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Impact of acidosis on ζ-crystallin-mediated bcl-2 expression in leukemic cells

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“Inspiration is not the exclusive privilege of poets or artists generally.”

(W. Szymborska)

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1. ABSTRACT

The human antiapoptotic *bcl-2* gene is over-expressed in various malignancies, including leukemias/lymphomas. Its post-transcriptional regulation is orchestrated by an adenine-uracil rich element (AU-Rich Element, ARE) lying in the 3'-UTR of *bcl-2* mRNA which interacts with numerous ARE-binding proteins (AUBPs) in modulating mRNA stability or translation. In 2010, we identified a new *bcl-2* mRNA stabilizing AUBP, namely ζ-crystallin, and demonstrated that it contributed to the over-expression of *bcl-2* in acute lymphatic leukemia (ALL) cells as a result of its increased binding to the *bcl-2* ARE in T cells of ALL patients compared to normal T lymphocytes. Moreover, ζ-crystallin has been previously demonstrated to bind to specific AU-Rich RNA sequences, called pH-response element (pH-RE), lying in the 3'-UTR of some mRNAs whose genes code for proteins involved in the physiological maintenance of acid-base balance in renal cells. Acidosis, mainly consequent to the "Warburg effect", is a biochemical hallmark of the tumour microenvironment. Associating the above evidences with the recent report that acidosis increased the level of some antiapoptotic proteins, included Bcl-2, by a mechanism involving the acid-sensing G protein-coupled receptor (TDGA8), prompted us to hypothesize that the direct actor of *bcl-2* overexpression in acidosis is ζ-crystallin. Here, we report by which mechanisms acute acidosis impacts on ζ-crystallin-mediated *bcl-2* over-expression and on cellular resistance to apoptotic stimuli in Jurkat cells. We demonstrated that ζ-crystallin elevated *bcl-2* mRNA stability through a mechanisms involving acidosis, which increased its cellular levels. In the effort to unravel the mechanisms involved in the acidosis mediated ζ-crystallin up-regulation, we revealed that acidosis activated the p38 MAPK pathway which in turn could contribute to increase ζ-crystallin expression.

2. INTRODUCTION

After transcription, before translation: RNA processing and export

Our knowledge of the complexity and intricacy of gene expression regulation in eukaryotes have increasingly widened in the last decades, especially since the modulation acting at the post-transcriptional level has been discovered and studied in its multiple aspects. Each mRNA of a mammalian cell, after transcription and splicing processes, enters into the cytoplasm where a complex network of messenger ribonucleoproteins (mRNPs) determines its fate. Gene expression outcome is related to the performance of mRNP infrastructure. The enrollment, gating, processing and transit of each mRNA through mRNP network defines the proteomic composition of a cell (Keene J D., 2001). So, the post-transcriptional regulation of gene expression, involving the metabolism of messenger RNAs, emerges as a fundamental gene expression control (Ciafrè S. A. and Galardi S., 2013) (**Fig. 2.1**).

Moreover, studies revealed that consecutive steps in the post-transcriptional pathway are interdependent and influenced by each other. A mounting number of genetic studies have demonstrated the existence of functional links among factors which act in different post-transcriptional steps. This is supported by conventional biochemical and large-scale mapping of protein-protein interaction studies which have uncovered physical interactions among the various mRNPs. These events occur in the single cells integrated with each other and in a interdependent spatial and temporal context (Orphanides G. and Reinberg D., 2002; McKee A. E. and Silver P. A., 2007). It has been demonstrated, for example, that some factors link transcriptional termination of RNA polymerase II with pre-mRNA 3'-end processing (Birse C. E. et al, 1998), and the connection of pre-mRNA capping with two downstream transcriptional events as RNA Polimerase II elongation and alternative splicing (Lenasi T. et al 2011) or some splicing factors are required for correct mRNA localization into the cytoplasm (Wang E. T. et al,

2012). This organization of events may also introduce a series of quality control mechanisms, as it ensures that no individual step is omitted.

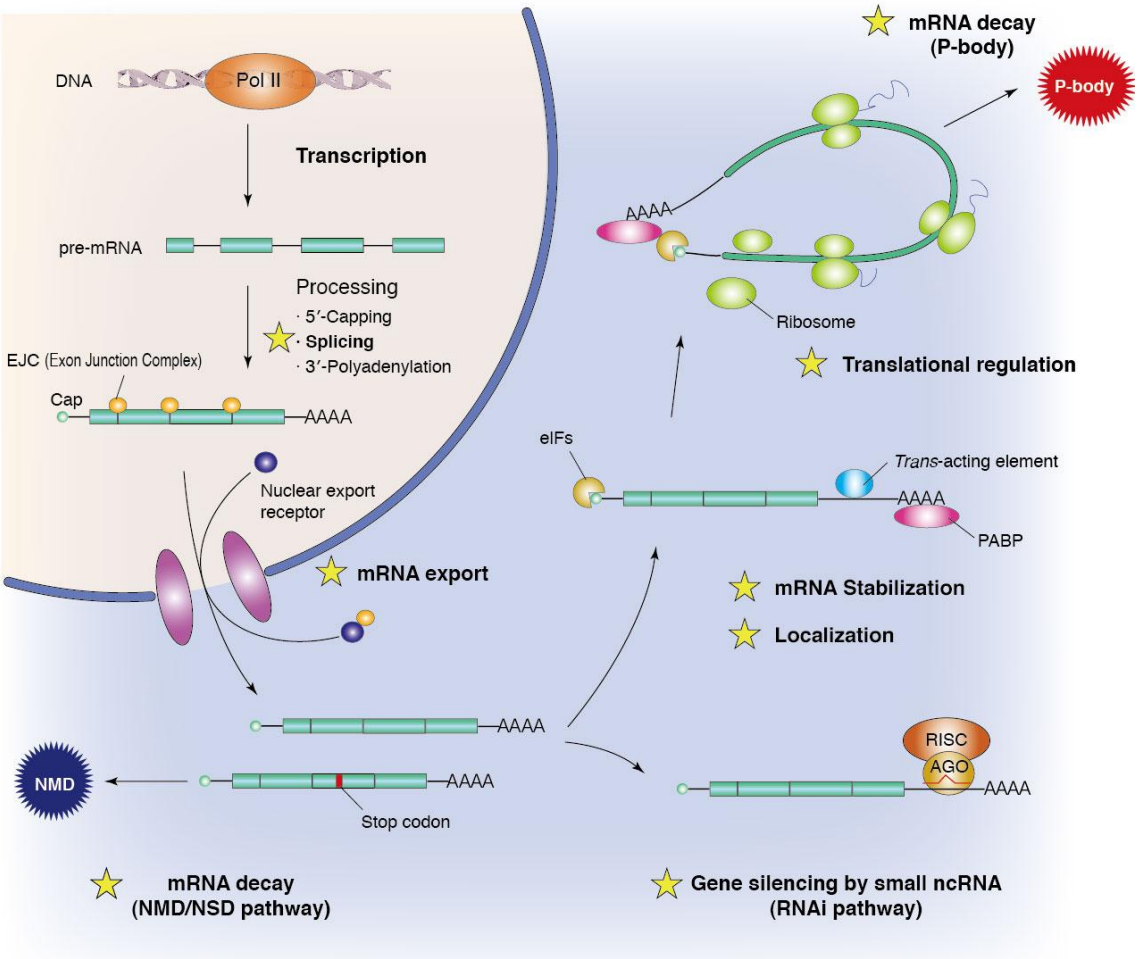


Fig. 2.1 Synthetic representation of post-transcriptional steps involved in gene expression control in eukaryotic cells (http://ruo.mbl.co.jp/e/product/epigenetics/RNAworld.html).

Before a transcript can be exported from the cell nucleus to become available for the translation machinery in the cytoplasm, it has to undergo a series of processing steps: the mRNA acquires a cap structure at the 5' terminus, introns are spliced out from the pre-mRNA and a polyadenilation in 3'end is generated. Concurrently with transcription, nascent pre-mRNA transcripts are modified by the addiction of a 5' m^7G cap structure when 20-30 nucleotides have been synthesized. In a three step reaction, the nascent pre-mRNA is hydrolyzed, the GMP moiety from GTP is added to its first nucleotide and GMP is methylated at position N7. In the nucleus, the m^7G cap is tied by the Cap Binding Complex (CBC) and after shuttling to the cytoplasm interacts with

translational initiation factor 4E to start translation process. So, 5'-cap structure is an important element for the binding with proteins involved in RNA stability, translational initiation, and nuclear export (Lackner D. H., Bähler J., 2008). Pre-mRNA contains introns which are removed by the Spliceosome complex at consensus sequence at exon-intron boundaries to generate mature and functional mRNA (Patel A. A and Steitz J. A., 2003). Spliceosome consists of five small ribonucleoprotin particles (snRNPs: U1, U2, U4, U5 and U6), each of which composed by a small nuclear RNA (snRNA), associated proteins and many accessory proteins. In fact, over hundred proteins are thought to be splicing factors (Jurica M. S., Moore M. J., 2003). So, splicing stage is carried out by numerous RNA-protein interactions. Alternative splicing contributes to increase the number of protein products (isoforms) from a single gene. Following, transcript termination mRNAs 3'-end is polyadenylated in two step: nascent mRNAs are cleaved at polyadenilation site and poly(A)tail is synthesized (Proudfoot N. and O'Sullivan J., 2002). In analogy to splicing, formation of the poly(A) tail requires a multi-subunit complex and *cis*-elements on pre-mRNAs. In mammalian cells, the cleavage sites lie mostly between an AAUAAA hexamer motif and a GU-rich downstream element (DSE) (McLauchlan J. et al, 1985). Cleavage and polyadenylation specifity factor (CPSF) binds to hexameric sequence while cleavage factors recognize DSE sequence. The principles of poly(A) tail formation are the same in yeast and mammalian cells and involved protein complexes have orthologous components, but also specific accessory factors that are only found in one of the species. Poly(A) tail is important for nuclear export and for beginning of translation. Moreover, cytoplasmatic polyadenylases can regulate the translational state and stability of various target mRNAs via modification of the length of respective poly(A) tails in cytoplasm (Stevenson A. L. and Norbury C. J., 2006). Functional mature mRNA is exported from nucleus to cytoplasm where it is translated in association with ribosomes. The passage through the nuclear membrane is possible via Nuclear Pore Complex (NPC) by interaction with RNAs binding proteins associated with RNAs (Stewart M., 2007). Furthermore, nuclear export is an important step in quality control, as wrong mature mRNAs are not only useless, but potentially unsafe, if translated in the cytoplasm. So, only functional mRNAs are exported into the cytoplasm and translated. This quality control crux is closely coupled to RNA processing and mRNP composition.

We could compare functionally the ensemble and the activity of mRNPs with bacterial operon, however in post-transcriptional circumstance. In a bacterial operon, genes of a same cluster are expressed in coordinated manner to respond to a specific stimulus (Keene J. D. and Lager P. J., 2005). The co-linearity between DNA and its encoded RNA polycistronic operon (polygenic) allows to a single transcriptional event to produce a set of functionally related proteins. In eukaryotes, mRNP particles "regroup" different monogenic RNAs in cytoplasm in only one physical context. Recent studies have highlighted how mRNA binding proteins interact with some subsets of mRNAs and how these subsets change dynamically in response to a wide range of endogenous or exogenous stimuli (Keene J. D. and Lager P. J., 2005; Tenenbaum S.A.et al, 2011). The hypothesis, now well established and accredited, is that higher eukaryotes have acquired the ability to post-transcriptionally orchestrate monocistronic subsets of mRNAs using similar sequence elements, situated in Untranslated region (UTR) at 3'- and/or 5'-ends of these transcripts, highly conserved called USER codes (Untraslated Sequence Elements for Regulation). Post-transcriptional operons represent a powerful, yet sensitive, mechanism to regulate and organize the genetic information hold in functionally related whole of monocistronic mRNAs.

mRNA stability and degradation

It is certain that mRNA stability is highly regulated since it is crucial to regulate different processes during cell life and that specific RNAs can be targeted for degradation by special machinery (Cairrão F. et al, 2005).

mRNA turnover in the cells is regulated by multiple mechanisms (**Fig. 2.2**). Deadenylation of the transcript is a crucial step in these regulatory mechanisms, and mRNAs are then decapped and degraded via exonuclease or alternatively, mRNAs can be degraded without decapping by the exosome complex. In certain cases decapped mRNAs can be degraded via endonucleolytic mechanisms without prior deadenylation (Fasken M. B., Corbett A. H., 2005), such as degradation via RNA interference (RNAi) machinery or as Nonsense-Mediated Decay (NMD), a mRNA quality control mechanism to degrade incorrect mRNAs with a premature stop codon. Instead, another mechanism, called Non-Stop Decay, degrades mRNAs which lack of correct stop codons without decapping by exosome (Vasudevan S. et al, 2002).

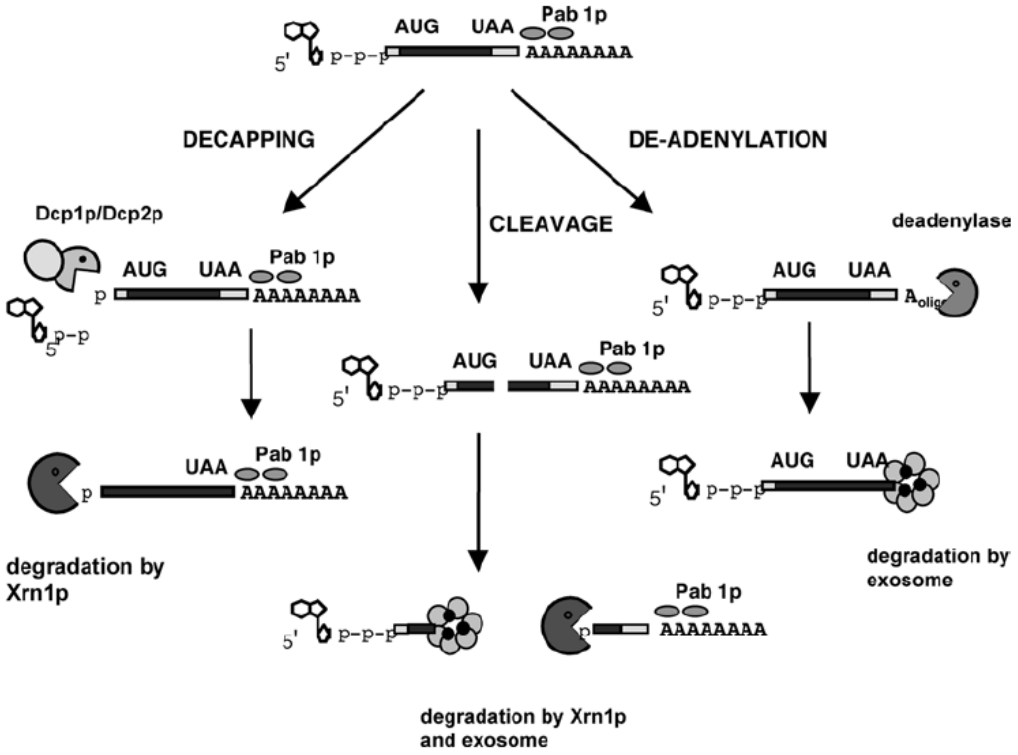


Fig. 2.2. Pathways of mRNAs degradation in eukaryotes (Newbury S. F., 2006).

The Xrn1p is the critical exoribonuclease in the 5'→3' degradation pathway. This enzyme is extremely conserved in all eukaryotes. Xrn1p exonuclease hydrolyses RNA from the 5'-end releasing mononucleotides. The enzyme is involved also in RNAi and NMD pathway (Newbury S. F., 2006). The existence of multiple exonucleases organized around a central cavity is explained by a their coordinate regulation and by the high specificity of particular exonucleases for particular RNA sequences/structures, so that this enzyme complexes can rapidly degrade even the most difficult RNAs (van Hoof A. and Parker R., 1999).

The exosome is a large protein complex containing multiple 3'→5' exonucleases that also functions in a variety of nuclear RNA-processing reactions. Structural analysis shows that the nine core exosome subunits form a ring structure which is likely to encircle the RNA. Most of the individual exosome subunits are highly conserved throughout eukaryotes and have also bacterial orthologues. Exosome activity is dependent on the helicase Ski2 and associated proteins Ski3p and Ski8p pathway (Newbury S. F., 2006). Ski2p unrolls RNA structures before digestion or promotes entry of the RNA into the central cavity of the exosome. The Ski2p–Ski3p–Ski8p complex seems to be recruited to the exosome through interactions with the cytoplasmic

specific subunit, Ski7p. The exoribonuclease Xrn1p or the exosome to access the body of the mRNA, first must be deadenylated, decapped or endonucleolytically cleaved (as summarized in **Fig. 2.2**). Deadenylation occurs via a variety of deadenylases, including the adenylase PARN (polyadenylatedribonuclease) that has the major deadenylase activity in mammalian cells. Another way to provide access for exonucleases is decapping mRNA through decapping complex Dcp1p-Dcp2p. The mechanism of action of the decapping complex is not fully understood, but it appears that Dcp2p cleaves the RNA and that this cleavage is stimulated by Dcp1p. Finally, a third way of accessing the mRNA is by endonucleolytic cleavage. In particular, in NMD, mRNAs are cleaved by a ribosome-associated endonuclease or by the ribosome itself. The resulting 5' fragment is degraded from the 3'-end by the exosome, whereas the 3' fragment is degraded from the 5'-end by Xrn1p.

Decapping enzymes and exonucleases are located in cytoplasmic processing bodies known as P-bodies, observed in different types of eukaryotic cells. P bodies are dynamic structures that change in size and number under different conditions (Newbury S. F., 2006). Factors of NMD pathway are also found in mammalian P-bodies (Unterholzner L. and Izaurralde E., 2004) However, it is not clear whether P-bodies are the only site of 5'→3' decay, as enzymes involved in this mechanism are also found in other site of cytoplasm of yeast or mammalian cells. It is also unclear whether mRNAs need to be deadenylated to enter P-bodies. Practically, several lines of evidence indicate that there are two states of mRNAs: actively translated and associated with polysomes or translationally repressed and associated with P-bodies. When yeast cells are exposed to stress stimulus translation is paused at the level of initiation, and as a consequence there is a strong decrease in polysomes and an increase in size of P-bodies (Coller J. and Parker R., 2005) With the restoration of normal conditions, P-bodies decrease in size and polysomes reform, even in the absence of new transcription. Therefore, P-bodies in yeast seem to serve as sites of mRNA storage (Lackner D. H., Bähler J., 2008). However, the exact mechanism of how mRNAs shuttle into P-bodies and become translationally repressed is not yet fully delineated.

Post transcriptional control

Two great classes of regulatory molecules working post-transcriptionally are microRNAs (miRNAs) and RNA-binding proteins (RBPs). We examine both of them more in below.

Micro RNA. MicroRNAs (miRNAs) are evolutionarily conserved, small (~21 nucleotides) non coding RNAs that are encoded within the genomes of almost all eukaryotes, from plants to mammals. miRNAs are processed from precursor molecules (pri-miRNAs), which are either transcribed from independent miRNA genes or are portions of introns of transcripts. A single pri-miRNA often contains sequences for several different miRNAs. Organisms express hundreds of miRNAs, which are involved in almost all known biological processes. In general, miRNAs, especially in animals, post-transcriptionally regulate protein synthesis by base pairing to partially complementary sequences in the 3'-untranslated regions (UTRs) of target mRNAs. miRNAs mediate mRNA repression by recruiting the miRNA-induced silencing complex (miRISC), a ribonucleoprotein complex, to target mRNAs.

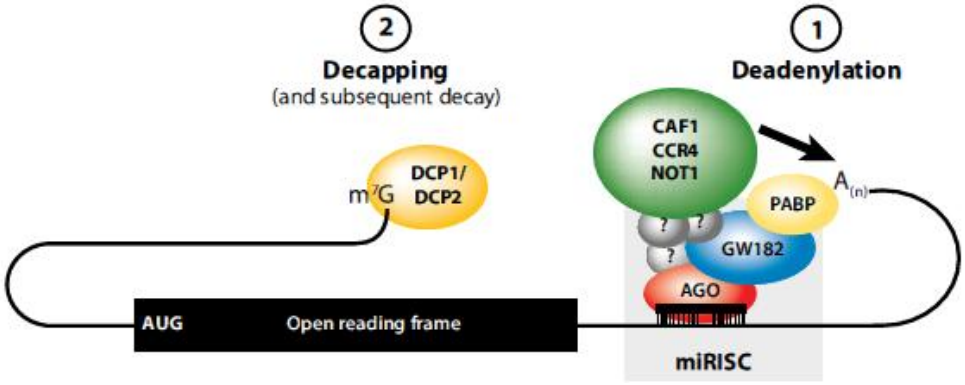


Fig. 2.3. Schematic diagram of miRNA-mediated mRNA decay (Fabian M. R. et al, 2010).

