were evolved to fit.

Lymphocyte and myeloid cells are predisposed to look after the immune system and to fight together against infections. Normally, these white blood cells repair and reproduce themselves in an orderly and controlled way. In leukemia, however, the process gets out of control, leading the cells to an intensive division but skipping normally acquired maturation. Therefore, immature dividing cells fill up the bone marrow and interrupt healthy blood cells production. Since the leukemic cells are immature, they cannot express proper functions and therefore, an increased risk of infection is present. Because the bone marrow cannot make enough healthy red blood cells and platelets, symptoms such as anemia, thrombocytopenia and neutropenia can occur at various degrees.

Most childhood leukemia are of acute type and are determined by rapid onset and deterioration in the absence of aggressive therapy. Acute leukemia can affect children of any age and is diagnosed equally in girls and boys. Further, acute leukemia can be subclassified according to morphology, genetic alterations, cell surface markers and other characteristics summarized in *Table 1*.

Table 1. Childhood leukemia types, subtypes, and features.

Type (%)	Subtype (%)	Morphology	Common genetic abnormalities (%)	Characteristics
ALL (74)	B progenitor (80–85)	L1, L2	t(12;21) (20) t(9;22) (4) 11q23 translocations (6) t(1;19) (5)	Precursor B-cell markers on cell surface, no surface immunoglobulin, ploidy abnormal in 35% of cells
	T cell (10–15)		7q35/TCRβ (3) 14q11/TCRαg (4) 9p deletions	T-cell markers on cell surface, higher median age of patients, higher white blood cell count, bulky disease, male predominance
	Mature B cell (1–2)	L3	t(8;14), t(2;8), or t(8;22) (2)	Surface immunoglobulin, same as Burkitt's lymphoma
AML (19)	Undifferentiated (2)	M0	Monosomy 5/7	
	Myeloblastic (45)	M1, M2	t(8;21)	
	Promyelocytic (10)	M3	t(15;17)	DIC (bleeding)
	Myelomonocytic (20)	M4	11q23/MLL	Infants, chloromas, secondary AML
	Myelomonocytic with eosinophilia[a]	M4Eo	Inversion 16	
	Monocytic (17)	M5	11q23 translocations	Infants, chloromas, secondary AML
	Erythroleukemia (1)	M6		Exceedingly rare in children
	Megakaryocytic (5)	M7	t(1;22)	Down syndrome, infants, myelofibrosis

DIC, disseminated intravascular coagulation.
[a]Percent not available.
ALL – Acute lymphoblastic leukemia, AML – Acute myeloid leukemia (taken from. *Brain DJ et al. Environmental Health Perspectives., 2003:111 (7): 962-970)*

1.2.1.A.. ALL - Acute Lymphocytic (or lymphoblastic) leukemia occurs when the lymphoid precursor cells (i.e., lymphoblasts) remain arrested in an early stage of development after the cancerous changes took place in the bone marrow. This arrest is usually caused by an abnormal gene expression, generally provoked by the chromosomal translocations or deletions (*Table 1*). Following, the lymphoblasts replace the normal marrow elements, resulting in a marked decrease in the production of normal blood cells, and can proliferate also in organs other than marrow, particularly in the liver, spleen and lymph nodes. In Europe, ALL accounts for around 80 % of all leukemia among children with age between 0–14 years.[35] In developed countries, more than 80 % of ALL is of the precursor B-cell subtype that is responsible for the pronounced peak of incidence in early childhood.[36,37]

1.2.1.B. AML – Acute Myelogenous (or myeloid) leukemia is a malignant disorder characterized by an uncontrolled overproduction of immature myeloid white blood cells. Accounting for almost 15 % of childhood leukemia, AML is the second most frequent leukemia type among European children. It has a fairly stable worldwide incidence of 5-9 cases per million per year.[38] At the same time, AML is the most common second malignancy (a different or second cancer found in a patient previously treated for cancer) in children. The exact cause of AML is still unknown. Research is ongoing into possible causes of this disease. It is known that children with certain genetic disorders, such as Down's syndrome or Li-Fraumeni syndrome, have a higher risk of developing leukemia. Brothers and sisters of a child with AML have a slightly increased risk of developing AML.[39,40] Other non-cancerous conditions, such as aplastic anemia or the myelodysplastic syndromes (MDSs), may increase a child's risk of developing AML.[40-43] Nevertheless, during the past decade, about 50-55 % of

AML were characterized by the presence of specific balanced chromosome abnormalities involved in the formation of specific fusion genes that encode for fusion proteins (*Table 1*).[44,45] These fusion proteins play a key role in the development of leukemia, effecting cell proliferation, survival and/or apoptosis.[46] There are different sub-types of AML, depending upon the exact cell type that has become leukemic, the stage of development (maturation, *BOX IV*) the cells are at, and whether the cells are differentiated or not. There are several classification systems for the sub-types of AML. The two most commonly used classification schemes for AML are the older French-American-British (FAB) system and the newer World Health Organization (WHO) system.

Although approximately 80 to 90 % of children with acute myeloid leukemia attain complete remissions (CR) with the use of current therapy, some of those patients have later recurrences.[47] Moreover, about 50 % of children with AML achieved long-term remissions after the introduction of intensified post-remission therapy.[48] However, additional study, which could assure the improved overall survival of the children with diagnosed AML, are more than appreciated.

1.2.2. TWO – HIT MODEL OF AML

AML is not caused by a single genetic alteration, but it requires a minimum of two genetic alterations for the pre-leukemia to develop into AML. This hypothesis is called the two-hit model of AML and is based on the studies carried out in mouse models with genetic alterations found in patients with AML.[49-51] The model hypothesizes that there are two classes of mutations that are required for development of AML:

(I) Class I mutations involves mutations in the genes responsible for cell proliferation leading to survival and proliferative advantage to the cells. This class includes genes such as FLT3, RAS, c-KIT etc. (*Figure 5*).

(II) Class II mutation involves the mutations in the genes responsible for differentiation. Therefore, these mutations block the differentiation and apoptosis of the cells. The examples of this class are the chromosomal translocations like AML1/ETO, PML/RARa, inv(16) etc. or transcription factors like C/EBP alpha or p53 (*Figure 5*).

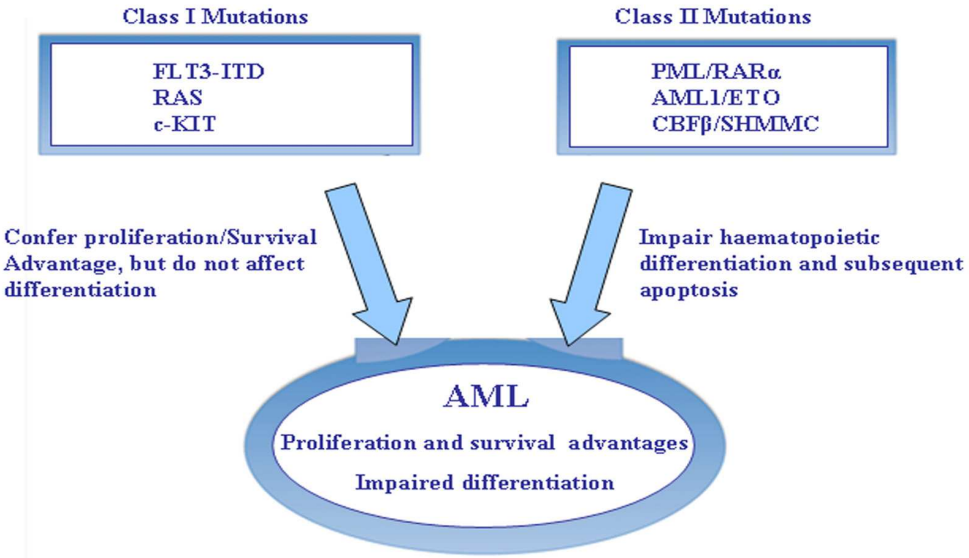


Figure 5: Two-Hit model for AML: The model states that two classes of mutations are required for development of AML; class I mutations that confers proliferative advantage to cells and class II mutation that blocks differentiation.

Recently, a new model of cooperative leukemogenesis for AML was proposed by Rocquain et al,[52] where the mutations of newly discovered genes, found to be co-involved in development of either MDSs or AML phenotype, were included (*Figure 6*).

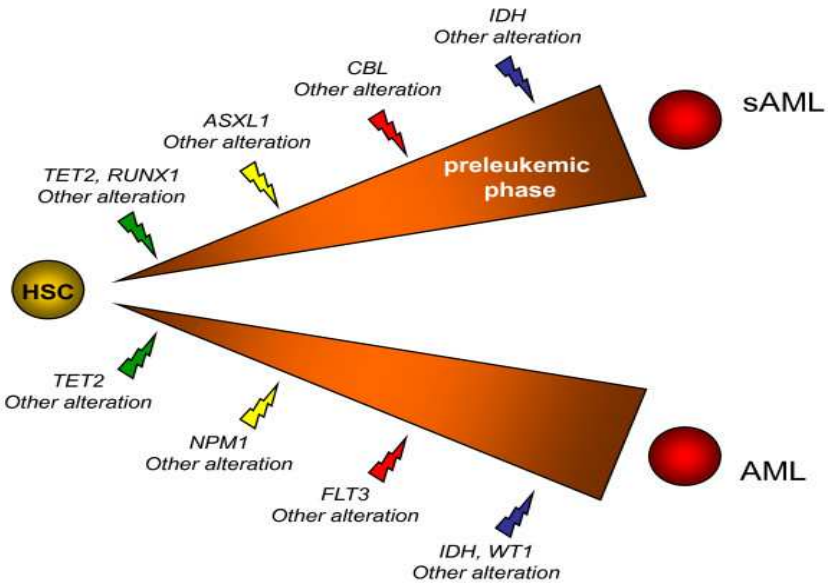


Figure 6. Hypothetical model summarizing frequent gene mutations in the development of two types of acute myeloid leukemia (AML). A haematopoietic stem cell (HSC) or a progenitor cell acquires a series of mutations beginning in class I genes (green). Different additional events (yellow, red and blue) induce clonal expansion and differentiation block and lead either towards secondary AML (sAML or MDS) via a patent preleukemic phase or AML without patent preleukemic phase. The order of events may vary.(Taken from Rocquain et al. BMC Cancer 2010, 10:401).

1.2.3. IMPAIRED CELLULAR PROCESSESS IN AML

The predisposition of the leukemic cells to acquire the multiplication capacity much intensive from the one regularly found in normal haematopoietic progenitor cells is due to an impaired regulation of cell homeostasis. This homeostatic misbalance usually occurs as a result of stimulated cell proliferation, blocked differentiation and/or inhibited apoptosis.[53]

1.2.3-a. BOX IV: Myeloid cells maturation

As mentioned previously, haematopoietic cells arise from pluripotent stem cells of the bone marrow and develop via different types of precursor cells, which become progressively committed to the different branches of the blood cell system. The same occurs for myeloid branch of the blood cells. In this case, maturation of the stem cell is accompanied by the changes in gene expression in a "myeloid specific" way. These changes can often be tracked by monitoring the presence of specific proteins on the surface of the cell. Each successive change limits further potential of the myeloid cell to become a different cell type (Figure 7).

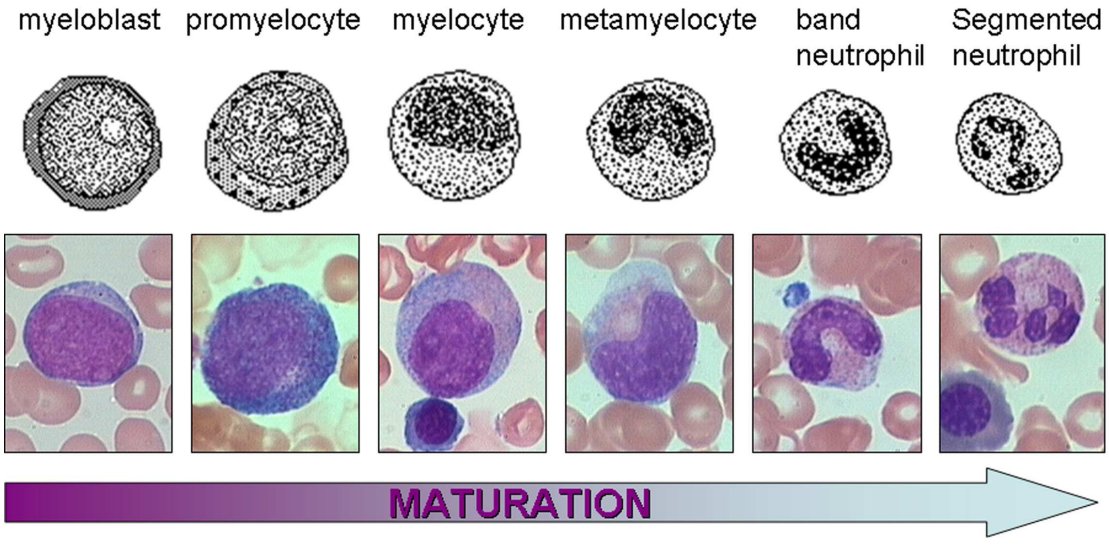


Figure 7. Phases of Myeloid maturation: Morphologic analysis after May – Grünwald – Giemsa staining of myeloid cells (taken from Childhood leukemia causation, by Nordbrock T. 2005).

1.2.3.1. PROLIFERATION

The mitogen-activated protein kinase (MAPK) pathway refers to a module of three serine/threonine - specific protein kinases: MAP kinases (MAPKs), MAP kinase kinase (MKK, MEK, or MAP2K) and MAP kinase kinase kinase (MKKK, MEKK or MAP3K). These kinases can activate each other in series by sequentially phosphorylation in response to a diverse range of extracellular stimuli, including cytokines, growth factors, neurotransmitters, cellular stress and cell adherence (*Figure 8*). Accordingly, the pathway plays a fundamental role in many key cellular processes, ranging from growth control, differentiation and survival/apoptosis till cellular adaptation to chemical or physical stress.[54-56]

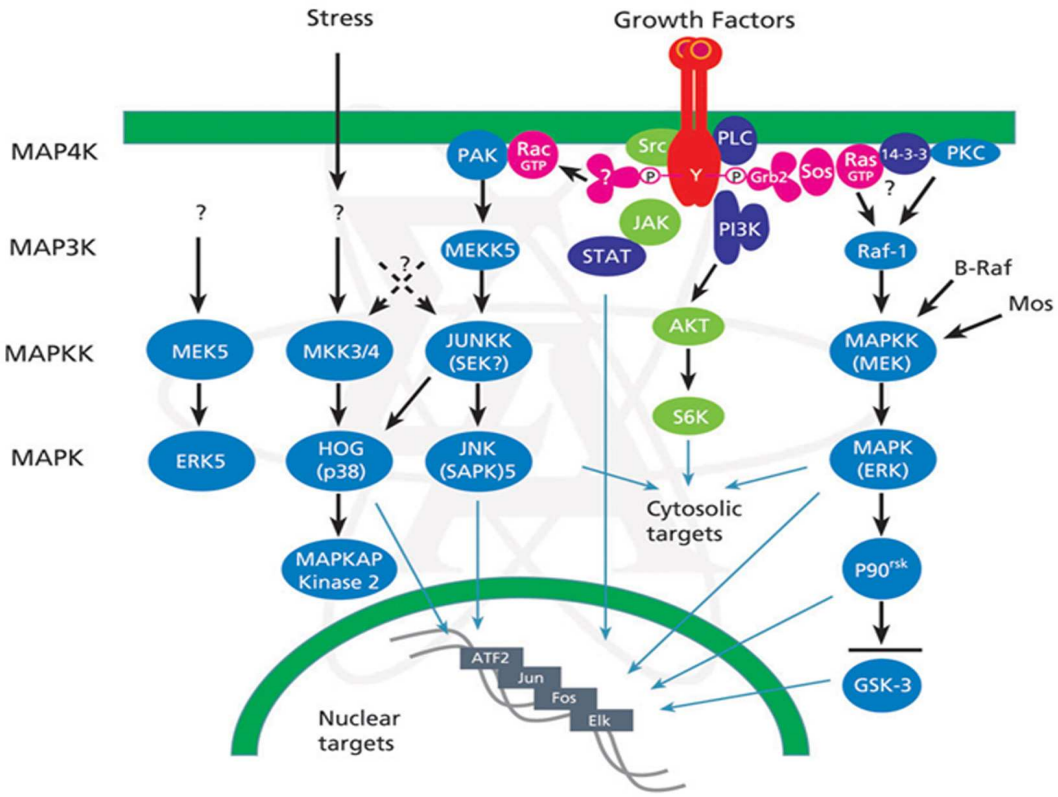


Figure 8. MAPK pathway: module of three serine/threonine - specific protein kinases: MAP kinases (MAPKs), MAP kinase kinase (MKK, MEK, or MAP2K) and MAP kinase kinase kinase (MKKK, MEKK or MAP3K) and adequate downstream target proteins.(taken from SIGMA-ALDRICH official sitefor Mitogen-activated Protein Kinase (MAPK) Cascades).

MAPKs, as a specific subset of three – tiered cascade, are evolutionary conserved enzymes important for the orchestration of the fundamental cellular processes mentioned above.[56,57]

In all currently known MAPKs cascades, the kinase immediately upstream of the MAPKs is a member of the MAP/ERK kinase (MEK or MKK) family. To date, four major groups of MAP kinases have been described in mammalian cells (*Figure 8*):

1) extracellular-signal-regulated protein kinases (ERK1 and ERK2)
2) c-Jun amino - terminal kinases (JNK1, JNK2, JNK3) or stress-activated kinases (SAPKs)
3) p38 kinases (p38 - α; ß; γ; δ)
4) Big MAP kinase 1 (BMK1) / ERK5

The mammalian ERKs (also referred to as p42/44 MAPK) are usually activated by growth factors and mitogenic stimuli, whereas JNK/SAPK and p38 MAPK are usually activated by ultraviolet (UV) irradiation, osmotic stress, pro-inflammatory cytokines, and anticancer drugs.[58,59] BMK1/ERK5 is the most recently identified member of MAPK family required for growth factor-mediated cell proliferation and cell cycle progression.[60]
ERK, a serine/threonine kinase which is a part of the Ras/Raf/MEK/ERK signaling transduction pathway, frequently results as interrupted in malignancies. Signal transduction along this pathway begins with the activation of the small GTPases Ras (and likely Rap) by receptor tyrosine kinases, G-protein-coupled receptors and/or integrins which covert Ras into its active conformation. Upon this activation, the small G-proteins recruit the MAP3K c-Raf (and, if present, A- and B-Raf; Raf stands for “Rapidly Accelerated Fibrosarcoma”) to the plasma membrane, where, by a complex processes of phosphorylation, changes in conformation and protein interactions, Raf becomes activated.[61] In following, Raf activates MEK-1/2 by phosphorylation of two serine residues. MEKs generally recognize only specific MAPKs as substrates. MEK-1/2 phosphorylates ERK-1/2 at threonine and tyrosine residues, activating ERK-1/2.[62] When activated, ERK can phosphorylate over 80 substrates in either cytoplasm or nucleus of the cell (*Figure 9*). It can regulate gene expression directly by phosphorylating, at serine and threonine residues, several transcription factors (Ets, Elk, and Myc), or indirectly by targeting substrates such as p90-RSK (ribosomal S6 kinase) family kinases, which can modify transcription factors and histones.[63]

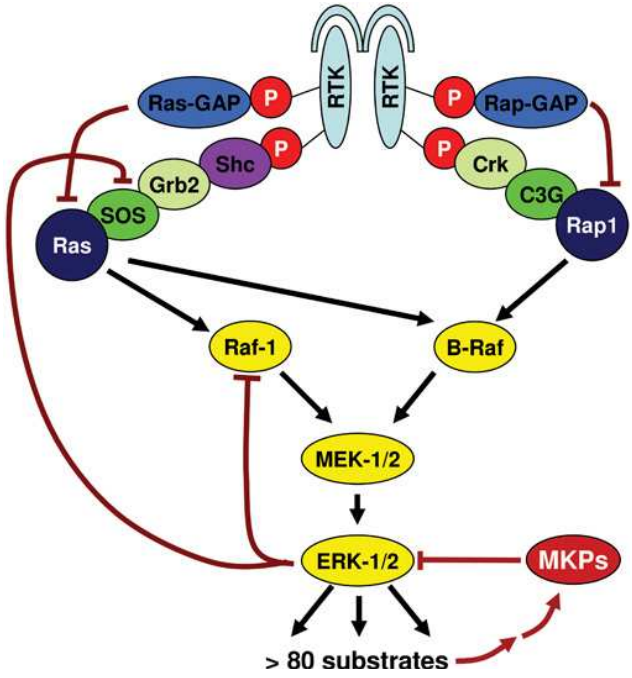


Figure 9. Structure of the ERK pathway: Upon ligand binding, RTK autophosphorylates (phosphates are shown as red circles) on tyrosine residues, which serve as docking sites for adaptor and signalling molecules. Ras and Rap1 are activated by the recruitment of guanosine-nucleotide exchange factors (SOS, C3G) via adaptor proteins (Shc and Grb2; Crk). Ras can activate Raf-1 and B-Raf; Rap1 presumably can activate B-Raf. Raf proteins phosphorylate and activate MEK-1/2, which in turn activate ERK-1/2 (indicated by black arrows). Negative-feedback loops (indicated by red lines) include the induction of MKPs by ERK as well as the inhibitory phosphorylation of Raf-1 and SOS. (taken from Orton JR. et.al. Biochem. J. 2005. 392; 249–261.)

Constitutive activation of RAS/RAF/MEK/ERK pathway is observed in the majority of AML patients[64,65] and its blockade with specific pharmacologic agents is shown to induce leukemic blast cell death.[66,67] Recent studies have provided clear evidence that a significant proportion of AML exhibit the activation of ERK which, simultaneously with changed activation of another proteins such as, AKT and PKCα, can lead to a worse prognosis, associated with short survival of AML patients.[68]

1.2.3.2. APOPTOSIS

The death and degradation of entire cells or some of their parts represent a normal aspect of the healthy life of eukaryotic organisms. The most common and profoundly studied form of cell death is so-called programmed cell death or apoptosis.[69] It represents a physiological process of controlled cellular self-destruction and was firstly introduced in a publication by Kerr, Wyllie and Currie in the year of 1972.[70,71] Apoptotic cells can be recognized by stereotypical morphological changes that include cell shrinking, loose of the contact with the neighboring cells, chromatin condensation, plasma membrane budding, cell fragmentation into compact membrane-enclosed structures ("apoptotic bodies"), "apoptotic bodies" engulfment by macrophages and removal from the tissue without an inflammatory response. (*Figure 10*).[72-74]

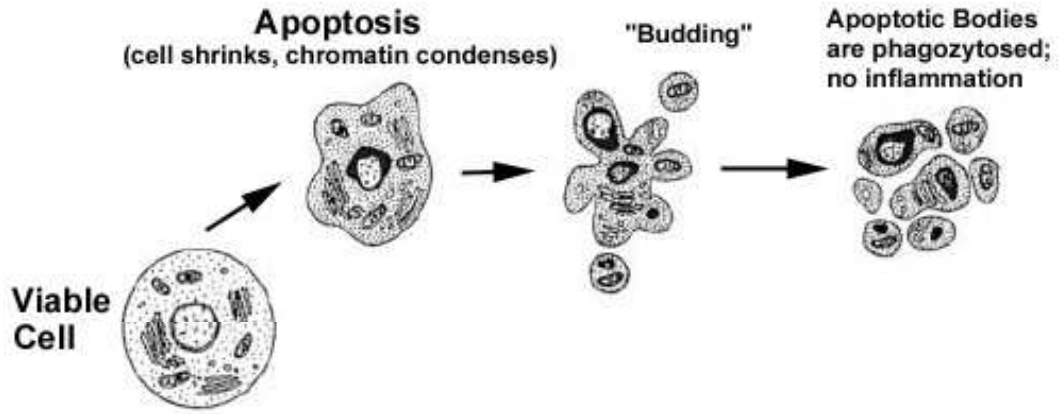


Figure 10: Hallmarks of the apoptotic cell death process. Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclearfragments and are phagocytosed without triggering inflammatory processes. (modified from Van Cruchten et al., Anat Histol Embryol 2002;. 31(4): 214-23)

The apoptotic mode of cell death is an active and defined process required for the maintenance of cell homeostasis, normal embryonic development and for the correct differentiation and function of haematopoietic cells.[75] There are at least two types of apoptosis which are determined by distinct signaling events. The first type, named Intrinsic or internal, involves mitochondria pathway while the second, known as Extrinsic or external *(Figure 11*), involves death receptor pathway.[76]

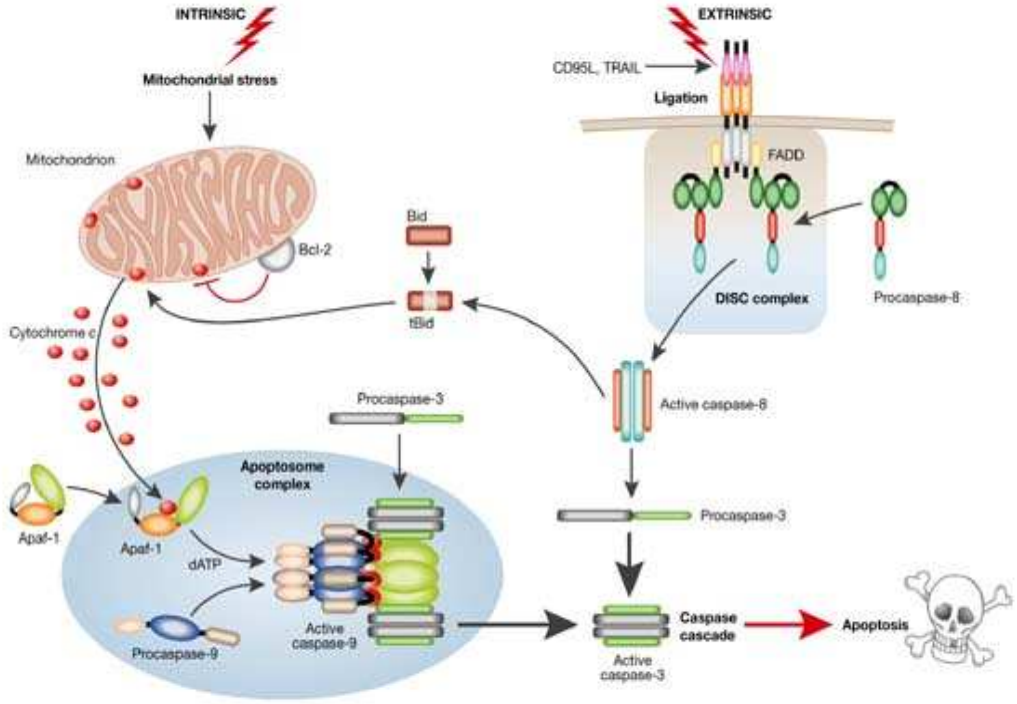


Figure 11: Apoptosis: Two major apoptotic pathways are illustrated: one activated via death receptor activation (extrinsic) and the other by stress-inducing stimuli (intrinsic). Triggering of cell surface death receptors of the tumour necrosis factor (TNF) receptor superfamily, including CD95 and TNF-related apoptosis-inducing ligand (TRAIL)-R1/-R2, results in rapid activation of the initiator caspase -8 after its recruitment to a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-associated death domain protein (FADD). In the intrinsic pathway, stress-induced apoptosis results in perturbation of mitochondria and the ensuing release of proteins, such as cytochrome c, from the inter-mitochondrial membrane space. The release of cytochrome c, from mitochondria is regulated in part by Bcl-2 family members, with anti-apoptotic (Bcl-2, Bcl-$X_{L,}$ Mcl-1) and pro-apoptotic (Bax, Bak and tBid) members inhibiting or promoting the release, respectively. Once released, cytochrome c binds to apoptotic protease-activating factor 1 (Apaf-1), which results in formation of the Apaf-1–caspase-9 apoptosome complex and activation of the initiator caspase- 9. The activated initiator caspases-8 and -9 then activate the effector caspases-3,- 6 and -7, which are responsible for the cleavage of important cellular substrates resulting in the classical biochemical and morphological changes associated with the apoptotic phenotype (reviewed in Adams, 2003; Danial & Korsmeyer, 2004)

1.2.3.2.A. Intrinsic type of apoptosis is activated in the moments of cell stress, when inner - cell inducers are formed and can cause the changes in mitochondrial membrane permeability. The major governing molecules of this process is Bcl-2 protein and its family members, which contribute to an increased release of pro-apoptotic factors involved in various aspects of apoptosis.[77] These factors include cytochrome c (cyto c;[78] second

mitochondria-derived activator of caspase (Smac/DIABLO;[79,80] apoptosis inducing factor (AIF)[81], and endonuclease G[82], which are normally found in the space between the inner and outer mitochondrial membranes.[83] When released in cytoplasm, cytochrome c interacts with Apaf-1 protein and procaspase-9, in ATP demanding way, and form the apoptosis complex known as “apoptosome”.[84] Following, apoptosome activates a set of caspases, starting with caspase-9, which, can cleave the executioner caspase-3 and caspase-7 (*Figure 11*), responsible for a cascade of proteolytic activity that leads to the digestion of structural proteins, DNA degradation, and ultimately phagocytosis.[85-87] In normal conditions caspases activation is suppressed by Inhibitor of Apoptosis (IAP) proteins (*Figure 12*).[88-90] When an apoptotic stimulus reaches the cell, causing the increase in mitochondrial membrane permeability, these proteins are inhibited by Smac/DIABLO after it has been released into cytoplasm along with cytochrome c. By binding IAPs, Smac/DIABLO relieves its activity, causing the "inhibition of the inhibitors” and thus prevents the apoptotic process arrest.[79,80] One of the widely expressed IAP members is X-linked inhibitor of apoptosis or XIAP. Smac/DIABLO can also bind directly to XIAP protein antagonizing caspase–XIAP interaction, thereby promoting apoptosis.[91] Beside XIAP, Smac/DIABLO can bind also bind cIAP1, cIAP2, survivin and Apollon in BIR dependent way.[92]

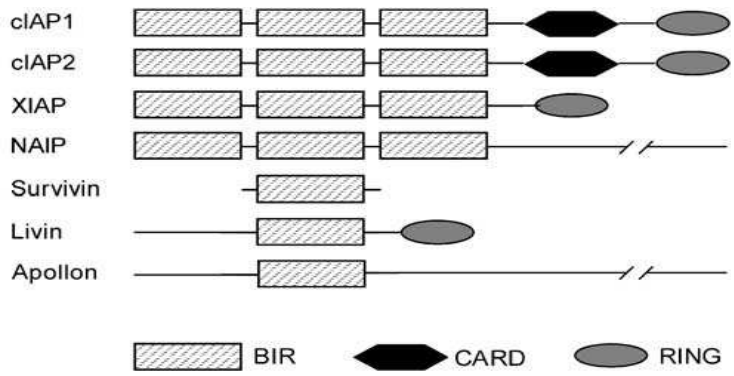


Figure 12. Schematic diagram of domain structure of human IAP proteins. BIR, Baculovirus IAP repeats; CARD, caspase-activating and recruitment domain; RING, ring zinc-finger.(taken from Fulda S, Oncogene (2006) 25, 4798–4811.)

