Distinct mechanisms of post-transcriptional control activated by inflammatory stimuli

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Erklärung zur Dissertation:

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel „Distinct mechanisms of post-transcriptional control activated by inflammatory stimuli“ selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe. Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

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ABSTRACT

Post-transcriptional mechanisms play a critical role in regulating the expression of numerous proteins that are involved in inflammation. Expression of these proteins can be influenced profoundly by alterations in degradation or translation of their mRNA, which often contain adenine/uridine-rich elements (AREs), but also other, less well characterized *cis*-elements in their 3'-untranslated regions.

Previously it was reported by our group that activation of the p38 MAP kinase/MAPKAP kinase 2 (MK2) pathway in HeLa cells can lead to stabilization of ARE-containing mRNAs, whereas UV-B light stabilizes mRNAs irrespective of an ARE and in a p38/MK2 independent manner. In light of these studies, behaviour of selected short-lived mRNAs, IL-8, IκBζ and IκBα mRNAs, which differ in their regulatory elements, were examined in response to UV-B light and IL-1. It was found that UV-induced stabilization of ARE-containing IL-8 mRNA by low doses requires the p38 MAPK pathway, whereas it was stabilized independently of this pathway at high doses, which suggests activation of other mechanisms and signal pathways in mRNA stabilization. Knock down of the candidate mRNA stabilizing protein HuR did not affect the stability of IL-8 mRNA mediated by UV light or IL-1. Both non-ARE IκBζ and IκBα mRNAs were not or only slightly affected by low and high doses of UV-B respectively, independently of p38 MAPK pathway. Initial results for mRNA stabilization by UV-B were also obtained in primary keratinocytes. Based on initial results in the group, IL-1-induced changes in the translational level of the selected mRNAs were studied. Of the three mRNAs, only IκBζ mRNA exhibits translational silencing which is reversed by IL-1. The translational silencing was identified by luciferase reporter assays to be executed by its 3' UTR region. Increased translation in response to IL-1 was independent of the p38 MAP kinase cascade. Mimicking the translational effect of IL-1 by IRAK proteins suggests involvement of other signalling pathway which apparently diverge downstream of IRAKs.

The results show that, depending on the stimulus and RNA, diverse post-transcriptional control mechanisms can be executed which selectively influence inflammatory gene expression.

Keywords: Regulatory elements, UV-light, mRNA stabilization, Translation

ZUSAMMENFASSUNG

Posttranskriptionelle Mechanismen spielen eine entscheidende Rolle bei der Expression zahlreicher Proteine, die in der Entzündung beteiligt sind. Die Expression diese Proteine kann durch Änderungen in der Degradation oder Translation ihrer mRNA stark beeinflusst werden. Diese mRNAs beinhalten oft Adenin/Uridin reiche Elemente (AREs), aber auch andere weniger charakterisierte *cis*-Elemente in ihren 3' untranslatierten Bereichen.

Wie früher von unserer Gruppe gezeigt, kann die Aktivierung des p38 MAP kinase/MAPKAP kinase 2 (MK2) Signalwegs in HeLa Zellen zur Stabilisierung von ARE-haltigen mRNAs führen, während UV-B Licht mRNAs unabhängig von einem ARE und dem p38/MK2 Signalweg stabilisierte. Angesichts dieser Beobachtungen wurde das Verhalten ausgewählter kurzlebiger mRNAs, IL-8, IκBζ und IκBα mRNAs, die sich in ihren regulatorischen Elementen unterscheiden, gegenüber UV Bestrahlung und IL-1 Stimulation untersucht. Es wurde festgestellt, dass UV-induzierte Stabilisierung der ARE-haltigen IL-8 mRNA durch niedrige Dosen den p38 MAPK Singnalweg benötigt, während diese Stabilisierung durch hohe Dosen unabhängig von diesem Signalweg verläuft, was auf sonstige mRNA stabilisierende Mechanismen und Signalwege hinweist. Der Knock down von mRNA stabilisierendem Protein HuR übte keine Wirkung auf die durch UV-B oder IL-1 vermittelte Stabilität der IL-8 mRNA aus. Die nicht ARE-haltigen IκBζ und IκBα mRNAs waren durch niedrige UV-B Dosen nicht und durch hohe Dosen nur leicht in einer p38 MAPK unabhängigen Weise beeinflusst. Anfängliche Ergebnisse für UV-vermittelte mRNA Stabilisierung wurden auch in primären Keratinozyten erhalten. Durch IL-1 induzierte translationelle Änderungen in den ausgewählten mRNAs wurden aufbauend auf erste Ergebnisse unserer Gruppe weiter untersucht. Von diesen drei mRNAs wies nur IκBζ mRNA eine translationelle Hemmung auf, die durch IL-1 aufgehoben wurde. Mit Hilfe von Luciferase Reporter Experimenten wurde festgestellt, dass dieser Hemmeffekt durch den 3' untranslatierten Bereich von IκBζ ausgeübt wird. Die Erhöhung der Translationsrate durch IL-1 war unabhängig von der p38 MAP Kaskade. Die durch IL-1 ausgeübte translationelle Wirkung wurde von IRAK Proteine nachgeahmt. Dies weist auf die Beteiligung weiterer Signalwege hin, die anscheinend "downstream" von IRAKs abzweigen. Diese Ergebnisse zeigen, dass abhängig von Stimulus und RNA mehrere posttranskriptionelle Kontrollmechanismen aktiviert werden können, welche die inflammatorische Genexpression selektiv beeinflussen.

Schlagwörter: Regulatorische Elemente, UV-Licht, mRNA Stabilisierung, Translation

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1 INTRODUCTION

1.1 Regulation of gene expression

The multistep pathway of eukaryotic gene expression is a fundamental cellular process, which involves a series of highly regulated events in the nucleus and cytoplasm. In the nucleus, genes are transcribed into pre-messenger RNAs which undergo a series of nuclear processing steps. Mature mRNAs are then transported to the cytoplasm, where they are translated into protein and degraded. Expression of a gene can be controlled at many levels, including:

- Transcription
- RNA processing (mRNA nuclear export and localization, mRNA splicing, mRNA stability and mRNA decay)
- Translation and post-translational events such as protein stability and modification.

The main focus of this study was to study the control of mRNA degradation and translation.

1.2 Mechanisms of mRNA decay in eukaryotes

The steady-state level of a eukaryotic messenger RNA is established by its relative rates of synthesis and degradation. The vast majority of eukaryotic mRNAs carry a 5' 7-methylguanosine cap structure and a 3' poly(A) tail of up to 200 adenosine residues in length. mRNA turnover is a regulated process, which can involve different pathways.

1.2.1 Deadenylation-dependent mRNA decay

A principal mRNA-degradation pathway used in both yeast and higher eukaryotes is initiated by removal of the 3' poly(A) tail, a process known as deadenylation (Shyu et al., 1991). Deadenylation-dependent mRNA decay is important for regulating transcript stability in mammalian cells (Shyu et al., 1991). The process starts with excision of poly(A) tail by deadenylase enzymes like poly(A) ribonuclease, PARN, (Gao et al., 2000) that was initially described and purified from mammalian cells (Aström et al., 1992; Körner et al., 1997). In yeast, the predominant deadenylase complex contains two nucleases Ccr4p and Pop2p and several accessory proteins, Not1-Not5p, Caf4p, Caf16p, Caf40p and Caf130p (Tucker et al., 2002; Tucker et al., 2001). Other deadenylases like Pan2p/Pan3p (poly(A) nuclease), are proposed to trim nascent poly(A) tails in the nucleus before export (Brown et al., 1996), but

might also participate in cytoplasmic deadenylation (Boeck et al., 1996; Tucker et al., 2001). The Ccr4p/Pop2p/Not protein complex (Draper et al., 1995; Dupressoir et al., 1999; Dupressoir et al., 2001) and the Pan2p/Pan3p deadenylase (Zuo et al., 2001), which were first discovered in yeast, are conserved in eukaryotic genomes. In Table 1 some human homologs of yeast RNA degradation factors are mentioned.

Protein Names in Yeast	Human Homologs (% Identity)	Information
Dcp1	DCP1B (34%), DCP1A (33%)	Member of decapping complex with Dcp2
Dcp2	DCP2 (37%)	Catalytic pyrophosphatase subunit of decapping complex
Xrn1	XRN2 (36%), XRN1 (35%)	Cytoplasmic 50 exonuclease
Ccr4	hCCR4 (18%)	Member of Ccr4-NOT complex
Pop2	CNOT7 (39%), CNOT8 (37%)	Member of Ccr4-NOT complex
Not1	CNOT1 (27%)	Member of Ccr4-NOT complex
Not5	CNOT3 (33%)	Member of Ccr4-NOT complex
Not3	unclear	Member of Ccr4-NOT complex
Caf16	CNOT3 (26%)	Member of Ccr4-NOT complex
Caf40, Caf130	unclear	Member of Ccr4-NOT complex

Table 1. Human Homologs of Yeast RNA Degradation Factors (modified from Houseley et al., 2009).

Once RNA is deadenylated, it can be degraded in either of two ways. One way is degradation in the 5'-3'direction, where the 5´cap is removed by decapping enzymes Dcp1 & Dcp2 (hDcp1 & hDcp2 are the mammalian homologues of the yeast) (Lykke-Andersen, 2002; Piccirillo et al., 2003; Wang et al., 2002) and the transcript is digested by exoribonucleases like Xrn1p (Muhlrad et al., 1994). The second way is degradation in the 3'-5' direction via a complex of exonucleases known as exosome (Jacobs-Anderson et al., 1998; Muhlrad et al., 1995) followed by hydrolysis of 5' cap structure by decapping enzymes (Fig.1).

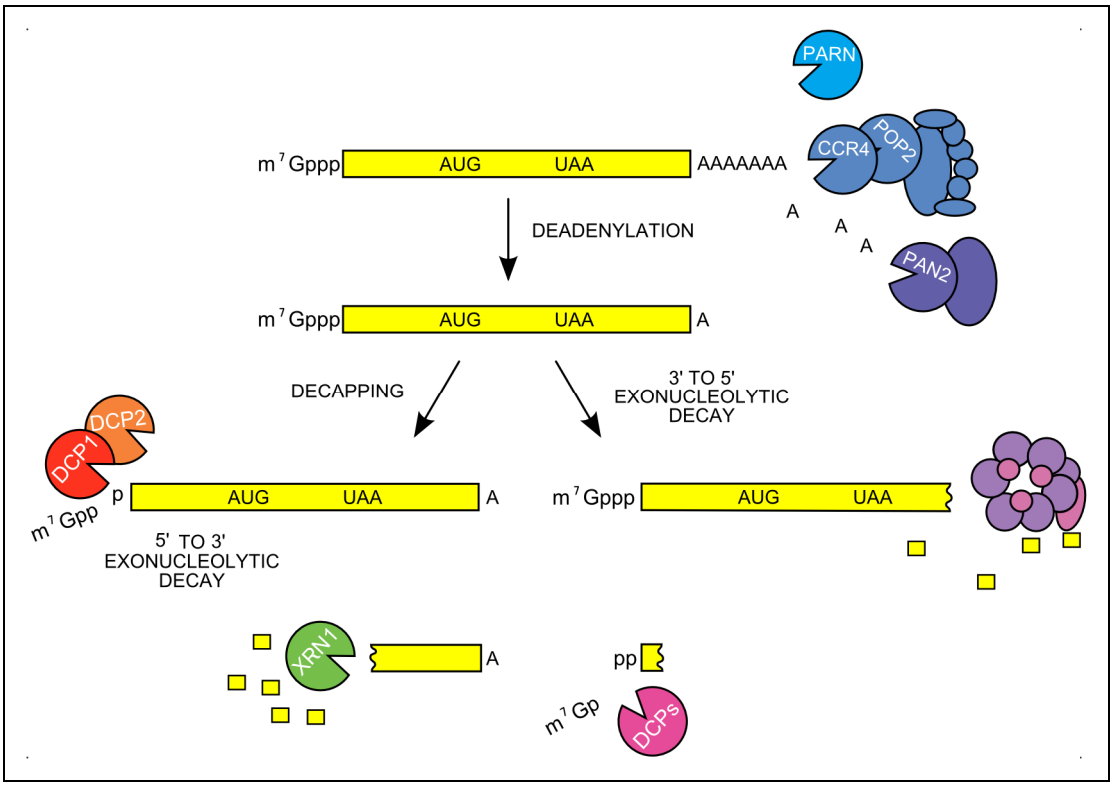


Fig.1 Eukaryotic mRNA decay mechanisms and enzymes. Two general mRNA decay pathways. Both pathways are initiated by deadenylation by the Ccr4/Pop2/Not complex or possibly by the alternative deadenylases, Pan2/Pan3 and PARN. Poly(A) tail shortening can lead to either 3'-5' exonucleolytic digestion by the exosome or decapping by the Dcp1/Dcp2 complex. Decapping is followed by 5'-3' exonuclease digestion by Xrn1. The residual cap structure resulting from exosome digestion is cleaved by the scavenger decapping enzyme DcpS (Decker et al., 2002).

Most enzymes and components of the deadenylation and decapping 5'–3'decay pathway (Dcp1/Dcp2 complex, exonuclease XRN1, CCR4-NOT deadenylase complex) come together in small cytoplasmic foci called **P bodies** (processing bodies). Therefore P bodies are thought to be places of mRNA degradation. In yeast, GFP-tagged Dcp1p, Dcp2p, and Xrn1p have been localized to P bodies (Sheth et al., 2003). Similarly, knockdown of XRN1 leads to the accumulation of polyadenylated mRNA at P bodies in mammalian cells (Cougot et al., 2004). Apart from P bodies **stress granules** are also non-membranous cytoplasmic aggregates, which are sites of mRNA triage, wherein individual mRNAs are dynamically sorted for storage, degradation, or translation during stress and recovery (Anderson and Kedersha, 2009). Proteins that promote mRNA stability like HuR or destabilize mRNA tristetraprolin (TTP) are also recruited to stress granules, suggesting that stress granules effect a process of mRNA triage, by promoting polysome disassembly and routing mRNAs to cytoplasmic domains enriched for HuR and TTP (Kedersha and Anderson, 2002; Anderson, 2008).

1.2.2 Deadenylation-Independent mRNA decay

1.2.2.1 Nonsense-mediated mRNA decay

Messenger mRNAs bearing premature translation termination codons (PTCs), which could give rise to truncated and potentially harmful proteins, are eliminated through nonsense-mediated mRNA decay (NMD). The NMD pathway is one of the best characterized mRNA surveillance mechanisms (Pulak et al., 1993). This pathway has been studied extensively in yeast, in which a premature stop codon is recognized during translation termination, resulting in rapid, deadenylation-independent decapping (Muhlrad and Parker, 1994). Four factors - Upf1, Upf2, Upf3 and Hrp1- are essential for nonsense-mediated mRNA decay in yeast. All interact with the translation- release factor RF3, and they are also involved in suppressing nonsense codons (Weng et al., 1996; Wang et al., 2001). The human homologue of these factors (UPF1, UPF2, UPF3) have also been identified and have functional characteristics similar to their yeast homologues (Bhattacharya et al., 2000; Serin et al., 2001; Mendell et al., 2000).

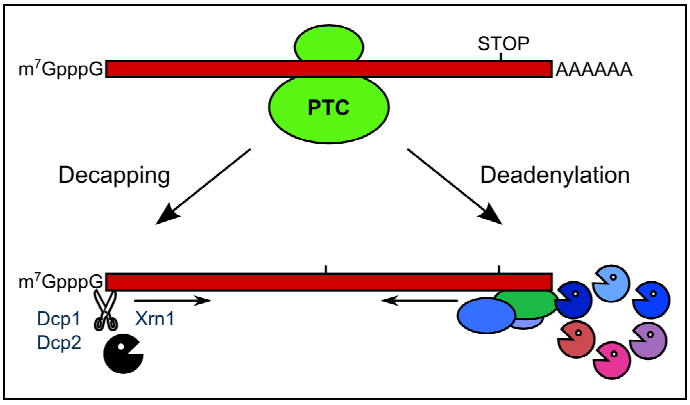


Fig.2 Decay of NMD substrates. In yeast and mammals decay of NMD targets is initiated by removal of the cap structure by the decapping enzymes Dcp1 and Dcp2. This exposes the mRNA to exonucleolytic digestion by the 5'→3' exonuclease Xrn1. An alternative pathway involves deadenylation followed by 3'→5' exosome-mediated decay (Lynne E. Maquat, 2006).