The core of the miRISC contains a miRNA-loaded Argonaute protein and a glycine-tryptophan repeat–containing protein of 182 kDa (GW182). The miRISC has been reported to inhibit cap-dependent translation at both initiation (by interfering with ribosome recruitment) and post-initiation steps (**Fig. 2.3**). The miRISC also engenders deadenylation and subsequent decapping and decay of target mRNAs. Exactly how the

miRISC elicits these events, the order in which they operate and recruit the proteins remains still controversial topic (Fabian M. R. and Sonenberg N., 2012; Fabian M. R. et al, 2010; Filipowicz W. et al, 2008).

The classic example of miRNA is lin-4 which regulates lin-14 mRNA in *Caenorhabditis elegans* through interaction with its 3'-UTR. This regulation does not involve changes in mRNA levels, but protein levels are dramatically altered. As lin-14 mRNA could be found associated with polysomes in both the active and the repressed state, it was suggested that translation of the mRNA is repressed at a point after initiation (Lackner D. H., Bähler J., 2008).

The importance of miRNAs arises from the fact that these molecules act within several processes. Indeed miRNAs regulate genes involved in cell proliferation, differentiation, development of an organism and even apoptosis and cancer (Bartel D. P., 2004). Meanwhile other types of small RNAs have been found in animals, plants, and fungi. These include endogenous small interfering RNAs (siRNAs) (Reinhart B. J. and Bartel D. P., 2002) and Piwi-interacting RNAs (piRNAs) (Aravin et al, 2007). Like miRNAs, many of these other RNAs function as guide RNAs within the broad phenomenon known as RNA silencing. However, miRNAs differ from these other classes of small RNAs in their biogenesis: miRNAs derive from transcripts that fold back on themselves to form distinctive hairpin structures (Bartel D. P., 2004), whereas the other types of endogenous small RNAs derive either from much longer hairpins that give rise to a greater diversity of small RNAs (siRNAs), or from bimolecular RNA duplexes (dsRNAs), or from precursors without any suspected double-stranded character (piRNAs) (Yoon J. H., 2013).

A particular mention should be made to another group of regulatory molecules called Long NonCoding RNA (lncRNA). Unlike other regulative RNA molecules lncRNA have lengths of over 200 nt. LncRNAs are transcribed by RNA polymerase II, even though many lncRNA genes contain histonic profile different from those of coding genes. After transcription, most lncRNAs are processed similar to protein-coding RNAs, including 5'-end capping, 3'-end polyadenylation, splicing of introns, and intracellular transport. Many lncRNAs have small open reading frames, but they are not predicted to codify for proteins (Yoon J. H., 2013).

Recently, a small number of lncRNAs have been reported to regulate gene expression post-transcriptionally in a variety of ways, which are summarized in **Fig. 2.4**.

There are more evidences of lncRNA to have role in growth, differentiation, and development.

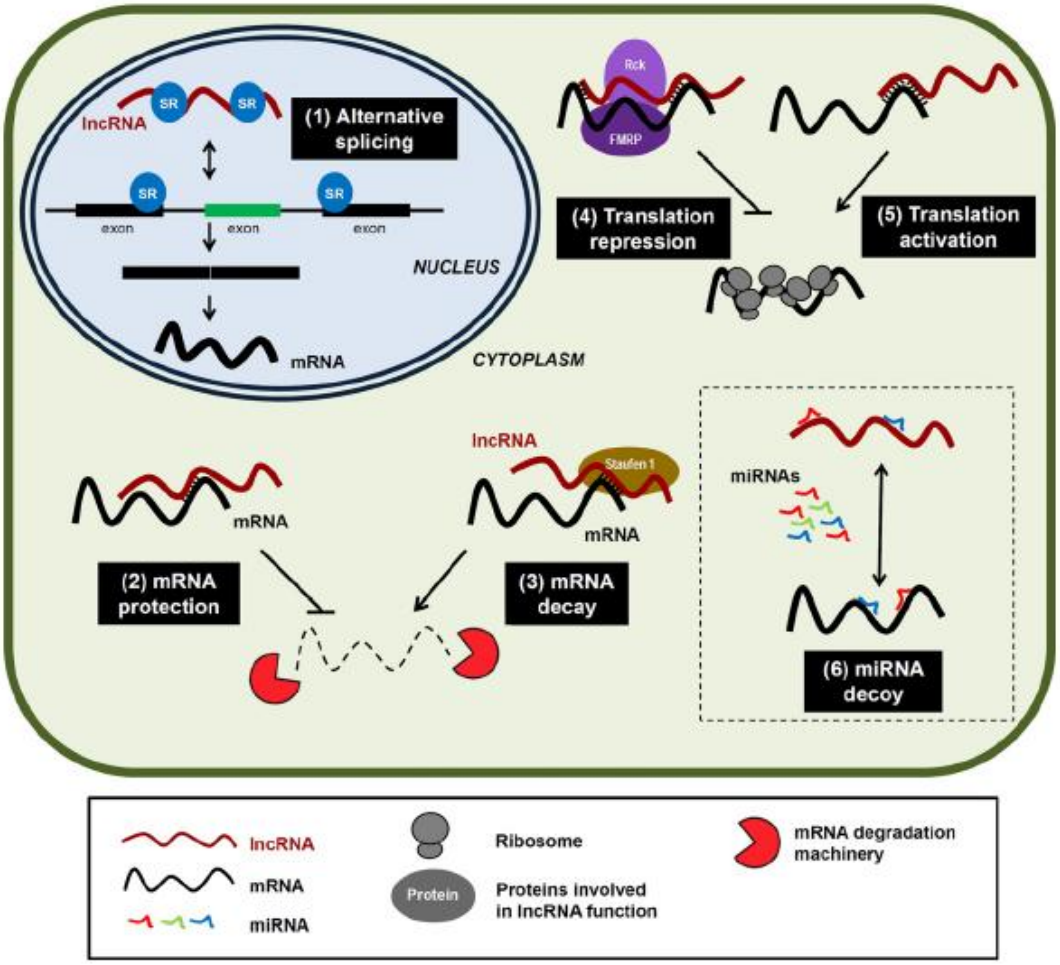


Fig. 2.4. Post-transcriptional processes influenced by lncRNAs (Yoon J-H. et al, 2012).

RNA Binding proteins. RNA Binding Proteins (RBPs) can recognize and bind specific mRNA sequences. These proteins are associated with different subsets of target mRNAs at various time points and in various compartments regulating the fate of targets in a time- and space-dependent manner . RBPs often provide a landing platform for the recruitment of additional factors and enzymes to the mRNA. In recent decades RBPs have emerged as master regulators of post-transcriptional gene expression. RBPs bind to specific sequences or secondary structures typically found in the untranslated regions (UTRs) but also in the open reading frame (ORF) of target

mRNAs (Pickering B. M. and Willis A. E., 2005).

UTR, in particular, have provided greater flexibility to the evolution because of upon such sequences there are not constraints of encoding a protein product. As a consequence, diverse and often conserved regulatory elements are present in the UTRs (Wurth L., 2012). In the 5'-UTR different elements are involved in monitoring of protein expression as ribose methylation of the cap structure, as well as 5' terminal polypyrimidine sequences or secondary structures such as internal ribosome entry sites (IRESs). Also in the 3'-UTR sequence elements regulate the stability of the mRNA, its translational efficiency and localization. Specific binding of regulatory proteins to these elements is achieved through RNA-binding domains (RBDs). More than 40 RBDs have been identified. Among them, the most prominent are RNA recognition motif (RRM), K-homology domain (KH), double stranded RNAbinding domain (dsRBD), zinc finger, Arginine-rich domain, cold-shock domain (CSD), and the PAZ and PIWI domains (Lunde B. M. et al, 2007). An RNA-binding protein can carry more than one of RBDs, which allow a high flexibility for interaction with different mRNA targets.
RBPs participate in every point of RNA's life from transcription, pre-mRNA splicing and polyadenylation to RNA modification, transport, localization, translation and turnover. The RBPs are involved in each of these steps and provide a functional link between them.

ARE-mediated post-transcriptional control. The most relevant *cis*-acting regulatory element involved in post-transcriptional control is an Adenylate- and Uridylate-rich (AU-rich) element (**ARE**) found in the 3'-untranslated regions (UTRs) of numerous mammalian mRNAs. AREs were first identified in early-response gene mRNAs, such as c-myc, junB, c-jun, and nur77, growing factors and cytokines. Since 1986, interest in the study of the ARE motifs has grown to deepen key determinants of the half-lives of many inducible mRNAs. Comparison of the ARE sequences of many mRNAs led to the identification of recurrent motifs within the 3'-UTRs which constitute an AU-rich stretch of 50–150 nucleotides. For instance, the AREs usually contain repeats of the pentameric sequence AUUUA (Caput D. et al, 1986; Shaw G. et al, 1986) or ARE motifs are fused in nonameric units constituting a crucial region for the interaction with trans-acting factors. Nonamer UUAUUUA(U/A)(U/A) forms minimal functional sequence but

it could not represent minimal consensus because AREs activity is modulated by the presence of U-rich sequences in the context of motif (Zubiaga A. M.,1995; Chen C. Y. et al, 2001). The ARE motif described also in 5'-UTR of transformant noncoding RNAs of Saimiri Herpes virus is the only known exception (Fan X. C. et al, 1998). There are three different subclasses of AREs (Chen C. Y., and Shyu A.B., 1995): class I contains one to three copies of the motif AUUUA flanked by U-rich sequences (early response genes c-fos, c-myc and IL-4 and IL-6 cytokines RNAs); class II contains ARES with multiple copies of the AUUUA sequence (GM-CSF, TNF, IL- 3 cytokines AREs); Class III assorts AREs that have no AUUUA motifs, but contain a long U -rich region (proto-oncogene c-jun and β-adrenergic receptor RNAs). Recent classification of genes with ARE motifs is contained in the database ARED (Bakheet T. et al, 2006), inside which are counted over 800 human mRNAs, belonging to several functional categories of genes, among which those of cytokines, growth factors and proto-oncogenes RNAs. AREs are also potent stimulators of both decapping and deadenylation processes. The stimulation of decapping by AREs requires sequence-specific ARE-binding proteins. Likewise, deadenylation is the first step in mRNA turnover, and ARE sequences direct the rapid removal of the mRNA poly(A) tract. A dozen of AU-rich RNA binding proteins (**AUBPs**) which directly interact with AREs, have been identified, characterized and cloned (Stumpo D. J., 2010). AUBPs modulate mRNA stability or translational efficiency, with either negative or positive effects, or can determine mRNA cellular localization (Bevilacqua et al, 2003). Multitude of studies bring out the fundamental importance of these sequence motifs revealing as the loss of ARE-mediated mRNA control leads to severe pathologies and then as post-transcriptional control ARE-mediated impacts on gene expression in a global scale (Hao S. and Baltimore D., 2009; Lu J. Y. et al, 2006; Ghosh M. et al, 2009; Katsanou V. et al, 2009; Taylor G. A. et al, 1996; Hodson D. J. et al, 2010; Gruber A. R. et al, 2011).

Although AREs are different in sequence, data show that most AREs are able to bind more than one AUBP (Barreau C., 2005). In some instances [e.g. c-myc, c-fos, GM–CSF, tumour necrosis factor α (TNF-α) and cyclooxygenase 2 (Cox-2)] the same ARE-containing mRNAs can bind to many of the known AUBPs. Furthermore, many of the AUBPs have been observed to bind to multiple mRNAs and this binding crosses the ARE classification limits. Therefore, at present no clear association between of certain

AUBPs, or structurally related AUBPs, with specific types of AREs appears possible. In a number of cases, the effects on mRNA stability or translation of AUBP binding to a specific mRNA have been examined, as summarized in **Table 2.1**. Some AUBPs such as AUF1, the Hu family, in particular HuR, Tristetraprolin (TTP) and KSRP have been characterized better and most extensively studied. HuR is a 36-kDa protein normally localized in the nucleus.

ARE-BPs	mRNA stability		Protein expression			
			Translational efficiency		Abundance	
	Increase	Decrease	Increase	Decrease	Up regulated	Down regulated
AUF1	c-myc (42) c-fos (42,67) PTH (56) GM–CSF (42) TNF-alpha (42)	c-myc (46) c-fos (53) p21 (48) Cyclin D1 (48) GM–CSF (53,54) IL-3 (55)				GM–CSF (55) IL-3 (55)
HuR	c-fos (59,63,67) MyoD (68) p21 (48,68,69) Cyclin A (70) Cyclin B1 (70) Cyclin D1 (48) NOS II/iNOS (64) GM–CSF (59) TNF-alpha (65,74,139) Cox-2 (71,139) IL-3 (55,66) VEGF (62) Myogenin (68)		p53 (99,137)	TNF-alpha (139) Cox-2 (139)	p21 (69) Cyclin A (70) Cyclin B1 (70) NOS II/iNOS (64) GM–CSF (55) Cox-2 (71,173) IL-3 (55) VEGF (173) p53 (99,137)	TNF-alpha (139)
Hel-N1	TNF-alpha (74) GLUT1 (72)		NF-M (73) GLUT1 (72)		NF-M (73) GLUT1 (72)	
HuD	GAP-43 (75–77)				GAP-43 (75,76)	
TTP		c-fos (90) GM–CSF (18, 81,83–85, 91) TNF-alpha (18,81,83–86,89,90) Cox-2 (87) IL-2 (82,90) IL-3 (18, 66, 83,84, 88)				GM–CSF (81) TNF-alpha (80) IL-2 (82) IL-3 (88)
BRF1		TNF-alpha (89,93) IL-3 (55,92,93)				GM–CSF (55) IL-3 (55)
TIA-1				TNF-alpha (120) Cox-2 (121)		TNF-alpha (120) Cox-2 (121)
KSRP		c-fos (90,93) NOS II/iNOS (102) TNF-alpha (90,93) IL-2 (90,93) c-jun (93)				NOS II/iNOS (102)
CUG-BP2	Cox-2 (150)			Cox-2 (150)		Cox-2 (150)
Nucleolin	bcl-2 (175)					
TINO		bcl-2 (176)				
PAIP2	VEGF (177)				VEGF (177)	

Table 2.1. Effect of RNA-Binding Protein on the stability and translation of ARE-containing mRNAs.

In response to various stress conditions, HuR affects the translation and enhances the stability of many mRNAs that contain an ARE (Fan X. C., 2008). However, the mechanism by which HuR mediates the stabilization of mRNAs is poorly understood. HuR ability to stabilize targets depends on its translocation from nucleus to cytoplasm in response to stress conditions, such as heat shock, UV irradiation, amino acid starvation, chronic ethanol exposure, hypoxia and ATP depletion (Lopez de Silanes I. et al, 2004; Wang W. et al, 2000). Besides, HuR binding affinity or its ability to associate with additional RNA stabilizing factors are modulated by post-translational

modifications of HuR protein (Lafarga V. et al, 2009; Gummadi L. et al, 2012). *AUF1* gene encodes for a family of four isoforms generated by alternative splicing distinguished by their apparent molecular weights in p37, p40, p42 and p45. AUF1 has either stabilizing or destabilizing activity of various mRNAs by binding to specific ARE sequences. In particular, the different role is cell specific, in relation to relative abundance of the AUF1 isoforms, or to various post-translational protein modifications (Raineri I. et al, 2004; Wilson G. M., 2003). HuR and AUF1 are AUBPs which influence each other because the two proteins interact physically and this dynamic interaction determines a complex which mediates a rapid turnover or facilitates stabilization of mRNA targets (Barker A. et al, 2012; Gummadi L. et al, 2012). The TTP belongs to a family of CCCH zinc finger AUBPs that trigger rapid mRNA decay by recruiting enzymes involved in mRNA turnover (Hau H.H. et al, 2006). TTP as a physiological regulator of gene expression was discovered in TTP knockout mice in which an increase of stability of TNF-α mRNA (TTP target) was responsible for elevated levels of protein in $TTP^{-/-}$macrophages and consequently to a strong chronic inflammation (Carballo E. et al,2008). TTP is posttranslationaly regulated: phosphorylation of TTP by MK2 primarily affects mRNA decay downstream of RNA binding by preventing recruitment of the deadenylation machinery (Clement S. L. et al, 2011). KSRP (KH-domain-containing ARE-BP) was first identified as an important component of a multiprotein complex involved in splicing process (Min H. et al, 1997) and than identified also as AUBP. KHs motif of KSRP interacts with RNA target promoting its decay via exosome or PARN. The ability of KSRP to promote mRNA decay correlates with its ability to bind ARE and associate with RNA-degrading enzymes (Gherzi R. et al, 2004).

Moreover, Pullmann et al have shown an interaction network among different AUBPs whereby each AUBP showing affinity for its cognate mRNA and for multiple other AUBP mRNAs (endogenous and recombinant in each case) (Pullmann R. Jr et al, 2007). This interesting circuitry suggests that the expression of these AUBPs likely has an important post-transcriptional regulative effect and that their expression levels could be tightly interdependent.

Post-transcriptional regulation in cancer. Modifications of mRNA stability and/or translational efficiency are increasingly reported in cancer. mRNA stability and

translation are controlled through a complex network of RNA/protein interactions involving recognition of specific target mRNAs by AUBPs.

c-fos is a transcription factor that heterodimerizes with members of the Jun family of transcription factors. The resulting AP-1 complexes are involved in a vast panel of physiological events and the function of *c-fos* is critical for malignant tumor development (Saez E. et al, 1995). *c-fos* mRNA is very labile and its stability is regulated also by an ARE-mediated mechanism. *v-fos*, the viral counterpart of *c-fos*, is the transforming gene of the FBJ-murine osteosarcoma retrovirus. *v-fos* gene is missing a part of the 3'UTR that contains the ARE and this may account in part for its oncogenic potential (Miller A. D. et al, 1984). *v-myc* is a retroviral-transforming gene. Alteration of its cellular counterpart *c-myc* affects cell proliferation, growth, metabolism and differentiation and it is associated to numerous forms of cancer. *c-myc* mRNA is regulated at post-transcriptional level by different mechanisms including ARE-mediated regulation that induces its instability. 3'-UTR mutation of *c-myc* mRNA can affect its stability: a human T-cell leukemia line missing a 61-nt ARE (Aghib D. F. et al, 1990) and in a human myeloma a 3'UTR translocation (Hollis G. F. et al, 1988), are responsible to stabilize mRNA suggesting the importance of this region in mRNA of oncogenes and so in cancer. Another extremely important gene for cell cycle and neoplastic progression regulation that presents a 3'UTR-mediated regulation is *cyclin D*. The 3'-UTR of *cyclin* D1 is rearranged in patients with mantle cell lymphoma and is truncated in line MBD MB-453 human tumor (Lebwohl D. E., 1994) In both cases, the loss of physiological regulation by this region brings to the increase of mRNA stability compared to physiological conditions (Audic Y. and Hartley R.S., 2004).

The over-expression of some AUBPs may contribute to tumorigenesis by stabilizing mRNAs of cytokines and other growth regulators. One of the examples is HuR binding to the p53 3'-UTR, which stabilizes p53 mRNA and enhances its translation. The translational regulation of p53 was first reported in 1984. *p53* mRNA contains a poly(U) translational repressor in its 3'-UTR and in response to stimulus (as gamma-irradiation), the poly(U) region mediates an increase in p53 translation that correlates with enhanced HuR binding, with no change in mRNA stability (Mazan-Mamczarz K. et al, 2003). HuR's function in directing mRNA translational activation is still not elucidated. HuR binds also an ARE in the COX-2 3'UTR stabilizing the mRNA and

thereby increasing protein levels (Cok S. J. and Morrison A. R., 2001). These data, coupled to the co-localization of the highest levels of HuR with growth factor up-regulation, suggest a role of stabilizator for mRNAs involved in growth of malignant brain tumors and colon cancer. Wang and colleagues showed that the expression and half-life of cyclins A and B1 mRNAs could be reduced in colorectal carcinoma (RKO) cells by knocking down HuR expression with antisense oligonucleotides. Cells with decreased HuR levels have reduced growth, indicating an oncogenic role for this RNA-binding protein inducing cyclins A and B1 mRNAs stabilization (Wang W. et al, 2000).

AUF-1 is up-regulated in numerous cancers compared with untransformed tissues. AUF-1 might have anti-tumorigenic roles as a destabilizing factor for some mRNAs as *bcl-2*, *cyclin D1* and proinflammatory molecules (e.g. IL-6, GM-CSF, Inos, COX-2). Upregulated levels of HuR have been found in different types of sarcomas or carcinomas. Moreover, it seems that AUF-1 plays a role in cancer because it regulates the production of miRNAs through regulation of the enzyme Dicer (Abdelmohsen K., 2012).