Furthermore, AIF and endonuclease G are released from the intermembrane space of mitochondria upon outer mitochondrial membrane permeabilization and translocate into the nucleus to contribute to nuclear chromatin condensation and large-scale DNA fragmentation.[83,93] In respect to cytochrome c and Smac/DIABLO, AIF and endonuclease G

induce apoptosis in a caspase-independent manner and are not going to be considered more profundity here.[81,94]

1.2.3.2.B. Extrinsic type of apoptosis occurs when outside - cell inducers start to send the "death messages" through the cell membrane receptors from tumor necrosis factor (TNF) receptor gene super-family. Members of this family share similar, cysteine-rich extracellular domains and, in addition, cytoplasmic domain of about 80 amino acids called "death domain" (DD), which plays a crucial role in transmitting the death signal from the cell's surface to intracellular signaling pathways (*Figure 11*). The best described inducers of apoptosis are Fas ligand (FasL) and Tumor Necrosis Factor alfa/beta (TNF-α/ß) that can bind to the TNF family of cytokine cell membrane receptors Fas (APO-1/Fas) and TNF (R1 and R2), respectively. Ligation of death receptors by their cognate ligands, or agonistic antibodies, results in receptor trimerization, clustering of the receptors' DD and recruitment of adaptor molecules (FADD for CD95, TRADD for TNFRI).[95] Specific adaptor proteins then lead to the activation of caspase-8 which unleashes a proteolytic cascade and, by the activation of the downstream effector caspase-3, causes, at final, the phagocytosis of the cell.[96,97] While initial events differ between extrinsic and intrinsic apoptotic pathways, they have the same later steps which begins with the activation of central effectors of apoptosis, a group of cysteine proteases called caspases. More precisely, at least two major pathways of caspase activation have been described till now:

1) the receptor-mediated apoptosis pathway, where the TNF family of death receptors activate upstream caspase-8
2) the mitochondrial-mediated apoptosis pathway, in which cytochrome c is released from the mitochondria and activates upstream caspase-9.

Both pathways culminate in the activation of a major downstream effectors caspases, but converge at the level of caspase-3 activation (*Figure 11*). In following, caspase-3 can cleave the structural and functional elements of the cell (eg, poly (adenosine diphosphate [ADP]-ribose) polymerase, shortly PARP), provoking previously-described morphological changes observed regularly in an apoptotic cell (*Figure 10*).

PARP is 113 kDa protein involved in a number of cellular processes including DNA repair and programmed cell death.[98] Normally, PARP is assisting in the repair of single-strand DNA nicks. In the case of apoptosis, PARP can deplete the ATP of a cell in an attempt to repair the

damaged DNA leading to lysis and cell death. Caspase activation during apoptosis can cleave PARP protein leading to appearance of the 89- and 24-kDa proteolytic fragments. This cleavage appears to be an universal part of the apoptotic process and has been accepted as one of the classical hallmarks of programmed cell death.[99,100]

At the biochemical level, the final stages of apoptotic cell death can be confirmed by the release of phosphatidylserine (PS), normally found on the cytosolic (inner) leaflet of the plasma membrane. During apoptosis, PS is redistributed to the extracellular membrane surface and can be recognized by the specialized receptors localized on the phagocytic scavengers, such as macrophages, that can carry out their disposal job in an orderly manner, without eliciting the inflammatory responses.[101,102] High affinity to bind PS is shown by the Annexin V (or Annexin A5) protein. The interaction between the members of annexin family of intracellular proteins and PS occurs in a calcium-dependent manner and is used for the development of Annexin V / PI test, which permits the evaluation of cell death.[103] More precisely, Annexin-V labeled fluorescently recognizes and interacts with PS, once it locates on the outer surface of the membrane of a dying cells, and the fluorescence can be measured by cytofluorimeter. On the other side, PI (PI for Propidium Iodide) represents an intercalating agent and fluorescent molecule, used for DNA staining. In early stage of apoptosis, the plasma membrane excludes this viability dyes, but became permeable for it during the late stage of apoptosis or necrosis (see material and methods section for details). Combination of these two dyes permits us to distinguish early or late cell death events from viable cells.

1.3. BCL-2 FAMILY PROTEINS

The founder of Bcl-2 protein family, the Bcl-2 proto-oncogene, was discovered at the chromosomal breakpoint of t(14;18) bearing human B-cell lymphomas.[104] First suggestion that Bcl-2 plays a role in cell survival came from a studies on the IL-3-deprivation-induced death of a lymphoid cell line.[105] The results presented subsequently reported that Bcl-2 could inhibit a cell death after application of various stimuli that normally induced it and were confirmed by the in vivo experiments on mice lacking the *BCL-2* gene.[106,107]

The Bcl-2 family of proteins is divided into three subfamilies.[108,109] All of the members share characteristic domains of homology entitled the Bcl-2 homology (BH) domains, which are known to be crucial for their function (*Figure 13*). The BH domains also serve to further

subdivide the pro-apoptotic group into those with several BH domains (e.g. Bax and Bak) or those proteins that have only the BH3 domain (e.g. Bid, Bim and Bad).[108] The site of action for the Bcl-2 family is mostly on the outer mitochondrial membrane (OMM), but they can be found in the cytosol, endoplasmic reticulum and nuclear envelope as well.[110-113] Depending on their function, once activated Bcl-2 proteins either prevent or promote cell death.[114] The pro-survival Bcl-2 family members include Bcl-2, Bcl-X_L, Mcl-1, characterized by the presence of three (Mcl-1) or four (Bcl-2, Bcl-X_L) BH domains.

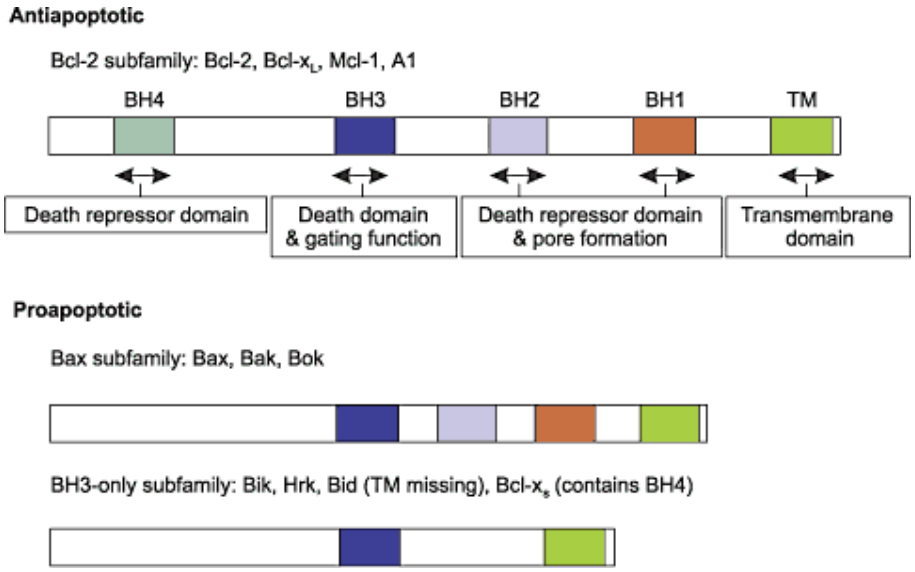


Figure 13. Domain structure of Bcl-2 family proteins. Bcl-2 homology (BH) and transmembrane (TM) domains are indicated.(taken from Ogata Y et al. Drug News Perspect 2003, 16(7): 446)

Pro-death Bcl-2 family members Bax, Bad and Bak, play crucial roles in cell survival control and loss of any of these proteins causes major deregulation of survival in most of the cell types. BH3-only proteins are essential for cell death initiation through the intrinsic pathway, whereas the Bax/Bak proteins play a key role in the progression of the apoptotic signaling stimuli at the level of permeabilization of the OMM.[115]

Bcl-2 is an anti-apoptotic protein that resides in the OMM and the membrane of the endoplasmic reticulum. Over-expression of Bcl-2 is known to block cytochrome c release, possibly through the inhibition of Bax and Bak.[116]

The Mcl-1 (for myeloid cell leukemia-1) gene encodes a 36 kDa protein with a carboxy terminal domain similar to Bcl-2. However, in contrast to Bcl-2, which is a long-living protein, Mcl-1 has a short half-life.[117,118] Mcl-1 is found in the early stages of differentiation

of the human myeloblastic leukemia cell line and normal haematopoietic precursors.[119] Like Bcl-2, Mcl-1 can form heterodimers with Bax and inhibits cell death in response to chemotherapeutic agents, UV irradiation and growth factor withdrawal.[120,121]

Bcl-X_L (for "B-cell lymphoma-extra large") is a transmembrane molecule localized mostly in the mitochondria, but can be detected also in the nuclear envelope and cytosol.[122,123] Its structure shows about 40 % of similarity with Bcl-2 protein and appears to function in the same apoptotic pathway as Bcl-2 does.[124]

Bcl-2 and Bcl-X_L dimerize with several Bcl-2 family proteins changing thereby the ratio of its members and altering the threshold of cell death.[125] Bcl-2 can inhibit the action of Bax/Bak by forming an inactive heterodimeric complex and preventing further release of cytochrome c.[116]

Bax has been implicated in apoptosis of many cell types. Under normal conditions, Bax is localized in the cytosol, but, in response to death stimuli, Bax undergoes a conformational change that triggers its translocation and insertion into the OMM.[123] This leads to permeabilization of the OMM and release of previously mentioned pro-apoptotic proteins (*Figure 14*).

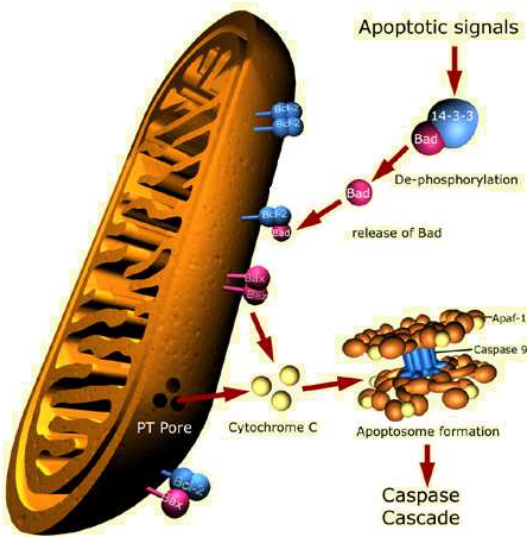


Figure 14. Schematically presented activation of Bcl-2 family proteins controlled intrinsic apoptosis, apoptosome formation and caspase cascade initiation.(taken from University of Reading web site, by Dash P, Mitochondria and the bcl-2 proteins,.)

High expression of Bcl-2 or Bcl-X_L has been reported to prevent Bax translocation and, hence, activation.[126,127] In contrast, Bak is always localized in the mitochondria as an integral membrane protein and has been reported to be maintained in an inactive conformation by anti-apoptotic Bcl-X_L and Mcl-1 proteins.[128]

Another characteristic shared by all three anti-apoptotic Bcl-2 family proteins, Bcl-2, Mcl-1 and Bcl-X_L, considers their proteasomal degradation which, at least in part, controls the level of proteins present inside the cell.[129-131] Moreover, this degradation is Ubiquitin (Ub)

dependent and is regulated by specific E3 ligases which can bind Ubs to proper target proteins. However, ubiquitylation is a reversible post-translational modification with key roles in various signal transduction cascades and in determination of protein stability. A link between protein and Ub can be disturbed by a type of proteins known as deubiquitinase. Opposite to E3 ligase, this class of proteins can remove Ubs attached to proteins and, hence, protect them from degradation via proteasome (*BOX V*). The balance between E3 ligase and deubiquitinase activity is important for keeping the homeostasis of Ubs inside the eukaryotic cells, where adequate ratio between free and captured Ubs is crucial for normal cell function. For example, Mcl-1 protein half-life is determined by the balance flanked by its own E3 ligase - Mule and deubiquitinase - Usp9X, which determine the Ub presence or absence on Mcl-1 protein and, therefore, its degradation or preservation, respectively.[132]

1.4. APOPTOSIS RELATED PROTEINS IN AML

Studies carried out during the last twenty years have demonstrated apoptosis as frequently impaired in cancer cells and have radically improved the understanding of the involvement of apoptotic mechanisms in tumorigenicity. The reduction in apoptosis correlates with enhanced growth of clonogenic leukemic cells and the key role of apoptosis in the pathogenesis of AML has been elucidated over the last years. In particular, some fusion proteins found in leukemic cells (eg. PML/RAR-alfa, CBF/SMMHC, AML1/ETO), are shown to interact with certain mediators of apoptosis, sending in that way anti-apoptotic signals that favor preferentially a survival of leukemic cells.[133-135] Over-expression of the anti-apoptotic Bcl-2 protein has the same consequence, resulting in cell resistance to a wide variety of pro-apoptotic stimuli, including radiation, chemotherapeutic agents and corticosteroids.[136,137] Bcl-2 was found to be over-expressed in AML patients which showed a low rate of complete remission (CR)[138] Furthermore, a high ratio of Bcl-2 to Bax was shown to be predictive of worse outcome.[139] It seemed conceivable that Bcl-2 down-regulation might lower the apoptotic threshold of leukemic cells and, through this mechanism, favor response to chemotherapy.[140] It has been also suggested that the Bcl-2/Bax ratio may be important in apoptosis determination since the outcome of cell death signaling pathways depends on a complex interplay involving physical interactions between the pro-survival and pro-death members.[136,141]

Bcl-2 is an important predictor of survival in multivariate analysis over other known prognostic markers, including age and white blood cell count.[136] On the other side, immunoblotting analysis of Bax expression in AML patients showed that increased Bax levels correlated with improved rates of overall survival (OS).[142] Moreover, high ratios of Bcl-2 to Bax protein conferred a poor prognosis with decreased rates of CR and OS.[143] In correspondence, a phase I study using an antisense to Bcl-2 in elderly AML patients showed promising results.[139]

The analysis of Mcl-1 protein expression in de novo AML patients showed its high expression.[144] Further, Mcl-1 levels have been reported as increased at the time of a leukemic relapse, showing 2-fold higher expression than at presentation in 53 % of patients.[145] The increase in Mcl-1 may partly explain the greater degree of drug resistance seen in relapsed leukemia.

AML blasts frequently express elevated levels of the anti-apoptotic Bcl-2 family member, Bcl-X_L. Elevated levels of Bcl-X_L in AML cells contributed to chemoresistance of these cells.[146] This finding is not surprising since *Bcl-X_L* is a target gene of Stat-5, whose constitutive activation is frequently observed in AML.[147] A widely expressed IAP member XIAP, is found to be highly expressed in most human cancer cells where its high levels confer tumor resistance to chemotherapy or irradiation XIAP was also found to be expressed in the large majority of adult cases of AML.[148-151]

In vitro assay, used to explore the downstream part of the mitochondrial pathway of caspase activation in AML patients, confirmed frequently impaired caspase activation in samples from patients with rapidly relapsing disease, but rarely in samples from patients with newly diagnosed AML. Similarly, the simultaneous detection of cytochrome c release, a key initial event in the activation of the intrinsic apoptotic pathway, and caspase-3 activation in leukemic cells, seems to represent an important approach to assess the functional integrity of apoptosis signaling in leukemic cells.[152,153]

1.4.1. BCL-2 – ASSOCIATED ATHANOGENE (BAG) PROTEIN FAMILY

Genes from Bcl-2-asscociated athanogene (BAG) family belong to an evolutionary conserved group with homologues found from yeast to animals. The first BAG gene was discovered in a screen of a mouse embryo cDNA library using recombinant human Bcl-2 protein as a bait to identify new Bcl-2 interacting proteins.[154] The ability of newly discovered gene to protect cells from the application of death signals, in a synergistic action with Bcl-2, justify the chosen name – Bcl-2 - asscociated "Athanogene" 1 (BAG-1; from the Greek word athánatos, meaning 'against death'). Soon after, several proteins with structure similar to BAG-1, were discovered and classified in a new BAG protein family. Till now, six BAG family members were found in humans.[155] Beside BAG-1, the family counts for BAG-2, BAG-3 (CAIR-1, Bis), BAG-4 (SODD), BAG-5 and BAG-6 (Scythe, BAT3) (*Figure 15*). All of these proteins contain at least one evolutionarily conserved domain, of roughly 110-124 amino-acid (AA) near the C terminus, called the "BAG domain" (BD).[156] The BD was shown to allow BAG family protein to bind and activate the Heat shock 70 (Hsp70) - family of molecular chaperones.[157,158] In addition to the conserved BD, several other domains within the BAG proteins have been identified and are likely to modulate both, target specificity and BAG protein localization inside the cell. In contrast to C terminus which is similar among BAG members, the proteins generally differ in their N termini and, hence, diverse the proteins they can interact with through this termini. Of the mammalian BAG-family proteins, only BAG-1, BAG-3, BAG-4, and BAG-6 have been investigated for their potential cellular functions.

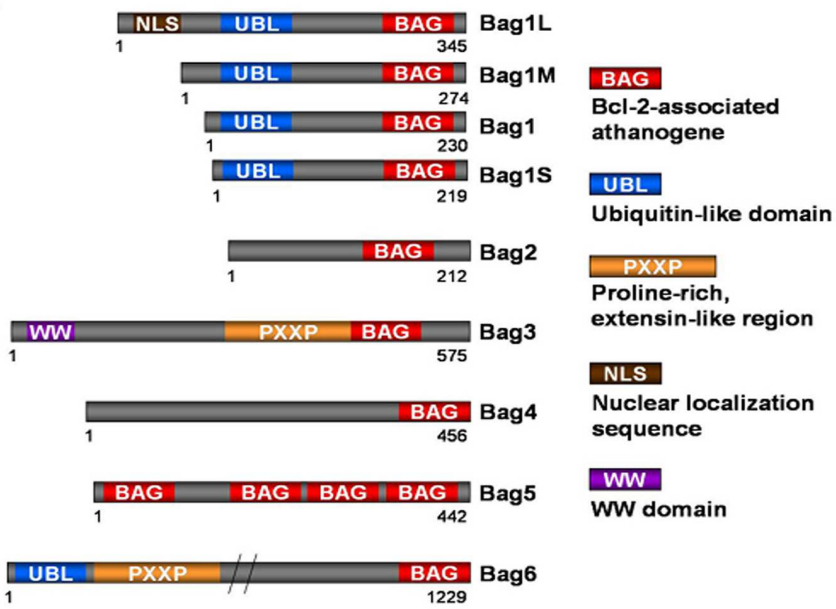


Figure 15. BAG family proteins in human, showing the conserved BAG domain, ubiquitin-like (UBL) domain, PXXP repeats, nuclear localization signal (NLS) and WW domain. Numbers shown below each BAG protein refer to a number of amino acids (AA) the protein is composed of.(Figure adapted from Sondermann et al, Science *2001; **291**: 1553–1557.)*

BAG-2 was shown to associate with carboxyl terminus of Hsc70-interacting protein (CHIP), an E3 ubiquitin ligase, previously identified as a co-chaperon protein, and inhibit CHIP-dependent ubiquitin ligase activity.[159,160] BAG-4, also known as silencer of death domains (SODD), has a similar domain-organization like BAG-2 and was also identified in a screen for Hsp70-interacting proteins.[161] Moreover, BAG-4 is thought to negatively regulate the function of TNF-R1 and death receptor 3 (DR3), preventing cell death signaling and NF-kB induction by suppressing ligand independent receptor oligomerization and maintaining an inactive monomeric state.[162] BAG-4 also confers protection from gamma-irradiation-induced effects in tumor cells.[163] BAG-5 is a unique member since it contains four BDs. Little is known about BAG-5 role in cells other then ability to bind Hsp70. There are some indication that BAG-5 could be important in development of Parkinson's disease, where it can express inhibiting capacities in respect to Parkin E3 ligase and Hsp70 chaperone activity.[164] BAG-6 is the longest BAG-like protein also called Scythe and Bat-3. It was proposed to play a role in apoptosis regulation, owing to its BAG domain, by trapping from release to the cytoplasm the pro-apoptotic factors.[165] BAG-6 was initially isolated from the human major histocompatibility complex and was found to be localized in nucleus.[166]

The human BAG-3 (Bis, CAIR-1) is a 74kDa cytoplasmic protein, which has a unique domain organization since it contains a WW domain, followed by a proline-rich region with

PXXP motifs (*Figure 15*). BAG-3 plays a role in modulating growth factor-induced activation of protein kinase C.[167] Other occupation of BAG-3 relates upon influence over Hsp70-dependent protein degradation of poly-ubiquitinated Hsp70 client proteins by favoring the induction of autophagy within the cell.[168,169] BAG-3 can bind Bcl-2 and c-Raf proteins and, in consequence, when over-expressed can sustain survival of cancer cells, impairing the response to therapy and hence constituting a potential target for anti-neoplastic therapies.[170]

1.4.2. BAG-1 – FOUNDING MEMBER OF BAG FAMILY

BAG-1 is the founding member of the BAG family. Within the cell, BAG-1 is expressed as multiple isoforms generated by alternative translation initiation from a single Mrna.[171] In humans, BAG-1 counts for four isoforms which differ in length. A large, BAG-1L (50kDa), isoform is translated from an upstream CUG condon, while other two in-frame downstream AUG codons give rise to median, BAG-1M (46kDa) and small, BAG-1S (36kDa) isoform, respectively (*Figure 16*). The fourth isoform, BAG-1 (29kDa), is believed to be generated by post-translational modification in human cells, and is not detected consistently inside all cells types.[171]

BAG-1 isoforms can be found differentially localized within the cells. Containing a NLS sequence, BAG-1L is predominantly localized in nucleus while BAG-1M and BAG-1S are distributed mainly in the cytoplasm.[172,173] However, in certain cell types, BAG-1S and BAG-1M were occasionally found in the nucleus, specially after the exposure to the stress conditions.[158]. As other BAG protein members, all BAG-1 isoforms possess BD, with which BAG-1 binds and actives the Hsp70-family of molecular chaperones. All of BAG-1 isoforms contain an ubiquitin-like domain (UBL), which can be used either for controlling protein turnover rates or as protein-interaction domains that mediate binding to other proteins, including subunits of the 26S proteasome (*BOX VI*). Indeed, some reports suggest a function of BAG-1 in chaperone-assisted 26S proteasomal protein degradation in cooperation with Hsp70 family molecular chaperones.[174] Together with BAG-1 and Hsp70, a part of this protein complex machinery is E3 ubiquitin ligase CHIP (C-terminus Hsp70 inhibition protein), which binds Ubiquitines to either proteins bound by Hsp70 and BAG-1 or to BAG-1 itself.[175]

Controversially, there are evidences that BAG-1 contributes also to a protection from proteasomal degradation of certain proteins.[176] These two opposite functions of BAG-1 protein indicate a complexity with which BAG-1 function is regulated and can be justified by

the presence of various domains (e.g. BD and UBL, diverse N-termini of BAG-1 isoforms) by which BAG-1 protein can associate with a wide range of proteins (*Table 2*), effecting in that way a high density of processes within the cell.

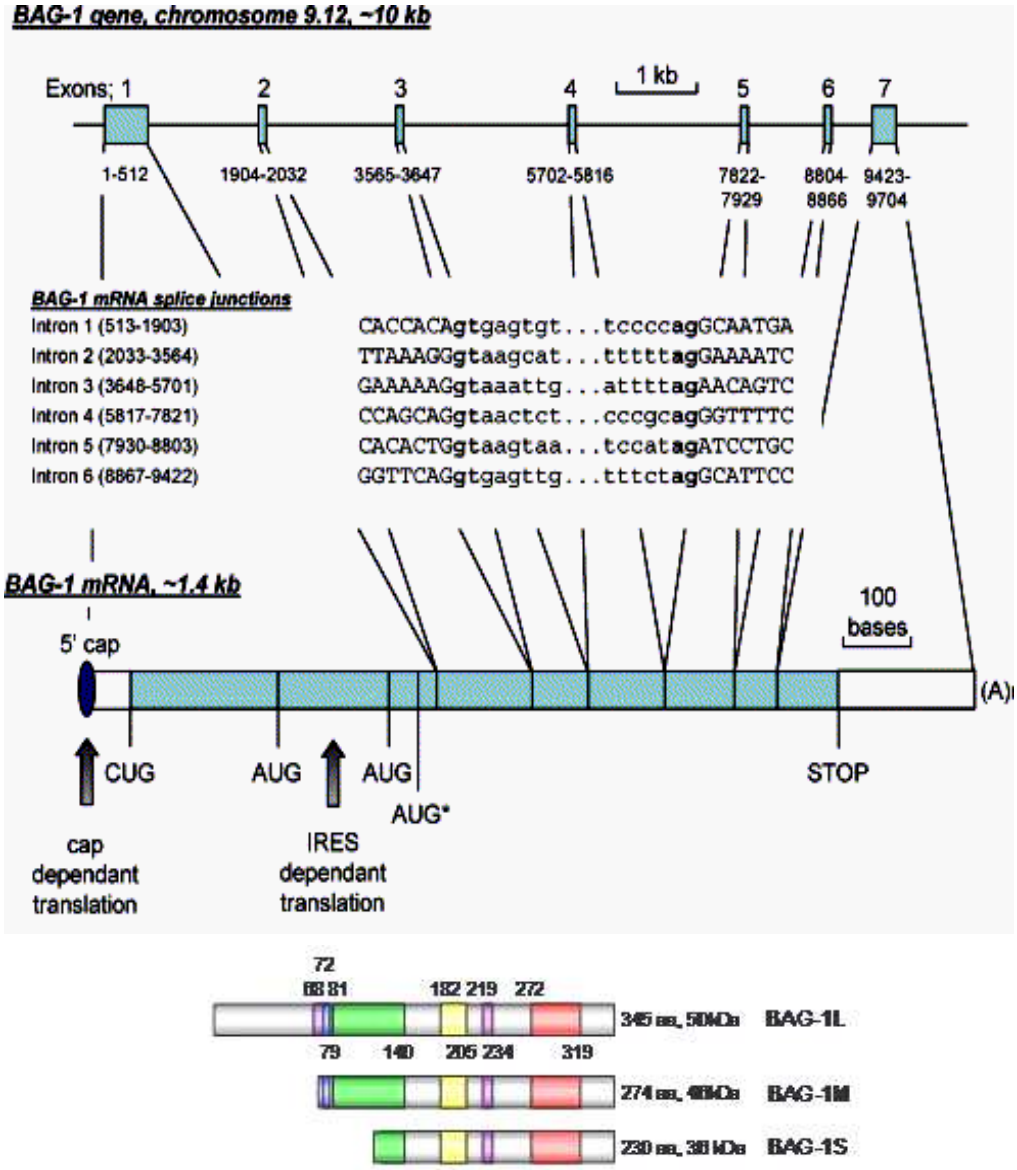


Figure 16 . BAG-1 isoforms and their domains. The human BAG-1 gene, located on chromosome 9p12, comprises seven exons which generate an mRNA product of approximately 1.5kb. Three major BAG-1 isoforms are produced from alternative translation start sites within BAG-1 mRNA. The 50kDa BAG-1L isoform is translated from the first in-frame CUG codon by CAP-dependent translation, while BAG-1M and BAG-1S are produced from two downstream AUG codons. BAG-1M is also produced by a CAP-dependent translation mechanism, while BAG-1S is generated by IRES-dependent translation resulting in highest abundance of this short isoform. BAG-1L contains a bipartite nuclear localisation sequence (NLS, purple), the amino-terminal portion of which overlaps with a DNA binding domain (blue). BAG-1M contains a partial NLS, while the NLS is completely absent from BAG-1S. An acidic serine/threonine rich region (green) has been implicated in protein-protein interactions. The ubiquitin-like domain (yellow) and BAG domain (red) are present in all three isoforms.(modified from Townsend AP, et al. Int J Biochem Cell Biol 2005. 37;251-259)