1.2.2.2 Endonuclease-mediated mRNA decay

Eukaryotic mRNAs can be degraded via endonucleolytic cleavage prior to deadenylation. Evidence for this mechanism comes from the analysis of transcripts such as mammalian insulin-like growth factor II, *IGF2*, (Nielson et al., 1992), transferrin receptor, *TfR*, (Binder et al., 1994), where mRNA fragments are detected *in vivo*. It was observed that mRNA degradation involved an endonucleolytic cleavage within the 3' UTR and did not involve

poly(A) tail shortening. Endonucleolytic cleavages have also been defined in vitro for the albumin mRNA (Dompenciel et al., 1995) and in the coding region of the *c-myc* mRNA (Bernstein et al., 1992).

Endonucleolytic cleavage can also be exhibited by Micro RNAs. Micro RNAs (miRNAs) are small endogenous noncoding RNAs that regulate gene expression post-transcriptionally (Bartel, 2004; Filipowicz, 2005). They control gene expression in animals, plants, and unicellular eukaryotes by promoting cleavage of complementary mRNA (Yekta et al., 2004) or repressing translation of target mRNAs (Lee et al., 1993). To accomplish their regulatory function miRNAs associate with the Argonaute proteins to form RNA-induced silencing complexes (RISCs) (Bartel, 2004; Filipowicz, 2005).

The existence of a link between the miRNA pathway and mRNA decay is supported by the observation that mammalian Argonaute proteins, miRNAs, and miRNA targets co-localize to P bodies (Jakymiw et al., 2005; Liu et al., 2005a,b; Meister et al., 2005; Sen et al., 2005).

1.3 Regulation of mRNA stability

The mRNAs are generally protected at their 5’ end through a 7-methylguanosine cap structure from exonuclease activity (Shatkin, 1976). At the 3’ end they are protected through a poly(A) tail and its interrelated poly (A)-binding proteins (PABPs) from nuclease decay (Bernstein and Ross, 1989). mRNAs lacking a 3’ poly(A) tract are unstable with or without added PABP (Bernstein et al., 1989; Ross et al., 1987).

PABP1 also interacts with a specific region of the translation-initiation factor eIF4G, which in turn forms a ternary complex with the cap-binding protein eIF4E (Gingras et al., 1999). This complex circularizes the mRNA (Wells et al., 1998), can promote translation, and stabilize mRNAs by preventing access of deadenylating and decapping enzymes to their targets.

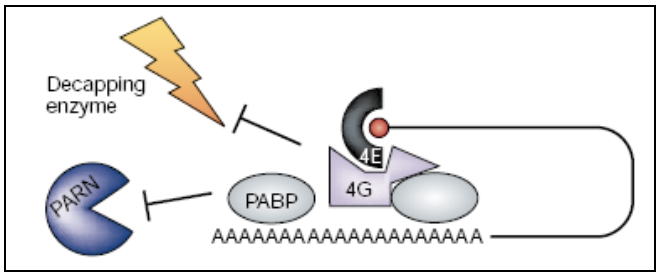


Fig.3 The ternary complex for translation and mRNA stability. During translation, the mRNA is thought to be circularized by its interaction with the translationinitiation factors eIF4E (4E), eIF4G (4G) and the poly(A)-binding protein (PABP). The eIF4E protein binds to the 5′ cap structure and this interaction is promoted by its binding to eIF4G which also binds to PABP on the poly(A)-tail. This conformation protects the 5′ and 3′ ends of the mRNA from attack by the deadenylase and decapping enzymes (Wilusz et al., 2001).

Several sequence elements can affect the stability of the mRNA transcript, either by promoting it (stabilizer elements) or by promoting mRNA decay (destabilizer elements). So far a few examples of stabilizer elements that block rapid mRNA decay have been identified. In yeast a stabilizer sequence that inactivates the deadenylation-dependent decay pathway has been found in the stable PGK1 mRNA (Decker et al., 1993). In mammals, a sequence in the 3'-UTR of the α-globin transcript slows down poly(A) shortening and rapid decay of the mRNA (Wang et al., 1999).

The mRNA half-lives vary considerably among different species of transcripts. Half-lives of most mRNAs are influenced by specific *cis*-acting elements within the mRNA molecule frequently located in the 3' untranslated regian (3' UTR) of the mRNA. One of the best studied and most prevalent *cis*-acting elements is the AU-rich element (ARE).

1.3.1 AU-rich elements (AREs)

AREs are sequence elements of 50–150 nt that are rich in adenosine and uridine bases. These motifs were first identified within the 3' UTR of many short lived mRNAs encoding several cytokines or lymphokines (Caput et al., 1986). The first direct evidence that the ARE can function as a potent mRNA destabilizing element came from a study in which a conserved region of 51 nucleotides containing AUUUA motifs from the 3' UTR of human granulocyte-macrophage-colony-stimulating factor (GM-CSF) mRNA was inserted into the 3' UTR of ß-globin mRNA; the otherwise stable β-globin mRNA was destabilized with a half-life of less than 30 min (Shaw and Kamen, 1986). Similarly the 3'UTR of the *c fos* mRNA, which contains a 69 nucleotides ARE, was also observed to reduce the stability of the β-globin mRNA (Chen et al., 1995). Since then, inserting a putative ARE into the 3' UTR of an otherwise stable reporter RNA has become the classical experimental approach to study

cellular or artificial AREs. Many of these studies used tetracycline and its derivates (tet-off system) or actinomycin D to block transcription and thereby allow degradation rates to be measured (Xu et al., 1998).

Numerous mRNAs including IL-2, IL-3, IL-8, IL-6, TNF-α were shown to contain ARE as instability determinants (Lewis et al., 1998; Lindstein et al., 1989; Stoecklin et al., 2000; Winzen et al., 1999). It has been estimated that 5–8% of human genes code for ARE-containing mRNAs; the corresponding proteins perform a variety of functions implicated in numerous transient biological processes (Bakheet et al., 2001; Bakheet et al., 2003).

Based on the number and the distribution of AUUUA pentamers, AREs have been grouped into three classes (Chen et al., 1995). Class I AREs contain several dispersed copies of the AUUUA motif within U-rich regions. Class II AREs possess at least 2 overlapping UUAUUUA (U/A)(U/A) nonamers. Bakheet and co-workers have subsequently constructed a database containing class II AREs and these regulatory elements were divided into five groups (Bakheet et al., 2001; Bakheet et al., 2003). The classification in this ARE database is based on the repetition pattern of the AUUUA pentamer. Class III AREs are much less well defined, they are U-rich regions but contain no AUUUA motif. The best documented example of a type III ARE is that situated within the 3’ UTR of c-jun mRNA (Chen et al., 1994; Peng et al., 1996; Xu et al., 2001).

Group	Motif	Examples
I	WAUUUAW and a U-rich region	c-fos, c-myc
IIA	AUUUAUUUAUUUAUUUAUUUA	GM-CSF, TNF-
IIB	AUUUAUUUAUUUAUUUA	Interferon-
IIC	WAUUUAUUUAUUUAW	cox-2, IL-2, VEGF
IID	WWAUUUAUUUAWW	FGF2
IIE	WWWWAUUUAWWWW	u-PA receptor
III	U-rich, non-AUUUA	c-jun

Table 2. Classification of AREs. Where W can be either A/U. (Wilusz et al., 2001).

The AREs are conserved among different species. Although a significant proportion (>25%) of human genes differ in their ARE patterns from mouse and rat transcripts (Halees et al., 2008).

AREs are shown to stimulate deadenylation processes *in vivo* (Wilson et al., 1988) and *in vitro* (Ford et al., 1999a). Other *in vitro* decay studies indicate that ARE-mRNAs are degraded primarily in the 3’–5’ direction by the exosome (Chen et al., 2001; Mukherjee et al., 2002), or by decapping (Gao et al., 2001). Similarly a 5’−3’ Xrn1 pathway to degrade ARE-mRNAs in

mammalian cells was also reported (Stoecklin et al., 2006). It has been shown that ARE binding protein TTP promotes the deadenylation of ARE containing mRNAs by PARN (Lai et al., 2003). Altered control of ARE-mediated mRNA turnover has been shown to result in aberrant gene regulation and to promote disease. For example, oncogenic alleles of the c-*fos* gene have deletions in the AU rich 3' UTR sequence (Schuler, 1988; Raymond et al., 1989; Schiavi et al., 1992). Immunological disorders caused by accumulation of TNF-α mRNA and increased TNF-α protein production have been shown to involve deregulation of ARE-mediated decay of the TNF-α mRNA (Taylor et al., 1996; Kontoyiannis et al., 1999). It has been observed that many human cancer and inflammatory diseases are linked to ARE defects (Conne et al., 2000).

1.3.2 ARE-binding proteins (ARE-BP)

The mRNA stability is a product of not only the *cis*-acting sequences such as the ARE but also transacting factors, the RNA-binding proteins, that bind directly or indirectly to the *cis*-acting elements and promote the deadenylation and degradation of the mRNA. *In vitro* studies have revealed that ARE-containing mRNAs undergo 3'-5' degradation by the exosome (Mukherjee et al., 2002; Liu et al., 2002; Chen et al., 2001). The destabilizing activity of AREs can be increased or decreased as a result of interactions with ARE-binding proteins. It has been reported that ARE-BPs can regulate the movement of ARE-containing RNA into and out of stress granules and P-bodies in a way that regulates mRNA translation and decay (Anderson, 2008). Table 3 shows how several ARE-BP can interact with one ARE-containing mRNA. Several ARE-BPs have been described over the past decade. Here some are introduced which are well known to modulate turnover of ARE-mRNAs.

Class	mRNA	ARE-BP
I	c-myc	AUF1, HuR
	c-fos	AUF1, HuR, KSRP, TTP[1]
	Interferon-γ	Hsp70
	MyoD	HuR
	iNOS	HuR, KSRP
	Cyclin A, B1, D1	AUF1, HuR
II	GM-CSF	AUF1, HuR, TTP, TIAR
	TNF-α	AUF1, HuR, TTP, TIAR, TIA-1, KSRP
	Interferon- α	hnRNP
	COX-2	AUF1, HuR, TTP, TIAR, TIA-1, KSRP
	Interleukin-2	AUF1, HuR, TTP
	Interleukin-3	HuR, BRF1
	Interleukin-8	HuR[2], KSRP[3], TTP[4]
	VEGF	HuR, TTP, KSRP
III	c-jun	KSRP, hnRNP
	p53	HuR
	Hsp70	HuR
	Myogenin	HuR

Table 3. ARE-mRNAs and their interacting ARE-binding proteins. [1]Chen et al., 2001; Hau et al., 2007, [2]Winzen et al., 2004, [3]Winzen et al., 2007, [4]Suswam et al., 2008; Winzen et al., 2007. Modified from Barreau et al., 2005.

AUF1 (AU binding factor 1) was the first ARE-binding protein to be identified, and was isolated on the basis of its ability to induce c-*myc* mRNA decay *in vitro* (Zhang et al., 1993). AUF1 promotes mRNA decay (Zhang et al., 1993; Loflin et al., 1999). It is reported that AUF1 mediates ARE mRNA degradation by recruiting the exosome (Chen et al., 2001). Tristetraprolin or TTP is a prototype of a group of CCCH tandem zinc finger domains (Thompson et al., 1996). TTP was identified to promote the decay of ARE-containing TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF) transcripts by direct binding to their AU-rich element (Lai et al., 1999; Carballo et al., 2000). Knockout strains of mice harbouring a deletion of the tristetraprolin gene show increased stability of both TNF-α and GM-CSF mRNAs (Carballo et al., 1998; Carballo et al., 2000). Like AUF1, TTP is thought to degrade ARE-containing mRNA by recruiting the exosome (Chen et al., 2001).

1.3.2.1 KSRP

K-homology splicing regulatory protein was originally identified as a component of a complex, assembled on an intronic enhancer required for neuronal specific c-src splicing (Min et al., 1997). KSRP was first purified by Chen and colleagues and it was found that it binds

specifically to the c-*fos* and TNF-α ARE and associates with the exosome, which can mediate rapid 3'-5' degradation of these mRNAs (Chen et al., 2001). It has a molecule weight of 75 kDa and contains four RNA binding K homology (KH) motifs. The KH domain was first identified in the hnRNP K protein and found to be necessary for the RNA binding of the hnRNP K and FMR1 proteins (Siomi et al., 1993, Siomi et al., 1994). These motifs are functionally essential since mutations within them lead to disease or differentiation defects in flies, worms, and mammals (Adinolfi et al., 1999). It was shown that KH domain 3 binds to the RNA with a significantly higher affinity than the other domains (Garcia Mayoral et al., 2008). Gherzi et al showed that KH domain 4 together with domain 3 mediate RNA binding, mRNA decay and interactions with the exosome and PARN (Gherzi et al., 2004).

KSRP activity is controlled through different signal pathways. For example it was reported that phosphatidylinositol 3-kinase (PI3K)-AKT phosphorylates KSRP at a unique serine residue, induces its association with the multifunctional protein 14-3-3, and prevents KSRP interaction with the exoribonucleolytic complex exosome (Gherzi et al., 2006). p38 MAP kinase also phosphorylates KSRP, thereby compromises its binding to ARE-containing transcripts (Briata et al., 2005). In our group it was shown that KSRP is involved in the degradation of IL-8 mRNA (Winzen et al., 2007).

1.3.2.2 HuR

Hu antigen R or ELAV-like 1 another ARE binding protein with a molecule mass of 36 kDa is a ubiquitously expressed member of the ELAV (embryonic lethal abnormal vision) family (Ma et al., 1996). In contrast to so far described ARE binding proteins HuR is believed to increase mRNA stability. An interaction of c-fos ARE and IL-8 ARE with HuR was described by Chen et al., 2001 and Winzen et al., 2004. It was demonstrated that HuR shuttles between the nucleus and cytoplasm via a nucleocytoplasmic shuttling domain and therefore may initially bind to ARE-containing mRNAs in the nucleus and provide protection during and after their export to the cytoplasmic compartment. (Fan and Steitz, 1998a; Ford et al., 1999). In another study, HuR is shown to interact with an endonucleolytic site in the $p27^{KIP1}$ mRNA and protect this site from endonuclease cleavage (Zhao et al., 2000). Brennan et al introduced protein ligands such as phosphatase 2A inhibitors which interact with HuR and modulate HuR's ability to bind its target mRNAs (Brennan et al., 2000). According to hypothesis of Yarovinsky et al early exposure of T lymphocytes to IL-4 positively regulates IL-4 mRNA stability via HuR (Yarovinsky et al., 2006). Katsanou and co-workers showed that in transgenic macrophages, HuR overexpression induced the translational silencing of specific

cytokine mRNAs despite positive effect on their corresponding turnover (Katsanou et al., 2005). The stabilizing effect of HuR on various mRNAs has been also studied with the help of siRNA knock-down experiments (Lal et al., 2004; Raineri et al., 2004). For example lowering of endogenous HuR levels through expression of antisense RNA (siRNA) inhibited stabilization induced by UVC light (Wang et al., 2000). Deletion of HuR in mice in recent studies reveals complex and essential roles of HuR in the development including thymic T cell maturation, placental branching morphogenesis, spleen ontogeny, intestinal integrity, and hematopoietic progenitor cell survival (Papadaki et al., 2009; Katsanou et al., 2009; Ghosh et al., 2009). HuR's ability to enhance expression of anti-apoptotic genes was investigated in another study in which HuR promoted the translation of prothymosin-α, an inhibitor of the apoptosome, in response to the ultraviolet light (UVC) (Lal et al., 2005).

As mentioned above HuR is predominantly localized in the nucleus and its translocation to the cytoplasm leads to stabilization of its target mRNA. Several signalling pathways such as ERK pathway (Yang et al., 2004), AMP-activated kinase (Wang et al., 2002), p38 pathway (Atasoy et al., 2003) and β-catenin (Lee et al., 2006) have been shown to be involved in its cytoplasmic localization. Phosphorylation of HuR by cell cycle checkpoint kinase (Chk2) was reported to regulate SIRT1 expression (Abdelmohsen et al., 2007). Other studies have indicated regulation of HuR via PKC-α-dependent phosphorylation or through phosphorylation by Cdk1 (Doller et al., 2007; Kim et al., 2008).

1.4 AREs as translational regulatory elements

Translational repression by AREs derived from several cytokine mRNAs was first discovered by microinjection of reporter mRNAs into Xenopus oocytes. It was observed that AU rich elements for interferon, GM-CSF and c-fos RNAs prevented mRNA translation (Kruys et al., 1989). The function of the TNF-α mRNA 3' UTR and the role of its ARE sequence was studied by Han et al. They found that the TNF-α ARE imposed a strong translational blockade in macrophages. However, this blockade was moderated when the cells were treated with LPS. (Han et al., 1990). Deleting the TNF-α ARE in mice affected mechanisms responsible for TNF mRNA destabilization and translational repression in hemopoietic and stromal cells which led to development of chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease (Kontoyiannis et al., 1999). ARE binding protein TIA-1 has been characterized to suppress the translation of TNF-α and Cyclooxygenase 2 (COX-2) (Dixon et al., 2003; Piecyk et al., 2000). Another ARE binding protein TIAR binds to the 3' UTR of mRNAs encoding translation factors and suppresses their translation, particularly in response to low

levels of short-wavelength UVC irradiation (Mazan-Mamczarz et al., 2006). Further studies notified that TIAR suppresses the translation of TIA-1; in contrast, HuR positively enhances TIA-1 expression (Pullmann et al., 2007). HuR was also reported to repress translation of CDK inhibitory protein p27 by reducing its IRES-dependent translation (Kullmann et al., 2002). It has been reported that overexpression of HuR can release selected ARE-containing mRNA molecules from P-bodies, allowing reinitiation of translation and polysome assembly (Bhattacharyya et al., 2006).