Finally, the RNA-binding proteins ability to bind ribonucleic acids could also regulate specific miRNAs. A relevant example of such interaction in cancer condition is miR-20a, whose negative regulation of TGFßR2 and the TGFβ pathway in glioblastoma is greatly enhanced by Quaking (QKI), an AUBP behaving as a tumor suppressor. Chen et al showed that p53 induces QKI in glioblastoma, where QKI associates with and stabilizes mature miR-20a, thus inhibiting the TGFß signaling (Chen A. J. et al, 2012).

The great interest dedicated by the scientific community to post-transcriptional mechanisms mediated by AUBPs and miRNAs is confirmed by the recent development of several specific web tools, such as the comprehensive PTRguide (www.ptrguide.org/doku.php), a continuously updated list of databases and tools for post-transcriptional regulation of gene expression analysis, and doRiNA (dorina.mdc-berlin.de/rbp_browser/dorina.html), focusing on the search for miRNAs and AUBPs co-regulating mRNAs (Dassi E. and Quattrone A., 2012).

Apoptosis and *bcl-2*

Apoptosis is a conserved genetic and biochemical program whose basic principles appear active in all metazoans. Apoptosis is vital for embryogenesis, tissue homeostasis and defense against pathogens. Notably, its deregulation can lead to cancer, as well as to autoimmune and degenerative diseases. In mammals, the apoptosis is controlled by two molecular programs each of which ultimately leading to the activation of members of the caspase family. Subsequently, cleavage of key cellular substrates drives to cell death. The two molecular programs are known as the extrinsic pathway operating downstream of death receptors, such as Fas and the tumor necrosis factor receptor (TNF) family, and the intrinsic pathway, which is activated by a diverse rank of stress signals that have as consequence the extrusion of cytochrome C from mitochondria. The "point of no return" in this pathway is defined by mitochondrial outer membrane permeabilization (MOMP), which leads to the release of cytochrome C into cytoplasm. Although the caspases proteolytic cascade represents a central point in the apoptotic response, its initiation is tightly regulated by a variety of other factors. Among these, Bcl-2 family proteins are known to play a pivotal role in the induction of caspase activation and in the regulation of apoptosis, Bcl-2 family proteins regulate MOMP and thereby determine the cellular commitment to apoptosis (Danial N. N. and Korsmeyer S. J., 2004; Adams J. M. and Cory S., 2007; Chipuk J. E.et al, 2006; Reed J. C. ,1994).

Bcl-2 superfamily consists of a set of evolutionarily conserved genes/proteins (**Fig. 2.5**), which regulate the cellular response to various intrinsic stresses, including growth factor deprivation, or damage caused by hypoxia, radiation, antineoplastic and oxidizing agents, and imbalance of Ca^{2+} (Reed J. C., 1998). This family includes proteins, which can promote either cell survival, such as Bcl-2, Bcl-XL, Mcl-1, A1, Bcl-W or cell death, like Bax, Bak, Bcl-X_S , Bok. The relative amounts or equilibrium between these pro- and anti-apoptotic proteins influence the susceptibility of cells to a death signal. Unrelated to their role in apoptosis, all Bcl-2 family members are characterized by containing at least one of four conserved Bcl-2 homology (BH) domains, designated BH1-BH4, which correspond to a-helical segments. In general, the anti-apoptotic members show sequence conservation in all four domains, while the pro-apoptotic molecules are characterized by absence of the first a-helical segment, BH4. The BH3

domain is also known as the minimal death domain required for binding to multi domain anti-apoptotic Bcl-2 family members and is the critical death domain in the pro-apoptotic Bcl-2 family members which contain only BH3 domain ("BH3-domain-only") and are all pro-apoptotic (Adams J. M and Cory S., 1998; Burlacu A., 2003). BH1, BH2, and BH3 domains of the anti-apoptotic proteins form a hydrophobic groove that binds to the hydrophobic face of the amphipathic α-helical BH3 domain from a pro-apoptotic binding partner.

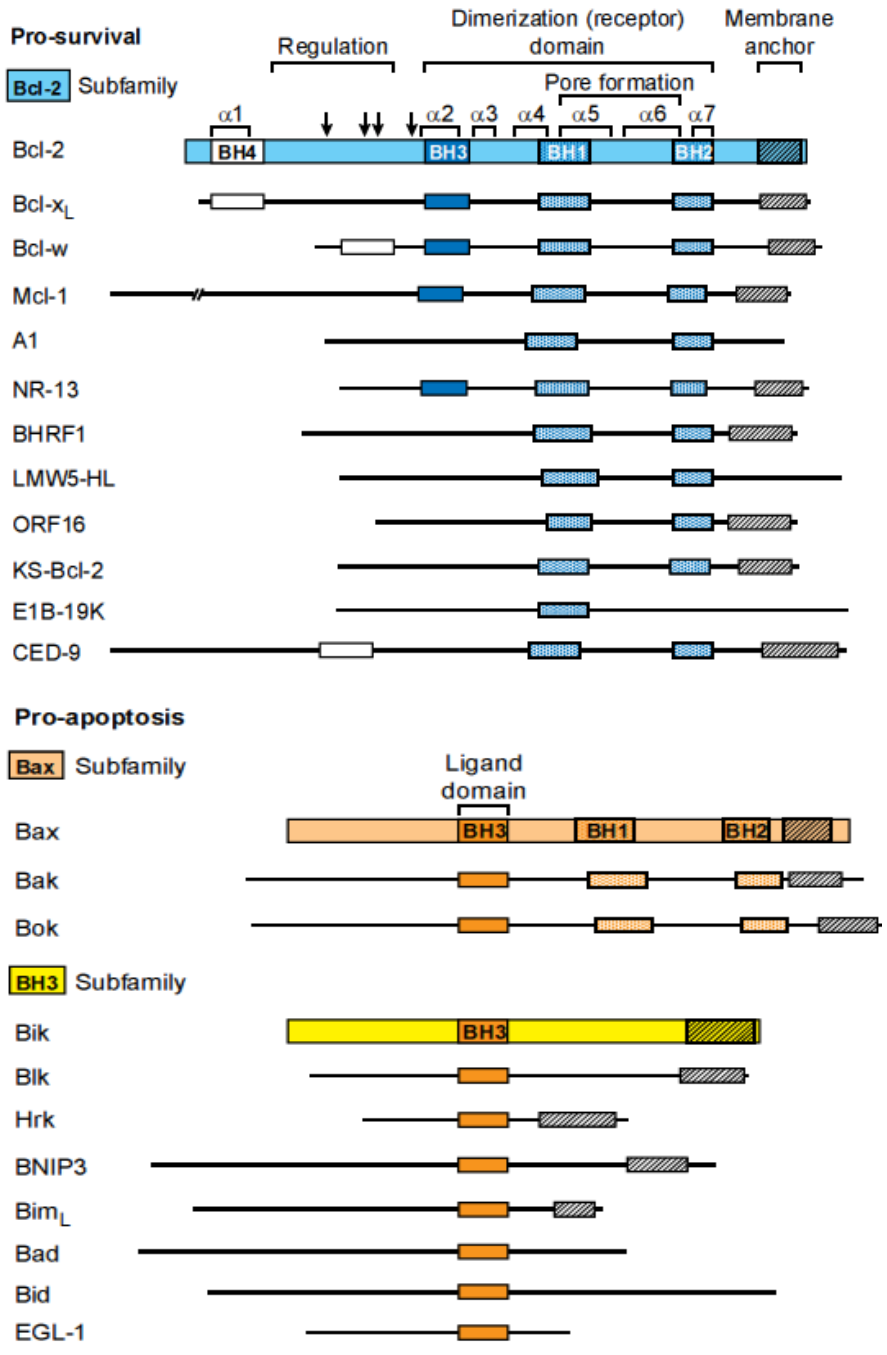


Fig. 2.5. The Bcl-2 Protein Family (Adams J. M. and Cory S., 1998)

The hydrophobic cleft formed by BH1-3 may be further stabilized by the BH4 domain. The unstructured loop between the BH3 and BH4 domains in Bcl-2 and Bcl-X_L is subjected to phosphorylation, leading to inactivation of their pro-survival activity. The other possible post-translational regulation mechanisms which can modify this domain include caspase-mediated proteolytic cleavage, which leads to the removal of BH4, rendering these proteins pro-apoptotic. In addition to BH domains, several Bcl-2 family members possess a trans membrane (TM) domain that enables them to localize in the sub-cellular membranes, including the mitochondrial outer membrane, endoplasmic reticulum (ER) and nuclear membranes (Danial N. N. and Korsmeyer S.J., 2004).

The *bcl-2* gene derives its name from B-cell lymphoma where it was first identified. In about 80-90% of cases of these cell-malignancies, the t(14;18) chromosomal traslocation actives the gene. Indeed, *bcl-2* gene is moved from its normal location on chromosome 18q21 into juxtaposition with the immunoglobulin heavy chain (IgH) *locus* on chromosome 14q32, thus placing the *bcl-2* gene under the control of the powerful transcriptional enhancer elements associated with the immunoglobulin heavy chain locus and leading to over expression of *bcl-2*. Bcl-2 protein is a membrane protein that is localized to the outer mitochondrial membrane, endoplasmic reticulum membrane, and nuclear envelope, whereby its NH2-terminal is pointing the cytosol. As many other members of its family, Bcl-2 has a hydrophobic domain at COOH-terminal (TM domain) that allows anchoring of the protein into membranes; this intracellular localization is important for their function. As already mentioned above, Bcl-2 family members form homo- as well as heterodimers, thus, Bcl-2 (a 239 amino acids protein, 26 kDa) forms heterodimers with Bax, a pro-apoptotic protein with ∼21% amino acid identity with Bcl-2. In addition to Bax, several other genes have been reported to encode proteins having sequence homology with Bcl-2 and capacity to form heterodimers with it. Among these, *bcl-X* is able to generate two proteins through an alternative splicing mechanism: Bcl-X_L (longer form) and Bcl-X_S (shorter form). Bcl-X_L is a 241 amino acid protein (with 43% sequence identity with Bcl-2)and suppresses cell death, while Bcl-X_S acts as a pro-apoptotic protein (Adams J. M. and Cory S., 1998). Another Bcl-2 homologous is Mcl-1, which shares ∼35% sequence identity with Bcl-2. It is involved in the regulation of apoptosis versus cell survival, and in the maintenance of viability interacting with Bax or Bak (Milot E. and Felep J. G., 2011). In absence of a

death signal, pro- and anti-apoptotic Bcl-2 family members are localized in distinct intracellular compartments, providing important clues for their function: anti apoptotic members are initially integral membrane while the large majority of the pro-apoptotic proteins is localized to the cytosol, but following a death signal, they undergo a conformational change that enables them to target and interact with anti-apoptotic protein, into mitochondrial outer membrane and to function as pro-apoptotic proteins (Burlacu A., 2003).

Bcl-2 inhibits the initiation of apoptosis directly, by acting on mitochondria to prevent the increase of permeability and indirectly, by interacting with other proteins that inhibit apoptosis. Bcl-2 is defined as a "scaffolding protein" or docking protein, because it is able to interact with a wide range of protein factors, such as the protein p53BP2 (p53-binding protein), R-RAS (small G protein monomer), and serine / threonine kinase c-Raf1. One of the main functions of Bcl-2 is to set the correct electrochemical homeostasis of mitochondria. At the level of the mitochondrial membrane Bcl-2 is a part of a complex of four proteins that form the structure so called "megapore", an ion channel denominated also "Permeability Transition Pore" (PTP). These proteins are hexokinase, the aqueous channel VDAC (Voltage Dependent Anionic Channel) through which flow molecules of ATP / ADP, the anti port ANT (Adenine Nucleotide Translocator) placed at the level of the inner mitochondrial membrane and cyclophilin D. "Megapores" are located in the points of contact between the inner and outer membranes of mitochondria. The fact that the inner mitochondrial membrane is almost impermeable is essential for the creation of an electrochemical gradient that allows the accumulation of H^+ ions inside the inter membrane space during respiration of mitochondria. Without this electrochemical gradient, cellular respiration would not be possible and there would not be ATP synthesis. Due to an apoptotic stimulus both membranes may lose their ability to selectively filter H^+ ions, causing the dissipation of the membrane potential. The mitochondrial external and/or internal membrane permeabilization is at least partly monitored to the complex of "megapore". The factor ANT has key role in permeabilization (Vieira H. L. et al, 2000) since under various stimuli, such as calcium internalization, it forms a non specific pore in the inner membrane. Due to these non specific pores H^+ ions enter in matrix, followed by the passage of water and glucose, which causes the swelling of the matrix to the outside

with distension of the cristae. Consequently a breaking of the outer membrane occurs with leakage of pro-apoptotic proteins located in inter membrane space including cytochrome C and the factor AIF (Apoptosis Inducing Factor). The over-expression of Bcl-2 does not prevent early gradual reduction in the transmembrane potential but, when expressed in the outer mitochondrial membrane it inhibits the permeability transition to several apoptosis inducers. In addition Bcl-2 also acts at the level of the endoplasmic reticulum where it is capable of increasing the permeability to calcium ions from the inside of the organelle to the cytoplasm, reducing ER stress. A moderate increase in the concentration of Ca^{2+} is associated with a protective effect against apoptosis (Foyouzi-Youssefi R. et al., 2000, Weston R. T. and Puthalakath H., 2010).

As already mentioned, Bcl-2 can bind numerous proteins, including Apaf-1. This bond protects the cells from apoptosis because sequesters Apaf-1 and inhibits its catalytic effect on caspase activation, even if the Cytochrome C is released by the mitochondria. The two mechanisms of anti-apoptotic activity of Bcl-2, that is direct prevention of the release of Cytochrome C and inhibition of the activation of caspases induced by Apaf-1 in spite of the release of Cytochrome C, are not mutually exclusive (Cotran R. S. et al, 1999). Moreover, studies performed on nematode *C. Elegans* model considering orhtologues gene of Bcl-2 and caspases (*Ced-3* and *-4* genes) have highlighted a direct regulation of caspases by Bcl-2. There are also studies that seem to show that Bcl-2 has also pro-apoptotic activity, being the substrate of caspase 3 that is able to cut Bcl-2 between the BH3 and BH4 domains, inducing pro-apoptotic activity of the BH3 domain (Cheng E. H. et al, 2001).

Overall, there is strong evidence that Bcl-2 and other family members, in addition to their well-described effects on apoptosis, are involved in the regulation of cell-cycle progression. Bcl-2 appears to affect cell-cycle either at the transition between G0/G1 and S phases or during G2/M phase. Inhibition of G1 / S transition by Bcl-2 over expression proceeds by modulating the level of proteins, such as p130 or p27, involved in the control of G1 / S transition (Bonnefoy-Berard N.et al, 2004; Vairo G. et al, 2000; Tsutsui S. et al, 2006; Greider C. et al,2002; Cory S.,et al, 2003).

No less important than transcriptional and post-transcriptional control is the regulation of Bcl-2 at post-translational level. A delicate balance between phosphorylation and de-phosphorylation events is apparently able to regulate Bcl-2

biological activity. Phosphorylation of Bcl-2 enhances cell survival, whereas de-phosphorylation leads to apoptotic induction (Agostinis P. et al, 2003). Among the sites well studied and characterized the amino acid Serine at position 70 plays an important functional role (Ruvolo P. P. et al, 2001), in fact its phosphorylation is linked to the ability of the Bcl-2 to suppress apoptosis (Takahiko I. Et al, 1997). Phosphorylation of Bcl-2 on Ser70 stabilizes its interaction with Bax, inhibits Bcl-2 degradation and blocks the binding with p53. Several growth-factor-activated protein kinases, including MAPK (mitogen-activated protein kinase), ERK1/2 (extracellular-signal-regulated kinase 1/2), JNK1 (c-Jun N-terminal protein kinase 1) and PKC (protein kinase C) could phosphorylate Bcl-2 (Willimott S. and Wagner S. D., 2010). It is evident that the type of stimulus, the regulatory pathways involved, and the degree and duration of phosphorylation at specific Bcl-2 residues produce different outcomes and are linked to cellular context. Furukawa et al have described another phosphorylation site in Threonine at position 56. Bcl-2 can be phosphorylated in this position by the cyclin dependent kinase CDC2 and consequently manifests inhibitory activity on the cell cycle resulting in the accumulation of cells in G2 / M phase (Furukawa Y. et al, 2001).

Involvement of Bcl-2 in Cancer. Bcl-2 promotes cell survival and so it considered a proto-oncogene because Bcl-2-mediated inhibition of apoptotic pathways is a critical step in tumorigenesis. For this reason, many studies have focused on the assessment of how *bcl-2* deregulation impacts on cancer development and maintenance. Bcl-2 transgenic mice develop spontaneous tumors (McDonnell T. J. et al, 1991) and Bcl-2 gene and protein over-expression has been discovered in various lymphatic malignancies, including acute and chronic lymphocytic leukemias (Tzifi F. et al, 2012), non-Hodgkin's lymphoma (Hussein M. R. et al, 2004), and solid tumors as small cell lung cancers (Brambilla E. et al, 1996), breast carcinomas (Vaillant F. et al, 2013), prostate carcinomas (McDonnell T. J. et al, 2008), melanoma (Leiter U. et al, 2000) and gliomas (Fels C. et al, 2000), and in some case it is correlated with prognosis. High expression of Bcl-2 correlates also with resistance to cancer therapeutics, including chemotherapy and radiotherapy (Reed J. C., 1997; Abdullah L. N. et al, 2013; Schulze-Bergkamen H. et al, 2008).

Anti-apoptotic Bcl-2 as target for treatment of cancer. On the basis of previous observations Bcl-2 is an interesting pharmacological target for the treatment of numerous cancers. The first strategy used was the treatment with synthetic molecules that mimic the BH3 domain of the pro-apoptotic Bcl-2 family members (as Bid, Bax, Bim). Bcl-2 inhibitor drugs were designed to potentially counteract the increase of anti-apoptotic Bcl-2 family members (as Bcl-2, Bcl-X_L e Bcl-w) and thus to induce cancer cell death. Examples include Gossypole and its derivates, AT 101, Obatoclax which are now undergoing clinical trials, all three demonstrating remarkable preclinical efficacy in a variety of cell systems (Liu Q. and Wang H-G., 2012). Notably, these Bcl-2 small molecule inhibitors kill chemoresistant cell lines as well as susceptible cell lines, demonstrating a distinct mechanism of action and clinical promise (Klymenko T. et al, 2011; Chauhan D. et al, 2007; Martin A. P. et al, 2009; Crawford N. et al, 2011; Hu W. et al, 2012; Balakrishnan K. et al, 2009).

The first drug developed to pharmacologically inhibit Bcl-2 was Oblimersen sodium (G3139, Genasense), an 18-mer antisense oligonucleotide designed to target the first six codons of human *bcl-2* mRNA (Klasa R. J. et al, 2002). Initial preclinical and clinical trials showed that the combination treatment of Oblimersen with a given anti-cancer drug increased the chemotherapeutic effect in various types of cancers with good tolerability (Moreira J. N. et al, 2006). However, after failing to result in survival differences in a pivotal melanoma trial this agent did not obtain US Food and Drug Administration approval. In addition, the treatment resulted in increased expression of other anti-apoptotic Bcl-2 family members probably caused by Bcl-2 down-regulation. To overcome these difficulties future efforts are directed to neutralize a broader range of the anti-apoptotic Bcl-2 family members (Weyhenmeyer B. et al, 2012).

Focusing on regulation of *bcl-2* expression

Mechanisms that regulate *bcl-2* expression have not yet been fully elucidated, although both classical transcriptional and post-transcriptional mechanisms have been described.

Transcriptional control. In 5' portion a negative control region of *bcl-2* gene was identified (Young R. L. et al, 1993) which seems to be the region which binds p53 in order to transcriptionally inhibit *bcl-2* expression (Miyashita T. et al, 1994).

Translational control. More recent studies have shown the existence of a region upstream of the start site of translation (from position -119 to -84 bp), in 5'-UTR of *bcl-2* mRNA, which exercises control at translational level, acting as a negative regulator of *bcl-2* gene expression. It encodes for a uORF (upstream Open Reading Frame) of 11 amino acids (Harigai M. et al, 1996) which produces a functionally inactive protein.

Another regulation mechanism al translational level is based on IRES sequence lying in 5'-UTR of *bcl-2* mRNA. In stress conditions, there is a strong reduction in the overall rate of protein synthesis, and this is due to caspase cleavage of proteins of the cap complex and consequent increased mRNA degradation. However, Bcl-2 can continue to be produced thanks to the presence of IRES element on its mRNA which recruits 40S ribosomal subunit. IRES activity is controlled by ITAFs (IRES trans-activating factors), that bind to the IRES to allow translation under specific conditions such as DNA damage or apoptosis (Spriggs, K. A. et al, 2005). Therefore, it appears that cells have a mechanism for maintaining production of Bcl-2 despite adverse conditions. IRES-mediated translation may represent a trick by which apoptosis can be delayed or by which the cell can be saved if the apoptotic stress is removed (Hellen C. U. and Sarnow P., 2001).