Interacting protein	Involvement of BAG-1	References
Hsp70 / Hsc70 molecular chaperones	BAG-1 acts as co-chaperone to regulate ATPase activity of heat shock proteins and inhibit chaperone activity	(Takayama *et al.*, 1997)
Raf-1 kinase	Binds to catalytic domain of Raf-1 kinase and activates it independent of Ras	(Song *et al.*, 2001, Wang *et al.*, 1996, Gotz *et al* 2005)
Glucocorticoid recetor (GCR) and other nuclear hormone receptors	BAG-1L interacts with and enhances androgen receptor function BAG-1M negatively regulates GCR- and hormone-induced apoptosis	(Froesch *et al.*, 1998, Kullmann *et al.*, 1998, Zeiner and Gehring, 1995,)
E3 ubiquitin ligases e.g. Siah1 and CHIP	BAG-1 negatively regulates the growth inhibitory ubiquitin ligase, Siah-1 BAG-1 accepts protein substrates from molecular chaperones and presents them to CHIP for ubiqitylation – cooperation to influence protein quality control	(Demand *et al.*, 2001, Matsuzawa *et al.*, 1998)
Proteasome	BAG-1 acts as a link between proteasome and chaperones, Hsp70 / Hsc70	(Luders *et al.*, 2000)
HGF and PDGF receptors	BAG-1 binds to receptor tail via C-terminal domain and enhances HGF- and PDGF-mediated protection against apoptosis	(Bardelli *et al.*, 1996)
DNA	BAG-1L and BAG-1M translocate to the nucleus and bind DNA to activate transcription upon heat shock BAG-1M can bind DNA and Hsp70 simultaneously and form subsequent substrate interactions possibly to act as a bridging molecule with transcription factors	(Niyaz *et al.*, 2001, Zeiner *et al.*, 1999)
Rb	Role for Rb in maintaining nuclear localisation of BAG-1 Nuclear BAG-1 inhibits apoptosis in adenomas to increase survival of colorectal tumour cells in vivo	(Arhel *et al.*, 2003, Clemo *et al.*, 2005, Barnes *et al.*, 2005)
Bcl-2	Association with BAG-1 enhances anti-apoptotic signalling of Bcl-2	(Takayama *et al.*, 1995)

Table 2. Summary of proteins that interact with BAG-1 and some of the reported cellular functions. BAG-1 associates with a wide range of proteins and through these interactions may have multiple cellular effects, any of which might contribute to cancerous cell phenotypes.[177-186,225]

The most frequently confirmed co-interacting partners of BAG-1 proteins are cytosolic Bcl-2, Hsp70 and Raf-1.[187] Under normal conditions, Raf-1 and Hsp70 compete for binding to the C-terminus of BAG-1, such that BAG-1 binds to and activates Raf-1, subsequently activating the downstream extracellular signal-related kinases (ERKs). The huge number of proteins with which BAG-1 can interact makes it capable for a modulation of a variety of pathways important for either normal or malignant cells, including signaling, proliferation, apoptosis, transcription, differentiation, embryogenesis, oncogenesis and motility (*Figure 17*).[188]

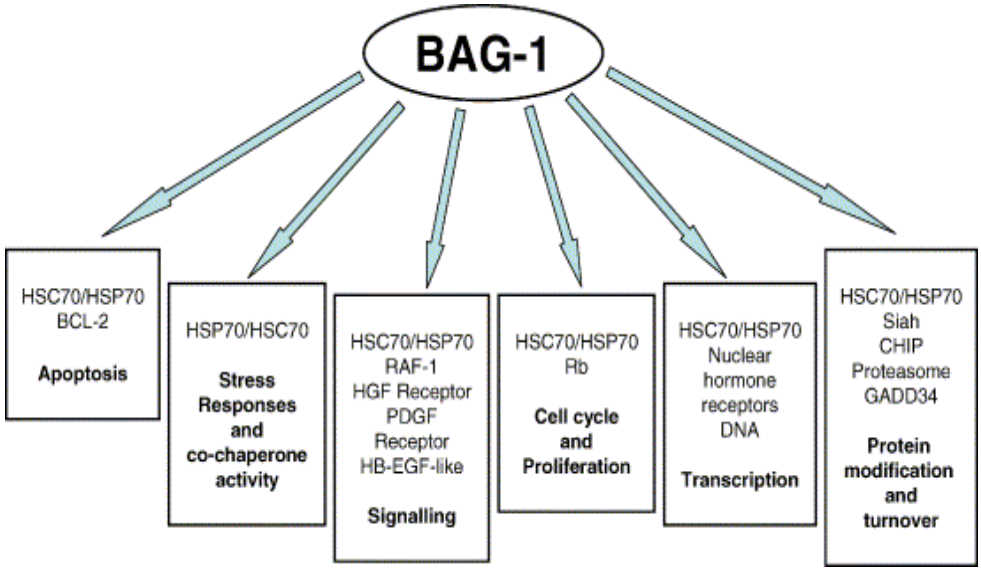


Figure 17. A scheme of the pathways where BAG-1 protein involvement has been elucidated.(from Townsend AP, et al. Int J Biochem Cell Biol 2005. 37;251-259)

Despite to an interesting suggestion that BAG-1 might regulate Bcl-2 function via induction of chaperone-mediated conformational change, there is no evidence demonstrating that Bcl-2 is an obligate effector of BAG-1 on survival. Furthermore, the function of BAG-1 in cell differentiation has been received with more attention, specially in neurons.[189] What's more, BAG-1 has been shown to be essential for haematopoietic cells survival as well.[190]

Another discovered characteristic of *BAG-1* gene is that its expression can be epigenetically regulated by methylation of CpG islands (CGI) located within promoter region (*Figure 18*). In a consequence, methylation status of *BAG-1* gene promoter determines the level of BAG-1 transcript expression.[191]

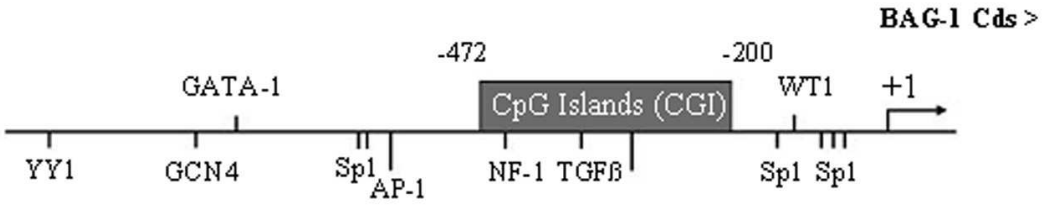


Figure 18. BAG-1 promoter region with Transcription Factors described to recognize and bind the region upstream of the +1 transcription start point. Cds = coding sequence. (modified from Sun L, et al. Cancer Res 2008;68(8):2726–35*)*

1.4.3. BAG-1 IN CANCER

BAG-1 expression is frequently altered in human cancers.[192] It has been shown to be over-expressed relative to normal cells in breast cancer[193], colorectal cancer[194], human squamous cell carcinoma[195], endometrial cancer[196], prostate cancer[197] and lung cancer.[198] Altered expression has also been found in pre-malignant lesions[199], implicating BAG-1 role in early tumor development. BAG-1 has been suggested to be involved in metastatic disease and has been shown to be a prognostic factor in a number of cancers.[200,201] In colorectal cancer, high nuclear BAG-1 expression resulted in a poorer prognosis and a higher risk of metastases.[194] In oesophageal[202] and breast cancer[203], over-expression of BAG-1 has been suggested to be a poor prognostic factor. On the other side, there is conflicting data with some studies which found that increased BAG-1 expression resulted in a good prognosis[204], or other that established no link between BAG-1 expression and prognosis of the disease.[205] The latter results could imply that BAG-1 role is a cell type specific and dependent of the developmental status cell is at. However, no many evidence could be found about the BAG-1 expression in childhood AML, where a great studies of other Bcl-2 family members had been performed. Accordingly, the aim of following study was to obtain evidence about BAG-1 isoforms presentation among leukemic cell lines and patients with diagnosed AML and to describe the role and way BAG-1 could contribute to leukemia lineage commitment.

1.4.3-a. BOX V Ubiquitin, Ubiquitination and deubiquitination

Over the past few decades, ubiquitination has emerged as one of the most versatile of post-translational modifications, engaged with the regulation of a diverse cellular processes such as cell-cycle control, DNA damage repair and membrane trafficking.[206] *Ubiquitin is conjugated through the sequential action of three components: E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and finally E3 ubiquitin ligases, which provide for substrate specificity. Polyubiquitin chains are formed through the serial addition of ubiquitin molecules linked to lysine residues in the preceding ubiquitin. A total of seven internal lysine residues (K6, K11, K27, K29, K33, K48, and K63) allows for diversity of chain configurations.*

Ubiquitination plays a central role in degradation of proteins both through proteasomal targeting and by direct sorting to the lysosome. However, it is becoming clear that reversible ubiquitination is also a crucial mediator within intracellular signaling cascades. Protein networks can be formed by interaction with specific ubiquitin binding domains of which there are at least 20 classes within the human genome.[207]

Reversibility of ubiquitination is accomplished through the action of deubiquitinases (DUBs). Approximately one hundred human DUBs fall into five classes: ubiquitin specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumour proteases (OTU), Josephins and the Jab1/MPN/MOV34 metalloenzymes (JAMM, also known as MPN+). Deubiquitinating enzymes are cysteine proteases that specifically cleave ubiquitin conjugates at the ubiquitin carboxy terminus (Figure 19).

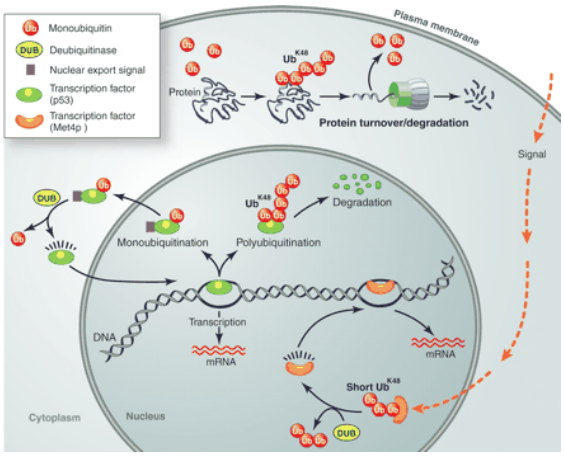


Figure 19. An example of Ubiquitinase and Deubiquitinase function: Protein modified by long (more than four ubiquitins) UbK48 chains is targeted to the proteasome for degradation. Short UbK48 chains can inhibit activity of a transcription factor (Met4p) without leading to its degradation. Deubiquitination activates the transcription factor. A transcription factor (p53) is imported into the nucleus. Monoubiquitination leads to its nuclear export, whereas polyubiquitination leads to its degradation.(taken from Mukhopadhyay D.et al. Science review,315; 201-205)

Deubiquitinating enzymes act at multiple levels in the ubiquitin pathway, regulating either free ubiquitin pools by recycling ubiquitin from branched-chain polyubiquitin; or removing ubiquitin from Ub-conjugated target protein, thereby regulating the localization or the activity of the target or trimming ubiquitin from a ubiquitinated target protein and rescuing therefore the protein from degradation by the 26S proteasome (before or after the ubiquitinated substrate interacts with the proteasome).

1.4.3-b. BOX VI Protesome machinery

Protein degradation is a critical regulatory process that allows cells to rapidly respond to intracellular signals during cell cycle, differentiation, stress responses and many other processes, by adjusting the levels of key effector proteins.[208,209] The major proteolytic pathway in eukaryotes is the ubiquitin/26S (S denotes Svedberg sedimentation coefficient) proteasome pathway. Here, proteins destined for degradation become modified by the covalent attachment of multiple ubiquitins. Ub is a 76-residue (8 kDa) protein whose covalent conjugation to other proteins, usually in the form of a multi-Ub chain, marks these proteins for a degradation by the 26S proteasome, an ATP-dependent multisubunit protease.[210-212] This pathway is comprised of several steps which require ubiquitin, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), ubiquitin-protein ligases (E3), and the 26S proteasome. The 26S proteasome is a giant (approximately 2000 kDa) multimeric protease present in the nuclear and cytosolic compartments of all eukaryotic cells.[210] Structurally, the 26S proteasome consists of a barrel-shaped 20S core complex made up of four stacked rings, capped by two polar 19S regulatory complexes.[213] Each of the two non-catalytic outer rings of the 20S core complex, called alpha, is composed of seven subunits. The catalytically-active inner rings, called beta, also contain seven subunits, three of which are proteolytically active. The 19S complex also participates in unfolding the target protein so it can interact with the active sites inside the core of the 20S complex (Figure 20.)[214] Moreover, the 19S particle represents not only a docking site for polyUb-proteins, but also for Ub-receptor proteins. These group of proteins possess Ubiquitin-like domains (UBL) which enable their binding to the 19S without being self-degraded. Several studies suggested that such Ub-receptor proteins (such as BAG-1) escort polyUb-proteins to the proteasome.[215] Such an "escort services" is thought to prevent protein aggregation while substrates are enrooted to the proteasome. However, these pathways are poorly understood and it is unknown whether distinct sets of proteins or even all proteasome substrates are transferred to the proteasome by escorting proteins

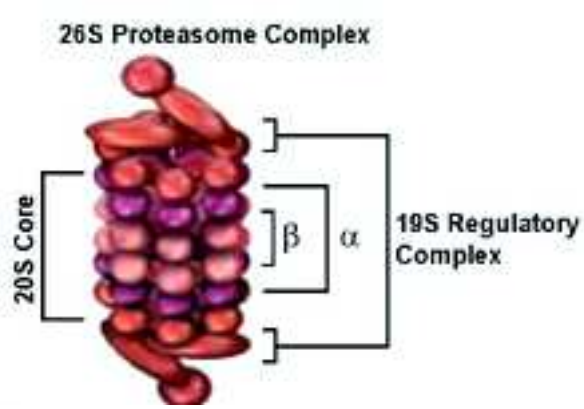


Figure 20. Diagrammatic representation of the 26S proteasome. The 20S catalytic core complex consists of two outer a rings and two internal and adjacent b rings. Each ring contains seven protein members (α and ß). In the center of the catalytic core is the axial catalytic chamber containing an unfolded ubiquitinated protein being degraded into small polypeptides. The 19S regulatory cap complex consists of 20 protein members. The average length of the 26S proteasome is 45 nm, with a width of 11-15nm. (Source: Cancer Control © H Lee Moffitt Cancer Center and research hinstitut,. Inc.)

CHAPTER II:

BAG-1 IN ACUTE MYELOID LEUKEMIA:

MODULATOR OF APOPTOSIS – RELATED PROTEIN

EXPRESSION

---

2. INTRODUCTION

Acute Myeloid Leukemia (AML) is a clonal disorder characterized by an accumulation of malignant hematopoietic progenitor cells blocked at various stages of normal myeloid development. Regular proliferation and survival of haematopoietic cells is largely regulated by apoptotic processes which, when severely impaired, could contribute to pathogenesis of leukemia.[216] Apoptosis-related B cell lymphoma-2 (Bcl-2) protein family, with its pro-apoptotic (Bax, Bak) and anti-apoptotic (Bcl-2, Mcl-1, Bcl-X_L) members, presents a series of provocative biochemical and cellular events ripe for targeting for novel molecular therapeutics.[217] When pro-apoptotic Bcl-2 family proteins prevail over anti-apoptotic ones, mitochondrial membrane permeability increases and release of pro-apoptotic factors, such as cytochrome *c* and Smac/DIABLO, occurs.[79,80,218] In AML patients, increased expression of anti-apoptotic Bcl-2 family proteins has been associated with resistance to chemotherapy, decreased rates of complete remission and shortened survival.[136,145,219-221]

Bcl-2 associated AthanoGene-1 (BAG-1) is a multifunctional protein firstly described as an enhancer of cell survival competent to delay cell death by a synergistic action with Bcl-2.[186] BAG-1 has been described as a part of apoptotic, transcriptional and proliferative pathways regulation, cell signaling and differentiation[155,188], although precise mechanisms that lay behind each of these processes are still an enigma. The implication in a variety of cellular functions is partially a result of different sub-cellular localization of the three major BAG-1 protein isoforms: nuclear BAG-1L (50 kDa), nuclear/cytosolic BAG-1M (46 kDa) and cytosolic BAG-1S (36 kDa).[172,222] All isoforms are generated from a single mRNA transcript through the use of at least three alternative translation-start sites.[171,223,224] As a result, BAG-1 isoforms share the same C-terminus characterized by a Heat shock protein 70 (Hsc70/Hsp70) - interacting BAG domain. BAG-1 isoforms differ in N-terminal extensions which contains ubiquitin-like (UBL) domain, responsible for a realization of BAG-1-proteasome interaction.[174,187]

BAG-3, another BAG family member, shares anti-apoptotic features with BAG-1 and interacts with a variety of partners in common, including Hsc70/Hsp70, Bcl-2 and Raf-1.[155,161,187] By cooperating with Raf-1, BAG-1 and BAG-3 might be considered together with the Mitogen-Activated Protein Kinase (MAPK) proliferation pathway [178,225], while capability to modulate Bcl-2 protein activity connects both proteins with cell survival-sustaining functions. [167,186] Separate studies confirmed either BAG-1 or BAG-3 de-regulation in diverse

cancer types, including some leukemia.[170,172,,190,226-228] In the present study, initial investigation of the potential BAG-1 association with development of AML was followed by the study of the mechanism by which BAG-1 contributes to leukemic phenotype sustaining. Particular emphasis is given to the apoptosis-related Bcl-2 family proteins, Bcl-2 and Mcl-1, and the way BAG-1 modulates their expression. Our data indicate that BAG-1, supported by BAG-3 protein, sustains proliferation and survival of leukemic cells by affecting protein degradation via proteasome either directly or indirectly. Moreover, Usp9X has been demonstrated as a novel protein partner that helps BAG-1 in the realization of described protective role.

Aveic Sanja[1] BS, Pigazzi Martina[1] PhD, Basso Giuseppe[1] MD

1) Department of Pediatrics, Laboratory of Hematology-Oncology, University of Padova, Padova, Italy

2.1. MATERIAL AND METHODS

2.1.1. Cell lines, primary cell cultures and patients' samples.

Human AML cell line HL60 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) 1 µg/mL of glutamine and 1 µg/mL of penicillin/streptomycin (GIBCO® - Invitrogen). Human myeloid cell lines NOMO1, NB4, THP1, MV4;11, ML2, lymphoid cell lines RS4;11, 697, JURKAT, REH, SEM and chronic myeloid line K562 (all from American Type Culture Collection) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 1 µg/mL of glutamine and 1 µg/mL of penicillin/streptomycin (GIBCO® - Invitrogen). Analysis of the RNA from bone marrow samples of 10 newly diagnosed cases of pediatric AML and ALL was performed. A series of 10 samples collected during remission phase and 10 healthy controls were included. A diagnosis of leukemia was done according to standard morphologic criteria based on immunohistochemical, immunophenotyping and cytogenetic studies following the AIEOP-2002 AML and ALL-2000 treatment protocols.[229] Informed consent in compliance with the Helsinki protocol was obtained from the parents. Primary cultures of bone marrow from newly diagnosed AML patients were considered. Cells were maintained in serum free RPMI medium (Invitrogen) with 1µg/mL of glutamine and 1µg/mL of penicillin/streptomycin (GIBCO® - Invitrogen), interleukin-3 (IL-3) and interleukin-6 (IL-6) (each 20 ng/mL; Inalco; Milano, Italy), stem cell factor (SCF), FMS-like tyrosine kinase 3 (Flt-3) ligand and thrombopoietin (TPO) (each 50 ng/mL; Inalco) for 24h and subsequently subjected to transfection as indicated in Transient transfection assay section. All leukemic cells, including primary cultures, were cultured in an incubator set at 37°C with 95% relative air humidity in the presence of 5% CO2 (for buffering).

2.1.2. RNA isolation and Reverse transcriptase polymerase chain reaction (RT-PCR).

Total cellular RNA from cell lines and patients' bone marrows was extracted with TRIzol reagent (Invitrogen) according to manufacturer's instruction. RNA quality was controlled using the Agilent 2100 Bioanalyzer (Agilent Technologies; Tokyo, Japan). Following, 1 µg of total RNA was reversely transcribed in cDNA using random hexamers and the Superscript II (Invitrogen) according to the manufacturer's instructions. The quality of synthesized cDNA

was confirmed by amplification of the abl housekeeping gene by standard PCR reaction (primers indicated in *Table R1*).

2.1.3. Polymerase chain reaction (PCR) and Sequencing.

From the synthesized cDNA, 1.5-2 µL was added in the reaction mix containing: 10x PCR buffer, MgCl2, dNTPs, Taq Polymerase and water, all calculated for the 25 µL of final volume and with adequate primer pairs from the *Table R1*. All primers were generated by Primer Express1.0 software. For *BAG-1* gene amplification, several sets of primers were created, comprising initial, median and final gene positions and run at the following program: initial denaturation 95°C 5 min 1 x; denaturation 95°C 1 min; hybridisation 58-62°C 1 min 30 x; elongation 72°C 1-2 min; terminal elongation 72°C 10 min 1 x .

Table R1. Set of primers used for PCR amplification.

PRIMER NAME	*forward*	*reverse*
BAG-1 148F-974R	*GGCTGCGCGCCCTT*	*TCTTTGAAATTTTCTGGCAGGATC*
BAG-1 346F-973R	*CTCGACCCGGAGCGAGGAG*	*TTGAAATTTTCTGGCAGGATCA*
BAG-1 173F-255R	*GGAGCCGCGCCAGTC*	*CCTGGTGGGTCGGTCATG*
BAG-1 526F-952R	*TGACTGTCACCCACAGCAATG*	*TCAGTGTGTCAATCTCCTCCAAGA*
BAG-1 173F-532R	*GGAGCCGCGCCAGTC*	*GCTTCTCATTGCTGTGGGTGA*
BAG-1 214F-789R	*CTCCCTCTGGGCGTCCA*	*TCTCCACAGACTTCTCCAAATGTTT*
ABL	*CCTTCTCGCTGGACCCAGTGA*	*TGTGATTATAGGCTAAGACCCGGAG*

For a determination of PCR product sizes, Agarose gel electrophoresis was performed. Concentration of the agarose gels (1-2%) was adjusted according to the size of the DNA fragments. The correct amount of powdered agarose (Gibco BRL, Germany) was added to a measured quantity of electrophoresis buffer (1 x TAE) and heated slurry in a microwave oven until the agarose was dissolved. After short cooling, ethidium bromide solution [0.2 µg/ml] (VWR International, Leicestershire, England) was added and warm gel was poured into a chamber. Once polymerization of the gel was completed, the electrophoresis was run inside the electrophoresis chambers loaded with 1 x TAE buffer. The visualization of DNA fragments was done under a UV light. To determine the size of the DNA fragments, a DNA

molecular weight marker VIII (Roche, Indianapolis, IN) with defined fragment sizes (19-1114 bp range) was applied.

TAE-buffer (50x): 242 g Tris base, 57,2 mL glacial Acetic acid, 100 ml 0,5 M EDTA (pH 8.0), for 1L of final volume

Remaining PCR products were sequenced for a confirmation of the genes, using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Forest City, CA). For one reaction, 2 μl of Big dye, 5 μL of water, 1 μL of primer (2μM) and 2 μL of purified PCR product, were used. Following, PCR was done as follows: 25 cycles at 96°C 10 sec, 55°C 5 sec, 60°C 4 min; finished on 4°C. The PCR products were purified with tubes (?) by centrifugation at 4000 rpm for 2 min. Then the supernatant was discarded and the pellet was kept till dry. DNA was dissolved in 12 μL of formamide, heated for 5 min at 95°C, then cooled on ice and subsequently analyzed in Prism 310 Genetic Analyzer.

2.1.4. Real-time quantitative RT-PCR (RQ-PCR).

RQ-PCR was performed on the Applied Biosystems 7900 HT Sequence Detection System using SYBR Green PCR Master Mixture Reagents (Applied Biosystems). Experiments were performed in triplicate and used for relative quantity study. Primers used for RQ-PCR analysis are listed in *Table R2*. All expression values were normalized using expression of GUS as an endogenous control.[230] The Human low density Immune array profile (Applied Biosystems) was also performed and results were interpreted by the comparative ΔΔCt method.[231]

Table R2. Set of primers used for RQ-PCR amplification.

BAG-1	CTCGACCCGGAGCGAGGAG	GCTTCTCATTGCTGTGGGTGA
BAG-3	CAGCCAGATAAACAGTGTGGAC	AGAGGCAGCTGGAGACTGG
GUS	GAAAATATGTGGTTGGAGAGC	CGAGTGAAGATCCCCTTTTTA
BCL-2	GGCCGTACAGTTCCACAAA	AGTACCTGAACCGGCACCT
MCL-1	CGACCCCCGCGAGGCTGCTTTTCT	CTGGCGGCGGCGTCGAGGGTAGT

*BAX gene amplification was done with the use of gene CARDS. Sequence for a single probes unavailable. Mcl-1 primers –ref n° 232

2.1.5. Transient transfection assay.

Transfection experiments were performed on HL60, RS4;11 and primary cell cultures. Cells were transfected with exogenous small interfering RNAs (siRNA) specifically recognizing BAG-1 (0.15 nmol) or BAG-3 (0.2 nmol) (Santa Cruz Biotechnology, Santa Cruz, CA) using the Amaxa Nucleofactor systems (Lonza; Cologne, Germany). Programs Q – 017, L – 017 and U – 015 were chosen for the transfection of HL60, RS4;11 and primary cell cultures, respectively. Non-silencing scramble siRNA (sc-siRNA; Santa Cruz Biotechnology) was used as a control. After transfection cells were maintained in DMEM (HL60), RPMI (RS 4;11) or RPMI supplemented with growth factors (primary AML cultures), all additionated with 10 % FBS. The efficiency of silencing was optimized to be up to 70 % with cell viability higher then 80 %.

2.1.6. Apoptosis assay and cell cycle analysis.

Cell death was quantified 24h, 48h and 72h after transfection using fluorescein labeled Annexin-V / Propidium Iodide (AnnV / PI; Immunostep S.L; Salamanca, Spain). The combination of AnnV, which binds with high affinity to exposed PS of the apoptotic cells, and PI, which can pierce only inside dead or damaged cells, allows “early apoptotic events” (AnnV+/PI-) to be distinguished from “late apoptotic/necrotic events” (AnnV+/PI+) and viable cells (AnnV-/PI-). For cell cycle analysis cells were washed with cold PBS and made permeable by the addition of 70% ethanol overnight at 4°C. The staining was done with 50 µg/mL PI and 10 µg/mL RNase A (Sigma; St Louis, MO). Both experiments were analyzed by Cytomics FC500 (Beckman Coulter; Brea, CA) for a minimum of 10 000 events. The cellular DNA content histogram and the %age of cells in the respective phases (sub-G_1, G_1, S and G_2/M) of the cell cycle were determined by Multicycle Wincycle software (Phoenix Flow Systems; San Diego, CA).

2.1.7. Preparation of total protein extracts and subcellular protein fractions.

Cells were collected and subsequently resuspended in cold lysis buffer (Biosource International; Camarillo, CA), supplemented with 0.5 mM PhenylMethaneSulfonyl Fluoride (PMSF, Sigma), mammalian protease and phosphatase inhibitor mixture (1:100, Sigma), for 30 min on ice for total protein isolation. For the nuclear and cytosol protein isolation a combination of low salt buffer A (10 mM HEPES pH = 7.9; 1.5 mM $MgCl_2$; 10 mM KCL and

0,5 mM DTT) and high salt buffer C (20 mM HEPES pH = 7.9; 25 % v/v glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT) was used. Briefly, cell pellets were washed in cold PBS and resuspended in cold buffer A. After 10 min on ice, samples were centrifuged at 13000 rpm for 10 min at 4 °C. Supernatants were collected for cytosol protein fraction while pellets were gently washed once with cold 1 x PBS and re-resuspended in buffer C. After 30 minutes of incubation on ice, nuclear protein fraction was collected by centrifugation at 13000 rpm for 30 min at 4 °C. Total proteins or proteins from nuclear and cytosol fractions were immediately quantified with BCA Protein Assay Kit (Pierce; Rockford, IL).

2.1.8. Western blotting.

Aliquots of total, nuclear or cytosol lysates (10-20µg of proteins), were mixed with equal volumes of Laemmli sample buffer, incubated for 5 min at 99°C and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were hybridized with the following primary antibodies: BAG-1 (recognizing all three BAG-1 isoforms), BAG-3 (Santa Cruz Biotechnology), Phospho-c-Raf, total ERK1/2, Phospho-ERK1/2, caspase-3, PARP, Bcl-2, Mcl-1, Bcl-X_L, Bax, Smac/DIABLO, cytochrome *c*, XIAP, p27, p21, CDK2, Aurora-A, Usp9X (Cell Signaling; Danvers, MA) and cyclin D1 (BD Biosciences; San Jose, CA) in a concentration recommended by manufactures. Blots were then stripped and re-probed with β-actin or γ-tubulin (Sigma) antibody, used as the controls for a proper protein loading. As secondary antibody horseradish peroxidase (HRP)–conjugated anti-rabbit, anti-mouse or anti-goat IgG (Upstate Biotechnology; Lake Placid, NY) were used. Enhanced chemiluminescence (ECL) Western blotting detection reagents and films (GE Healthcare; UK) were used for protein bands acquisition and, when necessary, densitometry was done using ImageJ software.

2.1.9. Co-Immunoprecipitation.

For immunoprecipitation analysis $5x10^5$ HL60 and primary AML cells were harvested, washed once in cold 1 x PBS and resuspended in freshly prepared IP buffer (5 mM $MgCl_2$, 137 mM KCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl pH = 7.5 and 1% CHAPS) containing PMSF, protease and phosphatase inhibitors (Sigma). After 30 min of incubation on ice, the lysate was clarified by centrifugation and supernatant with total protein fraction was

pre-cleared with Protein A/G MicroBeads (Miltenyi Biotec, B. Gladbach, Germany) on ice for another 30 min. Lysate was passed over a µ-Columns and collected for immunolabeling with anti-BAG-1, anti-BAG-3, anti-p38, anti-Aurora-A or anti-Usp9X antibody 30 min on ice (Santa Cruz Biotechnology). Following, lysates were run through, retaining magnetically labeled protein in the µ-Columns and after four washes eluted with pre-heated (95 °C) 1 x SDS loading buffer. The immunoprecipitates were analyzed by SDS-PAGE.