1.5 Regulation of mRNA stability by signal transduction pathways

Several signalling pathways have been implicated in regulating the decay of specific mRNAs. Early reports revealed altered turnover of ARE-mRNAs in response to extracellular as well as intracellular signals, such as phorbol ester (TPA), antibodies recognizing T cell receptor (TCR) and the CD28 auxiliary receptor, and TNF-α (Gorospe et al., 1993; Lindstein et al., 1989). Extracellular stimulation like pro-inflammatory cytokines or other cell stresses can activate many signal transduction pathways including:

- NF-κB pathway
- Extracellular regulated kinase (ERK)
- Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK)
- p38 MAP kinase pathways
- PI3-AKT3 pathway
- Protein kinase C
- Wnt signaling pathway

These pathways also contribute to activation of gene transcription regulation (Baldwin, 1996; Karin et al., 1997). JNK activation was seen to be required for IL-2 and IL-3 mRNA stabilization in T-cell or mast cell lines (Chen et al., 1998; Ming et al., 1998). ERK was responsible for GM-CSF mRNA stabilization in TNF-α plus fibronectin-activated peripheral blood eosinophils (Esnault et al., 2002). Protein kinase C (PKC) was specifically implicated in the enhanced stability of many labile mRNAs, such as those encoding p21 and IL-1 (Gorospe et al., 1993; Park et al., 2001). It was also demonstrated that protein kinase-α dependent phosphorylation regulates shuttling of the mRNA stabilizing factor HuR (Doller et al., 2007). The Wnt β-catenin pathway activation can stabilize ARE-mRNAs. For example, β-catenin binds to the ARE region of the COX-2 mRNA and increases its stability and interacts

with HuR (Lee et al., 2006). The JNK pathway has been demonstrated to regulate human iNOS expression by stabilizing iNOS mRNA possibly by a TTP-dependent mechanism (Korhonen et al., 2007). The PI3 kinase pathway has been shown to be involved in the mRNA stabilization of several reporter genes linked to the ARE of IL-3, GM-CSF, TNF-α, IL-2, and IL-6 (Ming et al., 2001; Stoecklin et al., 2001). Phosphorylation of MAP-kinase-activated protein kinase-2 (MK2) by p38 MAPK induces stabilization of TNF-α, IL-6 and IL-8 mRNA (Kotlyarov et al., 1999; Winzen et al., 1999).

Relating to this study, the NF-κB and the Mitogen-activated protein kinases (p38 pathway) are explained more in details.

1.5.1 NF-κB pathway

The nuclear factor-κB was first described by Sen and Baltimore as a B cell nuclear factor that bound a site in the immunoglobulin κ enhancer (Sen and Baltimore, 1986a). NF-κB is a ubiquitous transcription factor which regulates gene expression during immune and inflammatory responses to various extracellular stimuli or stress, including inflammatory cytokines such as TNF-α or interleukin-1 (IL-1), lipopolysaccharide, phorbol esters and UV irradiation (Sen and Baltimore, 1986b; Baeuerle et al., 1996; Baldwin et al., 1996). NF-kB family members contain a conserved DNA binding and dimerization domain towards the N-terminus, called the Rel homology domain, which carries a nuclear localization signal (NLS) and interacts with IκB, inhibitor of NF-κB, proteins (May et al., 1998). Most members of the Rel family contain a C-terminally located transactivation domain (TAD) that is important for optimal transcriptional activity.

Mammalian cells contain five NF-κB subunits. They are RelA (p65), c-Rel, RelB, p50 and p52 which form various hetero- and homo-dimers and bind at κB sites in the DNA of target genes. The p50 and p52 subunits, which lack transactivation domains, are produced by processing of precursor molecules of 105 kDa and 100 kDa, respectively (Baeuerle et al., 1994; Siebenlist et al., 1994). IκB inhibitors have ankyrin repeats, each about 30-33 amino acids sequences, which form a unit able to interact with Rel regions. Activity of NF-κB results in liberation of NF-κB dimers from IκB following phosphorylation of IκBs by the cytokine-responsive IκB kinases IKK-α and IKK-β (Baldwin et al., 1996). Upon removal of IκBs, NF-κB enters to the nucleus to induce expression of coordinate sets of target genes, thereby controlling immunity, inflammation, cell growth and survival.

1.5.2 Members of IκB family proteins

All the isoforms of IκB - IκBα, IκBβ, IκBε, IκBγ, IκBζ, IκBNS, BCL-3, p100, p105- share a characteristic ankyrin repeat motif which binds to the dimerization domain of NF-κB dimers (Hayden et al., 2004). Different isoforms of IκB proteins exhibit differential affinity towards various subunits of the NF-κB family and thus control expression many genes. Except for Bcl-3, IκBNS and IκBζ, which are localized in the nucleus (Totzke et al., 2006; Zhang et al., 1994), all other IκB proteins are localized in the cytosol (Whiteside et al., 1997).

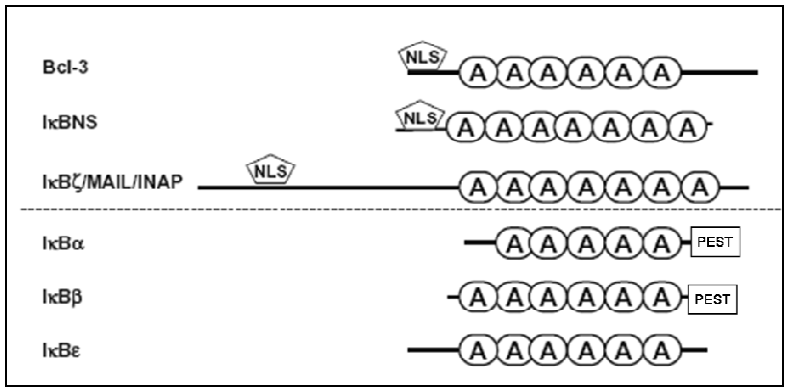


Fig.4 IκB family proteins. Distinct from cytoplasmic IκB proteins, Bcl-3, IκBNS, and IκBζ contain a nuclear localization signal (NLS). A: ankyrin motifs (Yamamoto et al., 2008).

The prototypical and most extensively studied member of the family is the 37-kDa protein IκBα. It is the first isoform of IκB family that was cloned and identified (Haskill et al., 1991; Davis et al., 1991). It inhibits the DNA binding activity of the NF-κB dimers (p50:p65 heterodimers, p50:c-Rel heteromers and c-Rel homodimers) in which it forms a complex with them (Davis et al., 1991). IκBα can be divided into three structural domains: a N-terminal region, an internal region that is composed of ankyrin repeats, and a C-terminal region that contains a so called PEST region. The PEST domain plays an important role in the inhibition of NF-κB DNA-binding activity (Ernst et al., 1995). Activation of NF-κB through stimuli leads to rapid phosphorylation of IκBα, followin ubiquitination by a ubiquitin ligase complex and degradation by the 26S proteasome (Chen et al., 1995; Alkalay et al., 1995). It has been shown that IκBα retains NF-κB in the cytoplasm through masking of the nuclear localization sequences (Baeuerle et al., 1994; Siebenlist et al., 1994; Beg et al., 1993). IκBβ is a 43-kDa protein which structurally is similar to IκBα. Like IκBα, IκBβ preferentially interacts with c-Rel/p50 or RelA (p65)/p50 heterodimers of NF-κB (Thompson et al., 1995). Unlike IκBα, which has both nuclear import and export sequences and hence has the ability to remove activated NF-κB from the nucleus, IκBβ does not enter the nucleus and can only bind to NF-κB in the cytoplasm. Where IκBα is targeted by a signaling pathway initiated by TNF, IL-1,

LPS; IκBβ reportedly is targeted only by pathways initiated by LPS or by IL-1 (Thompson et al., 1995). The 45-kDa protein IκBε shares many properties with IκBα along with structural homology. A major difference is that it forms complexes exclusively with p65 and c-Rel (Whiteside et al., 1997). IκBγ is a 70-kDa protein detected predominantly in lymphoid cells. It appears to be limited only to mouse B cells (Baeuerle et al., 1994; Siebenlist et al., 1994). IκBγ is shown to inhibit the sequence-specific DNA binding of NF-κB p50 homodimers (Inoue et al., 1992). BCL-3 is found in the nucleus associated with p50- and p52-containing homo- and heterodimers of NF-κB (Franzoso et al., 1993). IκBNS, also named IκBδ, is similar to BCL-3 a nuclear protein and contain ankyrin repeats. It was originally cloned as a gene rapidly induced by T cell receptor stimulation in thymocytes (Fiorini et al., 2002). IκB/NF-κB precursor proteins p105 and p100 have been categorized both as IκB protein family and NF-κB family members due to the presence of a Rel homology domain (RHD) at the N-terminal and ankyrin repeats at the C-terminal. The RHD of these proteins interact with complementary regions of NF-κB subunits like c-Rel and p65 (Rice et al., 1992; Scheinman et al., 1993).

1.5.2.1 IκBζ

A new member of the IκB protein family IκBζ was also termed as MAIL (molecule possessing ankyrin-repeats induced by lipopolysaccharide, Kitamura et al., 2000) or INAP (IL-1- inducible nuclear ankyrin-repeat protein, Haruta et al., 2001). IκBζ contains 6 ankyrin repeats in its C-terminal portion and thereby shares about 40% homology with those of IκB proteins such as IκBα and Bcl3, whereas its N-terminal portion showed no homology with any other proteins (Kitamura et al., 2000). In contrast to IκBα or β, IκBζ is localized in the nucleus and therefore does not affect the translocation of NF-κB. Instead, it inhibits the DNA binding of NF-κB by interacting mainly with the NF-κB subunit p50 in the nucleus (Yamazaki et al., 2001). It was demonstrated that IκBζ gene expression was rapidly induced by IL-1 and LPS (Kitamura et al, 2000; Haruta et al, 2001; Yamazaki et al., 2001) but not by TNF-α (Haruta et al., 2001), all of which are known to activate the NF-κB signalling pathway. However Totzke et al described a weak induction of IκBζ by TNF-α (Totzke et al., 2006). It has been observed that Overexpression of MyD88 (myeloid differentiation factor 88) and TRAF6 but not TRAF2 led to induction of IκBζ (Eto et al., 2003). According to Kitamura and co-workers IκBζ is an activator of IL-6 production (Kitamura et al, 2000). Deletion of the IκBζ gene in mice resulted in impaired expression of IL-6, GM-CSF and the p40 subunit of IL-12 (Yamamoto et al, 2004). Overexpression of IκBζ inhibited the transcriptional activity of

Signal transducer and activator of transcription 3 (STAT3) (Wu et al., 2009). Similarly other studies reveal that IκBζ can suppress expression of certain genes while it may be required for the expression of others (Matsuo et al, 2007; Kayama et al, 2008; Yamazaki et al, 2008). It was shown that IL-17A up-regulated IκBζ and knockdown of IκBζ significantly diminished the expression of IL-17A-induced human β-defensin 2 (Kao et al., 2008). IκBζ mediated gene regulation appears to be crucial for the accomplishment of a specific inflammatory response.

1.5.3 Mitogen-Activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) compose a family of protein kinases which phosphorylate specific serines and threonines of target protein substrates and thereby mediate a number of processes such as gene expression, metabolism, cellular proliferation, division, differentiation and apoptosis. Its function and regulation have been conserved during evolution from unicellular organisms such as yeast to complex organisms including humans (Widmann et al., 1999). The MAPK are regulated by phosphorylation by upstream kinases, MKKs. MKK-catalyzed phosphorylation activates the MAPK and increases its activity in catalyzing the phosphorylation of its own substrates. MKKs are phosphorylated and activated in turn through MAPK kinase kinases (MKKKs).

Four different MAP kinase pathways have been described: extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPKs) comprising the c-Jun N-terminal (JNK) and p38 MAP kinases, respectively, and ERK5/big MAP kinase 1 (BMK1).

1.5.4 The p38 MAPK pathway

The mammalian p38 MAPK family consists of four different proteins, p38α, p38β, p38γ and p38δ, which are most similar to the Hog1 MAPK of budding yeast (de Nadal et al., 2002). The p38 MAPK is activated mainly by dual phosphorylation of threonine and tyrosine in the Thr-Gly-Tyr activation motif by three upstream MAPK kinases MKK3, MKK4 and MKK6. p38 MAPK can also be directly activated, independently of MKKs by MKKK candidates, such as TAK and TAB1 (Moriguchi et al., 1996; Ge et al., 2002). The p38 MAPKs phosphorylate Ser/Thr residues of their substrates. Downstream substrates of p38 MAPKs are mostly protein kinases such as MK2, MK3, MNK1, MNK2 and casein kinase 2 (CK2), transcription factors such as activating transcription factor1, 2 and 6 (ATF-1/2/6), p53 and CREB, cell cycle regulators like Cdc25B, Cdc25C, cyclin D1/2/3, and cytoskeletal proteins such as keratin 8 and microtubule-associated protein Tau (all reviewed in Zarubin et al., 2005). The identification of physiological substrates for p38 MAP kinases has been facilitated

by the availability of specific pyridinyl imidazole inhibitors such as SB203580 and SB202190 (which specifically inhibit p38α and p38β isoforms), and the recently reported inhibitor of all four p38 isoforms (BIRB0796) (Kuma et al. 2005).

Stress signals, such as lipopolysaccharides (LPS), heat shock and ultraviolet light or pro-inflammatory cytokines like IL-1 or TNF-α can activate p38 MAPK pathway by its phosphorylation. Activation of p38 MAPK regulates mRNA stability in multiple cell types. Many ARE-containing mRNAs involved in inflammation and cancer are known to be stabilized by this pathway, for example, COX-2, TNF-a, IL-3, IL-6, IL-8, GM-CSF, VEGF and c-fos (Stoecklin et al., 2000, Brook et al., 2000; Winzen et al., 1999). However it is noticeable that not all the ARE-mRNAs regardless of there ARE class are targets for the MAPK p38 pathway (Frevel et al., 2003). Activation of p38 MAPK pathway leads to phosphorylation of KSRP leading to loss of function of this RNA-binding protein, subsequently ARE-mediated mRNA stabilization (Briata et al., 2005). It has been reported that MK2-mediated phosphorylation of TTP promoted the assembly of TTP: 14-3-3 complexes followed by inhibition of ARE-mRNA degradation and stress granule association (Stoecklin et al., 2004). Selective activation of the p38 MAPK pathway by MAPK kinase 6 induces mRNA stabilization of IL-8 (Holtmann et al., 1999). MK2 can regulate IL-6 at the levels of mRNA stability, and of TNF-α mainly through TNF-ARE-dependent translational control (Neininger et al., 2002). It has been reported that p38 MAPK phosphorylates HuR which could compete with TTP and it can regulate IL-3 mRNA decay (Ming et al., 2001). The p38 MAPK regulates HuR localization and subsequently HuR mRNA targets that are involved in chronic inflammation and cancer such as cyclin-dependent kinase inhibitor $p21^{kip1}$ and COX-2 (Lafarga et al., 2009; Dixon et al., 2006).

1.6 Regulation of mRNA stability by UV light

Ultraviolet light is a potent inducer of inflammation which can trigger signal transduction cascades and thereby lead to changes in the gene expression. High doses of UV radiations cause physical, cellular and molecular damage, resulting in erythema (sunburn), immunosuppression and carcinogenesis. Over 1 million new skin cancers are diagnosed yearly in the United States which include approximately 40% of all new cancer cases. The UV radiation component of sunlight consists mainly of UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–290 nm). Among all these three radiations UV-B radiation is the most effective inducer of sunburn, immediate tanning and cancers of keratinocytes (Nickoloff et al.,

2002). UV-C radiation is absorbed by the ozone layer (Madronich et al., 1998), UV-A radiations affects the oxidative status of target molecules (Vile et al. 1993).