Post-transcriptional control. As also mentioned above, *bcl-2* expression is regulated also at post-transcriptional level. 3'-UTR of *bcl-2* mRNA is strongly implicated in the regulation of gene expression. mRNA stability is orchestrated by interactions between sequence and/or structural elements (*cis* elements) lying on mRNAs and specific *trans*-acting factors that recognize these elements. Indeed, the 3'-UTR of *bcl-2* mRNA can control its nuclear export, state of polyadenylation, sub-cellular localization, speed of translation and degradation of mRNA. In general, the mRNA 3'-UTRs may be considered as a regulatory regions essential for an appropriate expression of thousands of human genes. Alterations in the functions mediated by the 3'-UTR might influence the expression of a gene (if the gene carries a mutation in its 3'-UTR for example) or more genes (if there are changes in the trans-acting elements regulating the fate of different mRNAs).

In our laboratory, a new mechanism of post-transcriptional regulation for the *bcl-2* gene expression has been disclosed. Already known for other genes, this mechanism is based on functional cooperation between *cis*-acting motive ARE in the 3'-UTR of the

bcl-2 mRNA (Schiavone N. et al, 2000) and a set of proteins that bind specifically to this motive (*trans*-acting factors) called AUBPs governing the stability of mRNA. *bcl-2* ARE (belonging to class II) has destabilizing activity, modest in physiological conditions, early and drastically increases after apoptotic stimulus, in association with modification of expression profile of specific AUBPs (Donnini M. et al, 2001).

As described above, AREs are able to accelerate deadenylation, and so degradation, of mRNAs. This is possible because of the recruitment of Exosome that is dependent on the simultaneous binding of specific cellular proteins to both ARE sequence and Exosome subunits. **Fig. 2.6** summarizes principal *cis* and *trans* agents present on *bcl-2* mRNA.

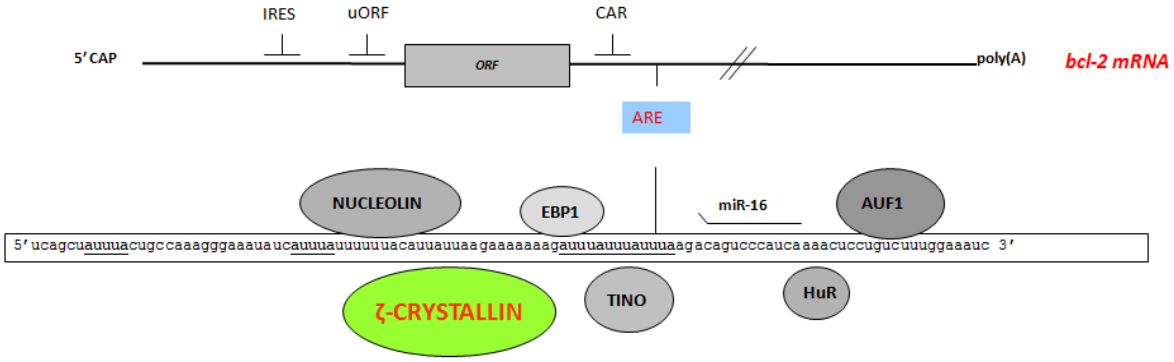


Figure 2.6. *cis* and *trans* acting factors of *bcl-2* mRNA.

To date several *bcl-2* mRNAs AUBPs have been identified. AUF1 is a heterogeneous nuclear ribonucleoprotein protein implicated in mRNA stability regulation, and demonstrated to shuttle dynamically between the nucleus and the cytoplasm. Lapucci et al have demonstrated that AUF1 binds ARE of *bcl-2* mRNA *in vivo*, stabilizing the relevant mRNA (Lapuci A. et al, 2002). Spicer's group identified two stabilizing AUBP: Ebp1 and Nucleolin, members of the same bcl-2 mRNP complexes (bcl-2 messenger ribonucleoprotein). A possible role for Ebp1 in the post-transcriptional regulation of *bcl-2* expression includes the modulation of mRNA stability; however, Ebp1 may also influence protein translation efficiency and the transport of *bcl-2* mRNA from the nucleus to the cytoplasm (Bose S. K. et al, 2006). Recent studies in HL60 cells have identified Nucleolin as *bcl-2* mRNA-stabilizing protein binding specifically *bcl-2* ARE and inhibiting ribonuclease-mediated *bcl-2* mRNA degradation (Sengupta T. K. et al, 2004;

Otake, Y. et al, 2005; Otake Y. et al, 2005 and 2007). Moreover, Ishimaru et al (Ishimaru D. at al, 2010) have proposed a possible mechanism of competition between Nucleolin and AUF1 which is balanced in normal conditions, while the presence of abnormal high levels of Nucleolin (i.e. in CLL) in the cytoplasm may cause a shift in the balance of mRNA regulation toward stabilization rather than degradation of *bcl-2* mRNA. Another component of bcl-2 messenger mRNP complex is HuR. It binds *bcl-2* mRNA in vivo together with Nucleolin but in a separate binding site. HuR has a positive role on *bcl-2* mRNA stability (Ishimaru D. et al, 2009). Donnini et al identified a new protein, Tino (and successively identified as RKHD1); Tino is able to destabilize a chimeric reporter construct containing the *bcl-2* ARE, endowed with a negative regulatory action on *bcl-2* gene expression at the post-transcriptional level (Donnini M. at al, 2004). In 2010, Lapucci et al identified a new bcl-2 mRNA AUPB, called ζ-crystallin (Lapucci A. et al, 2010) which I'll discuss in more detail in the next section.

bcl-2 mRNA decay is also modulated in part by the CA repeats in the 3'-UTR, which are located about 131 nucleotides from the stop codon and upstream of the previously characterized ARE. The *bcl-2* ARE has been known to mediate *bcl-2* mRNA decay during apoptosis, either by increasing binding of destabilizing factors or by reducing interaction with stabilizing factors while *bcl-2* mRNA decay does not significantly changes upon apoptosis induction. Therefore, CA repeat-mediated decay of *bcl-2* appears to contribute to maintaining appropriate Bcl-2 levels in normal conditions, while ARE-dependent destabilizing pathway may be activated by apoptotic stimuli to allow a rapid down-regulation of *bcl-2* level (Lee J. et al, 2004).

Post-transcriptional control of *bcl-2* expression contemplates also miRNAs. The chromosomal deletion 13q14.3 occurs in more than 50% of CLL cases, and it is also common in myeloma, mantle cell lymphoma and prostate cancer. *miR-15a* and *miR-16-1* have been mapped in this chromosomal region suggesting that their deletion may have a role in CLL onset and progression. Deletion of *miR-15a* and *miR16-1* in mice results in a lymphoproliferative disease similar to human CLL, thus providing strong evidence that deletion of these miRNAs is a cause of CLL (Calin G.A. et al, 2008). Over-expression of a genomic fragment containing *miR-15a* and *miR-16-1* sequences in a leukemic cell line expressing *bcl-2* significantly reduced Bcl2 expression, thus providing

direct regulation link between the miRNAs and *bcl-2.* So miRNAs can act as oncosuppressors or oncogenes (Willimott S. and Wagner S. D., 2010).

ζ-crystallin

ζ-crystallin is a highly conserved protein (Porté S. et al, 2009) endowed with pleiotropic functions. First discovered as a structural protein in the lens of guinea pigs (Huang Q. L. et al, 1987), camelids (Garland D. et al, 1991; Duhaiman A. S. et, 1995), and tree frogs (Fujii Y. et al, 2001), it was also found in plants (Mano J. et al, 2000) and yeast (Kranthi B. V. et al, 2006). Crystallin belong to a family of ubiquitous proteins (α, β and ζ-crystallins) ensuring the transparency and refractive properties of the ocular lens. In some taxa, up to 50% of the total lens proteins have also a metabolic activity, (Slingsby C. et al, 2013, Gonzalez P. et al, 1993). In particular ζ-crystallin also has oxidoreductase activity (Rodokanaki A. et al, 1989; Rao P. et al, 1992) and has been identified as a novel NADPH:quinone reductase (Rao P.V. and Zigler J.S., 1992; Gonzalez P. et al, 1993).

ζ-Crystallin is an example of "gene sharing," a term that was initially used to describe the recruitment of metabolic enzymes as structural proteins in the ocular lens (Piatigorsky J. and Wistow G. J., 1989). The generation of a new function in an existing gene is one of the evolutionary mechanisms through which organisms acquire new characteristics. In some instances, new functions apparently can be developed without the loss of the original function and without gene duplication (Gonzalez P. et al, 1993).

Tang A. and Curthoys N. P. (2001) and Ibrahim H. et al. (2008) identified ζ-crystallin as an RNA-binding protein in metabolic acidosis context. ζ-crystallin is able to stabilize rat *glutaminase* (*GA*) mRNA in conditions of metabolic acidosis and to increase relative protein expression. The mitochondrial Glutaminase catalyzes the initial reaction in the primary pathway of renal glutamine catabolism and its activity is increased 7- to 20-fold within the renal proximal convoluted tubule during chronic acidosis. ζ-crystallin stabilizes *glutaminase* mRNA by binding to a pH response element (pH-RE) lying in 3'UTR of transcript. The pH-RE is a repeat of an 8-base AU sequence sufficient to impart a pH-responsive stabilization to chimeric mRNAs and for its composition AU-rich, it could be considered an ARE. Ibrahim et al have confirmed that ζ-crystallin binds to an ARE sequence with high affinity and specificity (Ibrahim H. et al, 2008). Curthoy's proposed a possible model to explain the normal degradation and the pH-responsive stabilization of the *GA* (**Fig. 2.7**) for which ζ-Crystallin in low pH conditions ζ-crystallin

displaces destabilizing AUF-1 and binds pH-RE element. Schroeder J. M. et al. (2003) found that ζ-Crystallin also stabilizes rat *glutamate dehydrogenase* (*GDH*) mRNA by binding to peculiar motifs of its 3'-UTR by the same mechanism that affects an increase in *GA* mRNA that is in pH-RE-mediated way. GDH is also an enzyme involved in renal ammoniagenesis and gluconeogenesis, two processes that maintain acid-base balance.

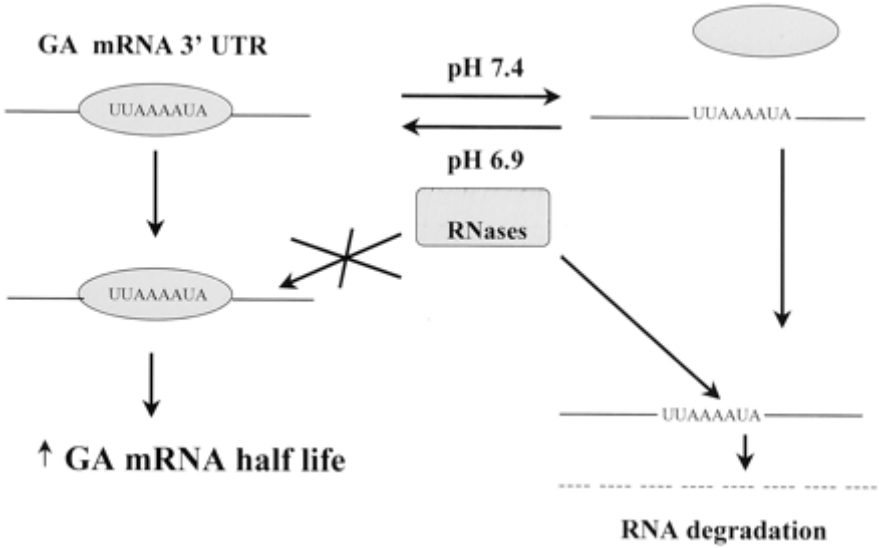


Fig. 2.7. Proposed model for the mechanism by which the onset of metabolic acidosis leads to a stabilization of the renal GA mRNA. The 8-base adenosine and uridine-rich pH-RE serves as a binding site for AUBP to recruit a specific endoribonuclease (Laterza O. F and Curthoys N. P., 2000).

Moreover, Fernandez M. R. et al. (2007) have recently reported that human and yeast ζ-crystallins specifically bind a synthetic A(UUUA) pentameric probe indicating that both enzymes are ARE-binding proteins and that this property has been conserved in evolution. This supports a role for ζ-crystallins as trans-acting factors that could regulate the turnover of certain mRNAs.

Last, Portè et al. (2009) have further characterized the RNA binding properties of ζ-Crystallin, demonstrating that NADPH efficiently competed against the A(UUUA) pentameric probe for binding to human ζ-crystallin, which suggests that the NADPH-binding site is involved in the binding of ζ-crystallin to RNA. Szutkowska et al demonstrated that acidification of extracellular medium increased the expression and the activity of BSC1/NLCC2 ($Na^+/K^+/2Cl^-$ co-transporter) through a post-transcriptional mechanism which involves ζ-crystallin in renal thick ascending limb cells. Acidic

medium increases the expression of ζ-crystallin and consequently of this target BSC1 (Szutkowska M. et al, 2009). In particular exogenous ζ-crystallin modulation impacts on *BSC1* mRNA stability by an ARE-mediated mechanism suggesting that ζ-crystallin as a key element in adaptation of renal tubular cell types in response to acidosis. This data support that ζ-Crystallin plays an essential role in the stabilization of Na(+)/K(+)/2Cl(-) co-transporter mRNA in the medullary thick ascending limb. However, other experiments carried out by using small interfering RNA to down-regulate ζ-crystallin in proximal tubule and thick ascending limb cells suggest that additional proteins must contribute to the rapid turnover and selective stabilization of the various mRNAs during metabolic acidosis, as speculated by Curthoys (Curthoys N. P., 2009).

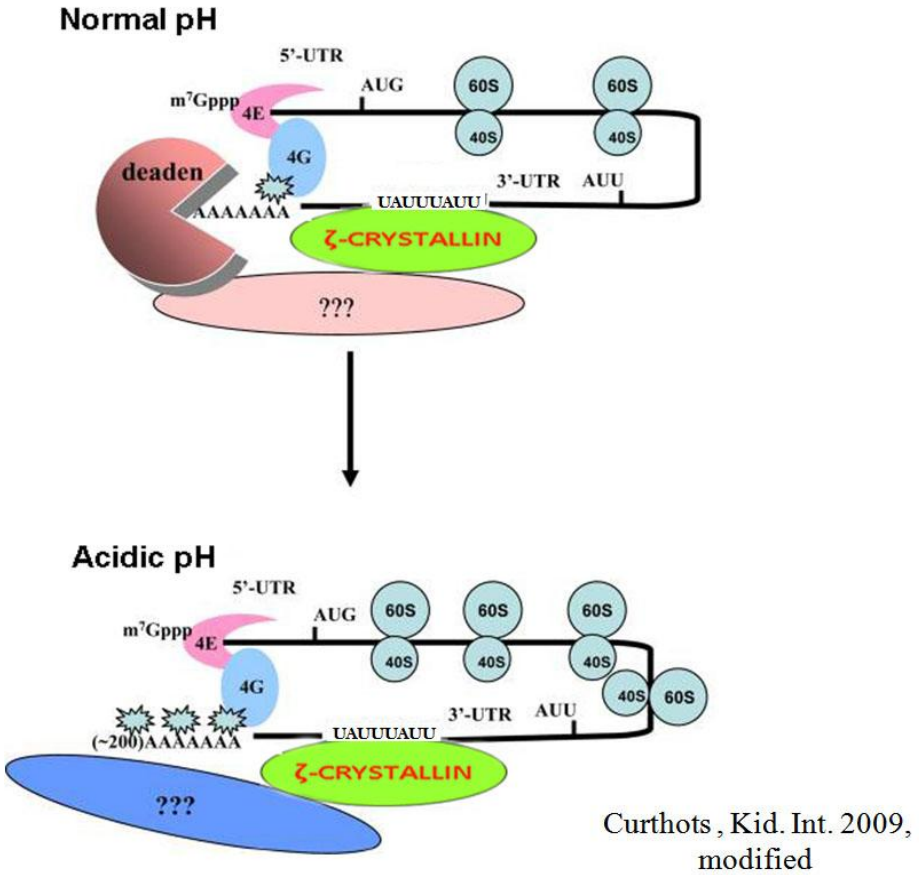


Fig. 2.8. ζ-Crystallin is involved in pH-responsive stabilization of different mRNAs in acidosis by binding to an AU-rich pH-responsive element located in 3'UTR of several pH-responsive mRNAs. In a pH normal condition protein complex bound to ARE recruits deadenylase and promotes rapid degradation of target mRNAs. In acidosis, activation of a signaling pathway leads to increased expression of ζ-Crystallin and remodeling of proteins bound to ARE.

Increasing binding of ζ-Crystallin and additional protein leads to stabilization and increased translation of mRNA targets (Curthoys N. P., 2009).

In a pH normal condition protein complex bound to ARE recruits deadenylase and promotes rapid degradation of target mRNAs. Decrease of pH leads to activation of a signaling pathway that results in increased expression of ζ-Crystallin and remodeling of proteins bound to ARE. In turn, increasing binding of ζ-Crystallin and additional protein leads to stabilization and increased translation of mRNA targets by Curthoys (Curthoys N. P., 2009) (**Fig. 2.8**).

In our laboratory ζ-Crystallin was identified as new AUBP of *bcl-2* mRNA, able to bind *bcl- 2*-ARE-both in cell-free models and in T-cell Acute lymphoblastic leukemia (ALL) cell lines. We showed a positive correlation of Bcl-2 and ζ-crystallin expression in different ALL-T-cell lines and in PHA-activated (phytohemagglutinin-activated) T lymphocytes. Moreover, PHA-activated T lymphocytes displayed higher levels of both ζ-crystallin and Bcl-2 proteins compared with non activated T lymphocytes and reached levels similar to those observed in ALL T-cell lines. With exogenous modulation of ζ-crystallin we demonstrated a stabilizing activity of ζ-crystallin on *bcl-2* mRNA in a *bcl-2* ARE-dependent manner. We have also demonstrated a higher *bcl-2* mRNA stability in T-cell ALLs respect to normal T-lymphocytes, which results in a stronger association of ζ-crystallin to *bcl-2* ARE in leukemic T lymphocytes respect to normal T-lymphocytes, which results in the increased Bcl-2 protein level. In our system, cytoplasmic levels of ζ-crystallin did not differ in normal PHA-activated T lymphocytes and leukemia T cells, indicating that the varying concentration-independent binding of ζ-crystallin to the *bcl-2* ARE in leukemia T cells might depend on other parameters. Therefore, we propose two possible alternative mechanisms to explain the differential interaction of ζ-crystallin with the *bcl-2* ARE in normal T lymphocytes and ALL T cells (**Fig. 2.9**). The first is that qualitative modifications undergone by ζ-crystallin in ALL T cells respect to normal T lymphocytes increase its binding to *bcl-2* mRNA, consequently altering the *bcl-2* AUBPs pattern. The second is that modifications of the *bcl-2* AUBP pattern in ALL T cells could favour the ζ-crystallin interaction with the *bcl-2* ARE. As presented above, there are different *bcl-2* AUBPs and for this their altered interactions or possible deregulation pathways could be characteristic of pathological situation. Furthermore, because the binding of one *bcl-2*

AUBP could prevent the binding of another AUBP and possible interactions among different AUBPs which bind the *bcl-2* ARE are not completely elucidated, the resulting effects on *bcl-2* mRNA fate are unpredictable *a priori* (Lapucci A. et al, 2010).

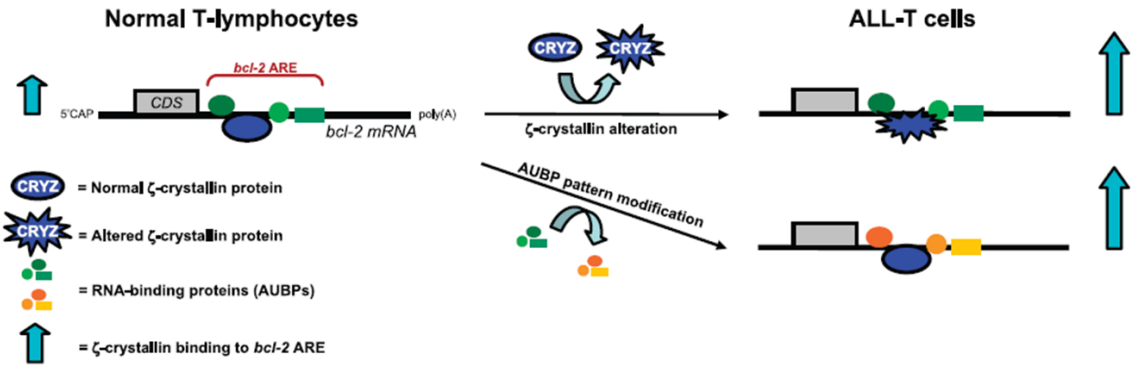


Fig. 2.9. Proposed model of bcl-2 mRNA and ζ-crystallin differential interaction in normal T lymphocytes and ALL T cells (Lapucci A. et al, 2010).