2.1.10. Cell viability assay.

Cell viability was determined by trypan-blue (GIBCO® - Invitrogen) exclusion assay. While viable cells are not permissive to trypan blue, dead cells are, and become stained in blue after the application of this dye. This characteristic permits dead cells to be distinguish from viable ones, which remain unstained when looked under the phase-contrast microscope. For the determination of the dead/viable cell number, a mixture of cells and typan blue dye (1:3 in PBS) was placed on the standard Neubauer chamber and following formula was used:

cells / ml = number of viable cells / 4 x dilution factor x 10,000

2.1.11. Reagents and treatments

Cells were treated with 3 µM and 5 µM aza-2-deoxycytidine (AZA; Sigma) and de-methylation effect on BAG-1 protein expression is checked 2h and 24h later. Proteasome inhibition was obtained using selective and reversible proteasome inhibitor (benzyloxycarbonyl)-leu-leu-phenylalaninal (Z-LLF-CHO; 20µM, Santa Cruz Biotechnology). Cellular stress/apoptosis was generated using *Etoposide* (*Eto;* 2µM, 3µM; Sigma).

2.1.12. Statistical analysis.

Statistical analysis is performed using Prism 4.02 (Graph Pad). All experiments were performed minimum as triplicate and mean values are presented as mean ± standard deviation (SD) or as mean ± standard error of the mean (S.E.M.) of replicate experiments. Statistical significance was evaluated by One-way ANOVA followed by Newman-Keuls multiple comparison post Test for validation of intergroup diversity or unpaired Student's t-test, for the

comparisons between two groups. Differences were considered significant for $p < 0.05$ and presented as $*p < 0.05$; $**p < 0.01$, $***p < 0.001$.

CHAPTER III

RESULTS PART I:

BAG-1 mRNA and protein expression in cell lines and leukemic patients

3. RESULTS

3.1. Validation of BAG-1 mRNA presence in diverse leukemic cell lines by PCR analysis. Lack of data about BAG-1 expression in acute leukemia constricted us to open our research with basic molecular investigation for the eventual *BAG-1* mRNA expression in diverse leukemic cells. Therefore, in a smaller cohort of myeloid and lymphoid cell lines (Jurkat, HL60, REH, THP1, NOMO1, 697, NB4, RS 4;11, ML2), we firstly went to confirm the presence of *BAG-1* mRNA. A standard PCR analysis was performed using a diverse sets of primers as indicated on the *Figure R1A (the primer sequence could be found in Table R1 of Material and methods).*

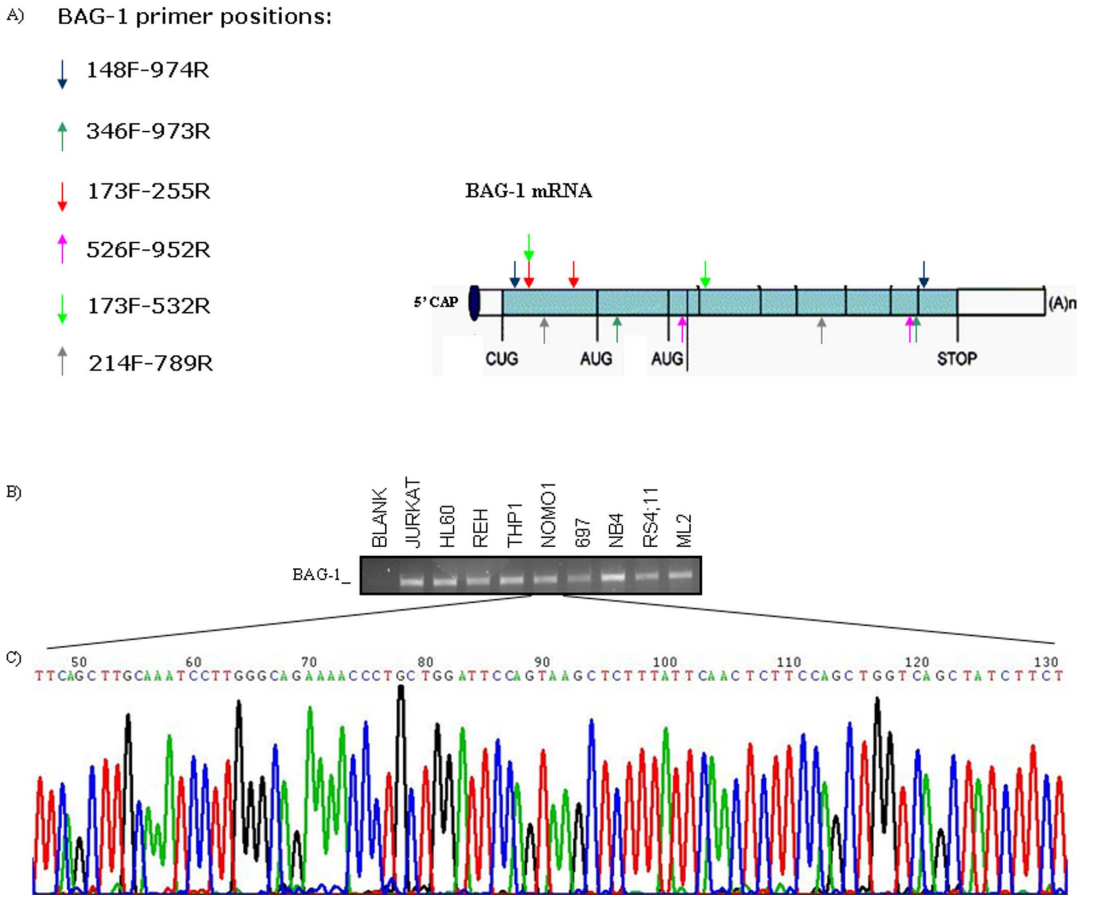


Figure R1. Amplification of BAG-1 gene by PCR: A) Primer pairs and their positions (indicated with colored arrows; while the numbers outline the initial base pair of each primer) at BAG-1 mRNA (and hence reversely transcribed cDNA). B) Agarose gel electrophoresis run after the BAG-1 gene PCR amplification for the control of its presence in leukemic cell lines. Blank denotes a control sample reaction done for a pure mix, without cDNA addition. C) Part of the BAG-1 gene sequence.

The results confirmed BAG-1 mRNA presence in all examined cell lines with no exceptions (*Figure R1B)*. Each of the amplified PCR products was confirmed by sequencing as BAG-1 gene (only a short sequence of the BAG-1 gene was introduced on the *Figure R1C).*

3.2. BAG-1 mRNA and protein expression in leukemic cell lines. After initial confirmation that BAG-1 gene was actively transcribed in all selected leukemic cell lines, we measured its relative expression in a larger cohort of leukemic cell lines, using real-time PCR (RQ-PCR). The cells were divided in two groups: myeloid cell lines HL60, NOMO1, NB4, THP1, MV4;11, ML2 were referred hereafter as "AML" group and acute lymphoid RS4;11, 697, JURKAT, REH, SEM and chronic myeloid K562 cell lines were referred as "non-AML" group. As it could be seen in *Figure R2A,* BAG-1 mRNA was heterogeneously expressed in all samples examined, showing similar fluctuation profile among AML and non-AML cells. Therefore, the mean values of relative BAG-1 mRNA expression, calculated for AML and non-AML groups separately, did not differ considerably (AML RQ BAG-1/GUS = (1.37 ± 0.70), non-AML RQ BAG-1/GUS = (1.27 ± 0.60), n = 3; p = 0.8; *Figure R2A on right*).

In contrast to only a single BAG-1 mRNA which could be detected by RQ-PCR, Western blot analysis permitted to all three of BAG-1 protein isoforms, BAG-1L, -1M and -1S, to be distinguished. The protein expression study showed BAG-1S and -1M as the most abundant and commonly expressed in all examined leukemic cell lines (*Figure R2B*). At our surprise, BAG-1L was undetected in the total protein extracts isolated from three AML cell lines (ML2, THP1, NOMO1), all characterized by the presence of Mixed Lineage Leukemia (*MLL*) gene rearrangements. Densitometric analysis, done for the quantification of each BAG-1 protein isoform expression in each of the leukemic cell line, revealed that BAG-1L had the most heterogeneous as well as the lowest mean value in myeloid cell lines (AML vs non-AML = (0.13 ± 0.07) vs (0.37 ± 0.06), n = 3, *p = 0.03). On the contrary, in AML cell lines with respect to non-AML ones, a lightly higher expression of BAG-1S (AML vs non-AML = (0.67 ± 0.06) vs (0.58 ± 0.07), n = 3, p = 0.4) and BAG-1M (AML vs non-AML = (0.31 ± 0.06) vs (0.28 ± 0.08), n = 3, p = 0.8) isoform was found.

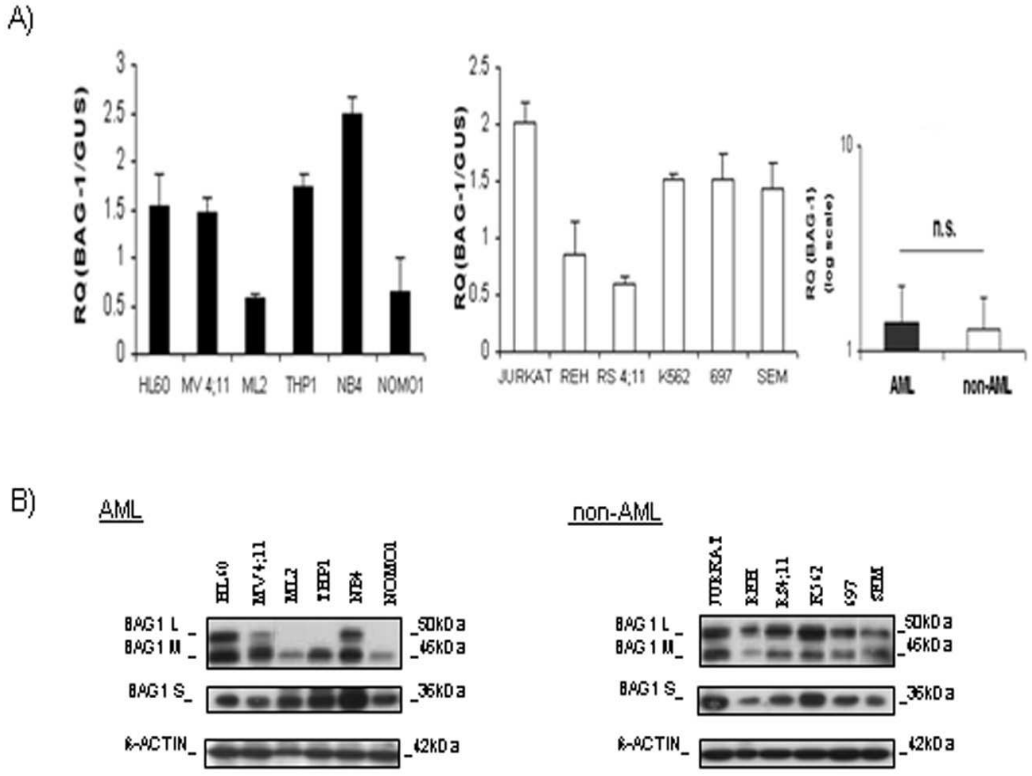


Figure R2. BAG-1 expression in leukemic cell lines. A) RQ-PCR for the cohort of AML and non-AML cell lines normalized for GUS endogenous control. Mean values for the two groups presented at log scale (at right) varied non-significantly (n.s.). B) Western Blot (WB) analysis of three BAG-1 protein isoforms (BAG-1L, -1M and -1S) expression in a group of AML and non-AML cell lines. ß-actin was used as a protein loading control.

3.3. De-methylation of BAG-1 gene promoter. As described previously, methylation status of BAG-1 gene promoter can determine the expression level of *BAG-1* mRNA, keeping it less expressed when CpG islands are methylated, and highly expressed if de-methylated. To test if intense methylation status of *BAG-1* gene promoter was responsible for a lower expression of BAG-1L isoform in ML2, THP1 and NOMO1 cell lines, we chosen to treat ML2, as a deputy of the three AML cell lines with *MLL* gene rearrangements, and HL60, where BAG-1L was present, with AZA, known de-methylating agent. When we checked if AZA-dependent removal of methyl groups from CpG islands of *BAG-1* gene changed the expression of BAG-1 protein isoforms in two selected lines, in the case of HL60 we detected an increase in expression of all three BAG-1 isoforms (BAG-1L,BAG-1M and BAG-1S; *Figure R3).* In the case of ML2 cell line, the expression of two isoforms, BAG-1M and BAG-1S, augmented, but BAG-1L still remained undetectable in the whole cell protein lysates (wcl). Presented results confirmed that low expression of BAG-1L protein isoform in some of the myeloid cell lines

was not, probably, dependent on impaired methylation of *BAG-1* gene promoter, and future studies will be done in order to explain different BAG-1 isoforms expression in AML.

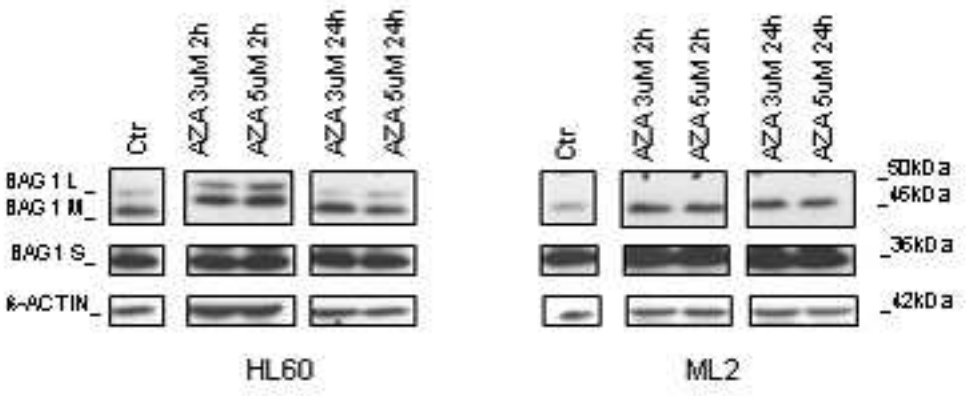


Figure R3. WB analysis for the BAG-1 protein expression after 2h and 24h of AZA treatment in HL60 and ML2 leukemic cell lines. ß-actin was used as a protein loading control.

3.4. Sequencing of +1 CUG region of BAG-1 mRNA. Next, we decided to check for the eventual mutations in the sequence of *BAG-1* gene at the region upstream of the translation start site (recognized as +1 CUG at BAG-1 mRNA), because preserved native sequence of this region is known to be crucial for the correct interaction between mRNA and translation machinery.[233] In fact, a mutated sequence is weakly bound by the translation complex and could be a cause of impaired production of BAG-1L protein isoform. These sequence in BAG-1 gene shows high similarity to Kozak consensus sequence (gcc)gccRccA(C)UGG (R- is placed three base pairs upstream of +1 AUG, or less frequently CUG, start codon and can be either adenine or guanine) in which at the nucleotide position +4 (4 base pairs (bp) downstream of +1 CUG) and position -3 (3 bp upstream of +1 CUG) the consensus sequence must be preserved for an adequate binding of translation complex.[234] For that purpose, we designed primers (marked with yellow at the *Scheme 1*) which allowed us to successfully amplify sequence of BAG-1 mRNA upstream and downstream of +1 translation start site in ML2, THP1, NOMO1, and additionally HL60, K562 and RS 4;11 cell lines. However, no mutations on native sequence (*Scheme 1*) of BAG-1 gene was found in the cell lines investigated, indicating that impaired +1 surrounding sequence was not a motive for lower BAG-1L expression.

```
  1 aggccggggc ggggctggga agtagtcggg cggggttgtg agacgccgcg ctcagcttcc
 61 atcgctgggc ggtcaacaag tgcgggcctg gctcagcgcg gggggcgcg gagaccgcga
121 ggcgaccggg agcggctggg ttcccggctg cgcgcccttc ggccaggccg ggagccgcgc
181 cagtcggagc ccccggccca gcgtggtccg cctccctctc ggcgtccacc tgcccggagt
241 actgccagcg ggcatgaccg acccaccagg ggcgccgccg ccggcgctcg caggccgcgg
```

Scheme 1. The sequence of BAG-1 gene upstream and downstream of the translation initiation start site +1 CUG (CTG when reversely transcribed to a cDNA) marked in red. The yellow marked letters sign the position of the primers used for the PCR amplification. Numbers on left indicate the base pair (bp) positions.

3.5. BAG-1L protein isoform expression in subcellular protein fractions. Knowing that BAG-1 protein isoforms are differentially localized inside the cell, we performed a separate isolation of nuclear and cytosol protein fractions and looked for the BAG-1L presence within each of them. In all three cell lines (indicated with asterisk on *Figure R4A*), which did not express BAG-1L in wcl, BAG-1L resulted as present in the nuclear protein portion (*Figure R4B*). Moreover, in all three cell lines (ML2, THP1, NOMO1), BAG-1M isoform resulted to be mostly nuclear, while in HL60 cells, BAG-1M was localized in the cytosol. This result confirmed that very low expression of BAG-1L rather then its complete absence, caused it to be undetectable in wcl.

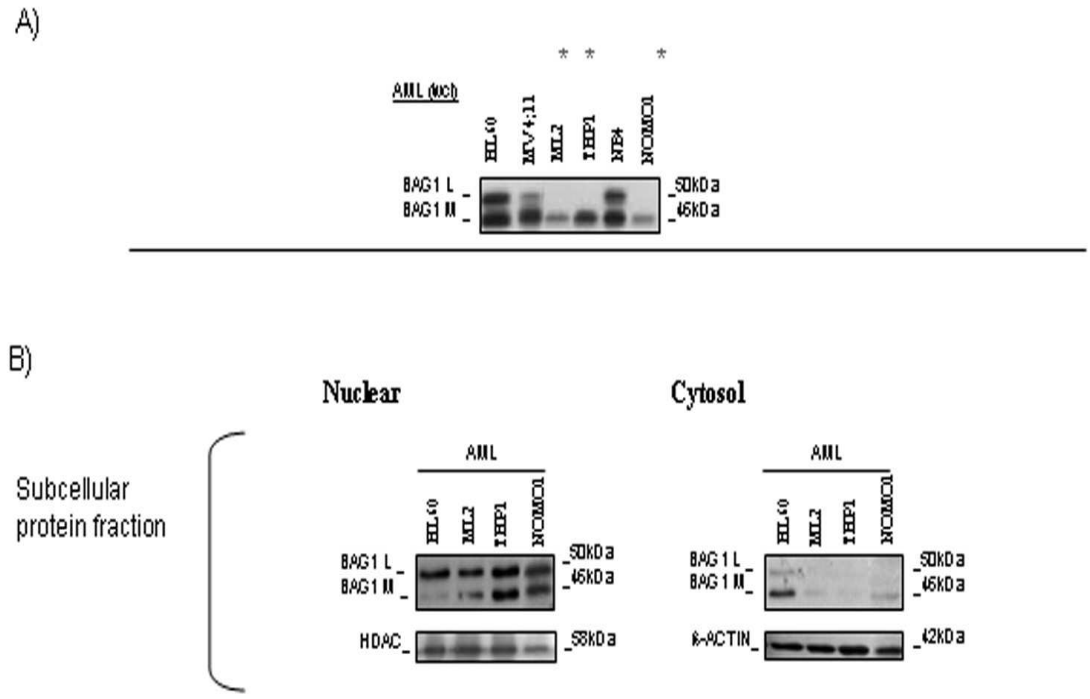


Figure R4. WB analysis for BAG-1 protein isoforms expression in AML cell lines. A) whole cell lysate (wcl) and B) nuclear and cytosol protein fraction of HL60, ML2, THP1 and NOMO1 cell lines. HDAC and ß- actin are the controls for adequate protein loading. Asterisks show lines with MLL rearrangements.

All results done so far, and knowledge that three BAG-1 protein isoforms are translated from a single RNA, brought to a conclusion that in myeloid cells that carry MLL gene rearrangements, translation occurs less frequently from the first translation CUG site of BAG-1 mRNA, using preferentially second and first regular AUG sites, respectively, for BAG-1 protein production, while translation in lymphoid cells occurs equally from all three translation start sites. Furthermore, we could conclude that expression of BAG-1 protein isoforms within leukemic cells is primarily governed by the post-transcriptional control mechanisms and is not linearly correlated with BAG-1 mRNA expression.

3.6. BAG-1 expression in AML and ALL patients. In following, we sought to study BAG-1 expression in patients with acute leukemia. A group of 10 AML and 10 ALL samples were firstly screened for BAG-1 mRNA expression. A large variability of BAG-1 mRNA expression was found among either AML or ALL patients at the moment of diagnosis. When mean BAG-1 mRNA value for AML or AML group was compared to a mean value for a pool of healthy bone marrow (hbm) samples, no significant discrepancy could be seen. However, differences in mean BAG-1 mRNA expression between AML and ALL group of patients resulted as significant when calibrated to a mean mRNA expression for the pool of healthy samples (BAG-1 expression RQ mean for AML = (0.91 ± 0.34), RQ mean for ALL = (1.47 ± 0.63), n = 3, *p = 0.03; hbm RQ = 1; *Figure R5A*).

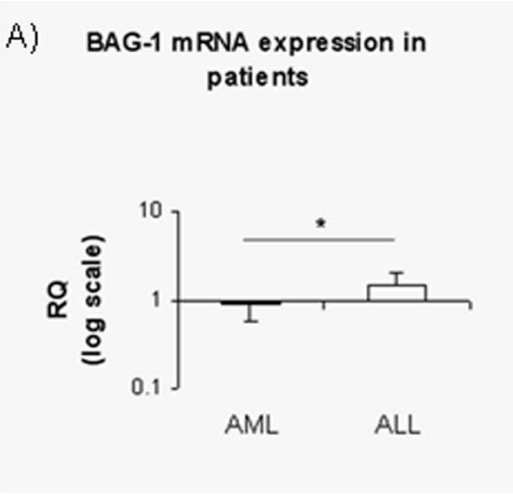


*Figure R5A. The mean values of relative mRNA expression for AML and ALL patients at the moment of diagnosis presented in logarithmic scale. The relative expressions for BAG-1 mRNA varied significantly (*p<0.05) between AML and ALL patients. However, BAG-1 mRNA expression for both groups did not vary significantly in respect to BAG-1 mRNA expression found in hbm.*

Next, we went to verified a pattern of BAG-1 protein isoforms expression in the group of AML and ALL patients, collecting samples during leukemia detection (diagnosis) or later, during remission. We were particularly curios to see if the therapy application could impair the BAG-1 protein expression in leukemic cells. As a control sample, we used cells isolated from healthy bone marrow specimens, where a negligible level of BAG-1protein was revealed (*Figure R5B*).

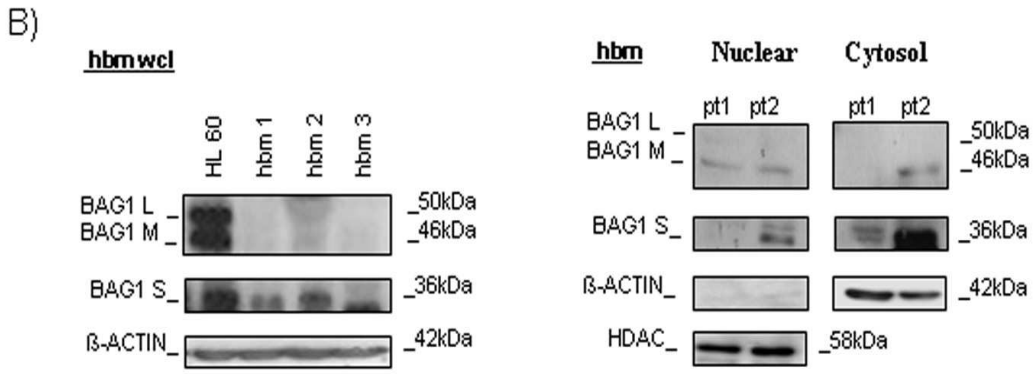


Figure R5B. BAG-1 protein isoforms expression in healthy bone marrow (hbm) samples from wcl or nuclear and cytosol protein fractions. HL60 was used as a control for a good WB efficiency and ß-actin or HDAC as the controls for a proper cytosol and nuclear protein loading, respectively.

In contrast to hbm samples, in patients with acute leukemia we could find different expression of the three BAG-1 protein isoforms. In particular, in the moment of AML diagnosis, preferentially expressed isoform was shown to be BAG-1S, while BAG-1L and -1M isoforms were, in general, weakly expressed. We went to consider BAG-1 expression after therapy. At the moment of leukemia remission, BAG-1S protein levels decreased in wcl of AML patients (*Figure R5C, upper*) and detected BAG-1 protein profile resembled a lot the profile seen in hbm (Figure R5B). Repeated study for the subcellular (nuclear and cytosol) protein fractions of AML patients at diagnosis demonstrated that BAG-1L was often present in nuclear extracts, and the same was seen for the BAG-1M isoform (*Figure R5C, lower*). During leukemia remission, analysis of BAG-1 protein isoforms distribution in subcellular fractions showed a tendency of BAG-1M to move from nucleus to cytosol. Once again, we confirmed that, even in patients like in some cell lines previously, lower expression rather than absence of BAG-1 isoforms left them to be undetected in total protein lysates. BAG-1S was observed to be in major part located in cytosol, but in some cases even small isoform resulted to be

eventually present inside the nucleus. Importantly, the results presented here open for the further studies the role of each BAG-1 isoform in AML pathogenesis.

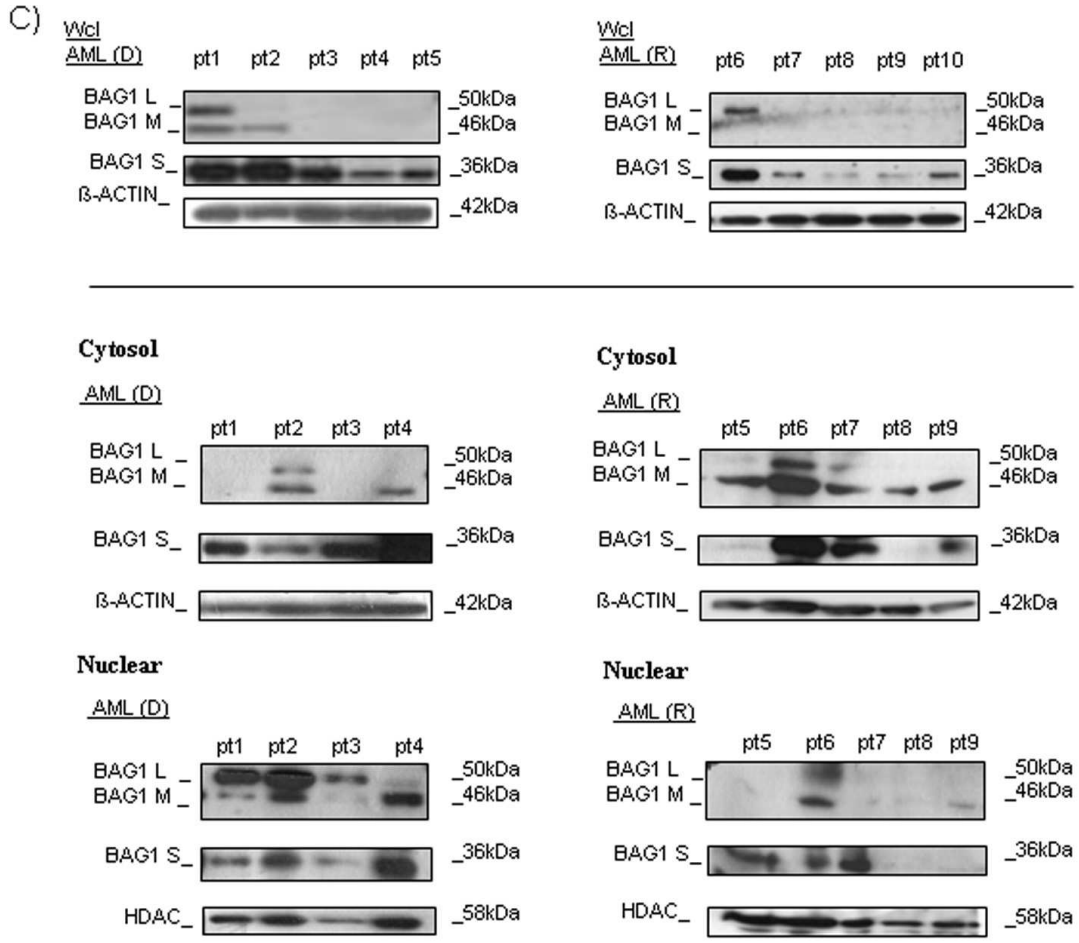


Figure R5C. BAG-1 protein isoforms expression in the AML patients at the moment of diagnosis (D) and remission (R). Wcl and nuclear and cytosol protein fractions are examined. ß- Actin and HDAC were the controls for a proper cytosol and nuclear protein loading, respectively.