UV radiation is known to induce the activation of stress-inflammation signal transduction pathways such as p38, JNK, ERK and NFκB (Tyrrell, 1996; Chouinard et al., 2002; Baldwin 1996). Activation of these signaling cascades can result in a number of cellular responses that include apoptosis, proliferation, inflammation, differentiation and development (Bode et al., 2006). Activation of JNK pathway in response to UV-C irradiation promotes apoptosis by the proapoptotic gene *hid* (Luo et al. 2007). It has been shown that p38 kinase mediates UV-induced phosphorylation of p53 protein (Huang et a., 1999). It was reported that UV-B regulates the expression of COX-2 in human keratinocytes via p38 MAP kinases and ERK (Chen et al., 2001). UV light induces stabilization of short-lived mRNAs such as VEGF, c-fos, c-jun and c-myc in mammalian cells (White et al., 1997; Blattner et al., 2000; Bollig et al., 2002) and inhibits degradation of reporter RNAs containing AREs but also RNAs without AREs (Bollig et al., 2002). This mRNA stabilization was indicated to be in a p38 MAPK dependent or independent manner (Bollig et al., 2002). Furthermore it was reported that the increased mRNA stability induced by UV light is due to inhibition of deadenylation (Gowrishankar et al., 2005). The ARE-binding protein HuR is reported to be involved in UV-C mediated stabilization of p21 mRNA (Wang et al., 2000). Similarly binding of HuR to p53 following UV-C irradiation has been indicated to increase the translation of this mRNA (Mazan-Mamczarz et al., 2003). UV irradiation also induces changes in the translational mechanism. UV light elicits phosphorylation of eukaryotic translation initiation factor 2 (eIF2α), which leads to the assembly of stress granules by decreasing the availability of the eIF2–GTP–$tRNA^{Met}$ Ternary complex that is needed to initiate protein translation (Kedersha and Anderson, 2002).

1.7 Aims of the study

The aim of this work was to investigate the mechanism of post-transcriptional regulation of gene expression particularly in response to the pro-inflammatory cytokine IL-1α and to UV irradiations. UV light strongly activates stress signaling pathways, including the p38/MK2 pathway (Iordanov et al., 1997). From our previous studies we know that UV light stabilizes short-lived ARE- and non-ARE containing mRNAs in a p38 MAPK/MK2 pathway independent manner (Bollig et al., 2002). In light of these studies, behaviour of different mRNAs containing AU-rich elements such as IL-8 mRNA (Winzen et al., 1999), or those without classical AREs such as IκBζ and IκBα mRNAs (Bakheet et al., 2001) towards UV-B

radiations and involvement of the p38 MAPK pathways was compared. Using the HeLa tTA cell line, the stability of endogenous mRNAs was detected by actinomycin D- chase experiments, while that of the reporter mRNAs, with defined regulatory sequences, was determined with the help of the tet-off system. In addition to HeLa cells the stability of endogenous mRNAs was investigated in primary keratinocytes as well. It was also of interest to understand if and how these mRNAs are controlled at the translational level. Taking IL-1α as an inducer and mediator of inflammation we analyzed the changes in the translational level of mRNAs mentioned above, specifically IκBζ 3' UTR mRNA, which showed changes in the ribosomal occupancy induced by IL-1 based on a microarray screen. Towards this aim, a luciferase reporter construct containing 3' UTR of IκBζ mRNA was used. Furthermore regulation of the 3' UTR region of IκBζ mRNA by different signaling pathways and UV was examined. Comparing the stability and translation for the selected mRNAs and stimuli, IL-1 and UV-B light, in this study will further expand our information on the selectivity of post-transcriptional control mechanisms.

2 MATERIALS

2.1 Labware

Plasticware, disposable material- including 2ml, 5ml, 10ml and 25 ml pipettes, tips and eppendorf tubes, petri dishes for cell culture and cell culture flasks etc., were bought from the firms- Eppendorf, Sarstedt, Greiner and Nunc.

2.2 Laboratory equipment

Autoclave	sanoclav, Wolf
Axiovert 40 CFL (fluoroscent microscope)	Zeiss
BioDoc Analyze UV translluminator	Biometra
Centrifuges	
Laboratory centrifuge 3K30	Sigma
Tabletop centrifuge 1-15	Sigma
Tabletop centrifuge 5415C	Eppendorf
Tabletop centrifuge 5810R	Eppendorf
Ultracentrifuge J6 MC	Beckman
Gel-documentation systems	Digit-Store duo (INTAS)
Electrophoresis chamber for DNA gels	Peqlab
Electrophoresis chamber for RNA gels	BioRad
Electrophoresis chamber for SDS gels	Invitrogen
Electrophoresis Power supply- EPS600	Amersham Pharmacia Biotech
Electrophoresis Power supply- ST305	GIBCO BRL Life technologies
Electorblotter-iBLOT	Invitrogen
Electorblotter Semi-dry	Peqlab
Film Developer	Protec Optimax
Glassline (4ºC and -20ºC)	Liebherr
Horizontal shaker	B.Braun Biotech international
Hybridization oven- OVI	Biometra
Ice machine	Ziegra
Incubator C200 (cellculture)	Labotech
Innova 4230 (Refrigerated incubator shaker)	New Brunswick scientifi
Laminar flow- Hera safe KS	Thermo scientific
Luminometer-Lumat LB9501	Berthold
Magnetic stirrer- MR 3001K	Omnilab
Multilabel counter- Victor2 V 1420	Perkin Elmar
Microwave	Sharp R-939
Refrigerator (-80°C)	GFL

Scanner-scanjet G4050	HP
Spectrophotometer-NanoDrop ND-100	Peqlab
Test tube thermostat TCR 100	Roth
Thermomixer 5436	Eppendorf
Thermocycler	Landgraf
Thermocycler-7500 Fast Real-Time PCR System	Applied Biosystems
Thermocycler-primus 25 advanced	Peqlab
Vortex-MIXOMAT	Boskamp
Vortex-Genie 2	Bender and Hobein
Water bath with shaking- E100	Lauda
Water bath in cell culture	Grant OLS 200
Weighing scale	Omnilab

2.3 Chemicals and Solutions

All chemicals-powdered and solutions-, analytical grade or cell culture tested, were obtained from Fluka, Merck, Serva, SIGMA-Aldrich, BIOMOL, ROTH and J.T.Baker.

2.4 Buffers and Solutions

Luria Bertani (LB) medium

25 g Dissolved in 900 ml distilled water made up the volume to 1 liter, sterilized by autoclaving and stored at 4°C.

LB-Ampicillin Agar Plates

37 g of Standard Nutrient Agar was dissolved in 1000 ml water and autoclaved. After autoclaving, the medium was let to cool down to 55 °C and ampicillin was added to a final concentration of 100µg/ml. This medium was poured into 10 cm petriplates and left to solidify, then stored at 4°C.

Loading Buffer (DNA-, RNA- Gel electrophoresis)

50	% (v/v)	Glycerol
0.4	% (w/v)	Bromphenolblue
1	mM	EDTA pH 8,0

10X Detection solution (Northernblot)

0.1	M	Tris-HCl
0.1	M	Sodium Chloride (NaCl)

pH set to 9.5

2X HEBS

16.4	g/l	NaCl
11.9	g/l	HEPES
0.21	g/l	Disodium hydrogen phosphate (Na_2HPO_4)

pH set to 7.12

2X Maleic acid solution

0.1	M	Maleic acid
200	mM	NaCl

pH set to 7.5

10X MOPS

200	mM	3-(N-Morpholino) propanesulphonic acid
50	mM	Sodium acetate
10	mM	EDTA

pH set to 7.0

20X SSC

3	M	NaCl
300	mM	Tri-sodium citrate

pH set to 7.0

20X SSPE

3	M	NaCl
200	mM	Sodium dihydrogen phosphate (NaH_2PO_4)
20	mM	EDTA

pH set to 7.4

50X TAE

2	M	Tris
0,1	M	EDTA

pH set to 8.0 using acetic acid

TSS solution (Preparation of competent bacteria)

10	% (w/v)	Polyethylenglykol 8000
5	% (v/v)	dimethylsulfoxide (DMSO)
50	mM	Magnesium sulphate ($MgSO_4$)

in LB-Medium

10X electrophoresis buffer (for SDS gels)

250	mM	Tris
2	M	Glycine
1	%(w/v)	SDS

pH set to 8.3

10X TBS buffer (for western blot)

100	mM	Tris-HCL. pH 7.5
1.5	M	NaCl

Transfer buffer (for semi-dry western blotting)

48	mM	Tris-HCL
39	mM	Glycine
0.0037	%(v/v)	SDS
20	%(v/v)	Methanol

3x Laemmli loading buffer

187.5	mM	Tris-HCl. pH 6.8
9	%(w/v)	SDS
15	%(v/v)	β-mercaptoethanol
30	%(v/v)	Glycerol

Coomassie Brilliant Blue solutions

staining solution:

7	%(v/v)	Acetic acid
3.6	%(v/v)	Methanol
0.1%	%(w/v)	Coomassie R250

Add H_2O for needed volume

De-staining solution:

7	%(v/v)	Acetic acid
10	%(v/v)	Methanol

Add H_2O for needed volume

Reporter-lysis buffer (for luciferase activity)

Buffer A (200mM KH_2PO_4)	4.25 ml
Buffer B (200mM K_2HPO_4)	45.75 ml
0.2% Triton X-100	200µl
H_2O	add to 100ml

Add inhibitors before using:

1µg/ml	Pepstatin
10µg/ml	Leupeptin
1mM	PMSF

Firefly Luciferase Buffer

25	mM	Potassium Phosphate buffer (pH 7.8)
15	mM	MgSO4.7H2O
1	mM	ATP
1	mM	DTT
4	mM	EGTA

Renilla Luciferase Buffer

100	mM	Potassium Phosphate buffer (pH 7.8)
500	mM	Sodium Chloride
1	mM	EDTA

2.5 Ready-to-use buffers and solutions

Dulbecco's modified eagle medium (DMEM)	PAA
Cryopan Freezing medium (for Keratinocytes)	PAN Biotech
EDTA 1%	PAN Biotech
Fetal Calf Serum	PAN
Keratinocyte Growth Medium with supplement Mix	Promocell
L-Glutamin (200 mM)	Gibco
PBS	PAA
Penicillin/Streptomycin	PAA
Trypsin-EDTA (10X)	PAA
NuPAGE LDS sample Buffer (4X)	Invitrogen
20X MOPS SDS Running Buffer	Invitrogen
20X Tris Acetat SDS Running Buffer	Invitrogen

2.6 Antibodies

Phospho-p38 MAPK (Thr180/Tyr182)	Cell signalling
p38 MAPK	Cell signalling
HuR 19F12	Santa cruze biotechnology

2.7 Cell lines

HeLa- tTA (stably transfected with the tetracycline sensitive Transactivator)	Gossen et al., 1992
Foreskin Keratinocytes	Hautklinik Linden, Hannover

2.8 Enzymes, Nucleotides and Standards

dNTP Set	MBI Fermentas
Oligo (dT)18 Primer	MBI Fermentas
rDNase	Macherey-Nagel
Restriction endonuleases	MBI Fermentas
Revert Aid M-MuLV Reverse Transcriptase (20u/µl)	MBI Fermentas
Taq-DNA-Polymerase (5u/µl)	MBI Fermentas

2.9 Escherichia coli strains:

DH5α
JM109
XL1 blue

2.10 Inhibitors and Stimulators

Actinomycin D	Sigma
Doxycycline	Sigma
SB203580	Calbiochem
rHu Il-1 α	Promokine
rHu IL-17A	R&D Systems
h TNF- α	Genentech
Wortmanin	Alomone Labs

2.11 Kits

FastSybrGreen-PCR-Mix	Applied Biosystems
TaqMan Fast Universal PCR Master Mix	Applied Biosystems
GENECLEAN® *Turbo* Kit	Qbiogene
GFX^{TM} for plasmid mini-preps from	Amersham Biosciences
MEGAscript® T7/T3 Transcription Kit	Ambion
Nucleospin RNA II (250 preps) for RNA isolation	Macherey Nagel
Nucleospin AX for plasmid maxi-preps	Macherey Nagel
PCR purification kit	Qiagen

2.12 Other materials and substances

Cell scrapper	Sarstedt
CSPD	Roche diagnostic
Coelenterazine 4mM	promega
D-Luciferin 25mM	Sigma
DIG-11-UTP	Roche diagnostics
Filter paper 3MM	Whatman
Hybond N Membran	Amersham
Immobilon PVDF membrane	Millipore
Neubauer cytometer	Superior Marienfeld
X ray film cassettes	Applied Gene Technology Systems
X ray films	Kodak

2.13 Plasmids

pEGFP-C1	Clontech
phRL-TK	Promega
ptetBBB	Xu et al., 1998
pCMV flag d.neg.p38	Winzen et al., 1999
pcDNA3 $MKK6_{2E}$	Winzen et al., 1999
pMir-Report	Ambion
pMir-IκBζ $_{2273-3885}$	Dhamija et al., 2010
pMir-IL-8 ARE	Dhamija et al., 2010
pCMV-HA-IRAK1	Kindly from Mark Windheim
pCMV-HA-IRAK2	Kindly from Mark Windheim
pFl-IRAK-1-DD	Neumann et al., 2007, Neumann et al., 2008

2.14 Probes and primers for Real time PCR

All TaqMan Probes were ordered from company Applied Biosystems with assay-ID as follows:

NFKBIA	Hs00153283_m1
GAPDH	Hs99999905_m1
IL8	Hs00174103_m1
JUN	Hs00277190_S1

SybrGreen sense and antisense primers:

IκBζ *sense*	5' gccaaccattccaagtcagg
IκBζ *antisense*	5' ttggtttgtgggtgtagtgtgg
GAPDH *sense*	5' tcaaggctgagaacgggaag
GAPDH *antisense*	5' atggtggtgaagacgccagt
Luciferase *sense*	5' gctgggcgttaatcagagag
Luciferase *antisense*	5' gccatccatccttgtcaatc

3 METHODS

3.1 Tissue culture methods

3.1.1 Passaging and Handling:

All cell culture techniques were performed under sterile conditions in the hood. Only disposable plastic ware-pipettes, 50ml falcon tubes, petri dishes, and culture flasks were used.

HeLa tTA cells: The medium that was predominantly used for the culturing of Hela cells was Dulbecco's modified eagle medium (DMEM). Fetal calf serum (FCS), to a final concentration of 5% or 10%, L-Glutamine, penicillin and streptomycin were added to usually 500ml of the ready-to-use DMEM medium just before use. This medium was then stored at 4°C for some weeks. The medium was always prewarmed to 37°C before use. Similarly the 1XPBS prepared from the 10X stock, and the 1X trypsin-EDTA solution were also stored at 4°C, and prewarmed to 37°C before use. The cells were normally cultured in medium supplemented with 5% FCS. For passaging, the cells were allowed to reach confluence, and then the medium was discarded. The cells were washed carefully with warm PBS twice. Then an appropriate amount of trypsin-EDTA (according to the size of the culture flask or petri dish) was added to the cells. The cells were then incubated at 37°C for 2-3mins until they detached from the surface of the culture vessel. Fresh medium was immediately added to the cells, and the cells were split into an appropriate number of culture flasks according to the need and the period before the next passage.

Keratinocytes: The culturing medium was Keratinocytes Basal Medium (serum free). To get the complete growth medium, a SupplementMix/$CaCl_2$-solution was added to the basal medium which includes:

	Final conc.
Bovine Pituitary Extract	0.004 ml / ml
Epidermal Growth Factor	0.125 ng / ml
Insulin	5 µg / ml
Hydrocortisone	0.33 µg / ml
Epinephrine	0.39 µg / ml
Transferrin, holo (human)	10 µg / ml
CaCl2	0.15 mM

To avoid contamination penicillin/streptomycin was also added. The complete medium was stored at 4°C. For passaging keratinocytes it is better that the cells have not reached full confluency. Here also medium, 1XPBS and trypsin-EDTA were prewarmed to 37°C before use. The medium was discarded. The cells were washed with 1XPBS twice. 4-5 ml of 0.02% EDTA was added to the cells, incubated for 5 min in order to separate the cells from each other. Then appropriate amount of trypsin-EDTA was added and the cells were incubated for 2 min. 10 ml of 1XPBS was added immediately to the cells. Cells were spun down for 3 min, 1100 rpm. They were then resupended in the appropriate amount of medium and allowed to grow at 37 °C.

3.1.2 Counting the cells

The cell count was determined using the Hemocytometer (Neubauer Chamber). An aliquot of the cell suspension obtained after trypsinizing the cells was diluted 1:1 with Trypan Blue (0.8% v/v in PBS). Trypan Blue is a cell permeable dye; while living cells are able to extrude the dye, dead cells are unable to do so and hence stain blue. To obtain an accurate count, the cells have to be uniformly distributed over the entire chamber. The chamber is divided into 9 squares. Each square has a surface area of 1sq.mm and the depth of the chamber is 0.1mm. To get the final count in cells/ml, first divide the total count by 0.1mm (chamber depth) then divide the result by the total surface area counted.