Bcl-2 and T-cell Acute Lymphoblastic Leukemia

T-cell Acute Lymphoblastic Leukemia (T-ALL), currently classified by the World Health Organization (WHO) as T lymphoblastic leukemia/lymphoma, comprises 15% of pediatric and 25% of adult acute lymphoblastic leukemia cases. Among these patients, only 70–80% of children and as few as 40% of adults reach long-term remission (Kraszewska M. D. et al, 2012). Recent intensive research into its molecular biology revealed significant heterogeneity of this type of leukemia. The heterogeneity of this disease reflects the variability of its biological features. Gene mutations, chromosome rearrangements and oncogene over-expression can be linked as mechanisms leading to a disruption of cellular pathways controlling lymphocyte differentiation and proliferation, phenomena occurring concurrently.

A number of studies have linked impaired apoptosis and deregulation of Bcl-2 family with the pathogenesis and treatment failure in ALL. The high expression of anti-apoptotic Bcl-2 could be implicated in leukemogenesis because a high frequency of *bcl-2* mRNA over expression is reported in ALL (Wojcik I. et al, 2005). Moreover, Bcl-2 expression in neoplastic cells from patients with precursor B-ALL, typical ALL and atypical ALL was found to be aberrant in 84%, 77%, and 75% of the cases, respectively, consistent with a diverse expression of Bcl-2 in the different types of ALL according to the stage of B-cell maturation (Menendez P. at al, 2004). However, different studies have failed to correlate the altered expression of these genes with survival and the results of the studies of lineage-dependent Bcl-2 expression in ALL are controversial. Furthermore, Bcl-2 levels influence the sensitivity of leukemic cells to therapy and it has been shown that differential regulation of pro- and anti-apoptotic Bcl-2 family members appears to be a key event in the execution of dexamethasone-induced apoptosis in ALL cell lines (Tzifi F. et al, 2010). These data suggest that an abnormal *bcl-2* gene expression influence the survival capacity of cell progenitors and contribute to malignant transformation or chemoresistance.

Acidic extracellular microenvironment and cancer

The hallmark of neoplasia is a constitutive and persistent increase in glycolysis that results in acute and chronic acidification of local environment. Indeed, a direct consequence of anaerobic glycolysis is the production of lactate from pyruvate, and acidic metabolites that cause drop in extracellular pH (pH_e), which may select more aggressive acid-resistant clones and favor tumor invasion (Gatenby R. A. and Gillies R. J., 2004). In fact, numerous *in vivo* studies have shown that extracellular pH of solid tumors is commonly acidic reaching pH value 6.5 or below (Mortensen B. T. et al, 1998). This phenomenon has been demonstrated in numerous solid tumors as well as in hematologic malignancies (Vaupel P. et al, 2009). Rapidly growing tumors need a complementary vasculature that can provide oxygen and nutrients necessary for survival. In addition, tumor mass develop faster then the blood supply, resulting in a hypoxic microenvironment. As result, increased metabolic acids such as carbonic acid and lactate are produced resulting in acidification of the microenviroment (Gatenby R. A. and Gillies R. J., 2004). Cellular lactate production by using the anaerobic glycolytic pathway, even in presence of sufficient oxygen rather than oxidative phosphorylation for energy production, is called “Warburg effect”.

The development of tumor-associated acidity depends on production of lactic acid, carbon dioxide, and on other factors such as inadequate blood supply, nutrient limitation and altered cellular mechanism. Recent studies have clarified that the aerobic glycolysis of cancer cells is commonly attributable to chronic over-activation of the transcription factor hypoxia-inducible factor-1 (HIF-1), which actives a set of glycolytic enzymes and pyruvate dehydrogenase kinase-1 (which function to inhibit pyruvate dehydrogenase and thus expedite conversion of pyruvate to Lactate) and promotes mitochondrial autophagy (Semenza G. L., 2007; Zhang H. et al, 2009) Cancer cells have to adapt to the stress caused by extracellular acidification to survive. Therefore dynamic mechanisms are required to ensure long-term pH homeostasis. Indeed, extracellular acidification leads to a decrease in intracellular (pH_i). pH_i is very important for control of cellular basic functions including membrane permeability, enzyme activity, metabolism, ATP maintenance, cell proliferation and apoptosis (Parks S. K. et al, 2011). Cancer cells activate an efficient membrane transport machinery that allows to eject H^+ and import HCO_3^- to maintain an elevated pH_i. Examples of transport

systems are Na^+/H^+ exchanger I (NHEI), Cl^-/HCO_3^-exchangers (CBEs), Na^+/HCO_3^- cotransporters (NBCs), H^+/lactate co-transporters (monocarboxylate transporters, MCTs), and CAII, IX, and XII. Thanks to these mechanism cancer cells develop an enhanced acid resistance to survive in the microenvironment where normal cells die (Fang J.S. et al, 2008). It imprints selection forces that drive cancer cells to evolve phenotypes with increased resistance to acid-induced cellular toxicity (Gatenby R.A. et al, 2006). The result is a cellular phenotype with a powerful advantage enhancing the evolutionary potential of the tumor population, which accelerates malignant progression and adaption to therapeutic strategies (Martínez-Zaguilán R. et al, 2006). Indeed, extracellular acidosis has also been blamed for a reduction in the cytotoxicity of drugs (Raghunand N. et al, 1999; Raghunand N. and Gillies G. A., 2000; Raghunand N et al, 2003) as chemotherapeutics. For example, an acidic pH inhibits cellular uptake of mitoxantrone and topotecan, so that elevation of pH_e in tumor tissue enhances those drugs' efficacy (Kato Y. et al, 2013; Adams D. J. at al, 2000). For this reasons, several studies have tried to manipulate extracellular pH as potential cancer therapy (McCarty M. F. et al, 2010). Acidic pH_e also plays a role in the resistance of tumor cells to drugs by increasing p-glycoprotein expression, thereby increasing drug efflux (Sauvant C. et al, 2008). Finally, under some conditions, low pH stimulates *in vitro* invasions and *in vivo* metastasis for solid tumors. Mechanisms responsible for such induction involve the metalloproteinases and/or cathepsins, which promote the deregulation of the EMC and basement membranes (Kato Y. et al, 2013). Moreover, acidosis has multiple effect on immune cells. On one side acidosis promotes inflammatory/immune response through activation of neutrophils (which themselves increase acidosis and hypoxia as result of their activity) and stimulation of monocytes and macrophages to release IL-1β and TNF-α respectively. On the other hand, many reports demonstrate that acidosis reduce NK cell activity and anergy of $CD8^+$ lymphocytes (Justus C. R. et al, 2013).

Acid pH is responsible for the phenotypic changes depending on the activation of transduction pathway, by which MAP kinases play a major role. Acidosis induces changes in MAPK activity via p38 and ERK1/2 phosphorylation. ERK1/2 seems to serve as a rescue program limiting necrotic cell death during milieu acidosis. Indeed, p38 phosphorylates the transcription factor CREB, a proto-oncogene that represents a link between tumor microenvironment and genomic changes. Thus, an acidic tumor

microenvironment can induce a longer lasting change in the transcriptional program, representing a memory effect which maintains the altered phenotype even when the cells leave the tumor environment (**Fig. 2.10**) (Riemann A. et al, 2012). However the mechanism by which extracellular acidosis affects the pathways (e.g. by G protein-coupled membrane sensors for H^+ concentration) and intracellular acidification has to be better elucidated.

The most interesting group of acid-sensing receptors current in cancer literature includes four homologous G protein-coupled receptors (GPCRs): GPR4, GPR65 [T cell death-associated gene 8 (TDAG8)], GPR68 [Ovarian cancer GPCR 1 (OGR1)] and GPR132 [G2 accumulation (G2A)]. This family of genes was only recently recognized to exhibit a pH-sensing function (Ludwig M. G. et al, 2003).

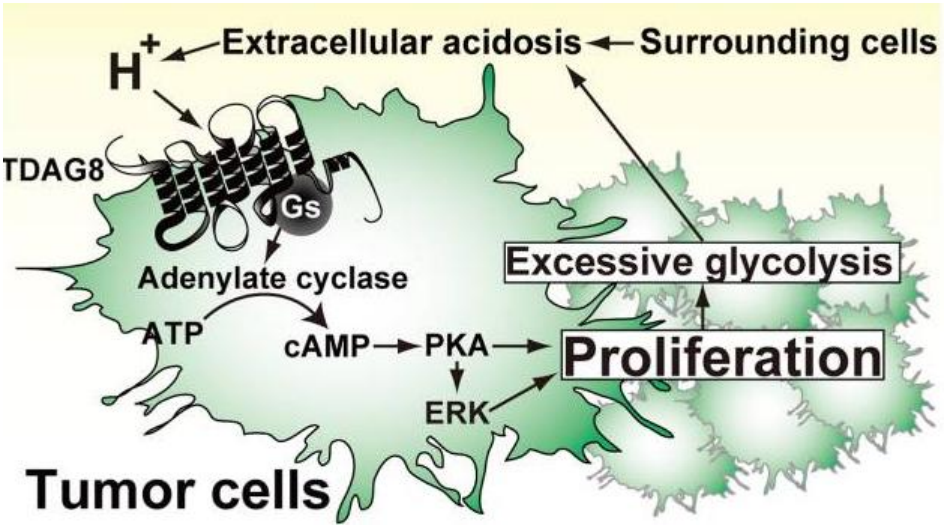


Fig. 2.10. Proposed mechanism mediated by G protein-coupled membrane sensors for tumor development. The excessive production of acidic glycolytic metabolites leads to the accumulation of protons in the extracellular space and subsequent acidification in the tumors, which in turn activates pH sensors, inducing activation of pathways kinases (e.g. ERK. p38) which trigger adaptations that allow the tumor cells to survive in an acidic environment and promote further tumor development (Ihara Y. et al, 2010).

Seuwen K. et al (2006) reported how G2A is however activated by oxidized lipids and not by acidic pH. The three validated pH-sensing members are differently expressed in normal tissues. GPR4 has demonstrated pH-sensing roles in kidney and endothelium (Chen A. et al, 2011; Sun X. et al, 2010). OGR1 functions as a proton sensor in osteoclasts and smooth muscle (Liu J. P. et al, 2010; Yang M. et al, 2006). TDAG8 seems

to be primarily limited in its expression to cells of the immune system (Radu C. G. et al, 2006). However, various cancers show elevated expression of GPR4 and/or TDAG8 (Ihara Y. et al, 2010; Sin W. C. et al, 2004). First results demonstrate the correlation between TDAG8 expression and tumor aggressiveness by promoting cells adaptation to the acidic environment. Another recent datum (Wyder L. et al, 2011) shows as tumor growth and tumor-associated vasculature are strongly reduced in GPR4$^{-/-}$ mice.
Some studies reported that TDAG8 couples with multiple G-proteins, probably Gαs and Gα12/13. Initially this receptor expression was restricted to lymphoid cells (Ishii S. et al, 2005), than it has been reported to be expressed in both lymphoid and non lymphoid tumors (Ihara Y. et al, 2010). As reported in Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/) and the BioGPS database (http://biogps.gnf.org/) strong expression of *TDAG8* mRNA, along with other pH-sensing GPCRs, is observed in a variety of human tumors, supporting the hypothesis that such receptors may be involved in tumor malignancy by sensing the acidic environment, mediating the activation of the metabolic pathways described above (Ryder C. et al, 2012; Yang L. V. et al, 2007).

Acidosis and Bcl-2 family

Ryder C. et al (2012) have demonstrated interesting data on the involvement of Bcl-2 family in acidosis to emphasize the increasingly evident importance of tumor microenvironment. They observed that incubation of lymphoma cell lines in acidic conditions minimizes apoptosis induced by multiple cytotoxic metabolic stress, including deprivation of glutamine or glucose and treatment with chemotherapic drugs as dexamethasone. Then they found that acidosis impacts on expression of Bcl-2 family members. At low pH anti-apoptotic *bcl-2* and *bcl-X_L* mRNA and protein expression increases while pro-apoptotic factors PUMA and Bim decrease. Moreover, the enhancement of anti to pro-apoptotic member ratio by acidosis makes cells more sensitive to the Bcl-2/Bcl-X_L antagonist ABT-737 (anti-apoptotic member inhibitor), suggesting that acidosis causes Bcl-2 family dependence. Authors speculate a possible implication of TDAG8 receptor, via MEK/ERK pathway.

3. AIMS

For more than 20 years, our group carried out studies aimed to elucidate post-transcriptional control of the major anti-apoptotic gene *bcl-2*, whose up-regulation is feature of various malignancies, including leukemias/lymphomas. Clarification of the mechanisms that regulate *bcl-2* expression is a topic of crucial importance given the key role that *bcl-2* assumes in tumor onset, development and chemo-resistance. *bcl-2* post-transcriptional control is mainly driven by interactions among a regulatory cis-acting adenine and uracil-rich element (ARE) located in the 3'-UTR of its mRNA and several related trans-acting ARE binding proteins (AUBPs). In 2010 we characterized the ζ-crystallin as a new AUBP of *bcl-2*, demonstrating that the specific association of ζ-crystallin with the *bcl-2* ARE was significantly enhanced in T cells of ALL cell lines and in T-cells derived from human ALL patients, which accounted for the higher stability of the *bcl-2* mRNA and suggested a possible contribution of ζ-crystallin to *bcl-2* overexpression occurring in this leukemia. Other than the *bcl-2* ARE, it has been demonstrated that ζ-crystallin binds also a specific RNA sequence called pH-response element (pH-RE), lying in the 3'-UTR of *glutaminase, glutamate dehydrogenase* and *BSC1* mRNAs, which code proteins involved in the maintenance of acid-base balance in renal cells. Importantly, ζ-crystallin stabilizes these transcripts via pH-RE binding during chronic acidosis, contributing to the response to acidosis of renal cells.. Numerous *in vivo* studies have demonstrated that the tumor microenvironment is characterized by a chronic acidification, mainly caused by the Warburg effect associated to rapid cellular growth, and which represents an important hallmark of cancer correlating with tumoral chemoresistance. Acidosis was recently found to inhibit apoptosis in lymphoma cell lines by enhancing Bcl-2 and Bcl-xL levels and lowering PUMA and Bim levels. Integrating the evidences reported above, the aim of this thesis is to unravel the impact of acidosis on ζ-crystallin-mediated *bcl-2*

overexpression occurring in T cell leukemia and to evaluate if this mechanism contributes to tumoral chemoresistance.

4. MATERIALS AND METHODS

Reagent and chemicals

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EPPS (3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid) and MES (2-(N-morpholino)ethanesulfonic acid) were purchased from VWR International LLC. The p38 MAPK inhibitor SB203580 was purchased from Life Technologies. Cycloheximide (CHX) and Perdrogen 30% H_2O_2 (w/w) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture and Treatments

Jurkat and Molt-4 human acute T-cell leukemia cell lines and HEK 293, Human Embryonic Kidney cell line (ATCC) were grown in RPMI 1640 medium (PAA) containing 10% fetal bovine serum (FBS) supplemented with 100 U/mL penicillin G, and 100 µg/mL streptomycin (Sigma) and 2mM of Glutamine (Lonza, Basel Switzerland) in a humidified incubator with 5% CO 2 at 37 °C. For pH treatment, cells were incubated in RPMI medium buffered with 7.5 mM HEPES, 7.5 mM EPPS and 7.5 mM MES (collectively known as HEM) at varying pH for different times. The pH of buffered medium was adjusted using HCl or NaOH (Yang L. V., 2007), using a standard pHmeter (PHM210, Meter Lab). For all studies, cells in suspension were seeded at 4–5 × 10^5 cells/ml.

For apoptosis studies cells were pre-incubated for 3 hrs in HEM at pH 7,4 or 6,3 and treated for 3 hrs with following apoptotic stimuli: 250 µM of H_2O_2 or 100 µM of Cycloheximide. For p38 inhibition experiments, Jurkat cells were treated with 10µM of inhibitor SB203580 for 30 minutes and than incubated with HEM-buffered RPMI medium (pH 7.4 or pH 6.3) for specific time courses.

Apoptosis Assay

Apoptosis was quantified with Annexin V and Propidium Iodide (PI) staining using Annexin-V-FLUOS Staining Kit (Roche) according to the manufacturer's protocol. Cells grown in acidic or physiological medium treated or not with stimuli were harvested and suspended in cold PBS and incubated in HEPES buffer with Annexin V/PI mix for 15 minutes at room temperature in the dark. Both Annexin V/PI fluorescence from live cells and PI fluorescence of fixed cells were measured using an FACScan (Becton-Dickinson, San Jose, CA, USA).

Western Blotting

Cells were lysed with ice-cold RIPA (radioimmunoprecipitation assay buffer) and protein concentrations were determined using Bredford method (Bio-Rad Laboratories, Hercules, CA, USA). Fifteen micrograms of total protein lysate were separated by 12% SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA, USA) and electroblotted (Trans-Blot Semi-Dry apparatus; Bio-Rad) onto Protran nitrocellulose (Schleicher & Schuell, Dassel, Germany) transfer membranes. Membranes were blocked in PBS with 3% of non fat dry milk buffer with gentle agitation for 1 hour at room temperature then, incubated in the same blocking buffer with primary antibodies. In the following are the antibodies used in the experiments: Polyclonal Antibody versus ζ-Crystallin was produced by Primm Custom Service (PrimmBiotech, Inc): pQE-TriSystem/HIS-Tag CRYZ expression vector was used to produce recombinant protein used for rabbit immunization.and monoclonal β-actin (Santa Cruz Technology, Inc., Santa Cruz, CA, USA), monoclonal Bcl-2 (clone 100, Upstate-Millipore), monoclonal α-tubulin (Sigma-Aldrich). Phospho-Tyrosine Mouse monoclonal (P-Tyr-102), monoclonal p38 MAPK and polyclonal Phospho-p38 MAP Kinase (Thr180/182,), (Cell Signaling Technology, Beverly, MA); monoclonal HuR was from Santa Cruz Biotechnology, Inc and AUF1 (5B9) was from Euromedex/Upstate Biotechnology.

The incubation with the primary antibody was performed overnight at 4 °C in continuous agitation. Followed by 4 washes of 5 minutes each with PBS + 0.1% Tween-20 and the subsequent incubation of the membrane for 1 hour at room temperature with secondary antibodies. The secondary antibodies included goat anti-mouse IRDye

800CW and goat anti-rabbit IRDye 800CW (Li-Cor Biosciences, Lincoln, NE, USA). The protein bands were analyzed by the Odyssey Infrared Imaging System (Li-Cor) using the software for protein quantification.

Immunopreciptation (IP)

Total protein extracted (500 μg) in non-denaturing lysis buffer (20 mM Tris HCl pH 8; 137 mM NaCl; 1% Nonidet P-40; 2 mM EDTA and Protease inhibitor cocktail) was added to mixture of monoclonal Antibody anti-ζ-Crystallin (Abcam) and protein G-coupled magnetic beads (Dynabeads Protein G) and were stirred overnight at 4°C. Immunoprecipitates were washed three times with lysis buffer and recovered with magnet, and suspended in sodium dodecyl sulfate (SDS)-sample buffer (187.5mM Tris Hcl pH 6.8, 6% w/v SDS, 30% glycerol, 150mM DTT, 0.03% w7v bromophenol blue). Immunoprecipates were separated with electrophoresis in 12% SDS (sodium dodecyl sulfate)-polyacrylamide gel and immunoblotted as described above.

Transfections

For transient transfection of recombinant ζ-crystallin, a 1016-nucleotide segment corresponding to the entire open reading frame of ζ-crystallin was PCR-amplified using 5'-GCGAAGCTTATGGCGACTGGACAGAAGTTG-3' and 5'-GCCTCGAGTAAGAGAAGAATCATTTTACCAGTAGCC-3' as forward and reverse primers, respectively, and cloned into the HindIII and XhoI sites of the pQE-TriSystem vector (Qiagen) to obtain the pQE-TriSystem/HIS-Tag ζ-cry expression vector. HEK 293 cells were transfected with pQE-TriSystem/HIS-Tag ζ-cry or the empty vector, using Lipofectamine 2000 reagent (Invitrogen) following protocol manufacturer's instructions, and analyzed for expression after 48 hrs by immunoblotting.

RNA Extraction, RT-PCR and Quantitative Real-Time RT-PCR

Total RNA was isolated using the TriReagent (Sigma-Aldrich), dissolved in RNase-free water, and RNA concentration was determined by Qubit Fluorimeter (Invitrogen, Carlsbad, CA, USA). Its quality was assessed by running 500 ng of sample on a 2% polyacrylamide gel. The RNA (1 μg of sample) were retrotranscribed using ImProm-II reverse transcriptase (Promega, Madison, Wisconsin, USA) and random examers.