Considering ALL patients, BAG-1 protein showed a high expression of all three isoforms at the moment of diagnosis when looked in the wcl. At the moment of leukemia remission, BAG-1L and -1M were found decreased in wcl, while BAG-1S isoform maintained its levels similar to the one observed at diagnosis (*Figure R5D, upper).* When BAG-1 protein expression was analyzed for nuclear and cytosol protein fractions, at diagnosis we could see general localization inside nucleus for BAG-1L and BAG-1M, but inside cytosol for BAG-1S (*Figure R5D, lower*). As it could be seen, even in the case of ALL, BAG-1M passed from mainly nuclear to mainly cytosolic after leukemia remission, while BAG-1S kept on to be cytosolic and expressed more in respect to BAG-1L and -1M proteins.

D)

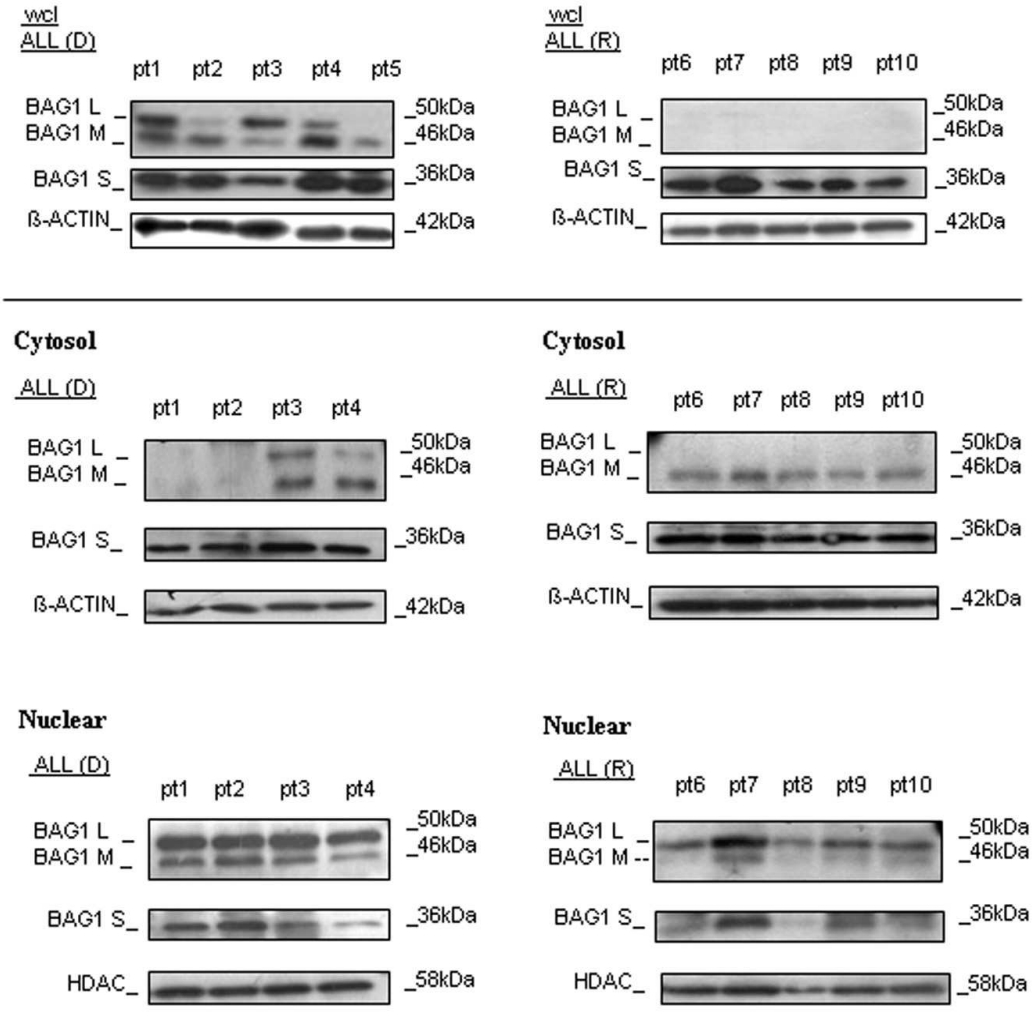


Figure R5D. BAG-1 protein isoforms expression in wcl or nuclear and cytosol protein fractions of ALL patients (D=diagnosis; R=remission). ß- actin or HDAC were the controls for a proper cytosol and nuclear protein loading, respectively.

All together, the results demonstrated here underline a putative different role of each BAG-1 isoforms in myeloid and lymphoid leukemia pathogenesis. More precisely, both parameters, either level of BAG-1 protein or its localization inside the leukemic cells, seem to be important in defining final effect on leukemia phenotype.

Being specifically interested in AML, in which BAG-1's role is not known, we continued with the experiments which could clarify putative role of BAG-1 in AML.

RESULTS PART II:

Study of BAG-1's function in AML cells: silencing approach

3.7. Study of BAG-1's role within AML cells. The absence of evidence about BAG-1 role and function in acute leukemic cells with myeloid origin, stimulated us to discover how BAG-1 might contribute to myeloid leukemia development. For the study, from the cohort of AML cell lines analyzed initially we chose HL60 line, since it expressed well either *BAG-1* mRNA or all three isoforms of BAG-1 protein. In this cell line, we performed a widely used silencing approach which allowed us to cause an artificial down-regulation of BAG-1 mRNA and in following, BAG-1 protein expression, and checked for the eventual effects provoked afterwards. For the silencing, we used commercially available small interfering RNA (siRNA), which specifically recognize and destroy BAG-1 mRNA, causing in a consequence a decrease in all three BAG-1 protein isoforms intensity. The silencing was followed by diverse functional analysis. At first, we elucidated the effectiveness of BAG-1 silencing in HL60 cells, which was confirmed at both, RNA and protein, levels (RQ = (0.24 ± 0.08); protein expression = (0.28 ± 0.07), n = 3, *p = 0.03, in respect to sc-siRNA = 1 for both values) (*Figure R6 A, B*).

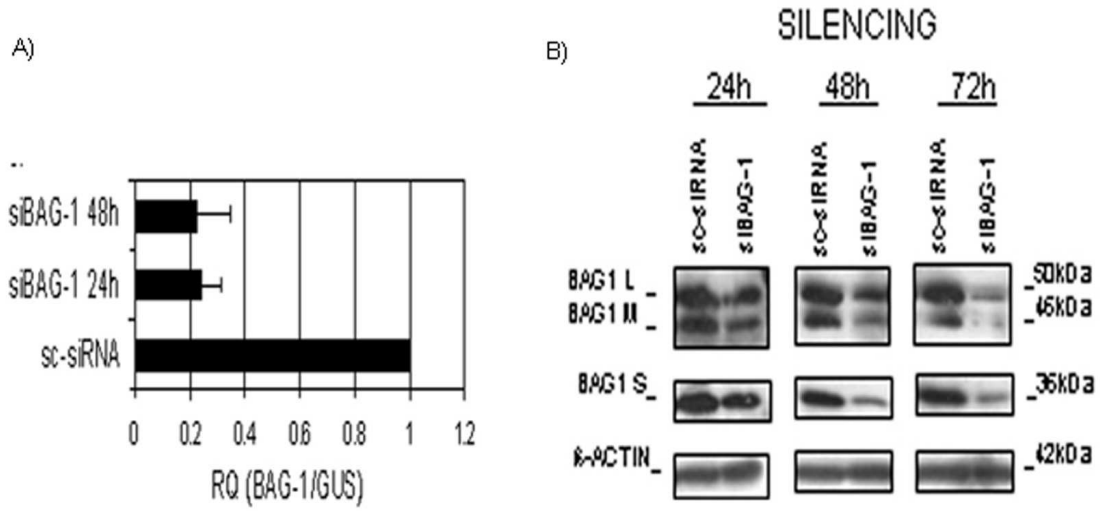


Figure R6. BAG-1 silencing in HL60 cell lines. A) RQ-PCR analysis of relative BAG-1 mRNA expression 24h and 48h after transfection normalized for a GUS as endogenous control. The silencing efficacy is calculated in respect to BAG-1 mRNA expression values obtained for sc-siRNA control, which corresponds to the RQ value 1 (100%) on x-axis. B) BAG-1 silencing efficacy at the protein level 24h, 48h and 72h after transfection compared to sc-siRNA control sample. ß- actin was the control of adequate protein loading.

As a response to BAG-1 silencing we could observed only a slight increase in apoptosis activation ⌈(sc-siRNA) vs siBAG-1 = (16±7)% vs (20±9)%, n=3, p>0.05; *Figure R7A*⌉ and small, but insignificant changes in cell cycle appearance ⌈sc-siRNA vs siBAG-1= (G1 (46 vs

42)%; S (12.1 vs 11.9)%; G2/M (41.9 vs 45.7)%; cell death shown as sub-G1 (10.7 vs 13.7)%; *Figure R7B*].

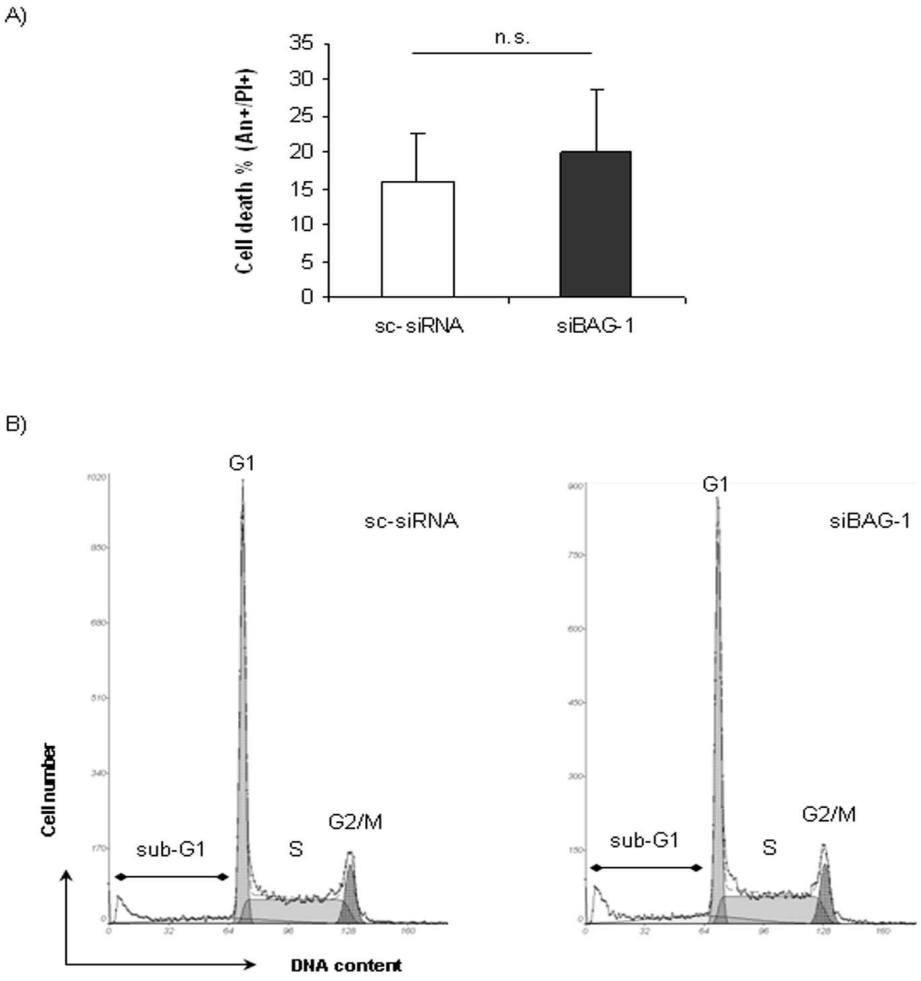


Figure R7. Effects of BAG-1 silencing on HL60 cells behavior. A) Annexin V/PI (AN/PI) assay was assessed 48h after silencing and results were presented as a % of dead cells found in sc-siRNA and siBAG-1 transfected HL60 line. Small but non significant (n.s.) differences in cell mortality was found when two samples were compared. B) Cell cycle appearance 48h after silencing: no drastic differences in appearance of any of the phases could be seen between sc-siRNA and siBAG-1 samples. Slight decrease in total cell number after siBAG-1 transfection could be noted at the y-axis.

Then, we went to check if silencing had any effects on the expression of some proteins. In particular, we were interested in Bcl-2 family belonging proteins and proteins from Mitogen-Activated Protein Kinase (MAPK) pathway, which have been both confirmed to have members that target BAG-1 protein. Interestingly, investigation of protein expression showed that certain changes occurred (*Figure R8A*). Specifically, we found a considerable decrease in Bcl-2 expression and slight decrease in Mcl-1 and Bcl-X_L apoptosis-suppressor proteins.

When we looked the apoptotic factor Bax, we confirmed no changes in its expression, while an increased expression of Smac/DIABLO was revealed. The XIAP protein, which like Bcl-2 or Mcl-1 has an inhibitory potential over apoptosis induction, but does not belong to Bcl-2 family, kept its expression unaffected after silencing. BAG-1 relationship with MAPK pathway was also impaired after its silencing. It is known that BAG-1 can interact with c-Raf, stimulating thereby additionally the activation of downstream signals of MAPK pathway. As expected, transient down-modulation of BAG-1 and, hence, impaired interaction with c-Raf, influenced MAPK pathway activation and cause a partial decreased of active, phosphorylated, ERK1/2 protein expression, which counted for a small reduction in cell proliferation. However, silencing did not influenced significantly the expression of phospho-c-Raf itself. Next, densitometry analysis was done for a quantification of protein expression (*Figure R8B*). Using densitometric values obtained for Bax and Bcl-2 proteins, we calculated Bax/Bcl-2 ratio, which has been shown as favorable survival feature in AML and an important parameter for indication of apoptosis rate. As it could be seen from the figure, Bax/Bcl-2 ratio was 0.86 for sc-siRNA and 1.88 for siBAG-1 transfected HL60 cells. This result indicated that slightly higher cell death observed after siBAG-1 transfection, could be a consequence of minor mitochondria-dependent apoptotic involvement, provoked through the modulation of Bax/Bcl-2 ratio, in favor to pro-apoptotic protein Bax.

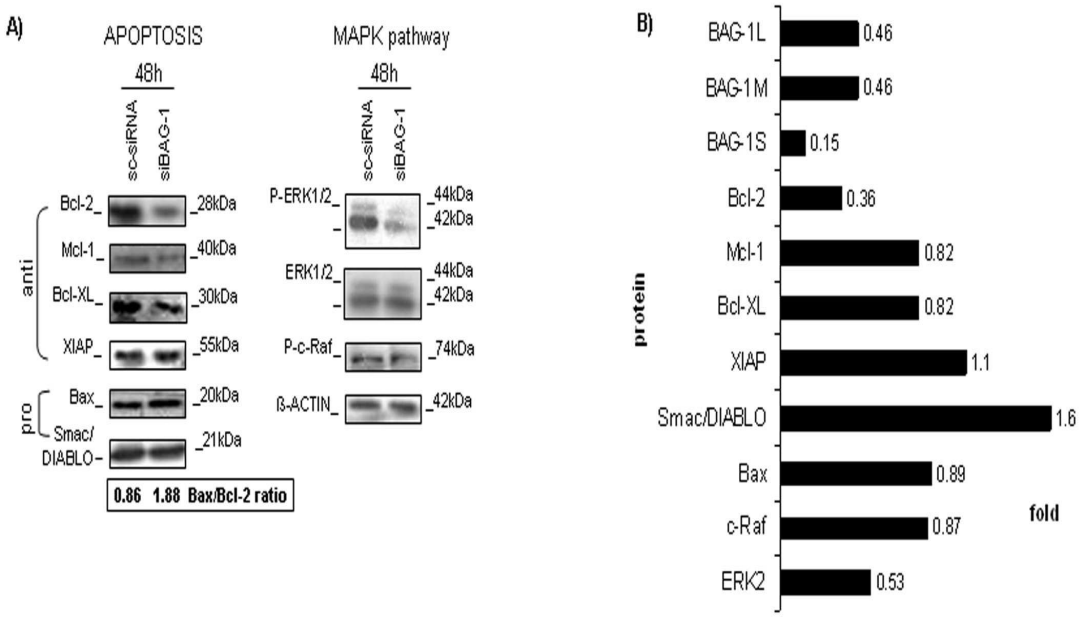


Figure R8. Effects of BAG-1 silencing on protein expression in HL60 cells. A) WB analysis of protein expression showed changes in Bcl-2, Mcl-1, Bcl-X_L and phospho-ERK1/2 protein levels. Bax/Bcl-2 ratio was calculated according to densitometric results presented at right part of the figure. B) Densitometric analysis of protein expression after BAG-1 silencing was normalized for ß- actin expression and calculated as a % of protein expression measured after sc-siRNA silencing, which was considered to have expression equal to 1 (100%).

A described changes at protein levels were not followed by the similar changes in mRNA levels of Bcl-2, Bax or Mcl-1 *(Figure R9*), indicating that BAG-1 acts at the protein level without affecting gene transcription.

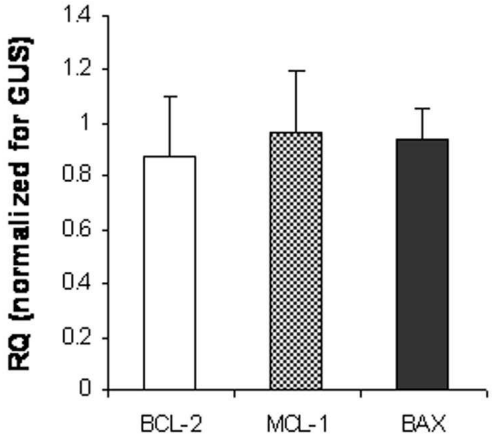


Figure R9. RQ-PCR analysis for the BCL-2, MCL-1 and BAX gene relative expression measure 48h after BAG-1 silencing. The results show the % of mRNA expression calculated for the control sc-siRNA samples which was considered to have the RQ=1 (100%).

3.8. BAG-1 and BAG-3 expression. From the results presented till now, we could determine two main spots in which BAG-1 realizes its function in AML: apoptotic, affecting Bcl-2 level, and proliferative, affecting phospho-ERK1/2 protein expression. Both of them were reported to be influenced by BAG-1 in other cancer types. However, in AML cells these events seem to be insufficient to make a considerably impact over leukemic phenotype. Nonetheless, additional minor decrease in expression of Mcl-1 and Bcl-X_L proteins, confirmed after transient BAG-1 silencing, made us to think that other molecular component might compensate for the induced deficiency of BAG-1, keeping cell processes functional. Therefore, we hypothesized that some of the proteins from BAG family could be a good candidate for this connotation and, considering the similar functions and interaction partners in common for BAG-1 and BAG-3 proteins, we controlled a BAG-3 expression (after standardization for *GUS* gene and normalization for the control sc-siRNA RQ value) after BAG-1 silencing and found it to be increased: siBAG-1 (24h, 48h, 72h): RQ Mean BAG-1 = (0.29, 0.32, 0.45), RQ Mean BAG-3 (1.25, 2.08, 2.6) (*Figure R10A*). To check if BAG-1 mRNA could be affected in the same way after BAG-3 silencing, we chose commercially siRNA specifically recognizing BAG-3 mRNA, and controlled BAG-1 mRNA expression after HL60 cells were silenced for BAG-3. We could confirm that BAG-3 silencing was also able to provoke an increase in BAG-1 mRNA expression (siBAG-3 (24 h, 48 h, 72 h): RQ

Mean BAG-3 = (0.65, 0.76, 1.1), RQ Mean BAG-1 (0.85, 1.5, 2.51) *Figure R8B*). Consistent with real-time PCR analysis, protein analysis verified that BAG-1 silencing provoked minor increase in BAG-3 protein level, and vice versa (*Figure R10C*). Notable, BAG-1 silencing was more efficient than BAG-3 in both, mRNA and protein reduction. We hypothesized that BAG-3 could compensate the induced lost of BAG-1, and hence, we proceeded by co-silencing both genes.

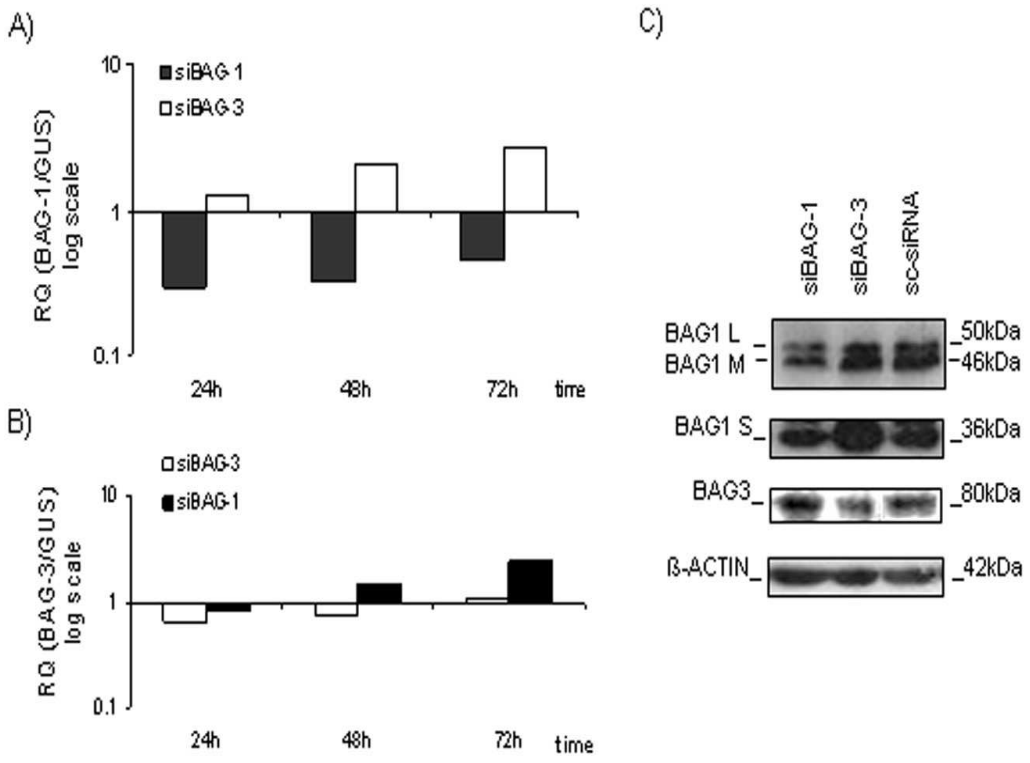


Figure R10. BAG-1 and BAG-3 single silencing in HL60 cells. A-B) RQ-PCR analysis of BAG-1 (black bars) and BAG-3 (white bars) mRNA expression 24h, 48h and 72h afterr silencing. Results are presented in logarithmic scale. C) WB analysis for BAG-1 and BAG-3 protein expressions compared to sc-siRNA control. ß- actin is used as a protein loading control.

3.9. BAG-1 and BAG-3 co-silencing. Applied co-silencing of BAG-1;3 (referred further as BCS) was confirmed at mRNA and protein level, at first (only results at the protein level demonstrated at *Figure R11A*). In concordance with our hypothesis, BCS provoked a considerable decrease in cell viability in respect to sc-siRNA transfected cells and was accompanied by increased apoptosis induction (viability after (24 h, 48 h, 72 h): mean values for sc-siRNA = (81.4 %, 84.3 %, 85 %); mean values for BCS = (73.7 %, 76.5 %, 75.8 %)

respectively, n = 3, *p < 0.05, **p < 0.01, *Figure R11B*; death rate measured by Annexin-V: (sc-siRNA) / (BCS) = (10 ± 2) % / (17 ± 3) %, n = 3, *p = 0.03). Protein analysis done after BCS confirmed a decrease in Bcl-2 protein level, while more evident decline of Mcl-1 and Bcl-X_L protein expression was observed in respect to single BAG-1 silencing described above. Additionally, the expression level of Bax and phospho-c-Raf proteins did not change significantly while phospho-ERK1/2 followed the same decreasing rate reported after single BAG-1 silencing (*Figure R11C*), indicating that proliferation pathway even in AML cells undergo only to a partial inactivation after BCS.

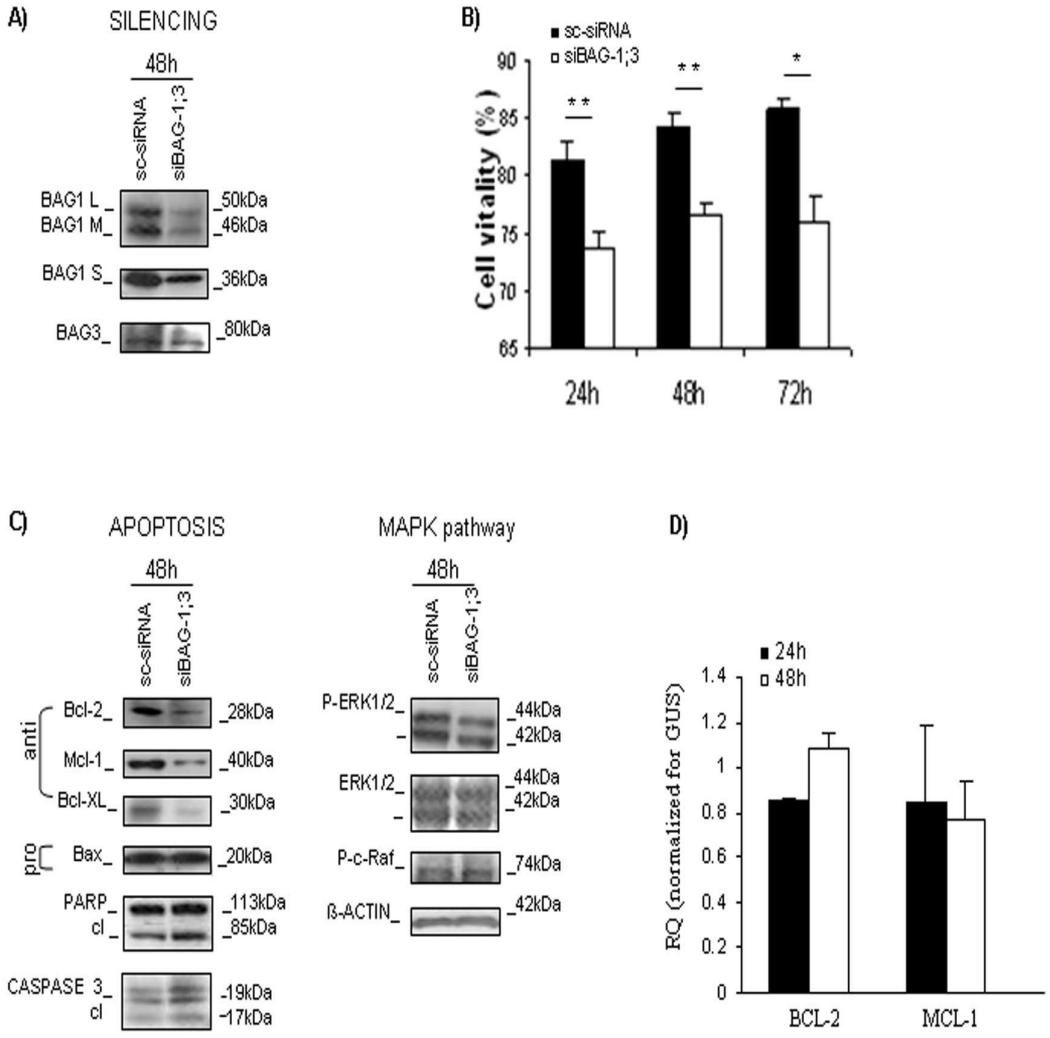


*Figure R11. BAG-1;3 co-silencing (BCS) effects on HL60 cells. A) WB analysis was done to check the silencing efficiency. Decrease in BAG-1 and BAG-3 proteins was confirmed. B) Cell vitality calculated 24h, 48h and 72h after BCS. Statistical significance, calculated for BCS samples and compared with sc-siRNA control, was marked with one (*p<0.05) or two (**p<0.01) asterisks. C) WB analysis for pro- and anti-apoptotic proteins and MAPK pathway proteins expression. ß- actin served as a control of correct protein loading. D) RQ-PCR analysis for relative Bcl-2 and Mcl-1 mRNA expression after BCS, calculated in respect to sc-siRNA control which corresponds to RQ=1 on epsilon axis, showing relative quantification results normalized for GUS gene.*

The observed misbalances in anti-apoptotic proteins turnover were not correspondent to a similar reduction of corresponded mRNA expression (*Figure R11D*), but explain previously observed cell behavior. The same results seen after BCS in HL60 cells were confirmed in a different cell line (RS 4;11) that was used in a control experiment (Figure R12 A-C).

The BCS supported the role of BAG-1 in maintenance and on progression of AML by participating with BAG-3, but likely with other important factors, which are to be discovered.