3.1.3 Freezing and thawing cells

HeLa tTA: Cells that are to be frozen are cultured until they reach confluence. One 75cm^2 flask of cells was prepared for one freezing ampoule. Each freezing ampoule was labeled with the name of the cell line and the date of freezing. The freezing medium contains:

70%	growth media
10%	DMSO
20%	FCS

The cells were trypsinized, and spun down by centrifugation. They were then resupended in the appropriate amount of ice cold freezing medium (1ml per ampoule) and transferred to the ampoule, which was then placed in a freezing box. The box was left at -80ºC for at least 24hrs. Thereafter the ampoules can be transferred to storage in liquid nitrogen.

For thawing cells, an ampoule was taken from the liquid nitrogen and allowed to thaw in warm water until only a small piece of ice is left. The cell suspension was transferred to a 15ml centrifuge tube. 1ml of cold FCS was added drop-wise to the cells, with gentle mixing. 10ml of growth medium containing 15% FCS was then added to the cells in portions with gentle mixing. The cells were spun down, and the cell pellet was resuspended in 1ml of the growth medium containing 15% FCS, after which the cell suspension was transferred to 75cm^2 flask containing 25ml of the same growth medium and allowed to grow at the appropriate growth temperature until they reach confluence.

Keratinocytes: Keratinocyte cells are to be frozen until they reach confluence of ca. 70%. When they are too full they hardly get separated from each other and form clumps. The freezing procedure is the same like passaging, only at the end the cell pellet is resuspended in cold serumfree freezing medium (PAN Biotech).

For thawing the cells, the ampule was thawed in warm water until only a small piece of ice clump is left. The cell suspension was transferred to 10ml 1XPBS and was spun down for 3 min, 1100 rpm. The cell pellet was resuspended in Keratinocytes complete growth medium and then transferred to 75cm^2 flask and allowed to grow.

3.1.4 Transient transfection

Transient transfection of the HeLa tTA cells was done using the calcium chloride-HEBS method. In brief, appropriate numbers of cells were seeded in petri dishes (see table above) the day before the transfection was to be done, such that the cells reach a state of sub-confluence on the day of the transfection. The next day the medium in the dishes was replaced with fresh medium containing 5%FCS at least an hour before the actual transfection was to be done. 250mM calcium chloride solution was prepared fresh from the 2.5M stock solutions. Then equal volumes of the 250mM calcium chloride solution and 2X HEBS solution (pH 7.12, set at RT) were tested against each other in a small glass tube for the formation of a thin white precipitate at the interface of the two solutions, taking care to add the pre-warmed 2X HEBS drop by drop along the sides of the tube. Now the required amounts of the plasmids (to be transfected were added to the appropriate amounts of the calcium chloride solutions aliquoted in the glass tubes. Then equal volumes of the 2X HEBS solution were added individually to each tube, making sure a precipitate was formed in each case. Each mixture was allowed to stand at RT for 1mins, mixed well and then added to the cells to be transfected in the petri dishes. 4h after the transfection, a glycerol shock was given to the cells using a 10% glycerol solution prepared in warm DMEM. An appropriate amount of the 10% glycerol solution was added to the cells, after removing the medium that was previously covering the

cells. The glycerol-medium mixture was allowed to stand on the cells for 3mins. Then the glycerol was removed and the cells were washed twice with warm PBS and fresh medium added. 2h after the glycerol shock the cells were trypsinized and reseeded in parallel cultures required for the assay to be carried out.

Medium	***In DMEM-Medium + 5% FCS + P/S, L-Glutamin***					
1.Day = seeding			**2.Day = Transfection + glycerol shock**			
Plates cm/cm²	cell number **Hela**	**Med.-vol.**	**µg plasmid**	each plate **1x CaCl**	each plate **2x HEBS**	each plate **10% Glycerol**
9 cm dish (56cm²)	$4x10^6$	10ml	20-30 µg	0.5 ml	0.5 ml	2 ml
6 cm dish (21cm²)	$1.5\text{-}2x10^6$	5ml	9-10 µg	0.25 ml	025 ml	1 ml
6-well dish (9.6cm²)	$5x10^5$	2ml	4 µg	0.125 ml	0.125 ml	0.35 ml

3.1.5 siRNA transfection

For siRNA transfection cells were seeded in the morning. 2.2 million cells were seeded in 6 cm plates. A gap for more than 6 h was kept between seeding and transfection. In the evening the cells were transfected with 200 pmol siRNA along with appropriate amount of empty and peGFP plasmid as mentioned in 3.1.4. The cells were incubated overnight without glycerol shock. On the second day the cells were washed few times with 1X PBS and splitted into 9 cm plates. Second siRNA transfection was done on the third day (500 pmol siRNA) followed by glycerol shock. Cells were incubated overnight without splitting. On the fourth day the cells were trypsinized and reseeded in parallel cultures required for the assay on the next day. Protein aliquots were also made additionally to check the efficiency of knockdown on western blot.

3.1.6 Preparation of lysates for RNA extraction

For the preparation of lysates, the transfected cells (about 24hrs after the transfection) were washed with cold 1XPBS. After adding appropriate amount of the RNA lysis buffer (from *Macherey Nagel*) the cells were scraped off from the petri dish. The lysates were frozen in liquid nitrogen and stored at -80°C.

3.1.7 Preparation of lysates for luciferase measurement

The transfected cells were washed with cold 1XPBS. After adding appropriate amount of the B-galactosidase buffer (see section 2.4) the cells were scraped off from the petri dish. The lysates were stored at -80°C.

3.2 Molecular biology methods

3.2.1 Preparation of competent E.coli cells:

3ml cultures of *E.coli* (JM109/XL1blue) were grown in LB overnight at 37ºC. The next day the 2ml cultures were inoculated into 120ml of LB each and allowed to grow at 37ºC in a shaker. In the meantime the transformation stop solution (TSS) was prepared fresh (see section 2.4). When the OD_{600nm} reached 0.3-0.4, the cultures were spun down at 2000 g, 4ºC for 10 min. To each pellet 1/10 the volume of 1XTSS (10ml for a 100ml culture) was added, the pellets resuspended in the same and incubated on ice for 2 min. Then 200µl aliquots were made and stored at -80ºC.

3.2.2 Determination of nucleic acid concentration

The concentration of the isolated DNA or RNA and the ratio of absorbance at 260 nm to 280 nm (A260/A280 ratio) were measured with the NanoDrop ND-1000 spectrophotometer. A ratio of A260/A280 between 1.8 and 2 monitored a sufficient purity of the DNA or RNA preparation.

3.2.3 Agarose gel electrophoresis of DNA

The size and purity of DNA was analyzed by agarose gel electrophoresis. Concentration of agarose used for analysis is inversely proportional to the size of the DNA of interest, that is, the larger the DNA the lower the concentration of agarose.

Agarose concentration (% [w/v])	Separation area (kb)
0.6	1-20
0.9	0.5-7
1.2	0.4-6
1.5	0.2-4
2	0.1-3

Agarose was weighed and dissolved in 1XTAE buffer by boiling in a microwave oven. The agarose solution was cooled to 60 °C and ethidium bromide was added to a final concentration of 0.5µg/ml. This was poured into the agarose gel cassette and allowed to polymerize completely. The sample DNA was mixed with gel loading buffer and loaded onto the gel. The gel electrophoresis was carried out at 100 V. Ethidium bromide is a fluorescent dye which intercalates between the stacked bases of the DNA and fluoresces under UV light at 254 nm. Hence DNA can be visualized. The gel was photographed using a gel documentation system.

3.2.4 Restriction endonuclease digestion of DNA

The activity of restriction enzymes is measured in terms of ‘Units’ (U). One unit of restriction enzyme activity is defined by the amount of restriction enzyme required to cut 1 microgram of bacteriophage lambda DNA to completion in a time of 1 hour.

Plasmid DNA	40µg
Enzyme buffer	20µl
Restriction enzyme	50 units
H2O	add to 200µl

The reaction mixture was then incubated at the suitable temperature recommended from the manufacturer for 2-4 hours. The digestion was checked by running the digested product with uncut controls of the same plasmid on a DNA agarose gel.

3.2.5 Dephosphorylation of DNA

To avoid undesired self ligation of plasmid DNA, the 5' phosphate groups of the DNA were dephosphorylated using calf intestinal phosphatise. 5 units of CIAP were added to the previously digested DNA and the mixture incubated for about 40 min at 37ºC. The dephosphorylated plasmid was purified using PCR purification kit from firm Qiagen.

3.2.6 Purification

To purify the digested plasmid or a PCR product 5-fold volume of buffer PBI was added. The sample was vortexed and transferred to the Qiagen-quick-column and centrifuged for 1 min at 14000 rpm. The flow-through was discarded and the filter- bound DNA was centrifuged for 1 min at 14000 rpm with 750µl of PE buffer. The flow-through was discarded again and the sample was centrifuged for another 1 min without addition of buffers to remove remaining ethanol. The elution of DNA was performed with 50µl elution buffer.

3.2.7 Purification of DNA by Gel extraction

To the previously purified linearised DNA, an appropriate amount of the DNA loading buffer was added. A 1% agarose gel was prepared and about 15µl of the Dye-DNA mixture was loaded in each of 3 wells. The gel was allowed to run until a good separation of the linearized DNA from the other fragments resulting from the digestion was achieved. The gel was now visualized in a UV transilluminator and neat incisions were made using a scalpel, just behind the linearised vector, on the sides and above. Gel piece was removed and placed in a clean eppendorf tube. The DNA was purified and eluted with GENECLEAN *Turbo* Kit from firm Qbiogene according to the manufacturer's instructions.

3.2.8 Ligation

Joining linear DNA fragments together by a phosphodiester bond between the 3'hydroxyl of one nucleotide and the 5' phosphate of another is called ligation. T4-DNA ligase is used for this purpose. The ligation reactions were set up taking either a 1:3 or a 1:5 ratio of molar concentrations of the vector: insert as follows:

Vector	100ng
Insert	depends the on size of insert
5X Ligation buffer	2 µl
T4 DNA ligase	0.5µl
ddH_20	to 10µl

The reactions were incubated at RT, 5 min. 5µl of the ligated mix was used for bacterial transformation.

3.2.9 Transformation of competent E.coli cells

10-50 ng of DNA or 5µl of the ligation mix was added to each 200µl aliquot of competent cells and incubated on ice for 30 min. Cells were subjected to heat shock by incubating at 42 °C for 45 sec and incubated on ice for 2 min. 0.8 ml of LB medium was added to the cells followed by incubation at 37 °C in the shaker for 1 hour. Cells were plated on LB agar plates containing appropriate antibiotic (z.B.100µg/ml of ampicillin).

3.2.10 Plasmid preparations

Mini plasmid preparations were done using the *GFX* (see section 2.11) kit according to the manufacturer's instructions. Maxi-preparations were also done using the Nucleobond kit from *Macherey Nagel*, according to the manufacturer's instructions.

3.2.11 Isolation of RNA from mammalian cells

RNAs were isolated from mammalian cells in culture using the Nucleospin kit from *Macherey Nagel*. The principle behind the isolation was very simple. Cells were lysed using the lysis buffer provided with the kit. The appropriate amount of the lysate was added to Shredder columns to homogenize the lysate. Freshly prepared 70% ethanol was then added to the homogenized lysates to bind the nucleic acids. This mixture was then added to Columns, which had silica gel matrices that bound the RNA from the lysates. After suitable washes to remove contaminating DNA and proteins, pure RNA was eluted in 40µl of RNase free water. If RNAs are to be used for DNA-*microarray* analysis or Real-Time PCR an additional DNase I treatment is needed.

3.2.12 RNA gels

The required amount of agarose, depending on the % required, was dissolved in deionized water by boiling. Then the required amounts of 10X MOPS solution and formaldehyde solution were added and the gel poured immediately into the gel platform.

Total gel volume	300ml
Deionized water	214.8ml
Agarose	3g (1%)
37% formaldehyde	55.2ml
10X MOPS	30ml

Prepare 5-20µg of total RNA in deionized water. A master mix of the loading buffer was prepared.

10X MOPS	1.5µl*X
37% Formaldehyde	3.75µl*X
1mg/ml Ethidium bromide	1µl*X
RNA loading dye	8.75µl*X

X is the no. of samples

Equal amounts of the RNA sample and the loading buffer were mixed with each other. The mixtures were vortexed briefly and then heated to 65ºC for 15 min, spun down, mixed well once again and loaded onto the gels. The gels were run at 120V until the dye front migrated halfway down the gel.

3.2.13 Northern transfer

The RNA gels were visualized under a transilluminator and photographed, so that a record of the quality of the RNA and the quantity (equal loading) can be made. Then the gels were rinsed in distilled water two times, 30 min each time to wash off the excess formaldehyde fumes. The gels were then rinsed in 10X SSC for about 20min and in the meantime the transfer apparatus set up, using a glass dish, 10X SSC and a whatmann wick. The gels were blotted onto 0.45µm nylon membranes. The membranes were cut to the sizes of the gels to be transferred, and soaked briefly in 10X SSC. The membranes were then placed over the gels that had previously been placed on the transfer apparatus, taking care to roll out air pockets if any. This was covered by two pieces of 3MM whatmann paper once again cut to the size of the gels which had also been briefly soaked first in distilled water and then in 10X SSC, followed by a stack of paper towels/diapers, and a weight was placed over the whole set-up.

The transfer was allowed to take place for about 20 h. Then the membranes were baked for about 10min at 80ºC, and crosslinked using either a UV transilluminator (1min 15secs) or simply using a crosslinker.

3.2.14 Hybridization of Northern blots

The blots were presoaked in 2X SSC. The blots were now incubated with the pre-hybridization solution at 68ºC for at least 2hrs the volume used depending on the size of the blots. After the pre-hybridzation, the blots were incubated with the hybridization solution at 68ºC O/N.

Pre-hybridization/Hybridization solutions (40ml)	
Formamide	20 ml
20X SSPE	10 ml
10X Blocking solution	8 ml
N-Laurylsarcosine	0.4ml
20% SDS	0.04ml
deionized water	1.52ml
*DIG labeled as RNA	100-200ng/ml

* only for the hybridization solution

The first time that the probe was dissolved in the hybridization solution it was heated to 95ºC for 5 min. Then the hybridization solution was stored at -80ºC. Thereafter the hybridization solution was thawed at 75ºC for 15 min just before use.

After the O/N hybridization the blots were first rinsed under high stringency conditions (2X SSC, 0.1% SDS) at RT two times, 5 min each time. Then the blots were washed under low stringency conditions (0.1X SSC, 0.1% SDS) two times at 68ºC, 15 min each time. The blots were now incubated with the blocking buffer for 30 min and then were incubated with the blocking buffer containing the anti DIG-AP (alkaline phosphatase) conjugated antibody at a dilution of 1: 10,000 for another 30 min. Now the blots were washed two times, 15 min each time with the washing solution (1X Maleic acid, 0.3% Tween 20). The blots were washed briefly in the 1X DIG detection solution (see section 2.4). The blots were now incubated with the substrate solution, CSPD for 5 min, then placed in the film cassettes, and are now ready for exposures. Exposures were made by placing X-ray films over the blots in the cassettes, and after a suitable exposure period, the films were developed using a developer.

3.2.15 Preparation of DIG labeled as RNA probe

The DIG labeled UTPs (DIG-11-UTP) were incorporated into an antisense RNA probe spanning a region of the gene of interest, which was generated by *in vitro* transcription using a linearized plasmid or a cDNA template containing the correct RNA polymerase promoter site (T7, T3) upstream of the sequence to be transcribed (gene of interest).

linearized plasmid template/	1µg
cDNA template from RT-PCR	200ng
10X transcription buffer	4µl
75mM ATP solution	2µl
75mM CTP solution	2µl
75mM GTP solution	2µl
75mM UTP solution	0.73µl
T7/T3 RNA polymerase	2µl
RNase free water	2µl (40 units)
	to 20µl

The transcription was allowed to take place for 1.5h at 37ºC. 1µl of Turbo DNaseI (2U/µl) was added and the mix incubated for 15 min at 37ºC. The RNA was than purified with "Mini Quick Spin™ Column" (Roche) with the following procedure:
The matrix was initially resuspended in the column buffer by gentle vortexing. Top cap and bottom tip are removed; the column bed is placed in a new tube and centrifuged for 1 min at 1000 x g. The flow-through is discarded. The sample is applied to the center of the column bed, which is placed into a new microcentrifuge tube. Furthermore the column is centrifuged for 4 min at 1000 x g and the RNA sample collected in the tube.