Regular RT-PCR was performed with GoTaqPCR enzyme (Promega) using specific primers: For *bcl-2*, forward 5'-TCAGCTATTTACTGCCAAAG-3' and reverse 5'-GATTTCCAAAGACAGGAG-3'; for ζ-crystallin forward 5'-CAATTGATTAGATTTTCAGAGTAGATTTAG-3' and reverse 5'-TACCAGACAAATGCAGATAATTCTT-3'; for 18S, forward 5'-CGGCTACCACATCCAAGGAA-3' and reverse 5'-GCTGGAATTACCGCGGCT-3'; for mature bcl-2, forward 5'-GAGGATTGTGGCCTTCTTTG-3' and reverse 5'-AGCCTGCAGCTTTGTTCCAT-3'; for immature bcl-2 (hnRNA), forward 5'-TGATGTGAGTCTGGGCTGAG-3' and reverse 5'-GAACGCTTTGTCCAGAGGAG-3'. All primers were purchased from Sigma. Amplification program was a two step protocol consisting of an initial 2 minutes denaturation step at 95°C followed by 40 cycles of repeated denaturation (15 seconds at 95°C), annealing/extension (1 minute at 60°C), and sequent dissociation step to execute melt analyses. Real-time PCR assays were performed using the 7500 Fast Real-Time cycler system (Life Technologies). Relative quantification was calculated using the ΔΔCt method with 18S rRNA as reference genes.

mRNA decay assay

Forty-eight hours after ζ-crystallin transient transfection HEK293 cells were incubated for 4 hrs in acidic conditions and then treated with actinomycin D at a final concentration of 5 μg/ml to block transcription and harvested at various time points (0-2-4-6 hrs). Total RNA was extracted, and the real-time PCR assays were performed as described above to measure *bcl-2* mRNA half-life.

The *bcl-2* mRNA and heterogenous nuclear RNA (hnRNA) for the *bcl-2* stability quantification was performed by PCR amplification, according to the strategy described by Otake Y. et al (2007), using primers and amplification conditions described in the previous paragraph. The amplification of mature and immature RNA was performed with 2 sets of primers (above described): a pair of primers that anneal to the first intron selectively amplify hnRNA and the others primers anneal to sequences in 2 adjacent exons selectively amplify spliced, mature RNA. Real Time PCR amplification of 18S rRNA were used as the normalizer.

In silico analysis

The NetPhos 2.0 server (Technical University of Denmark) is used to predict serine, threonine and tyrosine phosphorylation sites in human ζ-crystallin protein sequence (NCBI Reference Sequence: NP_001880.2) of 329 aminoacids:

MATGQKLMRA VRVFEFGGPE VLKLRSDIAV PIPKDHQVLI KVHACGVNPV
ETYIRSGTYS RKPLLPYTPG SDVAGVIEAV GDNASAFKKG DRVFTSSTIS
GGYAEYALAA DHTVYKLPEK LDFKQGAAIG IPYFTAYRAL IHSACVKAGE
SVLVHGASGG VGLAACQIAR AYGLKILGTA GTEEGQKIVL QNGAHEVFNH
REVNYIDKIK KYVGEKGIDI IIEMLANVNL SKDLSLLSHG GRVIVVGSRG
TIEINPRDTM AKESSIIGVT LFSSTKEEFQ QYAAALQAGM EIGWLKPVIG
SQYPLEKVAE AHENIIHGSG ATGKMILLL

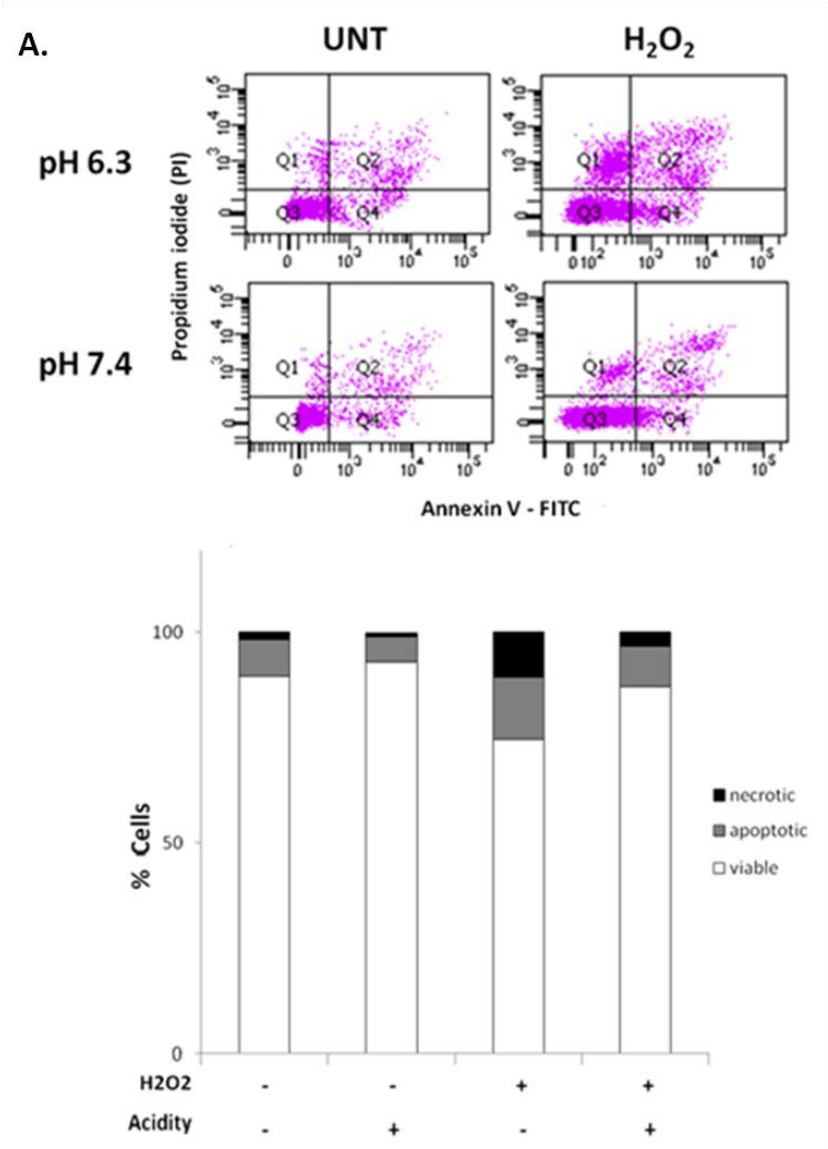
Statistical analysis

Statistical evaluation of the data was performed with a 2-tailed Student's t test using Primer analysis software. Differences were considered statistically significant when $P < 0.05$.

5. RESULTS

Acidic pH confers resistance against apoptotic stimuli in Jurkat cell line

We preliminary evaluated the influence of acidic conditions on the resistance of leukemic cells to apoptotic stimuli, using as experimental cellular model the human acute T cell leukemia Jurkat cell line, where first we characterized ζ-crystallin as a stabilizing *bcl-2* AUBP.



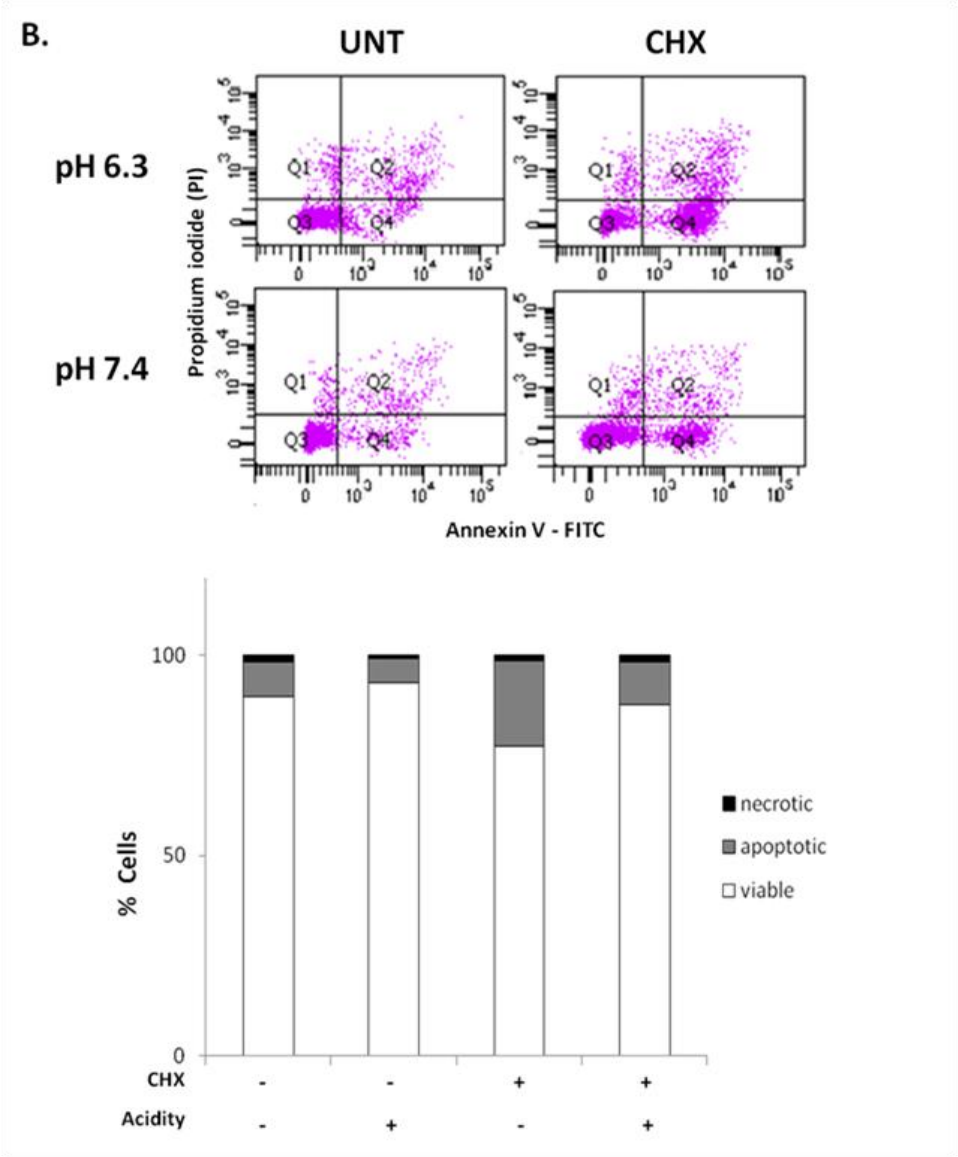


Fig. 5.1. Incubation in acid medium (pH 6.3) prevents cell death caused by apoptotic stimuli. Jurkat cells were subjected to different stimuli inducing apoptosis for 3 hrs with or without acidification of the medium for 3 hrs and then tested for viability and apoptosis with Annexin V and propidium iodide staining by flow cytometry. Jurkat cells were incubated with 250 µM H_2O_2 (**A**) or with 100 µM cycloheximide (CHX) (**B**). On the top of panels FACS plot are from a single experiment representative of three performed. On the bottom, histograms represent the percentage of viable cells (white bars), necrotic cells (black bars) and apoptotic cells (gray bars).

As reported in **Fig. 5.1** (**A** and **B**), normal culture conditions, that is in the absence of damaging agents, revealed a basal number of cells undergoing cell death, mainly attributable to apoptosis (evaluated by Annexin V test). Acidosis (pH 6.3) had minimal effect on this number. When cells were treated for three hours with different

apoptotic stimuli, which are the hydrogen peroxide (H_2O_2) and the protein synthesis blocker Cycloheximide (CHX), they underwent an increase in cell death, mainly attributable to apoptosis. In these conditions, pre-incubation for three hours in acidic medium is sufficient to significantly decrease the number of apoptotic and necrotic cells.

Bcl-2 protein and mRNA are up-regulated by acidosis in Jurkat cells

Ryder et al (2012) demonstrated that an acidic medium affects the regulators of the apoptotic process belonging to the Bcl-2 family in the WEHI7.2 lymphoma cell line. In particular, at pH 6.5 the expression of the anti-apoptotic genes *bcl-2 and bcl-*X_L underwent a significant increase, while the expression of the pro-apoptotic genes *PUMA* and *BIM* underwent a significant decrease. This rendered the cells more resistant to some metabolic stresses. Focusing on *bcl-2,* the above evidences prompted us to investigate if the major anti-apoptotic gene *bcl-2* underwent expression changes when cells were grown in acidic medium (pH 6.3) in comparison with physiological medium (pH 7.4). For this purpose we analysed by time course experiments the levels of Bcl-2 protein (**Fig. 5.2**) and *bcl-2* mRNA (**Fig. 5.3**) in Jurkat cells cultured in physiological (pH 7.4) or acidic (pH 6.3) conditions.

As shown in **Fig. 5.2** and **5.3**, acidic conditions induced a significant upregulation of *bcl-2* gene in Jurkat cells. In particular, just after 3 hours Bcl-2 protein levels were appreciably increased under acidic conditions (pH 6.3) when compared to physiological condition (pH 7.4), with an increase of approximately 50% ($p < 0.05$) after 6 hours. Furthermore, also the levels of *bcl-2* mRNA underwent an appreciable increase in acidic conditions, which was more than 50% after 9 hours ($p < 0.005$). This suggested that also in Jurkat cells the post-transcriptional control of *bcl-2* expression is dependent on the pH of the medium, stressing the possible role of ζ-crystallin.

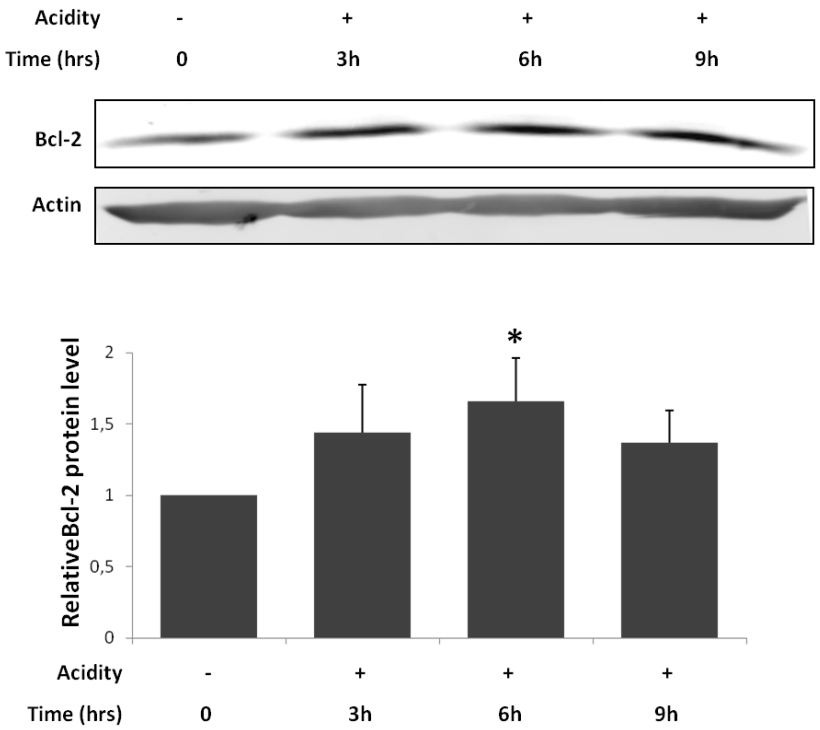


Fig. 5.2 Bcl-2 protein expression have been evaluated by quantitative WB in Jurkat cells maintained in acidic (pH 6,3) medium for 3, 6 and 9h. Upper panel shows a representative experiment of three performed. Values have been normalized to control conditions, represented by cells incubated in physiological conditions (pH 7.4). Actin is shown as loading control. Blots shown are presented as the means ± SD, n = 3. (*)$p < 0.05$ vs 0h (t-test).

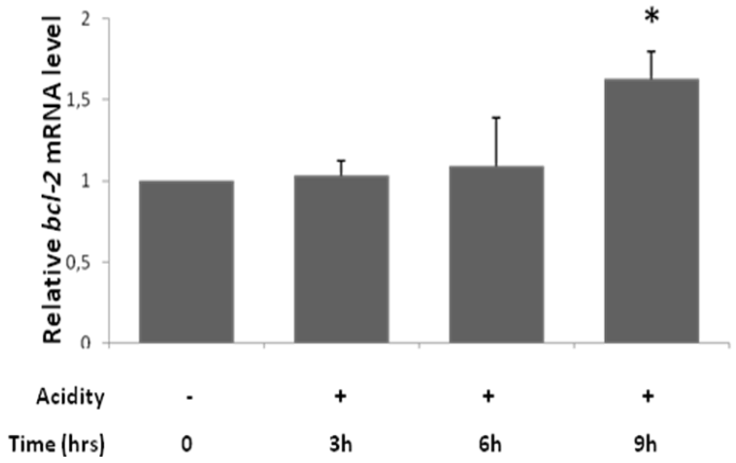


Figura 5.3. *bcl-2* mRNA levels have been evaluated by real time PCR in Jurkat cells maintained in acidic (pH 6,3) medium for 3, 6 and 9h. Values have been normalized to control conditions, represented by cells incubated in physiological (pH 7,4) medium. *18S* rRNA amplification was used as internal control. Blots shown are presented as the means ± SD, n = 3. (*)$p < 0.05$ vs 0h (t-test).

Acidosis increases ζ-crystallin protein and mRNA levels in Jurkat cells

To determine possible mechanism(s) controlling the up-regulation of *bcl-2* in acidosis, we considered one of the cellular actors that mediate the post-transcriptional regulation of *bcl-2* mRNA expression, that is ζ-crystallin, in normal (pH 7.4) and in acidic (pH 6.3) conditions. **Fig. 5.4** demonstrates that the ζ-crystallin protein levels were significantly higher in Jurkat cells grown under acidic culture conditions for 3 hours (over 50%; $p < 0.05$) and 6 hours (almost doubled; $p < 0.05$) respect to cells grown in physiological medium. This increase declined at the 9th hour.

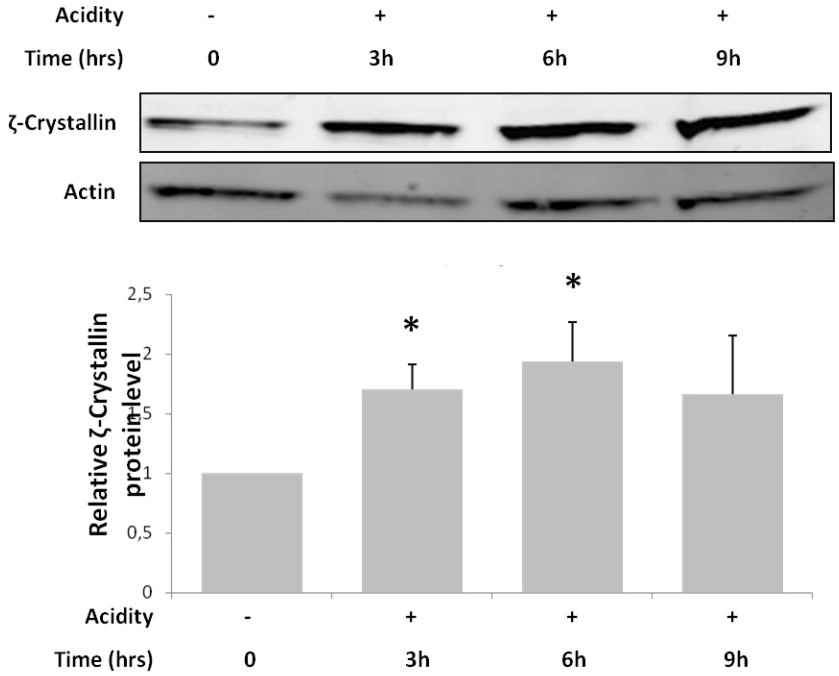


Fig. 5.4. ζ-crystallin protein levels have been evaluated by quantitative WB in Jurkat cells maintained in acidic (pH 6,3) medium for 3, 6 and 9 hrs. Upper panel shows an experiment representative of three performed. Values have been normalized to control conditions, represented by cells incubated in physiological conditions (pH 7.4). Actin is shown as loading control. Blots shown are presented as the means ± SD, n = 3. (*)$p < 0.05$ vs 0h (t-test).

As shown in **Fig. 5.5**, acidosis induced a robust increase of ζ-*crystallin* mRNA levels at 3 (+ 50%; $p < 0.05$), 6 (+ 80%; $p < 0.05$) and 9 (+ 150 %; $p < 0.05$) hours of incubation.

The increase of ζ-crystallin protein levels by acidic microenvironment was also observed in the Molt-T4 human acute T-cell Leukemia cell line (**Fig. 5.6**), which suggested that up-regulation of ζ-crystallin is probably a common phenomenon in ALL cells.