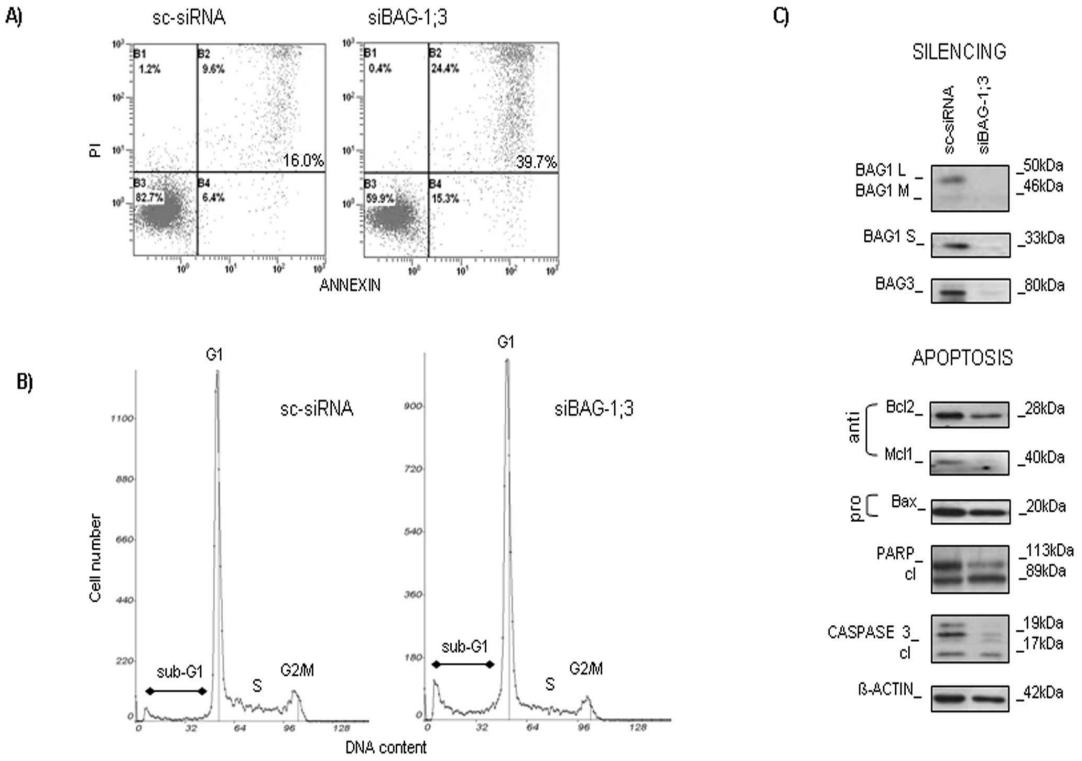


Figure R12. Effects of BCS in RS 4;11 cell line. A) Cell death determined with An/PI assay showed higher %age of dead cells after BCS in respect to sc-siRNA control samples. B) Cell cycle was affected after BCS, showing increase of the cells in sub-G1 [(sc-siRNA vs siBAG-1;3) = (3.8 vs 8.7) %] phase and lower number of the cells in G2/M phase[(sc-siRNA vs siBAG-1;3) = (10.2 vs 7.4) %)] and S phase [(sc-siRNA vs siBAG-1;3) = (10.8 vs 7.9) %)].G1 phase varied slightly [(sc-siRNA vs siBAG-1;3) = (75.1 vs 76) %)] C) WB analysis for silencing efficacy validation and pro- and anti-apoptotic proteins expression after BCS (BAG-1;3) or sc-siRNA transfection. ß- actin was used as a protein loading control.

3.10. BAG-1 and BAG-3 co-silencing (BCS) and cell sensitivity to Etoposide. Since BAG-1 and BAG-3 co-silencing caused down-regulation of anti-apoptotic proteins, in particular Bcl-2 and Mcl-1, which are well known protectors from cell death, we asked if HL60-BCS cells

would be more sensitive to drug addition. To test if this could be a case, we treated HL60-BCS and sc-siRNA transfected cells with *Etoposide (Eto),* drug widely used in leukemia treatment. Although we could detect a significant death induction in HL60-BCS compared to sc-siRNA HL60 cells [*Figure R13A*, mean death rate at 24 h of *Eto* treatment: (sc-siRNA vs BCS) = (22.9 vs 32.3) %; at 48 h: (28 vs 37.8) %; 24 h ctr (sc-siRNA vs BCS) = (10.5 vs 13.9) %; at 48h (14.6 vs 21.1) %; n = 3, *p < 0.05, **p < 0.01, ***p < 0.001], this death could be assigned in major part to BCS, while drug contribution was little (around 5 %), and increased lightly after dose augmentation. Consistent with An/PI analysis, 24 h of drug treatment at the protein level did not caused significant changes beside the ones already observed after BCS. Principally, enhanced proteolytical cleavage of caspase-3 and PARP, proteins considered as a specific sign of apoptosis, occurred, making thereby no additional influence over Bax or Bcl-2 protein level which were already affected by BCS (*Figure R13B*). However, with increased dose, the differences in death between two samples showed tendency for a slight increase, although this increase was not sufficient enough for a detection at the protein level as well.

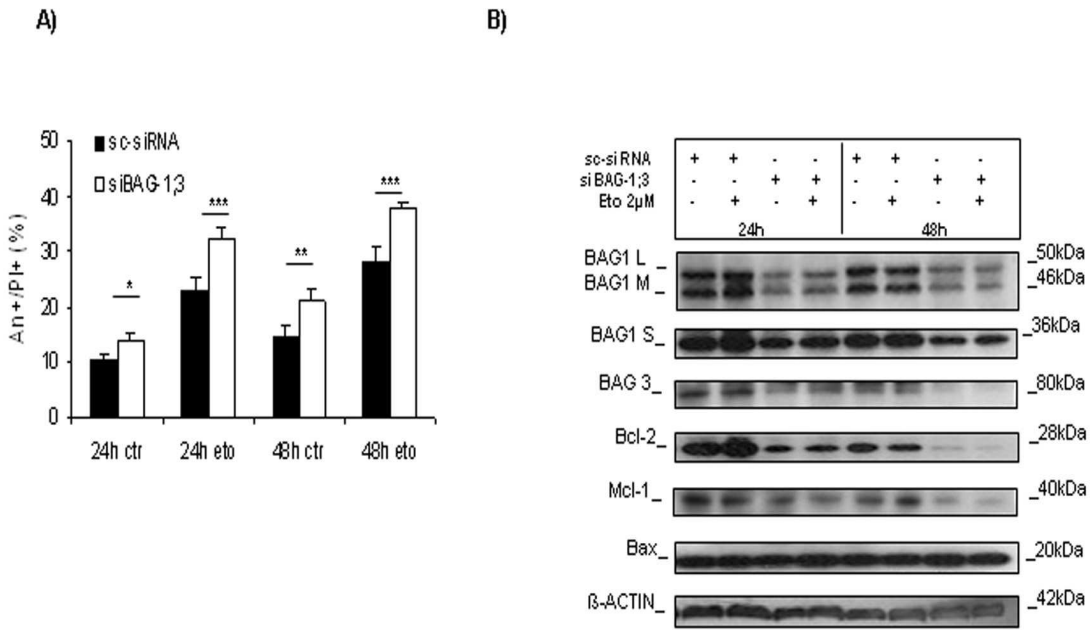


*Figure R13. Effects of BCS and Etoposide treatment on HL60 cells. A) Percent of AnV +/PI+ death cells after BCS (white bars) and sc-siRNA (black bars) transfection, calculated for three independent experiments 24h and 48h after Eto application (*p<0.05;**p<0.01;***p<0.001). B) WB analysis for the protein expression after Eto application comparing with no drug treatments. ß- actin was used as a protein loading control.*

3.11. BAG-1;3 co-silencing in primary AML cells. When BCS was applied at the HL60 cell line, we could identify the changes in leukemic cell behavior, assigning it to be caused mostly by BAG-1. We wondered if the same effect could be obtained after BCS of primary AML cells, derived from the AML patients at the moment of diagnosis. Therefore, after application of the BAG-1 and BAG-3 co-silencing approach used for the transfection of HL60 cell line, but optimized for primary cultures, we repeated functional analysis done previously. Once again, silencing was confirmed at mRNA and protein level (only protein decrease shown at the *Figure R14A*) and a more effective silencing was validated for *BAG-1* gene. Cleavage of caspase-3 and PARP proteins, that occurred more evidently after BCS (*Figure R14B),* was a sign of apoptosis activation. The expression of anti-apoptotic proteins Bcl-2, Bcl-X_L and Mcl-1, was decreased, while level of Bax protein remained unchanged (*Figure R14B*), confirming the results seen after BCS of HL60 cells. Repeated densitometric analysis confirmed a prevalence of Bax over Bcl-2 protein after BCS in respect to control sc-siRNA samples (Bax/Bcl-2 ratio for sc-siRNA = 0.70; Bax/Bcl-2 ratio for siBAG-1;3 = 1.05) which could explain the activation of mitochondrial type of apoptosis which was followed by the observed increase in cytochrome *c* release in cytosol of primary AML cells. When we checked the MAPK pathway we could confirm that even in AML primary cells BAG-1;3 silencing was able to influence the expression of only active ERK1/2 protein form, without changing total ERK1/2 expression (*Figure R14C*). In primary cell cultures, as in leukemic cell lines, after single BAG-1 and BAG-3 silencing a reciprocal correlation between BAG-1/BAG-3 mRNA expressions was confirmed (*Figure R14D*).

While single BAG-1 silencing did not influence cell cycle appearance (*Figure R15A-b*), BAG-1;3 co-silencing provoked increased number of the cells in sub-G_1 phase (25 ± 8 %) with respect to control sc-siRNA (16 ± 7 %; n = 3, p = 0.2; *Figure R15A-a,c*). A decreased percentage of the cells in G_2/M phase was also observed (BCS vs sc-siRNA = (7 ± 3) % vs (14 ± 4) %, n = 3, p = 0.07). Following, we checked if the expression of cell cycle regulatory proteins including cyclin D1, CDK2, p21 and p27 was influenced in AML-BCS cells. We could validate that the most influenced was the expression of cyclin D1, which decreased after BCS, while CDK2 was lightly changed, and p27 and p21 were almost unaffected. (*Figure R15B*).

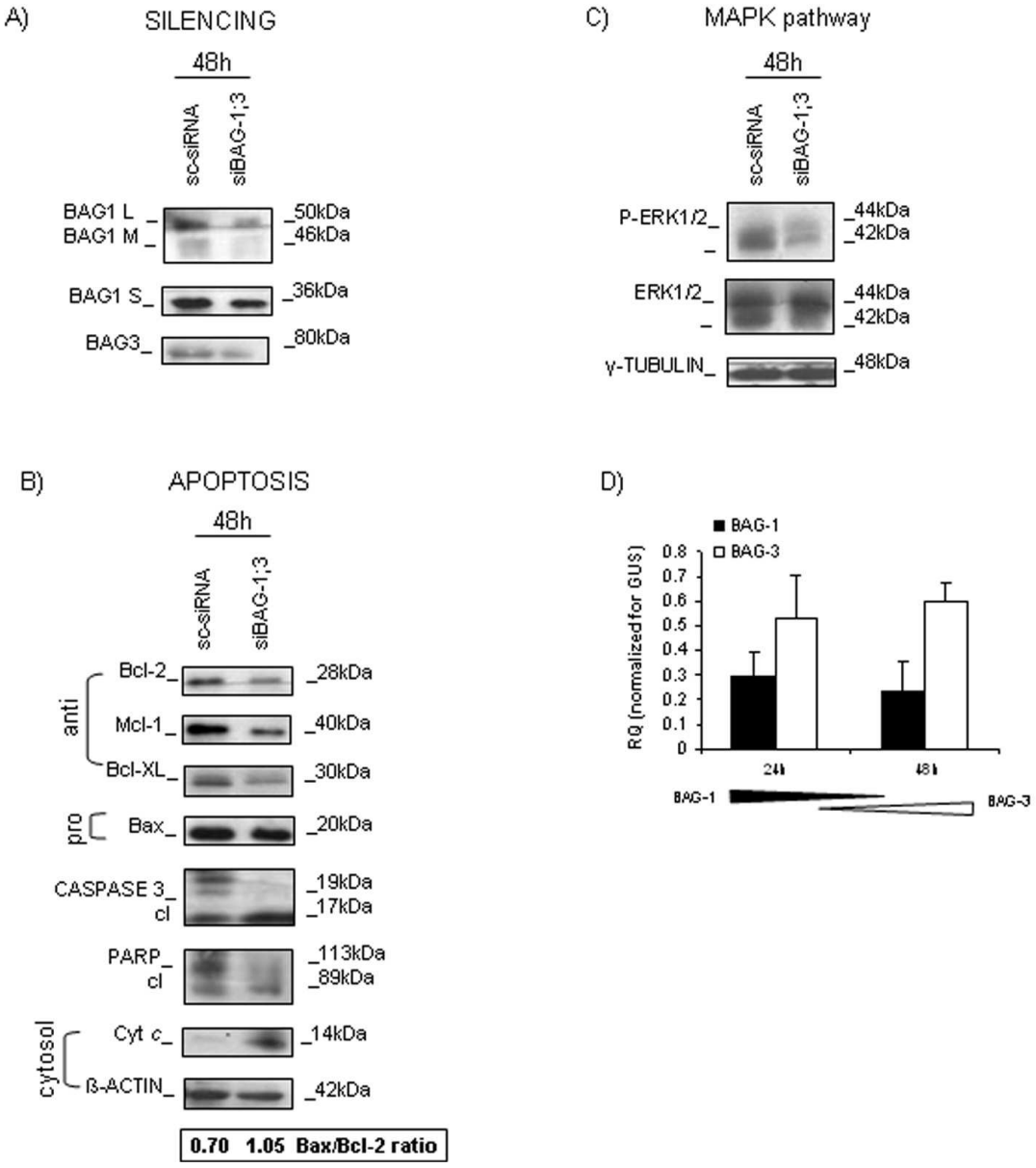


Figure R14. Effects of BCS on primary AML cell culture. A) WB analysis of BAG-1 and BAG-3 silencing efficacy. B) WB analysis for pro- and anti-apoptotic proteins and MAPK pathway proteins expression. D) RQ-PCR analysis for relative BAG- and BAG-3 mRNA expression after single BAG-1 and BAG-3 silencing, calculated in respect to sc-siRNA control. A reciprocally controlled expression between BAG-1 and BAG-3 mRNA are shown with black (BAG-1) and white (BAG-3) bottom arrows. ß- actin or γ- tubulin were used as controls for the adequate protein loading.

Since we were more interesting in defining BAG-1 role in apoptosis regulation, we did not perform more profound research on the way BAG could influence cyclin D1 level. However, since it has been demonstrated that BAG-3 can protect cyclin D1 from degradation, contributing in that way to cell cycle maintenance[168], we presumed that observed cell cycle alteration could be, at least in part, conferred to BAG-3 protein, but we can not exclude a

possibility that BAG-1 has some contribution itself. This is because it has been already shown that cyclin D1 is degraded by proteasome, which activity is mainly regulated by BAG-1.

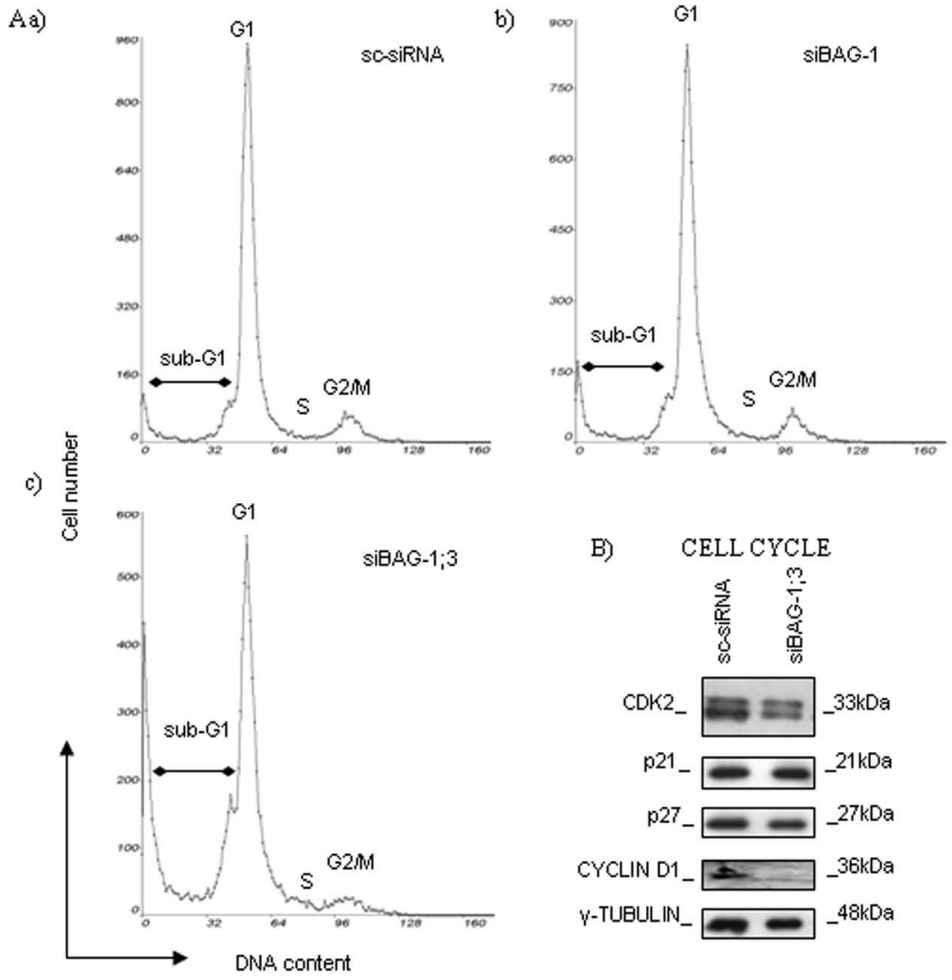


Figure R15. Effects of BCS on cell cycle of primary cell cultures. A) Cell cycle appearance after control silencing (a) single BAG-1 silencing (b) or BCS (c). Increased number of cells in sub-G1 phase and decrease % of the cells in G2/M phase could be seen only after BCS. B) WB analysis for the cell cycle regulatory proteins expression. γ- tubulin served as a protein loading control.

Next, we went to see what effect would have BCS in healthy bone marrow (hbm) specimens, where low expression of BAG-1 protein was detected. As we expected, down-modulation of BAG-1;3 had important impact on cell cycle appearance (*Figure R16*), confirming a basal BAG-1 expression as important for survival and proliferation of normal haematopoietic cells.

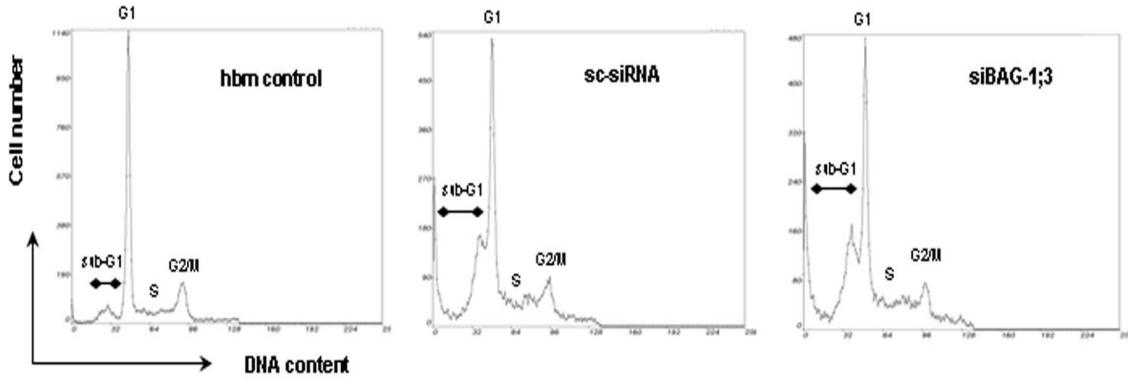


Figure R16. Effects of BCS on cell cycle appearance in hbm samples. Cell cycle was controlled 24h after transfection with siBAG-1;3 or sc-siRNA. The phases of cell cycle were influenced in respect to control non-transfected sample as followes: sub-G1 phase: (control vs sc-siRNA vs siBAG-1;3) = (5.6 vs 14.6 vs 20) %; G1 phase: (control vs sc-siRNA vs siBAG-1;3) = (69 vs 58 vs 54) %; S phase: (control vs sc-siRNA vs siBAG-1;3) = (9.6 vs 12.4 vs 12.2) %;G2/M phase: (control vs sc-siRNA vs siBAG-1;3) = (15.7 vs 14.9 vs 13.8) %.

RESULTS PART III:

Study of possible BAG-1's mechanism of action in AML

3.12. BAG-1 and proteasome-dependent protein degradation. The ability of BAG-1 to protect certain proteins from degradation, as Bcl-2 or Mcl-1, triggered us to determine whether decreased protein levels, observed after co-silencing, could be a consequence of their higher proteasome degradation. Therefore, we checked if the protein loss, observed after BCS for Mcl-1, Bcl -2 and Bcl-$X_{L,}$ could be restored after the artificial block of proteasomal function. Treatment of silenced cells with reversible proteasome inhibitor Z-LLF-CHO for 4h increased all three protein levels in either BCS HL60 or primary AML cells (*Figure R17*), although the increase was the most evident for Mcl-1 protein. This result can be explained by generally much faster degradation of Mcl-1 protein, while Bcl-2 and Bcl-X_L, which are more stable, degrade with a lower rate. We observed no significant changes in the expression of either BAG-1 or BAG-3 proteins after Z-LLF treatment. As a standard control of adequate Z-LLF function, a level of cell-cycle regulator p21 was checked, as it is confirmed to be rapidly degraded by the proteasome.[235]

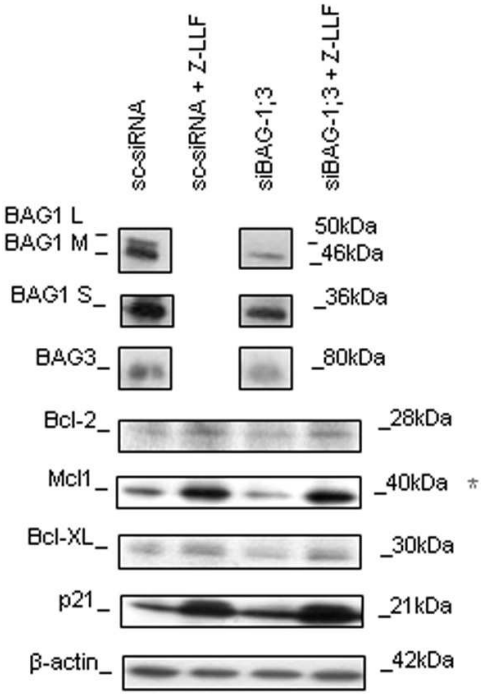


Figure R17. Effects of proteasome inhibition after BCS in primary cell cultures. The decrease in protein expression caused after BCS was restored after Z-LLF-CHO treatment . The effect was the most evident for the Mcl-1 protein, as indicate by asterisk. ß- actin was used as a control for adequate protein loading.

These results confirmed that BAG-1 is important in AML for a protection of the proteins, in particular Mcl-1 and Bcl-2, from a proteasome degradation machinery. The role we attributed specifically to BAG-1 owing to previously published data which describe BAG-1 as essential for effective degradation of polyubiquitinated (polyUb) proteins by the proteasome system,

while in contrast, BAG-3 was identified to stimulate the turnover of polyUb proteins by macroautophagy.

3.13. Interaction partners of BAG-1 protein. According to our results, BAG-1 is likely an important regulator of protein turnover inside the AML cell, where in the case of over-expression, it can significantly influence regular protein homeostasis. To determine if described "protective" function of BAG-1 was a result of direct interaction with anti-apoptotic proteins, we did co-immunoprecipitation analysis. Results proved a direct contact of Bcl-2 by BAG-1 protein, but no interaction between BAG-1 and Mcl-1 was found (*Figure R18*).

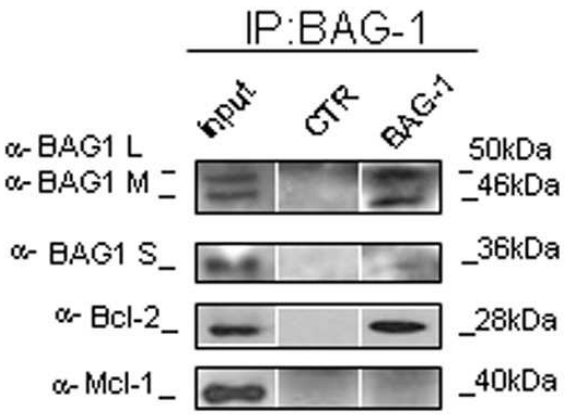


Figure R18. Immunoprecipitation (IP) analysis. BAG-1 primary antibody was used for IP, while WB, done afterwards, was used for a control of BAG-1, Bcl-2 and Mcl-1 presence. Only Bcl-2 was confirmed as a target for BAG-1 (the same results were seen after IP done with BAG-3 primary antibody).

From the last result, we could deduct that only in the case of Bcl-2, BAG-1 was directly involved in its protection from proteasome mediated degradation, making it rapidly degraded after BAG-1 suppression. Similarly, previous research confirmed BAG-1 over-expression to be responsible for increased Bcl-2 level. Both data speak in favor to BAG-1's protective role on Bcl-2 in AML, as demonstrated for other tissues.

3.14. Usp9X, a novel interaction partner of BAG-1. The immunoprecipitation analysis done for the control of eventual BAG-1 / Mcl-1 interaction resulted as negative. Therefore, a decrease in Mcl-1 protein level, which we confirmed to be proteasome-dependent itself, was not a result of direct BAG-1 involvement in our experimental model. We asked through which mechanism BAG-1 was able to regulate the expression of Mcl-1, protein which was reported to be important for AML phenotype sustaining. We presumed that some co-interacting molecule was involved, influencing together with BAG-1 the level of Mcl-1 protein. We focused our attention on two proteins, Aurora-A and Usp9X, as main targets by which BAG-1 could influence Mcl-1 protein expression. Both of these proteins have recently been described as important for Mcl-1 expression regulation, and both resulted to be connected with proteasome machinery in other cancers.

Aurora-A belongs to the Aurora protein kinases family which counts for three members named -A, -B and –C. Increased attention has been focused on Aurora-A kinase because of its suggestive role in tumorigenesis.[236-238]

This protein is also implicated in the cell cycle regulation and determination of the entrance in G1 phase. It has been confirmed that the level of Aurora-A, before cells progress into the G1 phase, is temporally regulated by Ub-dependent proteolysis which occurs via proteasome.[239]

Recently, from a study done in HeLa cells, a confirmation that Aurora-A has the ability to control the splicing of *Mcl-1* mRNA, favoring production of small, pro-apoptotic form of the Mcl-1 protein, has come out.[240]

In our analysis, while both proteins immunoprecipitated well with their own antibodies, co-immunoprecipitation between BAG-1 and Aurora-A controlled with reciprocial primary antibodies (α) gave no positive results for their eventual interaction, excluding this molecule to play a role with BAG-1 in the protein turnover process in AML (*Figure R19*).

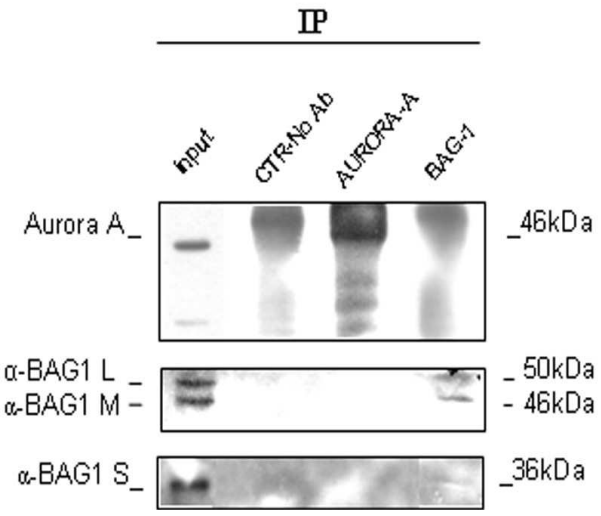


Figure R19. Immunoprecipitation analysis for BAG-1 – Aurora-A interaction in HL60 cells. Both, BAG-1 and Aurora-A, primary antibodies were used for IP analysis and, in following, WB was run to control for either Aurora-A and BAG-1 presence.

We moved on to another protein candidate named Ubiquitin-specific protease 9X (Usp9X). This protein is a deubiquitylating enzyme who can hydrolyze Ubs from substrates prior to proteasomal entry, extending thus a half-life of the certain proteins.[241] In different systems, as one of the target protein for Usp9X has been shown to be Mcl-1. Removal of Ubs protects Mcl-1 from proteasomal degradation, prolonging its existence within the cell and hence, protecting from apoptosis induction.[132] It has been seen that deubiquitinase commonly have to interact with some other protein in order to become active or their activity depends of direct interaction with proteasome.

To test if BAG-1 co-worked with Usp9X in Mcl-1 protection, we immunoprecipitated Usp9X and BAG-1 using correspondent primary antibodies and repeated western blot analysis using anti (α) - BAG-1 and α - Usp9X, respectively. As we could confirm, all the proteins resulted as well immunoprecipitated with their own primary antibodies, but also as positive was seen co-immunoprecipitation of Usp9X with α - BAG-1 antibody and vice versa (*Figure R20*).

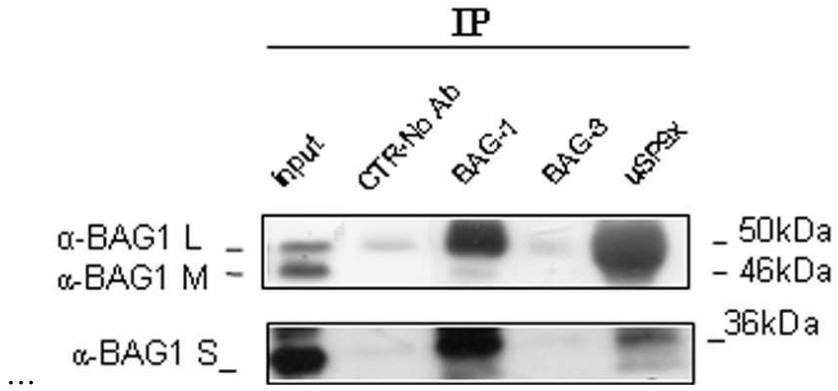


Figure R20. Immunoprecipitation analysis for BAG-1 – Usp9X interaction in HL60 cells. Both, BAG-1 and Usp9X, primary antibodies were used for IP analysis and, in following, WB was run for the control of Usp9X and BAG-1 presence, respectively.