3.2.16 RT-PCR

For the reverse transcription reaction, the following general protocol is followed:

Total RNA	1µg or 500ng for Real-Time PCR
5X RT buffer	4µl
0.1M DTT	2µl
10mM dNTPs	2µl
Oligo(dT)s	1µl
M-MLV reverse Transcriptase	0.5µl
H_2O RNase free	to 20µl

The reaction mix was then incubated in the thermocycler at:

23ºC	10 min
37ºC	60 min
95ºC	5 min

3.2.17 PCR

The reverse transcription can be followed by a classical PCR to amplify the generated cDNA.
PCR reaction:

cDNA (RT product)	10µl
10X PCR buffer	9µl
25mM $MgCl_2$	3.6µl
Sense primer	10 pmole
antisense primer	10 pmole
dd water	74.4µl
Taq polymerase	1µl
Total volume of 100 µl	

For each PCR reaction the annealing temperature was chosen depending on the melting temperature (Tm) of the primers. The extension step for *Taq* DNA polymerase is 1 min at 72°C for PCR products up to 2 kb. For larger products, the extension time is prolonged by 1min/kb.

The reaction is incubated in a thermocycler device where the temperature can be changed rapidly. Usually there is a preheating step of 5 min at 95°C, which is followed by switch to 85°C for 5 min. During this period enzyme is added. The cycles are as follows:

Condition	Temperature	Time	
	95°C	5 min	
	85°C	5 min	
Denaturation	95ºC	1 min	} X 40
Annealing	55ºC	1 min	
Extension	72°C	2 min	
	60°C	10 min	
	4°C	∞	

3.2.18 Real-Time PCR

Real-time PCR, also called quantitative PCR follows the general principle of polymerase chain reaction. The amplified DNA is detected as the reaction progresses in *real time*, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: probe-based and intercalator-based methods. Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers and an additional fluorogenic probe which is an oligonucleotide with both a *reporter* fluorescent dye and a *quencher* dye attached to the 3' and 5' ends. Intercalator-based method, also known as SYBRGreen method, requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and gives fluorescence.

Fluorescence is detected and measured in the real-time PCR thermocycler, and its geometric increase corresponding to exponential increase of the product is used to determine the threshold cycle (C_T) in each reaction.

Each sample requires an endogenous control. Typically, housekeeping genes such as ß-actin, glyceraldehyde-3-phosphate (GAPDH) are used as endogenous controls, because their expression levels tend to be relatively stable.

Protocol

First of all a reverse transcription was done to convert isolated RNA to cDNA. RNAs were then diluted to 250 ng. From this dilution 2 µl was added to the reaction mix (500 ng cDNA end concentration in the reaction). The expression of each gene was analyzed in duplicates. Values with standard deviations > 0.2 - 0.25 cycles were excluded from the analysis. The

mRNA units are calculated as $2^{-\Delta CT}$ x 10^3, based on GAPDH mRNA as a housekeeping mRNA control.

TaqMan reaction mix:		**SYBR Green reaction mix:**	
TaqMan Fast Master Mix	5µl	FAST SybrGreen MM	12µl
Assay On Demand	0.5µl	Sense Primer	1.44µl
H_2O	2.5µl	Antisense Primer	1.44µl
		H_2O	7.92µl
9.6µl is added in 0.5 ml Eppis		22.8 µl is added in 0.5 ml Eppis	
+ 2.4 µl of cDNA		+1.2 µl cDNA	
10µl is added into 96-well plate		20µl is added into 96-well plate	

The cycles are as follows:

TaqMan

95°C	20s
95°C	3s
60°C	30s

} X 45

SYBR Green

95°C	20s
95°C	3s
60°C	30s
95°C	15s
60°C	60s
95°C	15s

} X 40

3.2.19 DNA-Microarray-Analysis

The DNA-*Microarray*-analysis that has been done in this study was done in collaboration with Dr. Oliver Dittrich-Breiholz. The total RNA for this purpose was prepared using the Nucleospin kit from *Macherey Nagel* with an additional DNase I treatment. To detect differentially expressed genes, fluorescently labeled cRNA (Cy3-labeled) was generated. Labeled cRNAs were subjected to quality control and hybridized to the Whole Human Genome Oligo Microarray (G4112F, ID 014850; Agilent Technologies) as described in Winzen et al., 2007. Data were filtered according to a stringent multistep approach that accounted for quality of the measurements (hybridization performance), consistency among replicate assays, intensity range and fold change values.

3.3 Biochemical methods

The elecrophoretic separation of the proteins was performed through high-tris discontinual SDS polyacrylamide gel electrophoresis. A system with vertical oriented glass plate was used. The gels were prepared as follows:

The resolving gel (10 % acrylamide) was first put between glass plates and covered with a layer of water. After polymerisation of the gel was completed, water was removed and the space between glass plates was dried with Whatman paper. Then the stacking gel was put on top of the polymerised resolving gel and the combs for pre-forming the gel pockets were introduced immediately. After approximately 15 min polymerization was completed and the sample combs were removed. The glass plates with the gel between them were fixed inside the electrophoresis chamber and covered with 1X electrophoresis buffer (see section 2.4). Protein samples were mixed with Laemmli loading buffer 1:1, denatured by 95 °C for ca. 15 min and spun down. The protein samples were injected into the stacking gel pockets. Run time was approximately 50-60 min at 200 volts.

Ingredients	**Stacking gel (5%)**	**Separating gel (10%)**
30% Acrylamide solution (ml)	5.7	3.3
ddH_2O (ml)	1.7	4.1
0.5 M Tris-HCl, pH 6.8 (ml)	2.5	-
1.5 M Tris-HCl, pH 8.8 (ml)	-	1.5
10% w/v SDS (µl)	0.1	0.1
TEMED (µl)	10	5
10% APS (µl)	50	50

3.3.1 SDS-Polyacrylamide Gel Electrophoresis

Coomassie Blue staining is based on the binding of the dye Coomassie Brilliant Blue R250, which binds non-specifically to virtually all proteins. The gel is soaked in a 1:10 dilution of coomassie dye and de-staining solution for few hours.

3.3.2 Staining with Coomassie-Blue

Coomassie Blue staining is based on the binding of the dye Coomassie Brilliant Blue R250, which binds non-specifically to virtually all proteins. The gel is soaked in a 1:10 dilution of coomassie dye and de-staining solution for few hours.

3.3.3 Western blot (semi-dry)

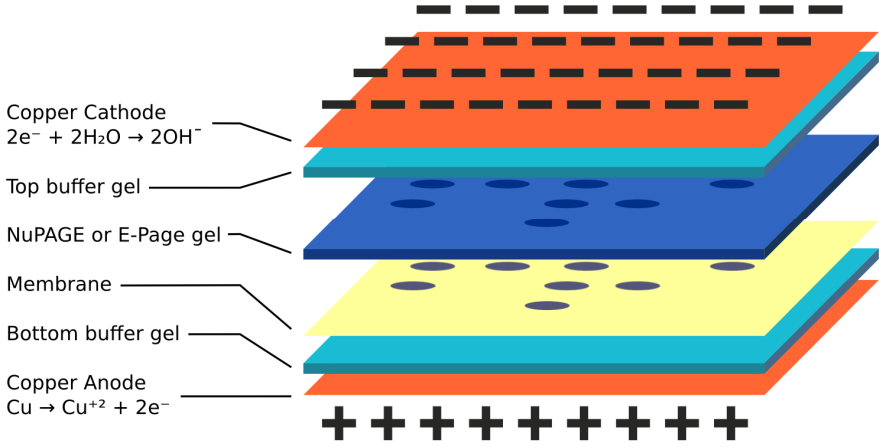
Six pieces of 3 mm Whatman paper and one piece of PVDF or nitrocellulose membrane were cut to the size of the SDS gel. Gel and membrane were equilibrated for 2 min in cathode buffer (PVDF membrane needs to be activated initially by soaking in methanol for 2 min followed by soaking in cathode buffer). The blot was assembled without air bubbles according to the following scheme:

cathode (-) ↓
3x papers soaked in transfer buffer
gel
membrane
3x papers soaked in transfer buffer
anode (+)

For transfer, the current was set to 1 mA/cm_2 gel size for 45-60 min. After transfer, the membrane was briefly washed with water, activated with methanol and rinsed briefly with water again. The membrane was stained with ponceau red and destained in water as required.
The blot was washed 4 times for 5 min each with 1X TBS (see section 2.4) containing 0.1%Tween 20. The blot was incubated in blocking solution (5% milk powder in 1XTBS/ 0.1% Tween 20) for one hour at room temperature. Subsequent incubation with the primary antibody diluted in blocking buffer, occurred either overnight at 4 °C or 2-3 hours at room temperature. For some proteins the blocking step was skipped and the blots were directly transfered into the primary antibody diluted in 1% BSA/ 1X TBS/ 0.1% Tween 20. The primary antibody is washed with 1X TBS/ 0.1% Tween 20 (4 times) for 5 min. Then the membrane was incubated with secondary antibody, also diluted 1:5000 in blocking solution for one hour at room temperature. The blots were now incubated with the substrate solution, CSPD for 5 min, then placed in the film cassettes, and are now ready for exposures. Exposures were made by placing X-ray films over the blots in the cassettes, and after a suitable exposure period, the films were developed using a developer.

3.3.4 iBlot® Dry Blotting System

For quick transfer of proteins another blotting system was performed with an iBlot gel transfer device (invitrogen). For this system ready-to-use NuPAGE 7% Tris-Acetat (31-400 kDa proteins) or 10% Bis-Tris (14-200 kDa proteins) gels were used. Protein samples were mixed with LDS sample buffer 1:1, denatured by 95 °C for ca. 15 min, spun down and loaded on the gel. Gel run was approximately 50-60 min at 200 V for 10% and 150 V for 7% gels. After electrophoresis the gel was removed from the cassette for transfer. The blot was assembled using anode and cathode stack provided by according to the following scheme:



For blotting program 3 was selected which transfers proteins of 30 kDa-150 kDa molecular weight in 7 min at 20 volt. Gel staining and the procedures after blotting is the same as described in 3.3.2 and 3.3.3.

3.3.5 Stripping for reprobing western blots

Stripping is the term used to describe the removal of primary and secondary antibodies from a western blot membrane. It is useful for the investigation of more than one protein on the same blot. For re-probing the same blot the primary and secondary antibodies should get washed off from the membrane. The blot is rinsed shortly in Methanol, incubated initially 5 min in 1XTBS, without Tween 20, next 30 min in 10 ml stripping buffer (25mM Glycin, 1% SDS, pH: 2.1) and finally 1 h in blocking solution (5% milk powder in 1XTBS/ 0.1% Tween 20 or 1% BSA/ 1X TBS/ 0.1% Tween 20).

3.3.6 Luciferase reporter gene assay

A reporter gene assay can be used to study the regulation of a gene of interest. Originally developed for studying the role of promoter elements on gene transcription, it was later adopted for studying post-transcriptional regulation by mRNA 3’ / 5’ UTR elements.

Luciferase is a generic term for the class of oxidative enzymes used in bioluminescence. Firefly luciferase and *Renilla* luciferase are the most commonly used luciferases for reporter gene assays.

The reaction catalyzed by Firefly luciferase takes place in two steps:

luciferin + ATP → luciferyl adenylate + PP_i
luciferyl adenylate + O_2 → oxyluciferin + AMP + light

The reaction catalyzed by Renilla luciferase is:

Coelenterazine + O_2 → coelenteramide + CO_2 + photon of light

Plasmids with the regulatory elements upstream / downstream of luciferase gene are transfected into cells, lysed after treatments and assayed for luciferase activity. A dual reporter assay could be carried out with a second luciferases construct under a constitutive promoter to normalise for experimental variations.

Protocol

Cells were transfected with luciferase expression vectors. For determining the effect of IL-1 or other stimulators, the cells originating from the same transfected culture dish were reseeded into parallel cultures, incubated over night. On the next day cells were stimulated or left untreated. For lysis the cells were washed with cold 1XPBS 2 times, lysed by adding 200µl of the Reporter lysis buffer (see section 2.4) and incubated on ice for 10 min. They were then centrifuged 15 min at 10,000 g and 4° C. The supernatant was collected and transferred into a new tube. The lysates were used directly for luciferase measurement or stored at -80°C.

Firefly Assay: 100 µl of firefly reaction buffer + appropriate volume of lysate (10-50µl) taken in the tube. 50 µl of luciferin solution (25µM D-luciferin in firefly buffer) is injected and measured by using a Lumat LB9501 luminometer.

Renilla Assay: 10 to 50 µl of lysate is pipetted directly into the tubes and 50 µl of substrate in Renilla buffer (4µM coelenterazine) is injected and measured.

4 RESULTS

4.1 Analysis of mRNA decay in cells

4.1.1 Actinomycin-D chase experiments to study mRNA decay

The classical approach for determining half-lives of endogenous mRNAs involve the blockade of transcription by the antibiotic Actinomycin-D (Act-D) and analysis of mRNA levels by northern blotting or RT-qPCR. In case of inducible genes, Act-D is added after stimulation.

4.1.2 The tet-off system to study mRNA decay

It is a well-established system used to determine half-lives of exogenously expressed mRNAs. It is helpful in identifying the cis-regulatory elements which regulate mRNA stability. In this system the gene of interest is cloned downstream of a tetracycline regulated promoter. In case of reporter based studies the cis regulatory elements or putative regulatory regions in the mRNA under investigation is cloned upstream (5’ UTR) / downstream (3’UTR) of a reporter mRNA. These constructs are then transiently transfected into cells that constitutively expresses the tetracycline transactivator (tTA). The addition of tetracycline or its analog doxycycline stops transcription. Lysates are made at appropriate time intervals thereafter, RNAs prepared and examined by blotting or PCR for half-life determination.

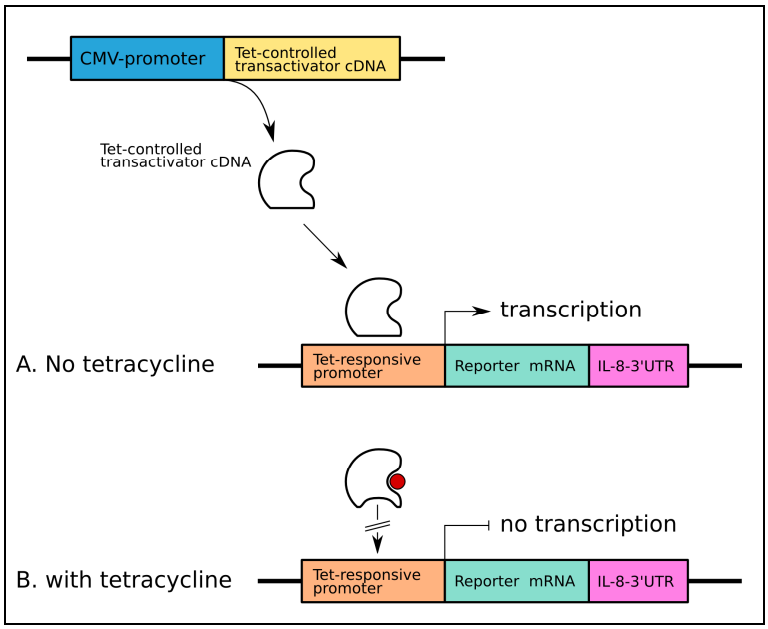


Fig.5 Schematic representation of the tet-off system: HeLa tTA cells obtained from Dr. Hermann Bujard, constitutively express the tetracycline controlled transactivator (tTA) (Gossen & Bujard, 1992). The transactivator (tTA) protein is a fusion between the tetracycline repressor (*tet*R) (from *E.coli*) and the activator domain of the *Herpes Simplex virus* transcription factor. The DNA of the reporter gene (example: β-globin) or another gene of interest is cloned under the control of a Tet-responsive promoter, which contains sequences for the binding of the tet repressor. The constructs are transfected in the HeLa tTA cells. In the absence of tetracycline or its analog doxycycline, tTA binds to and strongly activates the transcription of the reporter gene/gene of interest. In the presence of tetracycline/doxycycline the binding of the tTA to the promoter is abolished and transcription is stopped.