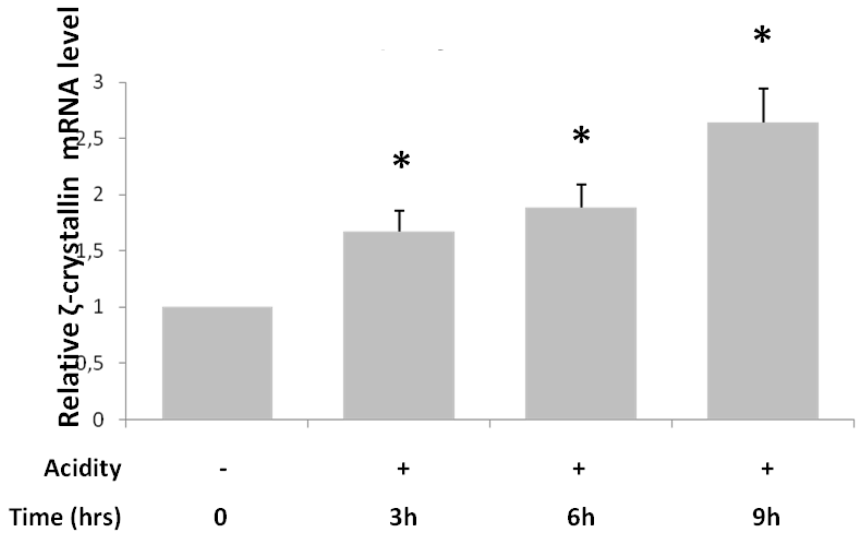


Fig. 5.5. *ζ-crystallin* mRNA in Jurkat cells maintained in acidic (pH 6.3) medium for 3, 6 and 9 hrs have been evaluated by real time PCR. Values have been normalized to control conditions, represented by cells incubated in physiological (pH 7.4) medium. *18S* rRNA amplification was used as internal control. Blots shown are presented as the means ± SD, n = 3. (*)$p < 0.05$ vs 0h (t-test).

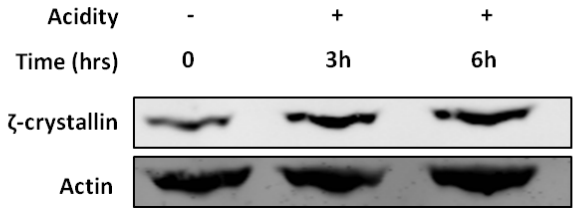


Fig. 5.6. ζ-Crystallin protein levels were evaluated by Western blot analysis in Molt-T4 cells grown with pH 6.3 or pH 7.4 for 3 and 6 hrs. Actin is shown as loading control.

We then investigated if acidosis impacted on the expression of others *bcl-2* mRNA AUBPs, particularly HuR and AUF-1. We did not observe any significant changes in HuR nor AUF-1 protein levels in Jurkat cells grown for 9 hours in acidic culture conditions (pH 6.3) respect to control (pH 7.4) (**Fig. 5.7**); this indicated that acidosis was not liable for quantitative alteration of HuR and AUF-1, although we could not exclude possible qualitative alterations at post-translational level.

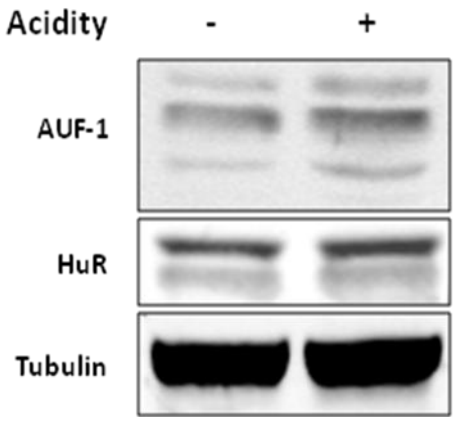


Fig. 5.7. AUF-1 and HuR protein levels have been evaluated by quantitative WB in Jurkat cells maintained in physiological (pH 7,4) or acidic (pH 6,3) medium for 9 hrs. Values have been normalized to control conditions. α-Tubulin is shown as a loading control.

Ratios of Bcl-2 and ζ-crystallin to β-actin protein levels plotted in **Fig. 5.8** indicate that they are significantly and linearly correlated as quantified by Pearson correlation coefficient equal to ρ= 0,98. This supported a strong relationship (ρ > 0,7) between Bcl-2 and ζ-crystallin proteins independently from their absolute levels.

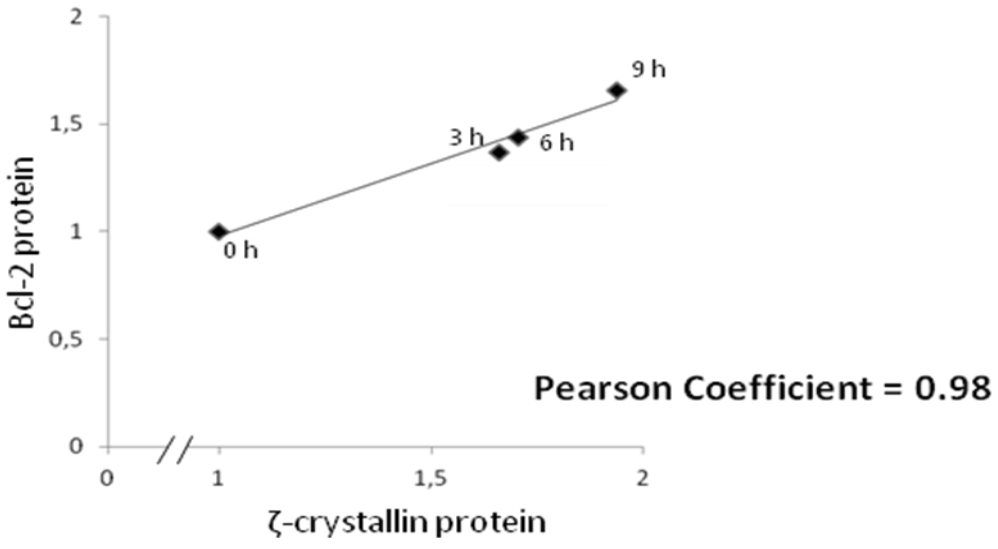


Fig. 5.8. Plot represents linear correlation between ζ-crystallin/β-actin and Bcl-2/β-actin protein levels in Jurkat cells incubated in acidity (pH 6.3) for 0, 3, 6 and 9 hrs. Pearson correlation coefficient is 0.98, calculated using 3 different experiments for each time point ($p<0.05$).

Acidic pH increases *bcl-2* mRNA stability in HEK293 cells

To unravel the mechanisms underlying the possible role of acidic pH on ζ-crystallin mediated *bcl-2* mRNA stabilization, we essayed the effects of acidic culture conditions on *bcl-2* mRNA stability exogenously modulating ζ-crystallin expression. Because repeated efforts to obtain satisfactory ζ-crystallin exogenous upregulation were unsuccessful in Jurkat cells, we used HEK293 (Human Embryonic Kidney) cells, where we had preliminarily verified that exposition to an acidic medium led to ζ-crystallin up-regulation (**Fig. 5.9**). Low pH conditions induced an increase of ζ-crystallin protein levels by 60% at the 6th hour ($p < 0.05$) and by 40% at the 9th ($p < 0.05$) respect to physiological pH. Consistently, a robust increase of *bcl-2* mRNA levels (+ 120%; $p < 0.05$) occurred at 4 hours of incubation of cells in acidic medium (**Fig. 5.10**).

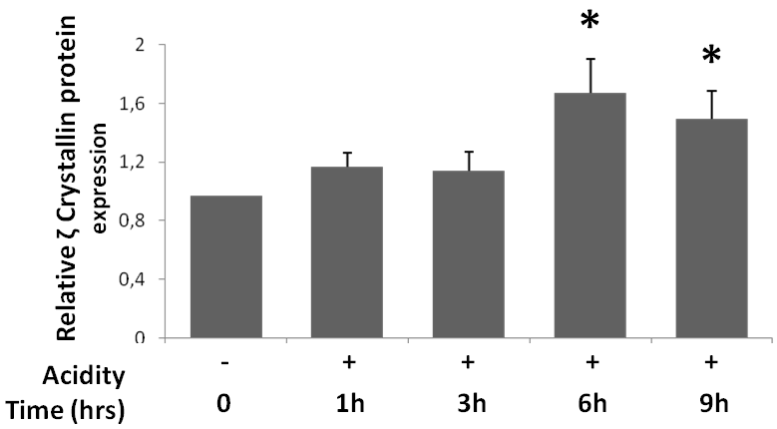


Fig. 5.9 ζ-crystallin protein expression have been evaluated by quantitative WB in Jurkat cells maintained in acidic (pH 6,3) medium for 1, 3, 6 and 9 hrs. Values have been normalized to control conditions, represented by cells incubated in physiological conditions (pH 7.4). Blots shown are presented as the means ± SEM, n = 3. (*)$p < 0.05$ vs 0h (t-test).

We then evaluated if the increased *bcl-2* mRNA levels observed in HEK293 incubated in low pH conditions were consequent to an increased *bcl-2* mRNA stability under acidic conditions. For this purpose, we have measured the levels of nascent, unspliced hnRNA and mature *bcl-2* mRNA in HEK293 incubated in low pH medium, a method that has been already used successfully to determine the relative rate of mRNA transcription and decay in a variety of cells (Otake Y. At al, 2007). The ratio of mature to immature RNAs gave an appraisal of mRNA stability. As shown in **Fig. 5.11**, the ratio

of *bcl-2* mRNA to *bcl-2* hnRNA measured in HEK293 grown in acidic conditions was about 3 fold higher respect to that measured in HEK293 grown in physiological conditions. These results indicate that acidosis is involved in *bcl-2* mRNA stabilization.

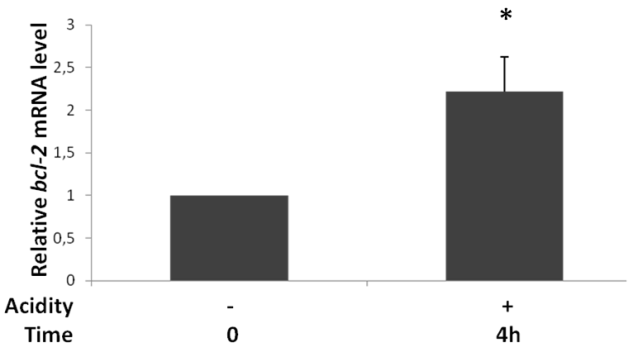


Fig. 5.10. *bcl-2* mRNA levels have been evaluated by real time PCR in HEK293 cells maintained in acidic (pH 6,3) medium for 4 hrs. Values have been normalized to control conditions, represented by cells incubated in physiological (pH 7,4) medium. *18S* rRNA amplification was used as internal control. Blots shown are presented as the means ± SEM, n = 5. (*)p < 0.05 vs 0h (t-test).

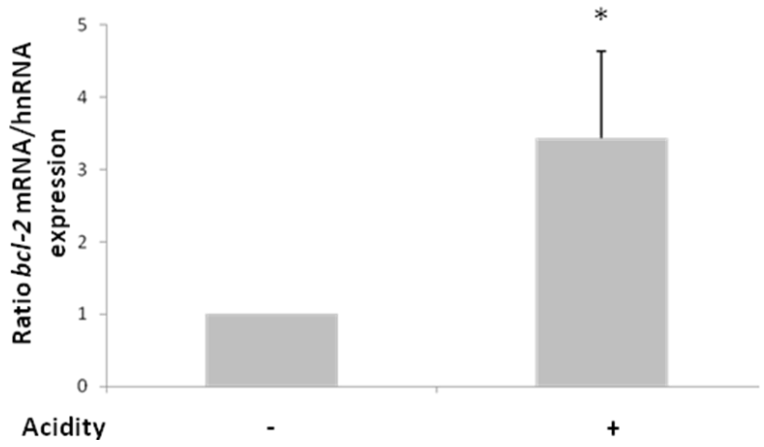


Fig. 5.11. Ratio of *bcl-2* mRNA/hnRNA levels obtained from Hek293 incubated in acidic conditions (pH 6.3) for 4 hrs determined by real-time PCR and normalized to the *18S* rRNA. Results are means ± SEM of 3 experiments. (*) $p < 0.05$ *vs* control conditions of cells incubated in physiological (pH 7.4) medium.

To better elucidate the positive effect of acidity on ζ-crystallin mediated *bcl-2* mRNA regulation we performed the over-expression of ζ-crystallin by transfection of a plasmid (p-ζ-cry)–harbouring the ζ-crystallin ORF, and using a mock vector as a negative control. Western blotting in **Fig. 5.12** shows the successful over-expression of

ζ-crystallin; in particular, the profile of the bands reveals the presence of the endogenous (below) and exogenous His-tagged (above) ζ-crystallin protein.

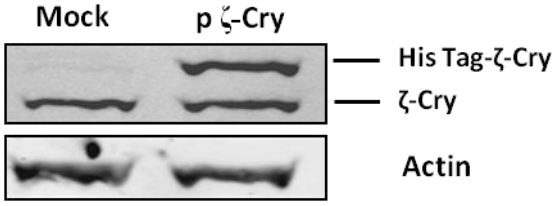


Fig. 5.12 Immunoblot analysis of ectopically expressed His-Tag ζ-crystallin in transfected HEK 293 cells after 48 hrs of transfection**.** Actin is shown as loading control.

Then, we evaluated the effects acid pH (6.3) combined or not with ζ-crystallin-over-expression on *bcl-2* mRNA stability, by measuring the ratio of *bcl-2* mature to immature transcripts (*bcl-2* mRNA/hnRNA) in HEK293 cells (**Fig. 5.13**). In physiological pH (7.4), ζ-crystallin over-expression caused an increase of *bcl-2* mRNA stability (∼12-fold; $p \leq 0.001$) respect to cells not over-expressing ζ-crystallin, as already demonstrated by our research group in Jurkat cells (Lapucci A et al, 2010). Importantly, combination of acidic culture medium (pH 6.3) with over-expression of ζ-crystallin led to more than additive effects, since it increased *bcl-2* mRNA stability by more than 20-fold ($p < 0{,}001$).

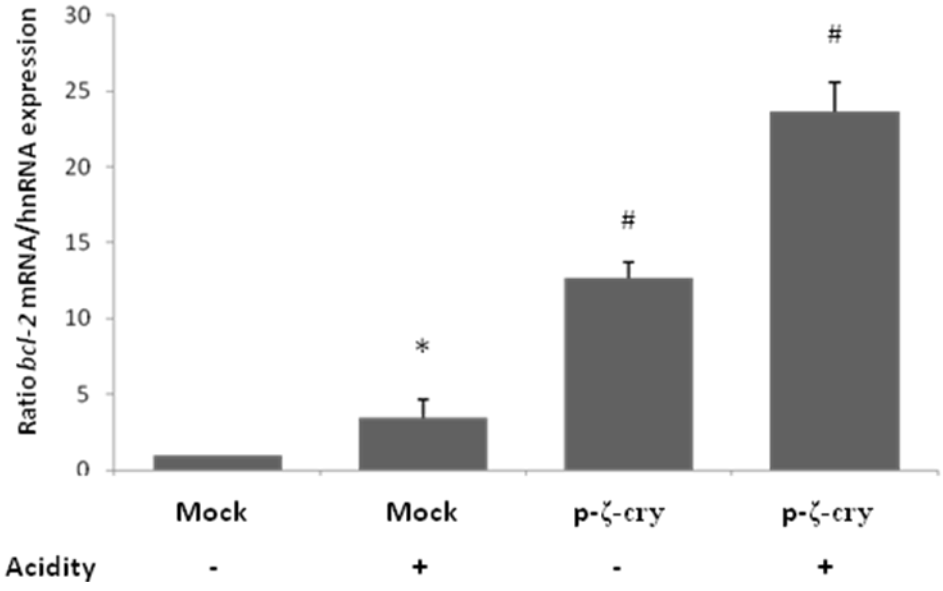


Fig. 5.13. Ratio of *bcl-2* mRNA/hnRNA levels obtained from Hek293 transfected with p-ζ-Cry or control vector (Mock) and incubated in acidic conditions (pH 6.3) for 4 hrs or not, determined

by real-time PCR and normalized to the *18S* rRNA. Results are means ± SEM of 3 experiments. (*) $p < 0.05$ and (#) $p < 0.001$ vs. control conditions of cells incubated in physiological (pH 7.4) medium.

To further confirm the stabilizing effect of low pH on bcl-2 mRNA, we measured *bcl-2* mRNA half-life (T½) in HEK293 cells subjected to the same treatments described above by a 4 hours acidity pre-treatment followed by a time-course experiments after blocking transcription with actinomycin D. Impressively, the impact of low pH and ζ-crystallin on *bcl-2* mRNA half-life was evident.

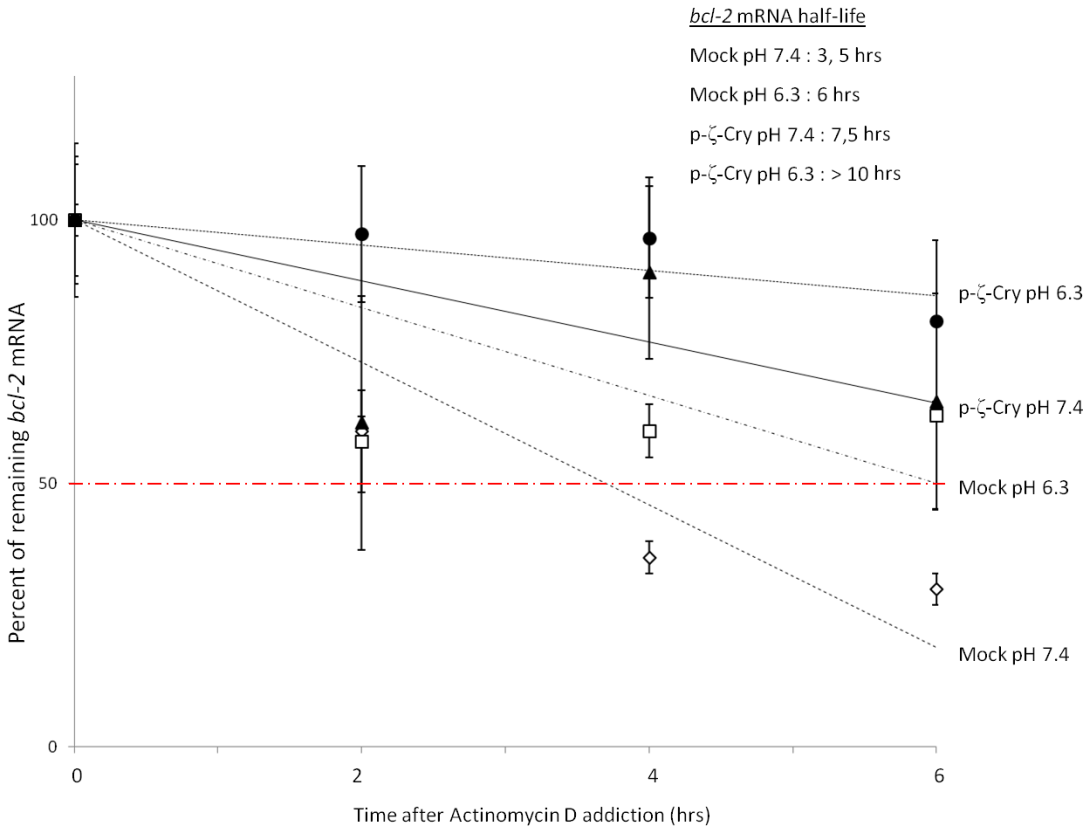


Fig. 5.14. *bcl-2* mRNA decay after ζ-crystallin or mock over-expression in HEK293 cells incubated for 4 hrs in pH 7.4 or 6.3, analyzed following transcriptional block using actinomycin D (5 µg/mL). Cells were harvested for RT-PCR analyses of *bcl-2* mRNA level at the times indicated (measured from the time of actinomycin D addition). The mRNA level at the time of actinomycin D was set to 1 for each condition. Approximate T½ for *bcl-2* mRNA: mock pH 7.4 ~ 3,5 hrs and pH 6.3 ~ 6 hrs; p-ζ-cry pH 7.4 ~7,5 hrs and pH 6.3 more than 10 hrs. Error bars represent SD of the mean of two individual experiments.

As shown in **Fig 5.14**, acidic conditions increased of *bcl-2* mRNA half-life from 3,5 hrs (at pH 7.4) to 6 hrs (at pH 6.3). On the other hand, in physiological pH conditions (pH

7.4) ζ-crystallin-over-expression increased *bcl-2* mRNA half-life from 3,5 hrs to about 8 hours, in agreement with what previously shown (Lapucci A. et al, 2010). ζ-crystallin-over-expression in HEK293 cell cultured in acidic conditions induced a further increase of *bcl-2* mRNA half-life, that is from ∼3,5 hrs to more than 10 hrs. These data support those reported in the previous figure. Furthermore, we can speculate that increased levels of Bcl-2 protein observed in acidic conditions by Ryder C. et al (2012) in WEHI7.2 lymphoma cell line is consequent to *bcl-2* mRNA stabilization by a mechanism of post-transcriptional involving ζ-crystallin expression.