The same results were obtained when IP analysis were repeated in ML2 and K562 cell lines (data not shown), confirming Usp9X as a novel BAG-1 interacting partner through which, most likely, BAG-1 controls Mcl-1 protein expression in AML. The possible scheme for a mechanism of BAG-1 action in AMl are presented below (*Scheme 2*), where a new protein partner, Usp9X, has been added.

Nevertheless, BAG-1;3 silencing did not cause the changes in Usp9X protein expression, and neither in the expression of CHIP E3 ligase (*Figure R21*) included on the *Scheme 2*, so we presume that BAG-1 could regulate the activity of Usp9X. In this way, BAG-1 might support the Usp9X function contributing therefore to a more efficient Mcl-1 deubiquitination. However, the last result presented here has opened a whole new amount of questions and functional experiments to work on and discuss about in some of the future chapters of BAG-1 story.

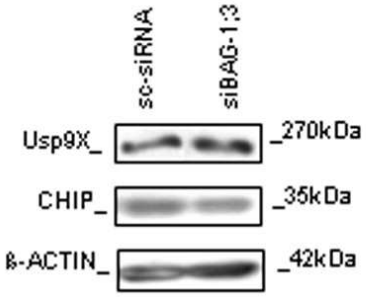
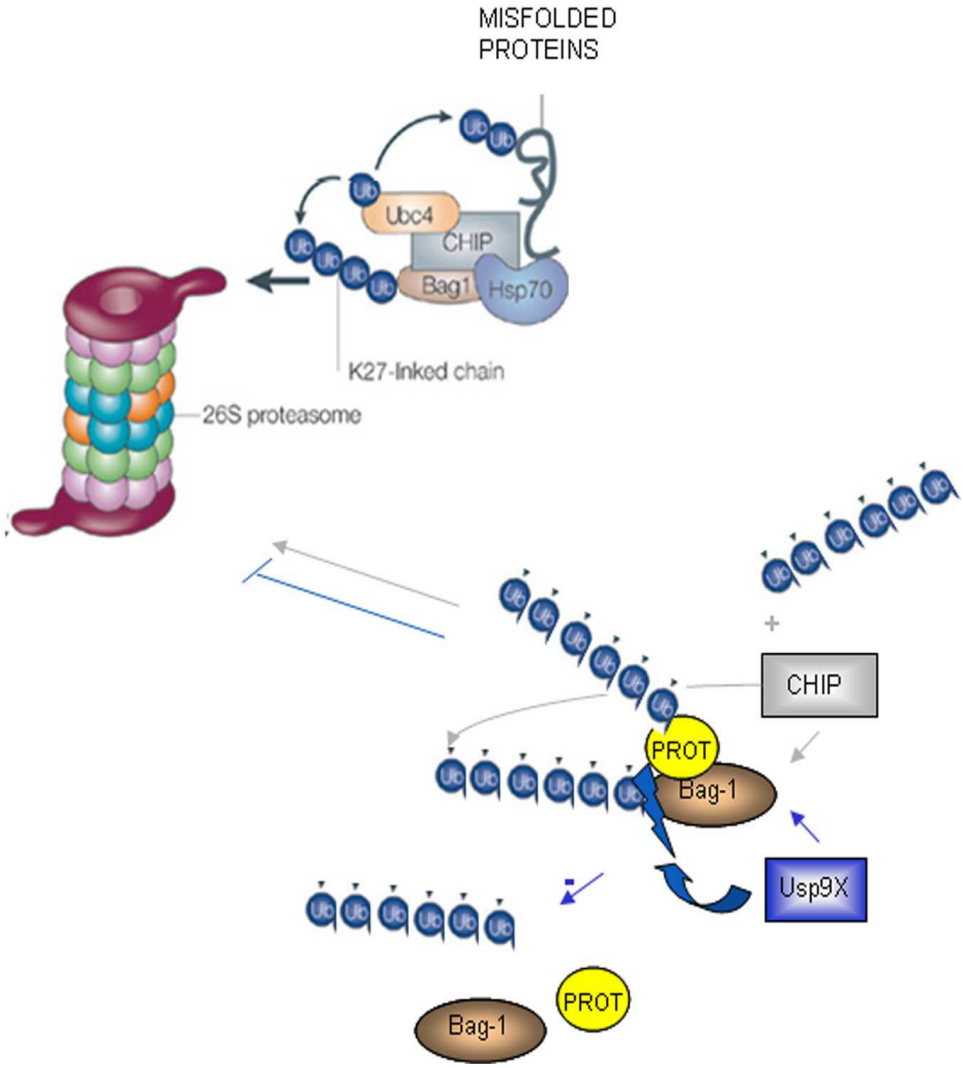


Figure R21.Effects of BCS on CHIP and Usp9X expression in HL60 cell line.



Scheme 2. Possible model of action inside AML cells for BAG-1. BAG-1 can interact with proteasome leading some certain proteins for degradation, but can also protect some proteins from proteasome derived degradation. A mechanism through which this role could be accomplished include E3 ligase CHIP, but, as we could see, likely deubiquitinase Usp9X as well, since both proteins show a capability to interact with BAG-1 protein. Therefore, a ratio between CHIP and Usp9X expression could be crucial in determination of the final BAG-1 action, regulating either Ub binding or Ub removing from the substrates. Also, it could be possible that BAG-1 supports Usp9X in its action. However, a future studies are opened to give the answer for the precise BAG-1/Usp9X connectivity.

DISCUSSION

Recent functional and expression studies reported BAG-1 as frequently over-expressed or deregulated in diverse cancer types where it provides a robust protection from cell death.[226,227] The essential role of BAG-1 for the survival and differentiation of haematopoietic and neuronal cells has been reported after BAG-1 knock-out experiments in mice.[190] However, little is known about BAG-1 expression and mechanism of action in leukemic cells of myeloid origin, where a great deal of research has been focused on apoptosis-related Bcl-2 family proteins, a challenging molecular therapeutic targets.[137,138,242] All members of Bcl-2 protein family have been reported to be important for the maintenance of AML phenotype. It has been confirmed that high Bcl-2 level or high Bcl-2/Bax ratio at diagnosis results in poor clinical outcome of patients with de novo AML[243], while elevated Mcl-1 expression has been detected in patients with relapsed AML.[145] Being associated with the Bcl-2 family, it is not surprising that BAG-1 can influence their function.

In present study, observed differences in expression of BAG-1 protein isoforms between a group of healthy donors on one side and patients with AML or ALL, on another, could indicate that BAG-1 has important role in leukemia lineage commitment. The changes in subcellular localization of BAG-1 isoforms at different moments of leukemia, explain us that, beside expression intensity, the position inside the cell is a parameter that should be considered as significant for the final outcome of the disease. Moreover, a nonlinear correlation between BAG-1 mRNA and protein levels verified in cell lines and in leukemia patients, implies that the post-transcriptional control mechanism plays a crucial role in dictating the expression of each BAG-1 protein isoform.

BAG-1 over-expression was previously reported to increase expression of Bcl-2 protein.[244] In our experiments, by the opposite silencing approach, artificially stimulated down-modulation of BAG-1 protein was able to reduce Bcl-2 expression in AML. At the same time, siRNA suppressed BAG-1 expression was able to influence the MAPK-pathway most likely due to diminished interaction with c-Raf, which in turn led to a partially phosphorylation and, hence, partial activation of downstream target ERK1/2, considered as hallmark of cell proliferation. However, as we could see, BAG-1 reduction influenced faintly AML cell proliferation by affecting RAF/MEK/ERK proliferative pathway. The major impact after BAG-1 silencing was observed at the level of apoptosis-related proteins, in particular Bcl-2, which expression was impaired dramatically. In addition, minor decline in expression was noted for the other

two Bcl-2 family proteins, Mcl-1 and Bcl-X_L. Even though functional analysis, done after single BAG-1 silencing, demonstrated that leukemic cell proliferation and survival were insignificantly damaged, they imply BAG-1's co-involvement in the control of these two processes.

Observed up-regulation of BAG-3 mRNA and protein, identified after BAG-1 silencing, and vice versa, pointed toward idea that close relation in expressional regulation of those two BAG family members existed in AML. It was not the first time that a reciprocal relation between BAG-1 and BAG-3 expression was suggested. The same results were demonstrated in aging model, where high level of one BAG member was accompanied with lower expression of opposed one.[169] This fact could also explain the ability of BAG-3 to compensate in function for BAG-1 deprivation. Indeed, BAG-1 and BAG-3 co-silencing was demonstrated to control significantly the leukemic cell faith, provoking more significant down-regulation of not only Bcl-2 and phospho-ERK1/2, but also of Mcl-1 and Bcl-X_L proteins, which was followed by the enhanced cell death in either cell lines or in primary AML. At the same time, massive cell death that we could observed after BAG-1;3 co-silencing of healthy bone marrow cells, specified that basal expression of BAG-1 protein was crucial for normal haematopoiesis to occur.

In contrast to the reduction at the Bcl-2, Mcl-1 and Bcl-X_L protein levels, BAG-1;3 co-silencing did not sustain reduction in mRNAs expression, implying that BAG proteins controlled foremost the protein levels without affecting gene transcription. Degradation of the Bcl-2 anti-apoptotic family proteins was shown to be actualized, at least in part, through proteasome. From these data and documentations that BAG-1 could regulate proteasome derived degradation of certain proteins, we presumed that in AML cells BAG-1 might have the ability to keep protected from degradation the pro-survival molecules, preventing their delivery to proteasome. As we demonstrated, this protection occurred to be direct in the case of Bcl-2, but indirect for the Mcl-1 protein. However, the number of proteins that BAG-1 can interact with, influencing in that way their function, is increasing constantly. Recently published data demonstrated that BAG-1 can recognize immature BCR-ABL protein and govern it to a proteasome for a degradation.[245] We expanded the list of BAG-1 protein partners and include deubiquitinase Usp9X, as a possible novel candidate through which BAG-1 realizes an indirect protection of Mcl-1, protein shown to have a role in maintenance of AML. Through this interaction, BAG-1 likely contributes to the protection from proteasomal degradation of Mcl-1 protein when over-expressed and in opposite, makes Mcl-1 easily degraded when reduced. In addition, a discovery that BAG-1 could interact with both,

ubiquitine ligase (such as CHIP) and deubiquitinase (Usp9X), indicates that regulation of protein protection/degradation by BAG-1 might be more complex then it was thought till now and should be studied more in depth.

Majority of tumor cells contain sufficient quantity of pro-apoptotic proteins to trigger effectively cell death. However, these proteins are blocked when anti-apoptotic ones are highly expressed. In concordance, Bcl-2 inhibits apoptosis by forming inactive heterodimers with Bax, described as dominant pro-apoptotic molecule for the release of cytochrome c from mitochondria.[125] By diminishing the expression of Bcl-2 and Mcl-1 proteins, BAG-1;3 co-silencing could favor the function of pro-apoptotic protein Bax, which expression remains non influenced in our model. Thus, in leukemic cells BAG-1, and possibly BAG-3, seems to modulate the balance between survival and apoptotic signals, assuring apoptosis activation when pro-apoptotic peptides of Bcl-2 family, prevail over anti-apoptotic ones.[11,45,46] contributing to an increased release of cytochrome *c* and Smac/DIABLO. These results indicate mitochondria-mediated apoptosis to be responsible for a death noted in AML.

The experiments done with etoposide showed a small additional pulse on cell survival along with the silencing-provoked death induction, but however is not to be considered as irrelevant. BAG silencing might provide an effective means to influence Bcl-2 and Mcl-1 protein stability, and hence improve the therapeutic effectiveness of anti-leukemic drugs in AML patients where high level of Bcl-2 or Mcl-1 confers for a drug resistance(ref 63 rad 2000) [248,249]

Taken all together, our results demonstrated that BAG-1, but also BAG-3, should be considered more in serious as the candidates that contribute to the AML maintenance. Likely, they are not sufficient to cause leukemia by them own, but, when over-expressed, sustain the leukemia phenotype. Therefore, we could say that BAG-1;3 present the important side players which should not be postponed. The results which indicate that the BAG-1;3 co-silencing modulates the expression of proteins important for cell survival, proliferation, but also cell cycle progression in AML, could propose BAG-1 and BAG-3 down-regulation as an approach for a degradation of proteins which often result as highly express in leukemia, making it less responsive to standard therapy.

CONCLUSIONS

Although over-expression of BAG-1 has been documented in many types of tumors, our is among the first reports about BAG-1 expression and potential therapeutic implication in human de novo AMLs. After initial confirmation that BAG-1 protein shows high expression in acute leukemia, we concentrated on defining the significance that BAG-1 has for the maintenance of the AML phenotype. We considered silencing approach as the most suitable for a determination of BAG-1's role in AML cells and applied it in our experimental models. Since transient BAG-1 silencing weakly influenced leukemia phenotype, we immediately include its family member, BAG-3, in silencing approach. By keeping BAG-3 expression low, and significantly decreasing BAG-1 level, we could see a considerable impaired cell vitality, caused by apoptosis induction. Beside apoptosis, we could confirm that cell proliferation was impaired after BAG-1 silencing, through a partial down-modulation of active ERK1/2 protein. Therefore, in AML, these two pathways seem to represent the main points where BAG-1 performs. Without doubt, the most important role of BAG-1 protein considers protein turnover regulation, specifically the one signed by proteasome system for Bcl-2 and Mcl-1 proteins.

In summary, we described a close relationship between BAG-1 and BAG-3 in AML and their down-regulation as restrictive for cell survival. Moreover, we enlarged the list of BAG-1 interacting proteins for one deubiquitinase, Usp9X, and opened a door for a new studies which could contribute to a better understanding of the complete mechanism by which BAG-1 express its function within AML. However, research should not stop here and further investigations of this molecule might help in better defining a potential effective therapeutic strategy involving BAGs silencing or antibodies mediated BAGs targeting.

ACKNOWLEDGEMENTS

To my family and close-to-heart friends

First and foremost, I would like to express my deep heartful gratitude to my entire family, who were always there during the time I have dedicated to my PhD thesis, encouraging me despite being hundreds of kilometers away. I would also like to thank to my friends all over the world, who were supporting me from a distance too.

During the previous three years of my PhD studying in Padova, at the Oncohematology Lab of Paediatric Department, I believe there was no person who did not help me at least once, or if I think better, two, three, four...times. People were coming and leaving and hence, situation was changing from a period to period, but no mater what, I have always been a part of a laboratory group. So, I cannot allow myself to leave a side any person, any name. Considering though that a list would be very long, I am extremely sorry beforehand if I should keep out deeper description and more precise thank message for the persons who have offered me help whenever I asked it for.

At first, I would like to take this opportunity and thank Prof. Dr Giuseppe Basso for providing me the opportunity to work in The Oncohematology Lab, following and believing in my thesis from the very zero we started from. Then, I would like to say thanks to Martina Pigazzi, a person who had to bear me every day during my development as a scientist. I was a stubborn sometimes but in spite of that her sustain was always present. I would also like to thank for giving me the wonderful opportunity to work in her group and for supervising my thesis in a way everyone should do, guiding me through this project in a good direction and encouraging me to continue even when I could see no way out.

In a chronological order, my adventure as a scientist began with Maddalena Elemana, Fran, Emmi, Manu Giarin, Minni, Sara, Elli, Ale Beghin, Ila, Silvia, Luca Trentin, Glori, Luisa, Benni, Piero, Robbi, Anto, Eppi, BB (Barbara), Marco, Truss, Claudia, Paola, Chiara, Luca, Elena Seganfreddo, Giulia, the girls from the first room, Silvia, Ale, Grazia, Barbara, Samuela , from cytogenetic Ana Maria, Vittoria, Sara, Anna, and at the end Andrea, without whom this thesis would have been "gone with the wind".

Words cannot describe the thanks that I owe to all the moments on work and the time outside the working hours that you all devoted to me. You made me feel like at home during my stay in Italy with all those nice spritz sessions and personal discussions we had together. I would like to thank to the ones who were there with me every time when my chips were down, when I had to share my happiness and moments when I was a little bit "giù".

This work would have been impossible without the active support of all the people mentioned here. Thanks you all guys.

DICTIONARY:

Aplastic anemia - condition where bone marrow does not produce sufficient number of new cells to replenish old blood cells.

Blasts - Immature blood cells.

Centrosome – cytoplasm organelle important for mitotic type of cell division and definition of opposite poles of the cell during mitosis.

Chaperone - Any protein that is required for the proper folding or assembly of another protein or protein complex without being a component of the final structure.

Clone - A cell, group of cells, or organism that is descended from and genetically identical to a single common ancestor, such as a bacterial colony whose members arose from a single original cell.

Complementary DNA (**cDNA**) – DNA synthesized from a mature mRNA template in a reaction catalyzed by the enzyme reverse transcriptase.

Granulocytes - A type of white blood cell filled with microscopic granules that are little sacs containing enzymes, compounds that digest microorganisms.

HeLa – stabile cell culture originally obtained from cancerous cervical tissue

Histone - A protein around which DNA coils to form chromatin.

Mixed lineage leukemia - type of childhood leukemia in which a piece of chromosome 11 has been translocated (broken off and attached itself to another chromosome).

Myelodysplastic syndrome - A group of bone marrow disorders characterized by the underproduction of one or more types of blood cells due to dysfunction of the bone marrow.

Neoplastic -An abnormal new growth of tissue in animals or plants; a tumor.

Neutropenia – counts for low number of neutrophils, a type of white blood cells.

Quiescent – state of cells inactivity or repose.

Remission - Disappearance of the signs and symptoms of cancer or other disease.

Relapse - The return of signs and symptoms of a disease after a patient has entered a remission.

Scenescent – growing old, aging

siRNA – counts for small interfering RNA, a class of double-stranded RNA molecules, 20-25 nucleotides in length, involved in major part in RNA interference pathway.

Thrombocytopenia - refers to a decreased number of platelets in the blood.

WW domain – a protein domain with two highly conserved tryptophans that binds proline-rich peptide motifs.

5' CAP - specially altered nucleotide on the 5' end of precursor messenger RNA (mRNA) important for normal mRNA maturation and adequate protein production (translation).

List of publications:

Colovic N, Tosic N, Aveic S, Djuric M, Milic N, Bumbasirevic V, Colovic M, Pavlovic S. Importance of early detection and follow-up of FLT3 mutations in patients with acute myeloid leukemia *Ann Hematol* 2007; 86:741–747.

Stojiljković M, Stevanović A, Đorđević M, Petručev B, Tošić N, Karan-Đurašević T, Aveić S, Radmilović M, Pavlović S. Mutations in the PAH gene: A Tool for population genetics study. *Archives of Biological Sciences* 2007; 59(3): 161-167.

Aveic S, Pigazzi M, Basso G: BAG-1 and BAG-3 as modulators of apoptosis - related proteins in acute myeloid leukemia. (manuscript in preparation)

Abstract to national / international congresses:

"BAG-1 IN ACUTE LEUKEMIA" - 15th Congress of EHA, Spain

"BAG-1 esspressione in leucemia acuta" - AIEOP in Lab workshop, Italy

"BAG-1 expression in acute leukemia" - ESH international Conference on "Mechanisms of cell death and disease, Portugal.

REFERENCES:

1. Graves M. Cancer – The Evolutionary Legacy. Oxford: Oxford University Press. 2000; 196-197.

2. Waxman S, Anderson KC. History of the development of arsenic derivatives in cancer therapy. Oncologist. 2001; 6: 3–10.

3. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med. 2006; 354:166–178.

4. Creutzig U, Zimmermann M, Ritter J, et al. Treatment strategies and long-term results in paediatric patients treated in four consecutive AML-BFM trials. Leukemia. 2005; 19:2030–2042.

5. Gibson BE, Wheatley K, Hann IM, et al. Treatment strategy and long-term results in paediatric patients treated in consecutive UK AML trials. Leukemia. 2005; 19:2130–2138.

6. Robertson S, Kennedy M, Keller G. Hematopoietic commitment during embryogenesis. Ann. N. Y. Acad. Sci. 1999; 872::9-15.

7. Kincade PW, Igarashi H, Medina KD, et al. Lymphoid lineage cells in adult murine bone marrow diverge from those of other blood cells at an early, hormone-sensitive stage. Semin. Immunol. 2002; 14: 385-394.

8. Muguruma Y, Yahata T, Miyatake H, et al. Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. Blood. 2006; 107: 1878-1887.

9. Fliedner TM. The role of blood stem cells in hematopoietic cell renewal. Stem Cells. 1998; 16: 361-374.

10. Domen J, Weissman IL. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol Med Today. 1999, 5: 201-208.

11. Domen J, Cheshier SH, Weissman IL. The role of apoptosis in the regulation of hematopoietic stem cells: overexpression of Bcl-2 increases both their number and repopulation potential. J Exp Med. 2000; 191: 253-264.

12. Orkin SH, Zon LI. Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. Nat Immunol. 2002; 3: 323-328.

13. Hao QL, Thiemann FT, Petersen D, et al. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. Blood. 1996; 88: 3306-3313.

14. Rogowski O, Sasson Y, Kassirer M, et al. Down-regulation of the CD62L antigen as a possible mechanism for neutrophilia during inflammation. Br J Haematol. 1998; 101: 666-669.

15. Crooks GM, Hao QL, Petersen D, et al. IL-3 increases production of B lymphoid progenitors from human CD34+CD38- cells. J Immunol. 2000; 165: 2382-2389.

16. Haylock DN, Horsfall MJ, Dowse TL, et al. Increased recruitment of hematopoietic progenitor cells underlies the ex vivo expansion potential of FLT3 ligand. Blood. 1997; 90: 2260-2272.

17. Lyman SD, Jacobsen SE. c-kit ligand and Flt3 ligand: stem/ progenitor cell factors with overlapping yet distinct activities. Blood. 1998; 91: 1101-1134.

18. Kinashi T, Springer TA. Steel factor and c-kit regulate cell-matrix adhesion. Blood. 1994; 83: 1033-1038.

19. Serafini A.. The Epic History of Biology. New York: Plenum. 1993: 395p.

20. Murray AW, Hunt T. The Cell Cycle: An Introduction. New York: Oxford University Press. 1993; 251p.

21. Vinella D, D'Ari R. Overview of Controls in the Escherichia Coli Cell Cycle. Bioessays 1995; 17(6): 527-36.

22. Forsburg SL, Nurse P.. Cell Cycle Regulation in the Yeasts Saccharomyces Cerevisiae and Schizosaccharomyces-Pombe. Annu Rev Cell Dev Biol. 1991; 7: 227-256.

23. Norbury C, Nurse P. Animal Cell Cycles and Their Control. Anuu Rev Biochem. 1992: 61: 441-470.

24. Gorbsky GJ. Cell Cycle Checkpoints: Arresting Progress in Mitosis. Bioessays 1997; 19(3): 193-197.

25. Clark BR, Keating A. Biology of bone marrow stroma. Ann NY Acad Sci. 1995; 770: 70-78.

26. Majka M, Janowska-Wieczorek A, Ratajczak J, et al. Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. 2001; 97:3075-3085.

27. Guenechea G, Gan OI, Dorrell C, Dick JE. Distinct classes of human stem cells that differ in proliferative and self-renewal potential. Nature Immunol. 2001; 2: 75-82.

28. Bruce WR, Van Der Gaag H. A quantitative assay for the number of murine lymphoma cells capable of proliferation in vivo. Nature 1963; 199, 79–80.

29. Bhatia M, Bonnet D, Murdoch B, et al. A newly discovered class of human hematopoietic cells with SCID repopulating activity. Nat Med. 1998; 4: 1038-1045.

30. McCulloch E. Stem cells in normal and leukemic hematopopoiesis (Henry Stratton Lecture). Blood. 1983; 62: 1-13.

31. Nowell P, Hungerford, D. A minute chromosome in human chronic granulocytic leukemia. Science 1960; 132, 1497-1498.

32. Baikie AG, Court-Brown WM, Buckton KE, et al. A possible specific chromosome abnormality in human chronic myeloid leukaemia. Nature 1960; 188: 1165–1166.

33. Lapidot T. A cell initiating human acute myeloid leukemia after transplantation into SCID mice. Nature 1994; 367: 645-648.

34. Steliarova-Foucher E, Stiller C, Kaatsch P, et al. Geographical patterns and time trends of cancer incidence and survival among children and adolescents in Europe since the 1970s (The ACCIS project): an epidemiological study. Lancet. 2004, 364: 2097–2105.

35. Coebergh JW, Reedijk AM, De Vries E et al. Leukaemia incidence and survival in children and adolescents in Europe during 1978–1997. Report from the Automated Childhood Cancer Information System project. Eur J Cancer. 2006; 42: 2019–2036.

36. Greaves MF, Colman SM, Beard ME, et al. Geographical distribution of acute lymphoblastic leukaemia subtypes: second report of the collaborative group study. Leukemia. 1993; 7: 27–34.

37. Stiller C. Childhood cancer in Britain: incidence, survival, mortality. Oxford, Oxford University Press, 2007.

38. Parkin DM, Stiller CA, Draper GJ, et al. International incidence of childhood cancer. Int J Cancer. 1988; 42(4): 511-520.

39. Hasle H, Clemmensen IH, Mikkelsen M. Risks of leukaemia and solid tumours in individuals with Down's syndrome. Lancet. 2000; 355: 165-169.

40. Gurney JG, Severson RK, Davis S, Robison LL. Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer 1995; 75: 2186-2195.

41. Stiller CA. Epidemiology and genetics of childhood cancer. Oncogene, 2004, 23: 6429–6444.

42. Belson M, Kingsley B, Holme A. Risk factors for acute leukemia in children: a review. Environmental Health Perspectives. 2007; 115: 138–145.

43. O'Connor SM, Boneva RS. Infectious etiologies of childhood leukemia: plausibility and challenges to proof. Environmental Health Perspectives. 2007; 115: 146–150.

44. Gilliland DG: Molecular genetics of human leukemias: new insights into therapy. Semin Hematol 2002; 39: 6-11.

45. Frohling S, Scholl C, Gilliland DG, Levine RL. Genetics of myeloid malignancies: pathogenetic and clinical implications. J Clin Oncol 2005; 23: 6285-6295.

46. Grimwade D, Walker H, Harrison G, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. Blood. 2001; 98: 1312-1320.

47. Hann IM, Stevens RF, Goldstone AH, et al. Randomized comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia: results of the Medical Research Council's 10th AML trial (MRC AML 10). Adult and Childhood Leukaemia Working Parties of the Medical Research Council. Blood. 1997; 89: 2311-2318.

48. Burnett AK, Goldstone AH, Stevens RM, et al. Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of MRC AML 10 trial. UK Medical Research Council Adult and Children's Leukaemia Working Parties. Lancet. 1998; 351: 700-708.

49. Grisolano JL, Wesselschmidt RL, Pelicci PG, et al. Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. Blood 1997; 89, 376-387.

50. He LZ., Tribioli C, Rivi R, et al. Acute leukemia with promyelocytic features in PML/RARalpha transgenic mice. Proc Natl Acad Sci U S A 1997; 94, 5302-5307.

51. Kelly LM, Kutok JL, Williams IR, et al. PML/RARalpha and FLT3-ITD induce an APLlike disease in a mouse model. Proc Natl Acad Sci U S A 2002; 99: 8283-8288.

52. Rocquain J, Carbuccia N Trouplin V, et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leuke mias. BMC Cancer 2010, 10:401.

53. Duhrsen U, Hossfeld DK. Stromal abnormalities in neoplastic bone marrow diseases. Ann Hematol. 1996; 73(2): 53-70.

54. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. 22 (2): 153–83. [1–31 Cobb, M. H. (1999) MAP kinase pathways. Prog. Biophys. Mol. Biol. 2001; 71, 479–500.

55. Widmann C, Gibson S, Jarpe MB et al. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. 1999; 79, 143–180.

56. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature (London) 2001; 410, 37–40.

57. Schaeffer HJ, Weber MJ: Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol 1999, 19: 2435-3444.

58. Lee JT Jr, McCubrey JA. The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. Leukemia (Baltimore) 2002; 16: 486– 507.

59. Hilger RA, Scheulen ME, Strumberg D. The Ras-Raf-MEK-ERK pathway in the treatment of cancer. Onkologie 2002; 25: 511–518.

60. Kato Y, Tapping RI, Huang S, et al. Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature. 1998. 395: 713-716.

61. Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem. J. 2000; 351; 289–305.

62. Yung Y, Dolginov Y, Yao Z, et al. Detection of ERK activation by a novel monoclonal antibody. FEBS Lett. 1997; 408: 292–296

63. Davie JR, Spencer VA. Signal transduction pathways and the modification of chromatin structure. Prog. Nucleic Acid Res. Mol. Biol. 2001; 65: 299–340.

64. Milella M, Kornblau SM, Estrov Z, et al. Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. J Clin Investig 2001; 108: 851–859.

65. Kim SC, Hahn JS, Min YH. Constitutive activation of extracellular signal-regulated kinase in human acute leukemias: combined role of activation of MEK, hyperexpression of extracellular signal-regulated kinase, and downregulation of a phosphatase, PACI. Blood 1999; 93: 3893-3899.

66. Lunghi P, Tabilio A, Dall'Aglio PP, et al. Downmodulation of ERK activity inhibits the proliferation and induces the apoptosis of primary acute myelogenous leukemia blasts. Leukemia (Baltimore) 2003; 17: 1783–1793.

67. Sebolt-Leopold JS. Development of anticancer drugs targeting the MAP kinase pathway. Oncogene 2000; 19: 6594–6599.

68. Kornblau SM, Womble M, Qiu YH, et al. Simult aneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. Blood 2006; 108:2358-2365.

69. White E. Life, death and pursuit of apoptosis. Genes Dev. 1996; 10: 1-15.

70. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basicbiological phenomenon with wide-ranging implications in tissue kinetics. Br.J. Cancer 1972; 26, 239-257.