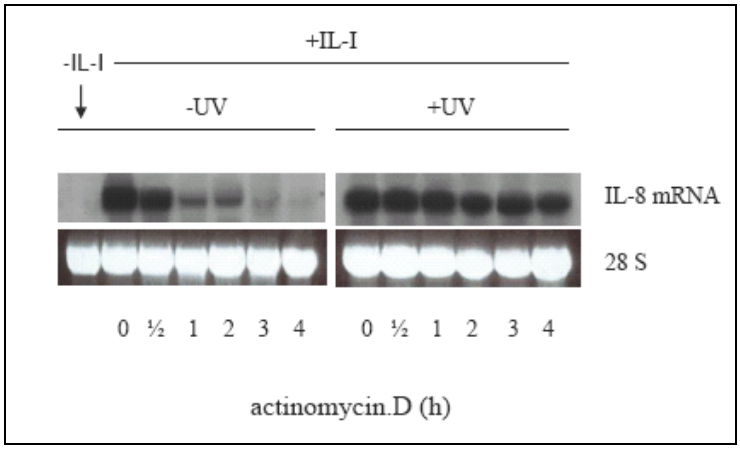
4.2 UV light induces stabilization of short-lived mRNAs in HeLa cells

UV light is a potent inducer of inflammation and induces expression of numerous genes including cytokines and oncogenes (Tyrrell, 1996, Herrlich et al., 1997) which is in part due to the stabilization of mRNAs (Blattner et al., 2000, Wang et al., 2000). UV light strongly activates stress signaling pathways, including the p38/MK2 pathway (Iordanov et al., 1997). Work from our group has shown that unlike the p38 MAPK/MK2 pathway, whose effect is limited to AU-rich trarnscripts, UV light stabilizes short-lived mRNAs irrespective of presence or absence of AU-rich elements in their 3´ UTR (Bollig et al., 2002). Here we wanted to investigate the behaviour of different mRNAs containing AU-rich elements (IL-8 mRNA with ARE class II, Winzen et al., 1999 and c-jun mRNA with ARE class III, Chen and Shuy, 1995) or those without classical AREs (IκBζ and IκBα mRNAs, Bakheet et al., 2001) towards UV-B radiations and involvement of p38 mitogen-activated protein kinase pathways in this manner.

4.2.1 Stabilization of IL-8 and IκBζ mRNA by UV light

Amounts of IL-8 and IκBζ in unstimulated cells were too low for detection by Northern blot (Fig.6A and B, first lane). Therefore expression of endogenous IL-8 and IκBζ mRNAs was induced by stimulation of the cells with the pro-inflammatory cytokine IL-1 for 2 hours. At that time transient activation of p38 MAP kinase and stabilization of IL-8 mRNA by IL-1 is no longer seen (previous results of the lab, Winzen et al., 1999). HeLa cells were exposed to high dose of UV-B light and transcription was stopped by adding Act-D. As observed it Fig.6 the half-life of the IL-8 mRNA (A) and IκBζ mRNA (B) was very short under the unstimulated control condition and was markedly increased by exposure to UV light.

A.



B.

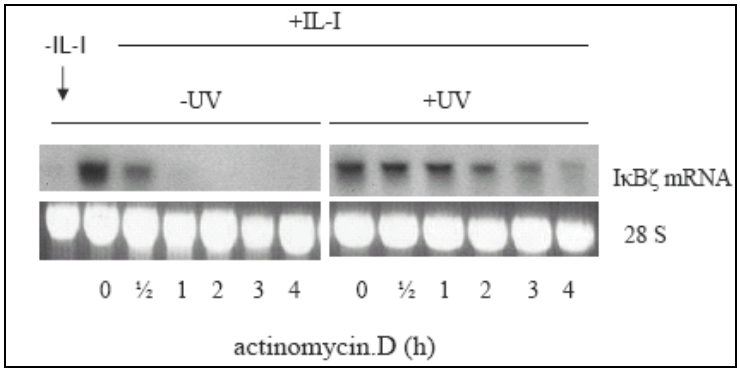


Fig.6 Effect UV-B light on the degradation of endogenous IL-8 (A) and IκBζ (B) mRNA in HeLa (tTa) cells. HeLa cells were treated with IL-1α (2ng/ml) for 2 hours. They were then exposed to high dose of UV-B light (ca. 2000J/m^2). Transcription was stopped by addition of 5µg/ml Act-D and the cells were lysed after the indicated times. Total RNA was isolated and analyzed by Northern blotting on 1% formaldehyde-agarose gel with digoxygenin-labeled antisense RNA probes for (A) IL-8 and (B) IκBζ mRNA. Ethidium bromide staining of the 28S rRNA is shown for RNA loading control.

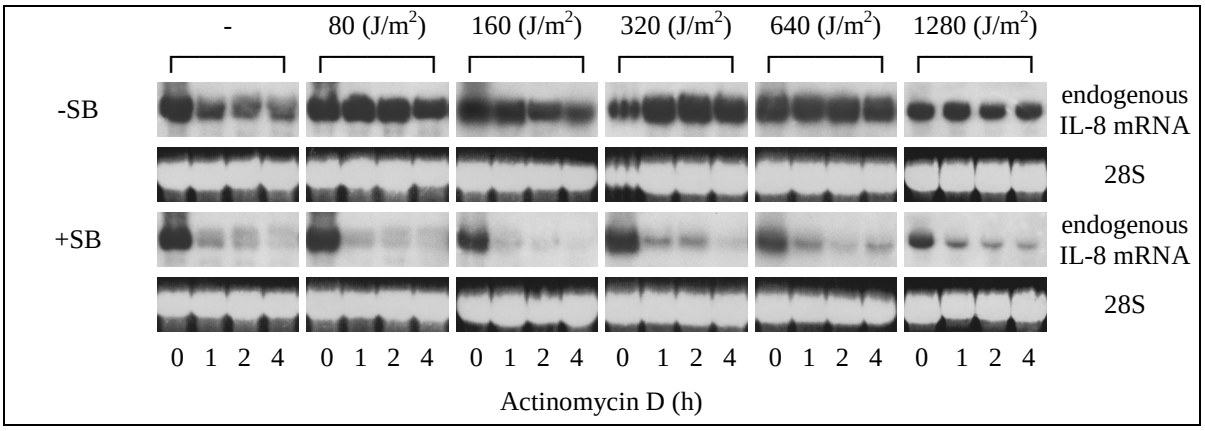
4.2.2 Effect of different doses of UV light and involvement of the p38 MAPK/MK2 pathway

4.2.2.1 IL-8 mRNA

Previously it was shown that UV-induced stabilization is independent of the p38 MAP kinase pathway (Bollig et al., 2002). To investigate this observation regarding low and high dose of UV light, the pyridinyl imidazole SB203580 (2µM), a selective p38 MAPK inhibitor, was applied to the cells prior to UV exposure. The cells were exposed to different doses of UV light, 80 J/m^2 – 1280 J/m^2. To investigate the effect of UV and SB203580 on endogenous IL-8 mRNA the cells were pre treated with IL-1α for 2 hours. Transcription was stopped by adding Act-D (Fig.7.A). Using the tet-off system (see 4.1) stability of the reporter B-globin-IL-8 ARE was monitored. The β-globin mRNA is a normally stable mRNA and is widely used to study the role of 3' UTR elements in regulation of mRNA stability (Shaw and Kamen, 1986). The B-globin-IL-8 ARE -reporter (BBB-IL-8 ARE) construct includes nucleotide sequence 1017-1076 of IL-8 mRNA (ARE) cloned down stream of the coding region of β-globin genomic DNA, under the control of tet responsive promoter. The β-globin mRNA is destabilised after addition of IL-8 ARE. Doxycycline was added to the cells, which stopped the transcription (Fig.7.B) of the reporter.

It was observed that stabilization of IL-8 mRNA at low dose UV light is mostly p38 MAPK dependent since samples treated with SB203580 show less stabilization compare to those without SB203580, whereas the stabilization at high dose UV is independent of p38 MAPK. Even though p38 MAP kinsase activation is blocked by SB203580 (Lee et al., 1994), samples treated with high dose UV (1280 J/m^2) maintain their stabilization (Fig.7)

A.



B.

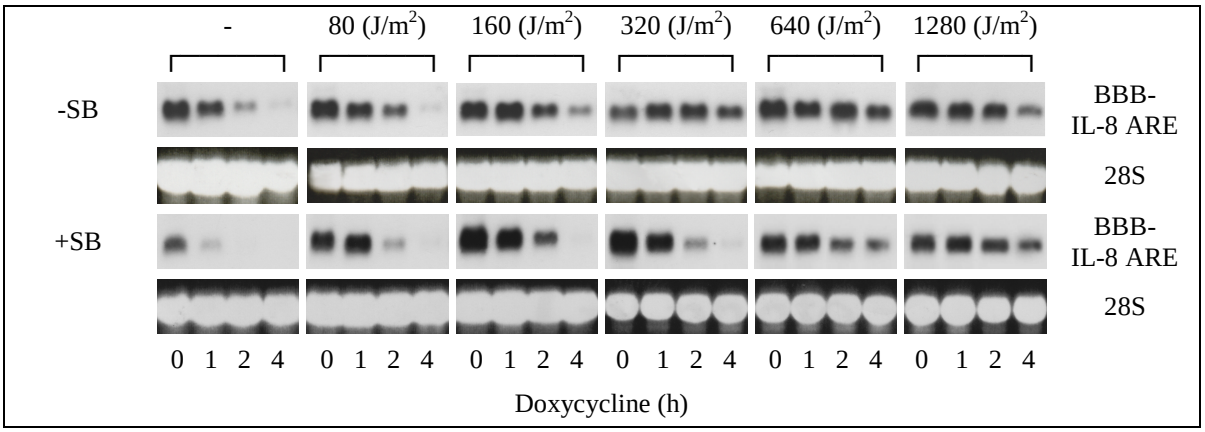


Fig.7 Kinetics of degradation of endogenous IL-8 mRNA (A) and Reporter BBB-IL-8 ARE (B) in response to different doses of UV-B light. (A) HeLa cells were treated with 2ng/ml IL-1α for 2 hours. After 90 minutes of IL-1 incubation 2µM of SB203580 was added to the cells for 30 min followed by exposure to UV light. Transcription was stopped by addition of 5µg/ml actinomycin D directly after UV irradiation. The cells were harvested at the indicated times thereafter. Degradation kinetics was determined as described in the legend to fig 4.1 with an antisense IL-8 RNA probe. **(B)** HeLa cells were transiently transfected by the calcium phosphate method (see chapter 3.1.4) with ptet-BBB-IL-$8_{1017-1076}$. To the transfected cells 2µM of SB203580 was added 30 min before exposure to different doses of UV light as indicated. After 15 min incubation the transcription was stopped by the addition of 3µg/ml doxycycline. RNA was extracted and degradation kinetics determined as described in the legend to fig 4.1 using an antisense β-globin probe.

4.2.2.2 IκBζ mRNA

As observed in Fig.4.2B IκBζ mRNA is stabilized with high dose of UV light. Next we wanted to check how IκBζ mRNA is regulated with low dose (160 J/m^2) and high dose (1280 J/m^2) of UV light compare to IL-8 mRNA. Interesting was also to find out whether this regulation is through p38 MAPK. Hence to determine the involvement of p38 MAP kinase SB203580 inhibitor was applied to the cells prior to UV exposure and the total RNA was isolated and analyzed by the Reverse transcription and quantitative PCR with SYBR Green

based sense and antisense IκBζ primers. The values were normalized to the housekeeping gene glyceraldehyde-3-phosphate (GAPDH).

According to Fig.8 there is no stabilization with low UV dose (160 J/m^2); however high dose (1280 J/m^2) stabilizes endogenous IκBζ mRNA to some extent (A). Similar to IL-8 mRNA blocking the p38 MAP kinase activation using the SB203580 inhibitor could not reverse the inhibition of mRNA degradation induced by high dose of UV light. However this is notable that the level of mRNA stabilization in the control (-SB) is not striking high to make a clear statement about the effect of SB203580 and role of p38 MAPK.

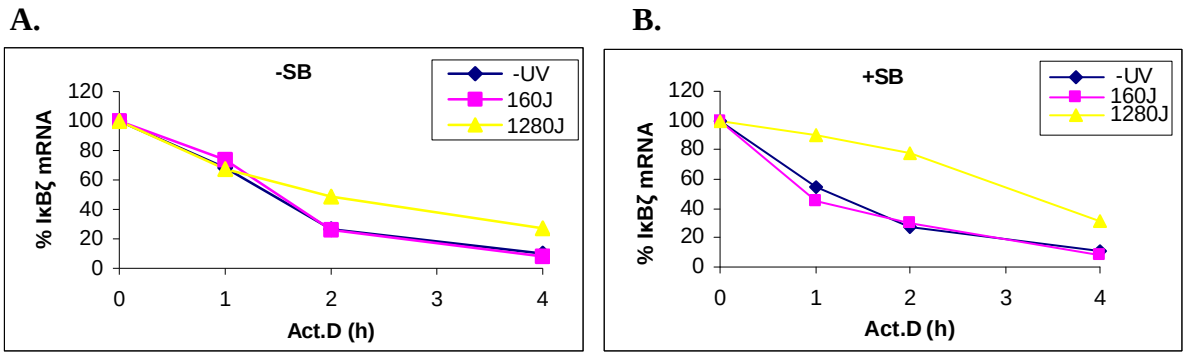


Fig.8 Effect of high and low doses of UV light on IκBζ mRNA. HeLa cells were treated with 2ng/ml IL-1α for 2 hours. After one and half hours of IL-1 incubation 2μM of SB203580 was added to the cells for an incubation time of 30 followed by UV- exposure. Transcription was stopped by addition of actinomycin D. SB203580 treated (B) and untreated (A) cells were harvested at the indicated time points. Total RNA was isolated and analyzed by the RT-qPCR with SYBR Green based sense and antisense IκBζ primers. The values were normalized to the housekeeping gene GAPDH which was used as an endogenous control. Results are expressed in percent of the amount at 0 min.

4.2.2.3 IκBα mRNA

Next we wanted to check how IκBα mRNA is regulated with UV-B light and to what extent is p38 MAP kinase involved. HeLa cells were treated with IL-1α for 2 hours and then exposed to different dose of UV-B light. The result demonstrates that similar to IκBζ mRNA UV-light moderately stabilizes IκBα mRNA and mostly only at high doses (1200J/m^2).

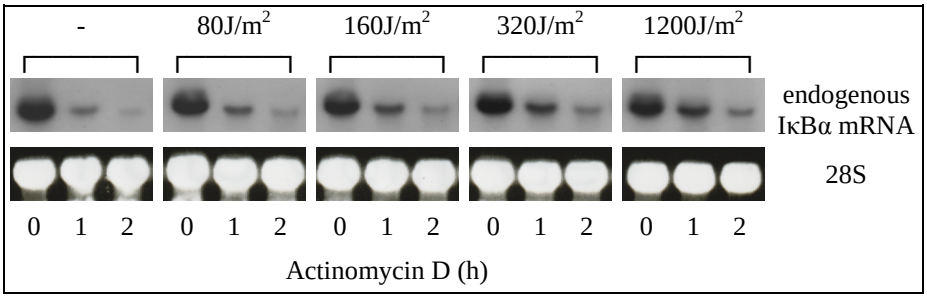


Fig.9 Kinetics of degradation of endogenous IκBα mRNA at low and high UV radiations. HeLa cells were treated with 2ng/ml IL-1α for 2 hours and then exposed to UV radiations. Transcription was stopped by addition of 5µg/ml Act-D. Decay kinetics was determined by Northern blotting as described in the legend to Fig.1 with an IκBα antisense RNA probe.

To investigate the role of p38 MAPK pathway in UV-mediated stabilization, samples treated with or without SB203580 were exposed to high and low dose of UV-B light. The RNA samples were analyzed using reverse transcription and quantitative PCR with a TaqMan probe for IκBα mRNA. The results illustrate that, similar to Northern blot results of Fig.9, a low UV dose (160 J/m^2) does not inhibit degradation of IκBα mRNA, whereas a high dose of UV has a slight stabilizing effect. Since there is no difference between SB203580 treated samples (Fig.10A) and those without SB203580 (Fig.10B), it can be concluded that p38 MAPK does not play any role in UV-mediated stabilization of IκBα mRNA.

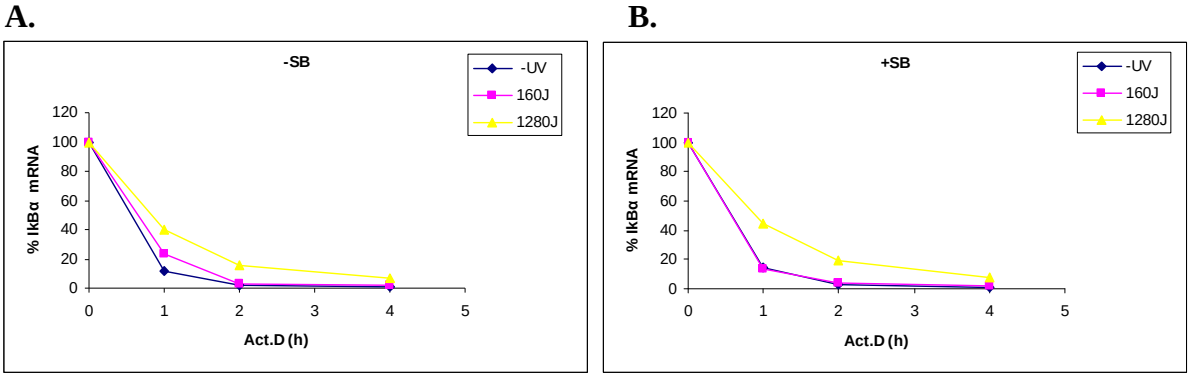


Fig.10 High dose of UV light stabilizes IκBα mRNA slightly and is p38 MAPK independent. HeLa cells were treated with 2ng/ml IL-1α for 2 hours. After one and half hours of IL-1 incubation 2µM of SB203580 was added to the cells for an incubation time of 30 following with the exposure to UV light. Transcription was stopped by addition of 5µg/ml Act-D directly after UV irradiation. The cells were harvested at the indicated times thereafter. Total RNA is analyzed by RT-qPCR quantification using a TaqMan IκBα probe with normalization to GAPDH mRNA.