Acidic pH activates p38 MAP kinase in Jurkat cells

Extracellular acidosis affects phenotypic changes of tumor cells in a process involving mitogen-activated protein kinase~~s~~ (MAPK), able to mediate the relaying of extracellular stimulations to intracellular responses. Riemann A. et al (2013) demonstrated that extracellular acidosis led to activation of p38 through its phosphorylation in different tumoral cell types. Based on this evidence, we evaluated the effects of acidosis on activation of p38 MAP kinase in Jurkat cells. **Fig. 5.15** shows that a 6 hour-treatment of Jurkat cells with extracellular acidosis (pH 6.3) leads to a marked phosphorylation/activation of p38 MAP kinase.

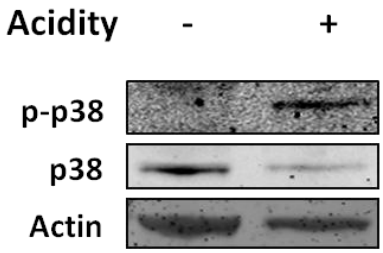


Fig. 5.15 Phosphorylated and overall protein p38 were evaluated by western blotting in Jurkat cells maintained in physiological (pH 7,4) or acidic (pH 6,3) medium for 6 hrs. Actin is shown as a loading control.

Identification of predicted phosphorylation sites within the human ζ-crystallin protein

The association between the increased ζ-crystallin protein and mRNA levels and the activation of p38 MAP kinase we found in Jurkat cells incubated in acidic conditions raised the hypothesis that the two phenomena could be related. The working

hypothesis was that p38 MAP kinase activation could led to the up-regulation and/or phosphorylation of ζ-crystallin protein and consequent modification of its activity as stabilizing *bcl-2* mRNA AUBP.

To verify if acidic condition induced the phosphorylation of ζ-crystallin, we first identified potential serine, threonine and tyrosine phosphorylation sites within the human ζ-crystallin amino acids sequence by using the NetPhos 2.0 Server (Technical University of Denmark). Among them, fourteen potential phosphorylation sites, indicated with an asterisk in the **Fig. 5.16**, had a high probability score.

```
  329 Sequence
MATGQKLMRAVRVFEFGGPEVLKLRSDIAVPIPKDHQVLIKVHACGVNPVETYIRSGTYSRKPLLPYTPGSDVAGVIEAV      80
GDNASAFKKGDRVFTSSTISGGYAEYALAADHTVYKLPEKLDFKQGAAIGIPYFTAYRALIHSACVKAGESVLVHGASGG     160
VGLAACQIARAYGLKILGTAGTEEGQKIVLQNGAHEVFNHREVNYIDKIKKYVGEKGIDIIIEMLANVNLSKDLSLLSHG     240
GRVIVVGSRGTIEINPRDTMAKESSIIGVTLFSSTKEEFQQYAAALQAGMEIGWLKPVIGSQYPLEKVAEAHENIIHGSG     320
ATGKMILLL                                                                              400
.....................................................Y....TYS.........S.........      80
.................S..S..Y.............................................................     160
.............................................Y.....................................     240
..........T.......T....S.........ST.....................................................     320
.........                                                                              400

Phosphorylation sites predicted:        Ser: 6  Thr: 4  Tyr: 4
```

Serine predictions

Pos	Context	Score	Pred
26	LKLRSDIAV	0.042	.
56	TYIRSGTYS	0.017	.
60	SGTYSRKPL	0.970	*S*
71	YTPGSDVAG	0.555	*S*
85	GDNASAFKK	0.005	.
96	RVFTSSTIS	0.038	.
97	VFTSSTISG	0.767	*S*
100	SSTISGGYA	0.953	*S*
143	ALIHSACVK	0.024	.
151	KAGESVLVH	0.031	.
158	VHGASGGVG	0.063	.
231	NVNLSKDLS	0.181	.
235	SKDLSLLSH	0.141	.
238	LSLLSHGGR	0.401	.
248	IVVGSRGTI	0.035	.
264	MAKESSIIG	0.546	*S*
265	AKESSIIGV	0.170	.
273	VTLFSSTKE	0.419	.
274	TLFSSTKEE	0.988	*S*
301	PVIGSQYPL	0.193	.
319	IIHGSGATG	0.004	.

Threonine predictions

Pos	Context	Score	Pred
3	--MATGQKL	0.041	.
52	NPVETYIRS	0.311	.
58	IRSGTYSRK	0.507	*T*
68	LLPYTPGSD	0.134	.
95	DRVFTSSTI	0.317	.
98	FTSSTISGG	0.077	.
113	AADHTVYKL	0.140	.
135	IPYFTAYRA	0.458	.
179	KILGTAGTE	0.022	.
182	GTAGTEEGQ	0.290	.
251	GSRGTIEIN	0.526	*T*
259	NPRDTMAKE	0.939	*T*
270	IIGVTLFSS	0.045	.
275	LFSSTKEEF	0.528	*T*
322	GSGATGKMI	0.189	.

Tyrosine predictions

Pos	Context	Score	Pred
53	PVETYIRSG	0.910	*Y*
59	RSGTYSRKP	0.815	*Y*
67	PLLPYTPGS	0.020	.
103	ISGGYAEYA	0.667	*Y*
106	GYAEYALAA	0.062	.
115	DHTVYKLPE	0.205	.
133	IGIPYFTAY	0.028	.
137	YFTAYRALI	0.023	.
172	IARAYGLKI	0.007	.
205	REVNYIDKI	0.931	*Y*
212	KIKKYVGEK	0.092	.
282	EFQQYAAAL	0.307	.
303	IGSQYPLEK	0.018	.

Fig. 5.16. Identification of potential serine-threonine-tyrosine phosphorylation sites within the human ζ-crystallin protein sequence. In the upper panel is reported amino acids sequence of ζ-crystallin protein and the position of potential phosphorylation sites. Fourteen potential serine, threonine and tyrosine phosphorylation sites within the human ζ-crystallin protein were identified and scored by use of the NetPhos 2.0 server. Those residues scoring above the 0.500 threshold are shown with asterisk.

Acid pH do not lead to phosphorylation of ζ-crystallin protein on tyrosine residues

The existence of fourteen phosphorylation sites with high probability score within the ζ-crystallin amino acids sequence prompted us to verify the possibility that ζ-crystallin was phosphorylated following exposition to an acidic microenvironment. To test this hypothesis, we conducted analyses in Jurkat cells incubated under acidic conditions for six hours and performed an immune-precipitation of ζ-crystallin with a specific monoclonal antibody. Successively, we probed immune-precipitated proteins in western blot with an antibody anti-phosphotyrosine. The preliminary results obtained indicated that ζ-crystallin is not phosphorylated in tyrosine residues neither in physiological conditions nor following incubation for 6 hours in acidic conditions (**Fig. 5.17**). Indeed, phosphorylation did not affect tyrosine residues on ζ-crystallin, but more analyses with anti-phosphothreonine and –phosphoserine antibodies should be performed to demonstrate conclusively that acidic conditions do not affect phosphorylation state of ζ-crystallin protein.

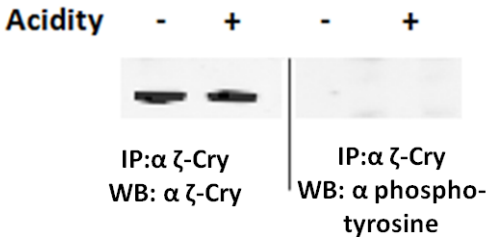


Fig. 5.17 Protein extracted by Jurkat cells cultured in acidic conditions (for 6hrs) were immunoprecipited by mAb ζ-Cry and analysed by western blotting with mAb ζ-Cry (on left) and pAb antiphosphotyrosine (on right).

P38 MAPK is involved in ζ-crystallin up-regulation in acidosis

On the basis of the evidence that phosphorylated p38 kinase triggers a transcriptional program through the activation of transcription factors, as CREB (DiGiacomo V. et al, 2009), we hypothesized that the involvement of the p38 MAPK pathway in upregulation of ζ-crystallin in acidosis could occur at transcriptional level. To verify this hypothesis, the possible role of p38 MAPK in enhancing *ζ-crystallin* gene transcription under low pH condition has been evaluated by inhibiting its activity with the p38 MAPK specific inhibitor SB202190. As shown in **Fig. 5.18**, treatment with 10 μM of SB202190

abrogated the up-regulation of ζ-*crystallin* under acidic conditions, indicating that p38 MAPK is involved in its up-regulation in acidic pH.

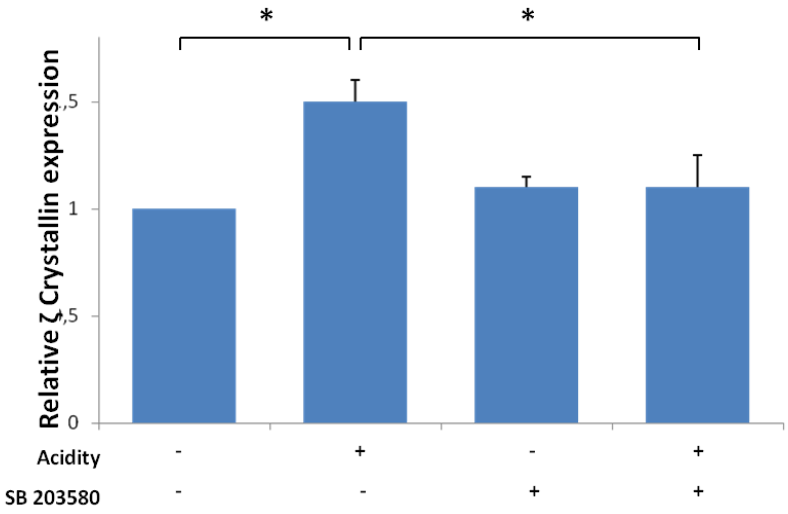


Fig 5.18. SB202190 (10 µM) was added Jurkat cells for 30 minutes before acidic exposition for 6 hours. After treatment, ζ-crystallin protein levels were evaluated by Western blot analysis of cell extracts. Values have been normalized to control conditions, represented by cells incubated in physiological conditions (pH 7.4). Actin is shown as loading control. Blots shown are representative of three independent replicates. (*)$p < 0.05$ vs 0h (t-test).

6. DISCUSSION

The constitutive increase in anaerobic glycolysis resulting in acute and chronic acidification of the local environment is an hallmark of neoplasia (Gatenby R. A. and Gillies R. J., 2004). Indeed, a direct consequence of anaerobic glycolysis is the production of acidic metabolites that cause a drop in extracellular pH reaching value of 6.5 or below (Yang V. et al, 2012), which in turn may select for more aggressive, acid-resistant clones and favor tumor invasion (Fang J. S. et al, 2008). Extracellular acidosis has also been blamed for a reduction in the cytotoxicity of chemotherapeutics agents (Kato Y. et al, 2013). Ryder et al. demonstrated the involvement of Bcl-2 family members in acidosis confirming that the tumor microenvironment plays a well-established role in modulating the survival of cancer cells (Ryder C. et al, 2012). They observed that acidic conditions minimize apoptosis induced by multiple cytotoxic stresses in lymphoma cells. Moreover, acidosis leads to up-regulation of the anti-apoptotic Bcl-2 and Bcl-X_L and to down-regulation of the pro-apoptotic PUMA and Bim factors. This implies an increase of cellular sensibility to the ABT-737, a selective inhibitor of Bcl-2/ Bcl-X_L interaction, suggesting that acidosis causes a dependence of cancer cells from the Bcl-2 family members. Yet, the molecular basis for this cellular response is not completely understood. Findings in this area promise to facilitate the discovery of possible novel therapeutic strategies that will succeed beyond *in vitro* studies. In this work we focalized on how extracellular acidity affected the expression of the major anti-apoptotic gene *bcl-2*, whose up-regulation is feature of various malignancies, including leukemias/lymphomas. To elucidate the mechanisms implicated in regulation of *bcl-2* expression is of crucial importance given the well known roles that *bcl-2* assumes in tumor onset, development and chemo-resistance (Juin P. et al, 2013).

bcl-2 expression is regulated not only at transcriptional, but also at post-transcriptional level through cooperation between an ARE located in the 3'-UTR of its mRNA

(Schiavone N. et al, 2000) and multiple ARE-binding proteins, *trans*-acting factors that recognize these elements. We identified some regulatory *bcl-2* mRNA AUBPs: AUF1, Bcl-2 itself, Tino/hMex-3D and ζ-crystallin (Lapucci A. et al, 2002; Bevilacqua A. et al, 2003; Donnini M. et al, 2004; Lapucci A. et al, 2010), while Nucleolin, HuR and Ebp1 have been added to the family of *bcl-2* AUBPs by others (Sengupta T. K., et al 2004; Bose S. K. et al, 2006; Otake Y. et al, 2007). One of these proteins, ζ-crystallin, in addition to its role as stabilizing *bcl-2* AUBP, was characterized also for its ability to recognize a specific RNA sequence, called pH response element (pH-RE) lying in 3'-UTR of some transcripts (Tang A. and Curthoys N. P., 2001) as *glutaminase*, *glutamate dehydrogenase* and *BSC1*, and to modulate their expression during chronic acidosis in renal cells (Schroeder J. M. et al, 2003 and Szutkowska M. et al, 2009). So, ζ-crystallin has a physiological role in the maintenance of acid-base balance during chronic acidosis in renal cells. Here, we clarify the role of acidosis on modulating the expression of ζ-crystallin and, accordingly, on ζ-crystallin mediated post-transcriptional regulation on *bcl-2* mRNA in the leukemia T cell line Jurkat. We first confirmed that acidosis minimizes apoptosis induced by multiple apoptotic stimuli, which are the hydrogen peroxide (H_2O_2) and the protein synthesis blocker Cycloheximide (CHX). Pre-incubation for three hours in acidic medium (pH 6.3) was sufficient to significantly decrease the number of apoptotic and necrotic Jurkat cells stimulated with the aforementioned cytotoxic compounds, suggesting that acidosis induced the acquisition of a more resistant phenotype in leukemic cells. To shed light on the molecular mechanisms underlying this phenomenon, we investigated if acidosis could modulate proteins involved in cell survival and resistance to apoptosis, particularly the anti-apoptotic protein Bcl-2. We observed elevation of both *bcl-2* mRNA and protein in Jurkat cells after as little as 6 hours of acidic treatment. Our results are consistent with similar observations obtained by Ryder C. et al in the WEHI7.2 lymphoma cell line. The expression of *bcl-2* is tightly regulated by transcription, control of mRNA stability and translation rate, and protein modification (Young R. L. et al, 1993; Willimott S. and Wagner SD, 2010). Focusing on the post-transcriptional regulators of *bcl-2* expression, we revealed that acidosis is able to positively modulate ζ-crystallin expression. We previously demonstrated a stabilizing effect of ζ-crystallin on *bcl-2* mRNA in a ARE-dependent manner and a stronger

association of ζ-crystallin to *bcl-2* ARE in leukemic respect to normal T lymphocytes, which results in the increase of Bcl-2 protein level (Lapucci A. et al, 2010).

Szutkowska M. et al, 2009 demonstrated that acidosis increased the expression of ζ-crystallin in medullary thick ascending limb cells in the physiological process of adaption of cells to renal acidosis. Here we found that low pH treatment increased the level of *ζ-crystallin* mRNA by approximately 50%, which is consistent with the level of ζ-crystallin protein rise. We showed a positive linear correlation of Bcl-2 and ζ-crystallin protein expression in Jurkat cells under different times of exposition to acidic conditions, supported by a high Pearson coefficient. This suggests that the acidic microenvironment affected the positive trend of expression of both proteins in cancer cells. We revealed that the up-regulation of ζ-crystallin occurred also in the leukemia T cell line Molt-T4, suggesting a similar behavior of ALLs in response to acidic pH. Moreover, among the *bcl-2* AUBPs, we evaluated also if acidosis could impact on the expression of AUF-1 and HuR, revealing that both proteins did not showed quantitative alteration in expression levels under acidic treatment in Jurkat cells. Obviously, we could not exclude possible qualitative alterations at post-translational level of AUF-1 and HuR. However, this observation prompted us to investigate the functional role of ζ-crystallin on the post-transcriptional regulation of *bcl-2* mRNA in response to acidosis. Indeed, we revealed that acidosis affect *bcl-2* mRNA stability and so its half-life. Hence, we investigated if ζ-crystallin takes part in this regulative process, by evaluating the *bcl-2* mRNA stability in HEK293 cells ectopically over-expressing ζ-crystallin. We demonstrated that low pH contributed to increase *bcl-2* mRNA stability of about 3-fold respect to physiological conditions and even ~~more~~ stronger (up to more than 20-fold) in over-expressing ζ-crystallin conditions. We had also confirmed these data estimating the *bcl-2* mRNA half-life performing transcriptional block with Actinomycin D and revealing its increase mediated by acidosis.

Extracellular acidosis affects phenotypic changes of tumor cells in a process involving mitogen-activated protein kinases (MAPK), able to mediate the relaying of extracellular stimulations to intracellular responses. In particular, Riemann et al demonstrated that extracellular acidosis led to activation of p38 through its phosphorylation in different tumoral cell types (Riemann A. et al, 2013). There are not literature data about the regulation of ζ-crystallin expression neither at transcriptional nor at translational

levels. Activated p38 has been shown to phosphorylate several cellular targets as transcription factors ATF/CREB, MEF2A, Sap-1, Elk-1, NF-κB, Ets-1, p53 and also several MAP Kinases, including MSK1 and -2, MNK1 and -2, and MK2 and -3 (Di Giacomo V. et al, 2009).

On these bases, we hypothesized the possibility of p38 pathway-mediated modulation of ζ-crystallin activity/expression in acidosis. First, ζ-crystallin could be regulated at post-translational level through direct phosphorylation by MAP kinases. Phosphorylation is a major post-translational modification of other crystallin family members as α- and B-crystallins (Li R. et al, 2011). Their p38 pathway mediated phosphorylation at Ser45 and Ser59 regulates their intracellular distribution, translocation, chaperone activity and ability of binding to substrates. Moreover, it is amply demonstrated that the activity of other AUPBs, as TTP, AUF-1 and HuR, is regulated by the phosphorylation state (Clement S. L. et al, 2011; Gummaidi L. et al, 2012) that affects their ability to bind and regulate half life of own mRNA targets. Here, we revealed that acidosis led to phosphorylation of the p38 MAPK. We can assume that the activation of MAPK pathway by acidosis occurs through acid sensing membrane receptors, including the four homologous G protein coupled receptors (GPCRs) and amongst them TDGA8, which is known to activate MAPK pathway (Yhara I. et al, 2010). We evaluated if the acidosis-mediated up-regulation and increase of activity of ζ-crystallin could be induced by activation of MAPK pathway. Our data indicated that the ζ-crystallin up-regulation is downstream to the p38 signaling in acidosis, supporting the possibility that p38 pathway transcriptionally regulates ζ-crystallin expression through activation of one or more transcriptional factors.

Considering the overall data we obtained in the present work, we can state that ζ-crystallin play an important role in the cellular response to acidosis through the stabilization of the *bcl-2* mRNA, and accordingly conferring more resistant phenotype and higher aggressiveness to cancer clones. Moreover, Bansal et al. recently revealed that p53 down-regulates ζ-crystallin expression in response to genotoxic stress in several cancer cell types (Bansal N. et al, 2011), and this led us to hypothesize that p53 may regulate apoptosis also via Bcl-2 down-regulation mediated by ζ-crystallin. This functional liaisons of p53, ζ-crystallin and Bcl-2 could be implicated in the acquiring of a resistant phenotype of cancer cells subjected to treatments with genotoxic

chemotherapeutic agents, which represents one of major limits of chemotherapy treatments. Lastly, ζ-crystallin could be also involved in altered cancer cell metabolism through its ability to bind and stabilize *glutaminase (GA)* and *glutamate dehydrogenate (GDH)* mRNAs (Ibrahim H. et al, 2008; Schroeder J. M. et al., 2003). Cancer cells are characterized by a rapid cellular growth in which glutamine becomes a conditionally essential nutrient: in this scenario, the glutamine pathway is pivotal for cancer metabolism (Hensley C. T. et al, 2013). To note, the up-regulation of key enzymes involved in glutamine catabolism, such as GA, has been revealed in several cancer cell type (Hensley C. T. et al, 2013).

The involvement of ζ-crystallin in *bcl-2* over-expression in leukemic cells and probably in acquisition of resistant phenotype in acidic microenvironment might constitute a significant molecular pathogenetic mechanism. The possibility that ζ-crystallin acts on glutamine-dependent proliferation and on apoptosis resistance of cancer cells in normal or acidic environment through *bcl-2* post-transcriptional control are now under investigation in our laboratory, in order to define the existence of an adaptive mechanism of cancer cells to tumoral microenvironment.

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