71. Wyllie AH, Kerr JF, Currie AR. Cell death: The significance of apoptosis. Int. Rev. Cytol. 1980; 68: 251-306.

72. Wyllie AH. Glucocorticoid-induced thymocyte apop- tosis is associated with endogenous endonuclease activation. Nature 1980; 284, 555-556.

73.Bøe R, Gjertsen BT., Vintermyr OK, et al. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp. Cell Res. 1991; 195: 237-246.

74. Van Cruchten S, Van Den Broeck W. "Morphological and biochemical aspects of apoptosis, oncosis and necrosis." Anat Histol Embriol. 2002; 31(4): 214-223.

75. Meier P, Finch A, Evan G. Apoptosis in development. Nature 2000; 407(6805): 796-801.

76. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004; 116: 205–219.

77. Green DR, Reed JC. Mitochondria and apoptosis.Science 1998; 281: 1309–1312.

78. Liu X, Kim CN, Yang J, et al. Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. Cell 1996; 86: 147–157.

79. Du C, Fang M, Li Y, et al. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 2000; 102: 33–42.

80. Verhagen AM, Ekert PG, Pakusch M, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. 2000; 102: 43–53.

81. Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397: 441–446.

82. Li Y, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 2001; 412: 95–99.

83. Saelens X, Festjens N, Walle LV, et al. Toxic proteins released from mitochondria in cell death. Oncogene 2004; 23: 2861–2874.

84. Cain K, Bratton SB, Langlais C, et al. Apaf-1 oligomerizes into biologically active ~700 kDa and inactive ~1.4 MDa apoptosome complexes.J Biol Chem 2000; 275: 6067–6070.

85. Budijardjo I, Oliver H, Lutter M., et al. Biochemical pathways of caspase activation during apoptosis. Annu. Rev. Cell Dev. Biol. 1999; 15, 269-290.

86. Degterev A, Boyce M, Yuan J. A decade of caspases. Oncogene 2003; 22: 8543–8567.

87. Bratton SB, Walker G, Srinivasula SM, et al. Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. EMBO J. 2001; 20: 998–1009.

88. Fesik SW, Shi Y. Structural biology. Controlling the caspases. Science 2001; 294(5546):1477-1478.

89. Deveraux QL, Takahashi R, Salvesen GS, et al. X-linked IAP is a direct inhibitor of cell-death proteases. Nature 1997; 388: 300–304.

90. Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J. 1998; 17: 2215–2223.

91. Goyal L. Cell death inhibition: Keeping caspases in check. Cell . 2001; 104: 805–808.

92. Vaux DL, Silke J. Mammalian mitochondrial IAP binding proteins. Biochem Biophys Res Commun 2003; 304: 499–504.

93. Cande C, Vahsen N, Kouranti I, et al. AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene 2004; 23: 1514–1521.

94. Ravagnan L., Roumier T, Kroemer G. Mitochondria – the killer organelles and their weapons. J. Cell. Physiol. 2002 ; 192: 131-137.

95. Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 2000; 256: 58–66.

96. Ashkenazi A, Dixit VM. Death receptors: Signaling and modulation. Science 1998; 281: 1305–1308.

97. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998; 17: 1675–1687.

98. Piskunova TS, Yurova MN, Ovsyannikov AI, et al. Deficiency in Poly (ADP-ribose) Polymerase-1 (PARP-1) Accelerates Aging and Spontaneous Carcinogenesis in Mice. Curr Gerontol Geriatr Res. 2008:754190.

99. D'Amours D, Desnoyers S, D'Silva I, et al. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem. J. 1999. 342, 249–268.

100. Smulson ME, Simbulan-Rosenthal CM., Boulares AH, et al. Adv. Enzyme Regul. 2000; 40, 183–215.

101. Savill J, Gregory C, Haslett C. Cell biology. Eat me or die. Science. 2003; 28: 302:1516-1517.

102. Wang X, Wu YC, Fadok VA, et al. Cell corpse engulfment mediated by C. elegans phosphatidylserine receptor through CED-5 and CED-12. Science. 2003; 28: 302:1563-1566.

103. Liemann S, Lewit-Bentley A.. Annexins: a novel family of calcium- and membrane-binding proteins in search of a function. Structure. 1995; 3(3): 233-237.

104. Strasser A, Harris AW, Bath ML, et al. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 1990, 348, 331–333

105. Vaux DL, Cory S, Adams JM. bcl-2 gene promotes haematopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 1998; 335: 440–442.

106. Tsujimoto Y. Stress-resistance conferred by high level of bcl-2a protein in humna B lymphoblastoid cell. Oncogene 1989; 4:1331–1336.

107. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2- deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 1993; 75:229–240.

108. Adams JM, Cory S. The Bcl-2 protein family: Arbiters of cell survival. Science 1998; 281:1322–1326.

109. Tsujimoto Y, Shimizu S.. Bcl-2: Life-or-death switch. FEBS Lett 2000a; 466: 6–10.

110. Gajkowska B, Wojewodzka U, Gajda J. Translocation of Bax and Bid to mitochondria, endoplasmic reticulum and nuclear envelope: possible control points in apoptosis. J Mol Histol 2004, 35: 11–19.

111. Germain M, Mathai JP, McBride HM, et al. Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis. EMBO J 2005; 24: 1546–1556.

112. Lithgow T, van Driel R, Bertram JF, et al.. The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. Cell Growth Differ 1994, 5: 411–417.

113. Zong WX, Li C, Hatzivassiliou G, et al. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 2003; 162: 59–69.

114. Green DR, Kroemer G. The Pathophysiology of Mitochondrial Cell Death. Science 2004; 305: 626–629.

115. Decaudin D, Marzo I, Brenner C, Kroemer G. Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy (Review) Int J Oncol 1998; 12: 141–152.

116. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 1999; 399: 483–487.

117. Yang T, Kozopas KM, Craig RW. The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. J Cell Biol. 1995; 128: 1173-1184.

118. Yang T, Kozopas KM, Craig RW. MCL-1, a member of the BCL-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. J Cell Physiol. 1996; 166: 523-536.

119. Kozopas KM, Yang T, Buchan HL, et al. MCL1, a gene espresse in programmed myeloid cell differentiation , has sequence similarity to BCL2. Proc. Natl. Acad. Sci. U. S. A. 1993; 90, 3516-3520.

120. Zhou P, Qian L, Kozopas K, et al. MCL-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 1997; 89:630–643.

121. Srivastava R, Mi Q, Hardwick J, et al. Deletion of the loop region of Bcl-2 completely blocks palcitaxel-induced apoptosis. Proc Natl Acad Sci USA 1999; 96: 3775–3780.

122. Gonzalez-Garcia M, Perez-Ballestero R, Ding L, et al. bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development 1994 , 120: 3033-3042.

123. Hsu YT, Wolter KG, Youle RG: Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci USA 1997, 94: 3668-3672.

124. Chao DT, Linette GP, Boise LH, et al. Bcl-XL and Bcl-2 repress a common pathway of cell death. J Exp Med 1995; 182: 821-828.

125. Oltvai ZN, Milliman CL, Korsmeyer K. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993; 74: 609–619.

126. Sedlak TW, Oltvai ZN, Yang E, et al. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc Natl Acad Sci USA 1995; 92: 7834–7838.

127. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, et al. Bax-induced caspase activation and

apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. J Biol Chem 1999; 274: 2225–2233.

128. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005; 19: 1294–1305.

129. Nilture SK, Jaiswal AK. INrf2 (Keap1) targets Bcl-2 degradation and controls cellular apoptosis. Cell Death Differ. 2010. 1-13.

130. Nijhawan D, Fang M, Traer E, et al. Elimination of Mcl-1 is required for the ignition of apoptosis following ultraviolet irradiation. Genes Dev. 2003; 17: 1475-1486.

131. Ji L, Chen Y, Liu T, Wang Z. Involvement of Bcl-XL degradation and mitochondrial-mediated apoptotic pathway in pyrrolizidine alkaloids-induced apoptosis in hepatocytes. Toxicology and Applied Pharmacology 2008: 231; 393-400.

132. Schwickart M, Huang XD, Lill JR, et al. Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. Nature. 2010: 463; 103-108.

133. Klampfer L, Zhang J, Zelenetz AO, et al. The AML1/ETO fusion protein activates transcription of BCL-2. Proc Natl Acad Sci USA 1996; 95:11863-11868.

134. Britos-Bray M, Ramirez M, Cao W, et al.. CBF-SMMHC, expressed in M4eo acute myeloid leukaemia, reduces p53 induction and slows apoptosis in hematopoietic cells exposed to DNA-damaging agents. Blood 1998; 92: 4344-4352.

135. Testa U, Grignani F, Samoggia P, et al. The PML/RAR fusion protein inhibits tumor necrosis factor-α- induced apoptosis in U937 cells and acute promyelocytic leukemia blasts. J Clin Invest 1998; 101: 2278-2289.

136. Campos L, Rouault JP, Sabido O, et al. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood. 1993; 81: 3091-3096.

137. Andreef M, Konopleva M. Mechanisms of drug resistance in AML. Cancer Treat Res 2002; 112: 237-262.

138. Banker DE, Radich J, Becker E. The t(8;21) translocation is not consistently associated with high Bcl-2 expression in de novo acute myeloid leukemias of adults. Clin Cancer Res 1998; 4: 3051-3062.

139. Venditti A, Del Poeta G, Maurillo L, et al. Combined analysis of bcl-2 and MDR1 proteins in 256 cases of acute myeloid leukemia. Haematologica 2004; 89: 934-939.

140. Marcucci G, Stock W, Dai G. Phase I study of oblimersed sodium, an antisense to Bcl-2, in untreated older patients with acute myeloid leukemia: pharmacokinetics, pharmacodynamics, and clinical activity. J Clin Oncol 2005; 23: 3404-3411.

141. Stoetzer OJ, Nussler V, Darsow M, et al. Association of bcl-2, bax, bcl-xl and interleukin-1-beta-converting enzyme expression with initial response to chemotherapy in acute myeloid leukemia. Leukemia 1996; 10: S18–S22.

142. Kornblau SM, Vu HT, Ruvolo P, et al. Bax and PKCa modulate the prognostic impact of Bcl-2 expression in acute myelogenous leukaemia. Clin Cancer Res 2000; 6: 1401–1409.

143. Del Principe M, Del Poeta G, Venditti A, et al. Apoptosis and immaturity in acute myeloid leukemia. Hematology 2005; 10(1): 25-34.

144. Xiang Z, Lio H, Payton JE, et al. Mcl-1 haploinsufficiency protects mice from Myc-induced acute myeloid leukemia. J Clin Invest. 2010; 120(6): 2109-2118.

145. Kaufmann SH, Karp JE, Svingen PA, et al. Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. Blood. 1998; 91(3): 991-1000.

146. Konopleva M, Zhao S, Hu W, Jiang S, et al. The antiapoptotic genes Bcl-X(L) and Bcl-2 are overexpressed and contribute to chemoresistance of non-proliferating leukaemic CD34+ cells. Br J Haematol 2002; 118: 521-534.

147. Birkenkamp KU, Geugien M, Lemmink HH, et al. Regulation of constitutive STAT5 phosphorylation in acute myeloid leukaemia blasts. Leukemia 2001;15: 1923-1931.

148. Sasaki H, Sheng Y, Kotsuji F, et al.. Downregulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. Cancer Res. 2000 ; 60: 5659–5666.

149. Holcik M, Yeh C, Korneluk RG, Chow T..Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene 2000; 19: 4174–4177.

150. Tamm I, Kornblau SM, Segal H, et al. Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin Cancer Res 2000; 6: 1796-1803.

151.Carter BZ, Kornblau SM, Tsao T, et al. Caspase-independent cell death in AML: caspase inhibition in vitro with pan-caspase inhibitors or in vivo by XIAP or Survivin does not affect cell survival or prognosis. Blood 2003; 102: 4179-4186.

152. Mohr A, Zwacka RM, Debatin KM, Stahnke K. A novel method fort he combined flow cytometric analysis of cell cycle and cytochrome c release. Cell Death Differ 2004; 11:1153-1154.

153. Meyer LH, Karawajew L, Schrappe M, et al.. Cytochrome c related caspase-3 activation determines treatment response and relapse in childhood precursor B-cell ALL. Blood 2006; 107: 4524-4531.

154. Takayama S, Sato T, Krajewski S,et al. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell 1999: 80; 279-284.

155. Doong H, Vrailas A, Kohn EC. What's in the 'BAG'?—A functional domain analysis of the BAG-family proteins. Cancer Lett. 2002; 188: 25–32.

156. Briknarova K, Takayama S, Brive L, et al. Structural analysis of BAG1 cochaperone and its interactions with Hsp70 heat shock protein. Nat Struct Biol 2001, 349-352.

157. Takayama S, Bimston DN, Matsuzawa S, et al.. BAG-1 modulates the chaperone activity of Hsp70/Hsc70. Embo J. 1997; 16: 4887-4896.

158. Zeiner M, Niyaz Y, Gehring U. The hsp70-associating protein Hap46 binds to DNA and stimulates transcription. Proc Natl Acad Sci U S A. 1999; 96:10194-10199.

159. Arndt V,Daniel C, Nastainczyk W, et al. BAG-2 acts as an inhibitor of the chaperone associated ubiquitin ligase CHIP. Mol. Biol. Cell 2005; 16: 5891–5900.

160. Dai Q, Qian SB, Li HH, et al. Regulation of the cytoplasmic quality control protein degradation pathway by BAG2. J. Biol. Chem. 2005; 280: 38673–38681.

161. Takayama S, Xie Z, Reed JC. An evolutionary conserved family of Hsp70/Hsc70 molecular chaperone regulators. J Biol Chem 1999. 274; 781-786.

162. Tschopp J, Martinon F, Hofmann K. Apoptosis: silencing the death receptors.. Curr Biol 1999; R381-384.

163. Gehrmann M, Marienhagen J, Eichholtz-Wirth H, et al. Dual function of membrane-bound heat shock protein 70 (Hsp70), Bag-4 and Hsp40: protection against radiation-induced effects and target structure for natural killer cells. Cell death Differ. 2005; 12: 38-51.

164. Kalia SK., Lee S., Smith PD., et al. BAG5 inhibits parkin and enhances dopaminergic neuron degeneration. Neuron 2004; 44: 931–945.

165. Thress K, Song J, Morimoto RI, et al., Reversible inhibition of Hsp70 chaperone function by Scythe and Reaper.EMBOJ 2001; 20: 1033-1041.

166. Banerji J, Sands J, Strominger JL, et al. A gene pair from the human major histocompatibility complex encodes large proline-rich proteins with multiple repeated motifs and a single ubiquitin-like domain. Proc.Natl. Acad. Sci. USA 1990; 87: 2374–2378.

167. Lee JH, Takahashi T, Yasuhara N, et al. Bis, a Bcl-2-binding protein that synergizes with Bcl-2 in preventing cell death. Oncogene. 1999; 18: 6183-6190.

168. Doong H., Rizzo K, Fang S, et al. CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation: accumulation of poly-ubiquitinated Hsp90 client proteins. J. Biol. Chem. 2003; 278, 28490–28500.

169. Gamerdinger M, Hajieva P, Kaya AM, et al. Protein quality control during aging involves recruitment of the macroautophagy pathway by bag3. Embo J. 2009; 28: 889–901.

170. Rosati A, Ammirante M, Gentilella A, et al. Apoptosis inhibition in cancer cells: a novel molecular pathway that involves BAG3 protein. Int J Biochem Cell Biol. 2007; 39: 1337-1342.

171. Yang X, Chernenko G, Hao Y, et al. Human Bag-1/Rap46 protein is generated as four isoforms by alternative translation initiation and over-expressed in cancer cells. Oncogene. 1998; 17: 981-989.

172. Takayama S, Krajewski S, Krajewska M, et al. Expression and location of Hsp70/Hsc-Binding anti-apoptotic protein BAG-1 and its variants in normal tissues and tumour cell lines. Cancer Res. 1998; 58: 3116-3131.

173. Schneikert J, Hubner S,Martin E. et al. A nuclear action of the eukaryotic cochaperone RAP46 in downregulation of glucocorticoid receptor activity. J Cell Biol. 1999; 146: 929–940.

174. Luders J, Demand J, Hohfeld J. The ubiquitin-related Bag-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J Biol Chem. 2000; 275: 4613-4617.

175. Alberti S, Demand J, Esser C, et al. Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. 2002; 277: 45920-45927.

176. Elliott E, Tsvetkov P, Ginzburg I. BAG-1 associates with Hsc70-Tau complex and regulates the proteasome Degradation of Tau protein. J. Biol. Chem. 2007; 282(51), 37276-37284.

177. Wang HG, Takayama S, Rapp UR, Reed JC. Bcl-2 interacting protein, Bag-1, binds to and activates the kinase Raf-1. Proc Natl Acad Sci USA. 1996; 93: 7063–7068.

178. Froesch BA, Takayama S, Reed JC. BAG-1L protein enhances androgen receptor function. J. Biol. Chem. 1998; 273, 11660-11666.

179. Kullmann M., Schneikert J, Moll J, et al. RAP46 is a negative regulator of glucocorticoid

receptor action and hormone-induced apoptosis. J. Biol. Chem. 1998; 273, 14620-14625.

180. Zeiner M, Gehring U. A protein that interacts with members of the nuclear hormone receptor family: identification and cDNA cloning. Proc Natl Acad Sci U S A. 1995; 92(25): 11465-11469.

181. Matsuzawa S, Takayama S, Froesch BA, et al. p53-inducible human homologue of Drosophila seven in absentia (Siah) inhibits cell growth: suppression by BAG-1. EMBO J. 1998; 17, 2736-2747.

182. Bardelli AP, Longati D, Albero S, et al. HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. Embo J. 1996; 15:6205-6212.

183. Niyaz Y, Zeiner M, Gehring U. Transcriptional activation by the human Hsp70-associating protein Hap50 J Cell Sci. 2001; 10:1839-1845.

184. Arhel NJ, Packham G, Townsend PA et al. Retinoblastoma Protein Interacts with BAG-1 in Human Colonic Adenoma and Carcinoma Derived Cell Lines. Int J Cancer. 2003; 106(3): 364-371.

185. Clemo NK, Arhel NJ, Barnes JD, et al. The role of the retinoblastoma protein (Rb) in the nuclear localization of BAG-1: implications for colorectal tumour cell survival. Biochem Soc Trans. 2005; 4:676-678.

186. Takayama S, Sato T, Krajewski S, et al. Cloning and functional analysis of BAG-1: a novel Bcl-2 binding protein with anti-cell death activity. Cell. 1995; 80: 279-284.

187. Takayama S, Reed JC. Molecular chaperone targeting and regulation by BAG family proteins. Nat Cell Biol. 2001; 3: 237-241.

188. Townsend PA, Cutress RI, Sharp A,. Bag-1: a multi functional regulator of cell growth and survival. Biochim Biophys Acta. 2003; 1603: 83-89.

189. Kermer P, Krajewska M, Zapata JM, et al. Bag1 is a regulator and marker of neuronal differentiation. Cell Death Differ. 2002; 9: 405-413.

190. Gotz R, Wiese S, Takayama S, et al. Bag-1 is essential for differentiation and survival of hematopoietic and neuronal cells. Nat Neurosci. 2005; 8: 1169-1178.

191. Sun L, Huang L, Nguyen P, et al. DNA Methyltransferase 1 and 3B Activate BAG-1 Expression via Recruitment of CTCFL/BORIS and Modulation of Promoter Histone Methylation) Cancer Res 2008; 68(8):2726–2735.

192. Townsend PA, Cutress RI, A. Sharp A, et al. BAG-1 prevents stress-induced long-term growth inhibition in breast cancer cells via a chaperonedependent pathway. Cancer Res. 2003, 63:4150-4157.

193. Tang SC, Shehata N, Charnenko G, et al. Expression of BAG-1 in invasive breast carcinomas.J Clin Oncol. 1999; 17: 1710-1719.

194. Kikuchi R, Noguchi T, Takeno S, et al. Nuclear BAG-1 expression reflects malignant potential in colorectal carcinomas. Br. J. Cancer., 2002; 87: 1136-1139.

195. Shindoh M, Adachi **M**.; Higashino F, et al. BAG-1 expression correlates highly with the malignant potential in early lesions.: Oral Oncol. 2000; 36; 444-449.

196. Moriyama T, Littell RD, Debernardo R, et al. BAG-1 expression in normal and neoplastic endometrium. Gynecol. Oncol. 2004: 94: 289-295.

197. Krajewska M, Turner BC, Shabaik A, et al. Expression of BAG-1 protein correlates with

aggressive behavior of prostate cancers. Prostate. 2006; 66:801-810.

198. Rorke S, Murphy S, Khalifa M, et al. Prognostic significance of BAG-1 expression in nonsmall cell lung cancer. Int J Cancer 2001; 95: 317–322.

199. Clemo NK, Collard TJ, Southern SL, et al. BAG-1 is up-regulated in colorectal tumour progression and promotes colorectal tumour cell survival through increased NF-KappaB activity. Carcinogenesis. 2008; 29(4): 849-857.

200. Hague A, Packham G, Huntley S, et al. Deregulated Bag-1 protein expression in human oral squamous cell carcinomas and lymph node metastases. J. Pathol. 2002: 197; 60-71.

201. Sharp A, Crabb SJ, Townsend PA, et al. BAG-1 in cancerogenesis. Expert Rev Mol Med 2004: 6;1-15.

202. Takeno S, Noguchi T, Takahashi Y, et al. Assessment of clinical outcome in patients with esophageal squamous cell carcinoma using TNM classification score and molecular biological classification. Ann Surg Oncol 2007; 14:1431–1438.

203. Krajewski M, Krajewska BC, Turner C, et al. Prognostic significance of apoptosis regulators in breast cancer . Endocrine-Related Cancer 1999; 6 (1) 29 -40.

204. Turner BC, Krajewski S, Krajevska M, et al. BAG-1: a novel biomarker predicting long-term survival in early stage-breast cancer. J Clin Oncol. 2001: 19; 992-1000.

205. Sjostrom J, Blomqvist C, von Boguslawski K, et al. The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res. 2002; 8(3): 811-816.

206. Welchman RL, Gordon C, Mayer RJ. Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nat. Rev. Mol. Cell. Biol. 2005; 6, 599–609.

207. Dikic I, Wakatsuki S, Walters KJ. Ubiquitin-binding domains—from structures to functions. Nat. Rev. Mol. Cell. Biol. 2009; 10, 659–671.

208. Hershko A; Ciechanover A. The ubiquitin system. Annu. Rev. Biochem. 1998; 67, 425–479.

209. Callis J, Vierstra RD. Protein degradation in signaling. Curr. Opin. Plant Biol. 2000; 3: 381–386.

210. Baumeister W, Walz J, ZuÈ hl F, et al. The proteasome: paradigm of a self-compartmentalizing protease. Cell 1998; 92, 367–380.

211. Scheffner M, Smith S, Jentsch S. In Ubiquitin and the Biology of the Cell, eds. Peters, J.-M., Harris, J. R. & Finley, D. (Plenum, New York), 1998; pp. 65–98.

212. Hochstrasser, M. Ubiquitin-dependent protein degradation. Annu. Rev. Genet. 1996, 30, 405–439.

213. Peters JM, Cejka Z, Harris JR, et al. Structural features of the 26S proteasome complex. J Mol Biol 1993; 234: 932-937.

214. Unno M, Mizushima T, Morimoto Y, et al. The Structure of the Mammalian 20S Proteasome at 2.75 A Resolution Structure 2002; 10 pp. 609.

215. Elsasser S, Finley D. Delivery of ubiquitinated substrates to rotein-unfolding machines. Nat. Cell Biol. 2005; 7: 742–749.

216. Testa U, Riccioni R. Deregulation of apoptosis in acute myeloid leukemia. Haematologica. 2007; 98: 81-94.

217. Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol. 2003; 195: 158-167.

218. Scorrano L, Korsmeyer SJ. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun. 2003; 304: 437-444.

219. Bradbury DA, Russell NH. Comparative quantitative expression of bcl-2 by normal and leukaemic myeloid cells. Br J Haematol. 1995; 91: 374-379.

220. Maung ZT, MacLean FR, Reid MM, et al. The relationship between bcl-2 expression and response to chemotherapy in acute leukemia. Br J Haematol. 1994; 88: 105-109.

221. Bensi L, Longo R, Vecchi A, et al. Bcl-2 oncoprotein expression in acute myeloid leukemia. Haematologica. 1995; 80: 98–102.

222. Packham G, Brimmell M, Cleveland JL. Mammalian cells express two differently localized Bag-1 isoforms generated by alternative translation initiation. Biochem J. 1997; 328: 807–813.

223. Coldwell MJ, De Schoolmeester ML, Fraser CA, et l. The p36 isoform of Bag-1 is translated by IRES following heat shock. Oncogene. 2001; 20: 4095-4100.

224. Pain VM. Initiation of protein synthesis in eukaryotic cells. Eur J Biochem. 1996; 236: 747-771.

225. Song J, Takeda M, Morimoto RI. Bag-1-Hsp70 mediates a physiological stress signaling pathway that regulates Raf-1/ERK and cell growth. Nat Cell Biol. 2001; 3: 276-282.

226. Brimmell M, Burns JS, Munson P, et al. High level expression of differentially localized BAG-1 isoforms in some oestrogen receptor-positive human breast cancers. Br J Cancer. 1999; 81(6): 1042-1051.

227. Kudoh M., Knee DA, Takayama S, Reed JC. Bag-1 proteins regulate growth and survival of ZR-75-1 human breast cancer cells. Cancer res. 2002; 62: 1904-1909.

228. Cutress RI, Townsend PA, Brimmell M, et al. BAG-1 expression and function in human cancer. Br J Cancer. 2002; 87: 834–839.

229. Pession A, Rizzari C, Putti MC, et al. Results of the AIEOP AML 2002/01 study for treatment of children with acute myeloid leukemia. Blood. 2009; 114[22].

230. Pigazzi M, Manara E, Baron E, Basso G. miR-34b Targets cyclic AMP-responsive element binding protein in acute myeloid leukemia. Cancer Res. 2009; 69(6): 2471-2478.

231. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods. 2001; 25: 402–408.

232. Aaron TJ, Lawrence JM. HSF1-mediated BAG3 Expression Attenuates Apoptosis in 4-Hydroxynonenal-treated Colon Cancer Cells via Stabilization of Anti-apoptotic Bcl-2 Proteins. J Biol Chem. 2009;284: 9176-9183.

233. Malys N, McCarthy JEG. Translation initiation: variations in the mechanism can be anticipated. Cellular and Molecular Life Sciences. 2010

234 Kozak M. An analysis of 5’-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 1987; 15 (20): 8125–8148.

235. Blagosklonny MV, Wu GS, Omura S, el-Deiry WS. Proteasome-dependent regulation of p21WAF1/CIP1 expression. Biochem Biophys Res Commun 1996; 227: 564-569.

236. Bischoff JR, Anderson L, Zhu Y, et al. A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J. 1998; 17: 3052–3065.

237. Shindo M, Nakano H, Kuroyanagi H, et al. cDNA cloning, expression, subcellular localization and chromosomal assignment of mammalian aurora homologues, aurora-related kinase (ARK) 1 and 2. Biochem. Biophys. Res. Commun. 1998; 244: 285–292.

238. Zhou H, Kuang J, Zhong L, et al. Tumor amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat. Genet. 1998; 20: 189–193

239. Littlepage LE, Ruderman JV. Identification of new APC/C recognition domain, the A box, which is required for the Cdh-dependent destruction of the kinase Aurora-A during mitotic exit. Genes dev. 2002; 16:2274-2285.

240 Moor MJ, Wang Q, Kennedy CJ, Silver PA. An lìalternative splicing network links cell-cycle control to apoptosis. Cell. 2010; 142: 625-636.

241. Hochestrasser M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradationCurr Opin. Cell Biol. 1995; 7:215-223.

242. Konopleva M, Tari AM, Estrov Z, Harris D, Xie Z, Zhao S, et al. Liposomal Bcl-2 antisense oligonucleotides enhance proliferation, sensitize acute myeloid leukemia to cytosine-arabinoside and induce apoptosis independent of other anti-apoptotic proteins. Blood. 2000; 95: 3929-3938.

243. Del Poeta G, Venditti A, Del Principe MI. Amount of spontaneous apoptosis detected by Bax/Bcl-2 ratio predicts outcome in acute myeloid leukemia (AML). Blood. 2003; 101: 2125-2131.

244. Cheng J, Xiong J, Liu H, Chernenko G, Tang SC. Distinct BAG-1 isoforms have different anti-apoptotic functions in BAG-1-transfected C33A hunan cervical carcinoma cell line. Oncogene. 2002; 21: 7050-7059.

245. Tsukahara F, Maru Y. Bag1 directly routes immature BCR-ABL for proteasome degradation. Blood. 2010; Jul 30 [Epub ahead of print].

246. Romano MF, Festa M, Pegliuca G, Lerose R, Bisogni R, Poggi V, et al. BAG3 protein regulates cell survival in childhood acute lymphoblastic leukemia cells. Cancer Biol Ther. 2003; 2: 508-510.

247. Bonelli P, Petrella A, Rosati A, Romano MF, Lerose R, Pagliuca MG, et al. Bag3 protein regulates stress-induced apoptosis in normal and neoplastic leukocytes. Leukemia. 2004; 18: 358-360.

248. Derenne S, Monia B, Dean NM, Taylor JK, Rapp MJ, Harousseau JL et al. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-XL is an essential survival protein of human myeloma cells. Blood. 2002; 100: 194-199.

249. Zhou P, Quian L, Kozopas KM, Craig RW. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood. 1997; 89: 630-643.