4.2.2.4 JUN mRNA

To check the changes in mRNA stabilization by UV light for multiple mRNAs a high density microarray analysis was carried out (see chapter 3.2.19). The effect of UV light on stabilization of mRNAs from HeLa cells was determined by comparing the differences between the transcriptomes with and without exposure to UV-B in the presence of act-D.
JUN mRNA was one candidate since it belongs to the ARE class III (Chen & Shuy, 1995), which showed increase in mRNA stability after irradiation.

HeLa cells						
Accesion no.	Gene name	+ IL-1 0	+ IL-1 3h act.D	+ IL-1 + UV 0	+ IL-1 + UV 3h act.D	Fold stabilization
NM_002228	JUN	**909**	**61**	**1530**	**489**	4.7
NM_002228	JUN	**939**	**69**	**1490**	**542**	4.9
NM_002228	JUN	**939**	**62**	**1573**	**472**	4.5
NM_002228	JUN	**950**	**58**	**1544**	**465**	4.9
NM_002228	JUN	**974**	**125**	**1532**	**502**	2.5
NM_002228	JUN	**1004**	**134**	**1546**	**536**	2.6
NM_002228	JUN	**1075**	**48**	**1940**	**520**	6.0
NM_002228	JUN	**1085**	**56**	**1466**	**499**	6.6
NM_002228	JUN	**957**	**65**	**1546**	**487**	4.6
NM_002228	JUN	**888**	**73**	**1521**	**503**	4.0

Table 4: Microarray analysis of UV-induced inhibition of JUN mRNA degradation in HeLa tTA cells. Cells were stimulated with 2ng/ml of IL-1 for 2h, and then exposed to high dose of UV-B (ca. 2000J/m^2). Transcription was stopped by the addition of 5µg/ml Act-D. Lysates were made before (0) and 3 h after Act-D addition and total RNA was prepared for microarray analysis. To provide a measure of technical precision in each hybridization one probe is usually spotted as replicates on the microarray slide (here in 10 different spots). The data for JUN mRNA is presented and the fold stabilization by UV exposure is shown.

In a separate experiment a similar microarray analysis was carried out using primary epidermal keratinocytes in order to check the effect of UV light on mRNAs before and after stopping the transcription with Act-D. The results for JUN mRNA are shown in table 5.

Epidermal Keratinocytes						
Accesion no.	Gene name	0	3h act.D	+ UV 0	+ UV 3h act.D	Fold stabilization
NM_002228	JUN	**19859**	**1156**	**17803**	**1873**	1.8
NM_002228	JUN	**19314**	**1079**	**18555**	**2276**	2.2
NM_002228	JUN	**17823**	**1063**	**14076**	**1904**	2.3
NM_002228	JUN	**20429**	**1177**	**17402**	**2125**	2.1
NM_002228	JUN	**17577**	**1119**	**18002**	**2209**	1.9
NM_002228	JUN	**18702**	**1113**	**17156**	**2198**	2.1
NM_002228	JUN	**19369**	**1170**	**17711**	**2149**	2.0
NM_002228	JUN	**18799**	**1149**	**17794**	**2199**	2.0
NM_002228	JUN	**19168**	**1204**	**17864**	**2126**	1.9
NM_002228	JUN	**18546**	**1050**	**17089**	**2184**	2.3

Table 5: Microarray analysis of UV-induced stabilization of JUN mRNA in primary keratinocytes. Primary keratinocytes were grown to 70-80% confluence with the serum free keratinocytes Growth Medium in 6-cm petri dishes. The cells were exposed to 160J/m^2 dose UV-B. Transcription was stopped by the addition of Act-D and lysates were prepared as described in the legend to table 4.

Comparing the result of HeLa cells (table 4) with those from keratinocytes (table 5) revealed a higher fold stabilization of JUN mRNA by UV-B light in HeLa cells than in primary keratinocytes, which is probably because the keratinocytes were exposed to lower doses of UV-B light(160J/m^2).

In a subsequent experiment HeLa cells treated with or without SB203580 were exposed to the high and low dose of UV-B light followed by addition of Act-D. Later the isolated total RNA was analyzed by RT-qPCR with a TaqMan probe for JUN mRNA. As it is shown in Fig 4.7 both low and high doses of UV light tend to cause an inhibition of JUN mRNA degradation with the difference that in high dose UV (1000 J/m^2) this effect is prolonged. Investigating the role of p38 MAPK in UV-mediated stabilization of JUN mRNA we could see that SB203580 blocks the stabilization of JUN mRNA induced with low dose of UV-B, which shows that this stabilization is p38 MAPK dependent, whereas it has no distinctive effect on the mRNA stabilization induced with high dose of UV light, which argues against a role of p38 MAPK.

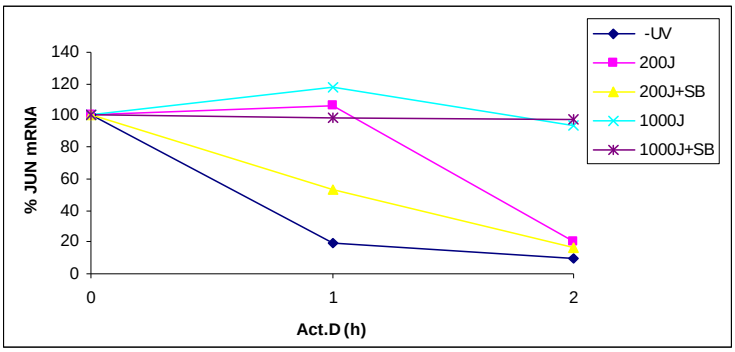


Fig.11 Stabilization of JUN mRNA in response to low and high dose of UV light and its dependence on p38 MAPK. HeLa cells stimulated with 2ng/ml IL-1α for 2h were treated with SB203580 (2μM) 30min before exposure to 200 and 1000 J/m^2 UV-B. Transcription was stopped by addition of 5μg/ml Act-D. Total RNA was analyzed by RT-qPCR quantification using a TaqMan probe for JUN RNA. The values were normalized to GAPDH mRNA.

4.3 Investigation of mRNA stabilization by UV-B light in Keratinocytes

Epidermal keratinocytes are the cell types in our body, which are maximally exposed to UV radiation. So far we detected the effect of UV-B light for the short-lived mRNAs in HeLa (tTA) cells. In the next part we wanted to analyse the influence of low and high dose of UV-B light in the epidermal keratinocytes isolated from neonatal foreskin for the three mRNAs IL-8, IκBζ and IκBα on the stability level. To determine involvement of p38 MAP kinase in parallel, SB203580 inhibitor was applied to the cells prior to UV exposure and the total RNA was isolated and analyzed by the Reverse transcription and quantitative PCR using either a TaqMan or a SYBR Green based probe.

4.3.1 IL8 mRNA

As it can be observed in Fig.12, low and high dose of UV light lead to an inhibition of IL-8 mRNA degradation in keratinocytes, which is comparable to HeLa cells (Fig.7). Blocking the p38 MAP kinase pathway with SB203580 did not impair the stabilizing effect of both low and high dose of UV radiations. However, the mRNA stability is already high at the basal level (Fig.12A, –UV) which is blocked by SB203580 (Fig.12B, –UV). This suggests the speculation that the keratinocytes are in a pre-activated condition. Exposure to UV-light leads to further stabilization, which is not blocked by SB203580.

A. **B.**

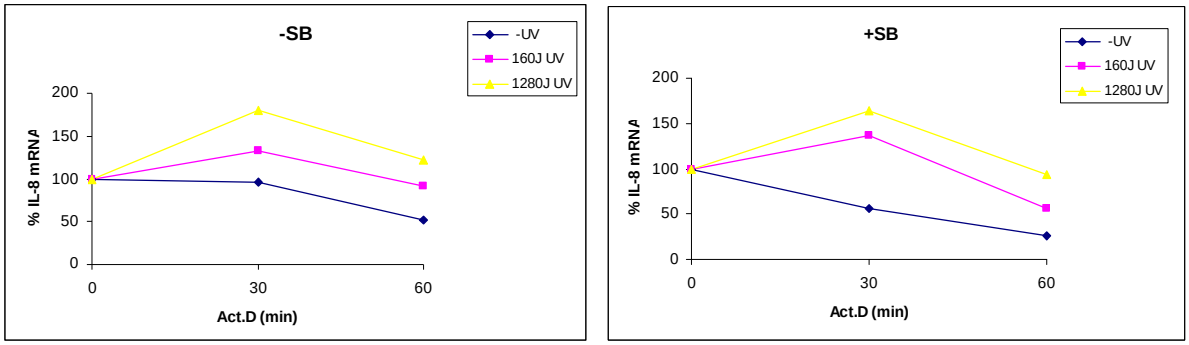


Fig.12 Stabilization of IL-8 mRNA by UV light in keratinocytes independent of p38 MAPK activity. Primary keratinocytes were grown to 70-80% confluence with the serum free keratinocytes Growth Medium in 6-cm petri dishes. After addition of 2µM of SB203580 to the cells (B), they were incubated for 30 min and then exposed to low dose (160 J/m^2) and high dose (1280 J/m^2) of UV-B. Transcription was stopped by adding Act-D (5µg/ml). At different time points thereafter, total RNA was isolated and analyzed by RT-qPCR for the amount of endogenous IL-8 mRNA using an IL-8 TaqMan probe. The values were normalized to GAPDH mRNA.

4.3.2 IκBζ mRNA

Next we investigated UV induced stabilization of IκBζ mRNA in keratinocytes. Primary keratinocytes were treated with SB203580 and then exposed to the indicated low and high doses of UV-B radiation. The total RNA was isolated and analyzed by RT-qPCR for endogenous IκBζ mRNA. As one can observe in Fig.13 there is a slight stabilization effect at 160 J/m^2. This effect is stronger at 1280 J/m^2. Neither basal nor UV induced mRNA stability was significantly dependent on p38 MAPK as shown by the p38 inhibitor.

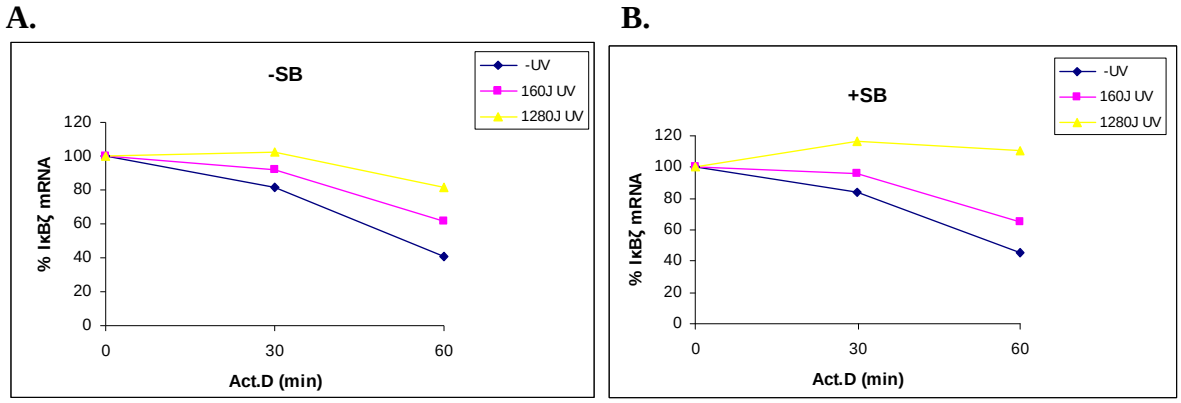


Fig.13 Stabilization of IκBζ mRNA by high dose of UV light in keratinocytes. The experiment performed as described in the legend to Fig.12. Total RNA was analyzed by SybrGreen based RT-qPCR for endogenous IκBζ mRNA using sense and antisense IκBζ primers. The values were normalized to GAPDH mRNA.

4.3.3 IκBα mRNA

To compare the UV-induced IκBα mRNA stabilization in HeLa cells with that of primary keratinocytes, we exposed keratinocytes to low and high doses of UV-B radiations. To study the role of p38 MAPK, the cells were incubated with SB203580 for 30 min before UV exposure. Low dose of UV light did not stabilize IκBα mRNA, whereas a minor stabilizing effect could be seen in the case of high dose of UV (Fig.14). This corresponds to the result observed in HeLa cells (Fig.10). Since the mRNA stabilization by high dose UV was low, we can not make a clear statement about the effect of SB203580 and the role of p38 MAPK pathway.

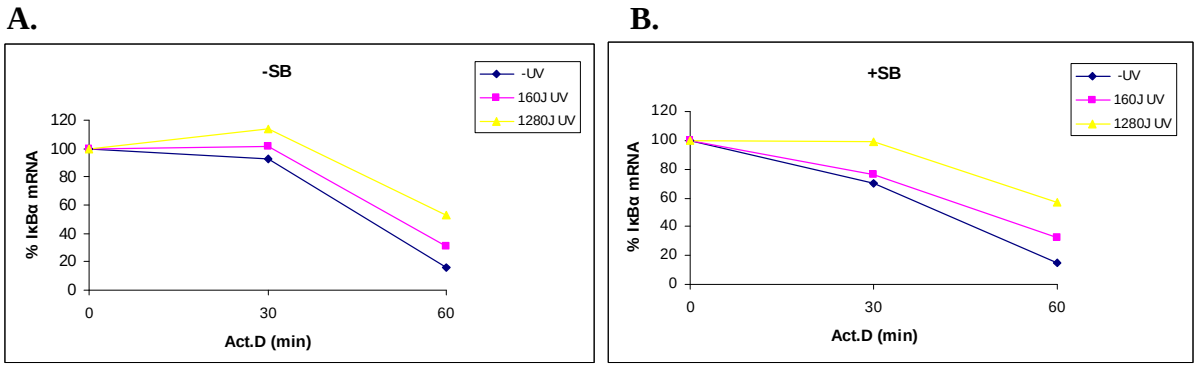


Fig.14 Minor stabilizing effect of UV light on IκBα mRNA in keratinocytes. The experiment was performed as described in the legend to Fig.12. Total RNA was analyzed by RT-qPCR for endogenous IκBα mRNA using a TaqMan probe. The values were normalized to GAPDH mRNA.

4.3.4 Microarray analysis of UV-induced mRNA stabilization

The effect of UV light on the stability of different mRNAs in keratinocytes was carried out by microarray analysis. The microarray data and the fold stabilization after UV exposure (low dose) for the selected mRNAs, IL-8, IκBζ and IκBα, are listed in Table 6. The profile obtained overlaps with those obtained from RT-q PCR experiments.

Epidermal Keratinocytes						
Accesion no.	Gene name	0	3h act.D	+ UV 0	+ UV 3h act.D	Fold stabilization
NM_000584	IL-8	**1701**	**676**	**1269**	**669**	1.3
NM_031419	NFKBZ	**13279**	**3745**	**9808**	**1920**	0.7
NM_020529	NFKBA	**83329**	**1137**	**76337**	**1189**	1.1

Table 6: Microarray analysis of UV effect on the three indicated mRNAs. Primary keratinocytes were grown to 70-80% confluence with the serum free keratinocytes growth medium in 6-cm petri dishes. The cells were exposed to 160J/m^2 UV-B. Transcription was stopped using 5µg/ml Act-D. Lysates were made before (0) and 3 h after Act-D addition and total RNA was prepared for microarray analysis.

4.4 Activation of p38 MAPK by UV-B light

Various cellular stresses such as ultraviolet light, lipopolysaccharides (LPS) and pro-inflammatory cytokines can initiate signalling cascades resulting in the activation of p38 MAPK by dual tyrosine/threonine phosphorylation. As a parameter for p38 MAPK activation we investigated the effect of different UV-doses on the phosphorylation of p38 MAPK (p-p38 MAPK) by western blot analysis (see chapter 3.3.4) with the help of a polyclonal antibody for phospho-p38 MAPK (Thr180/Tyr182).

In both the cell types we could detect activation of p-p38 MAPK by 160J/m^2. In HeLa cells the intensity of p-p38 MAPK bands increases in a UV dose-dependent manner, whereas this effect remains constant in the kerationocytes.

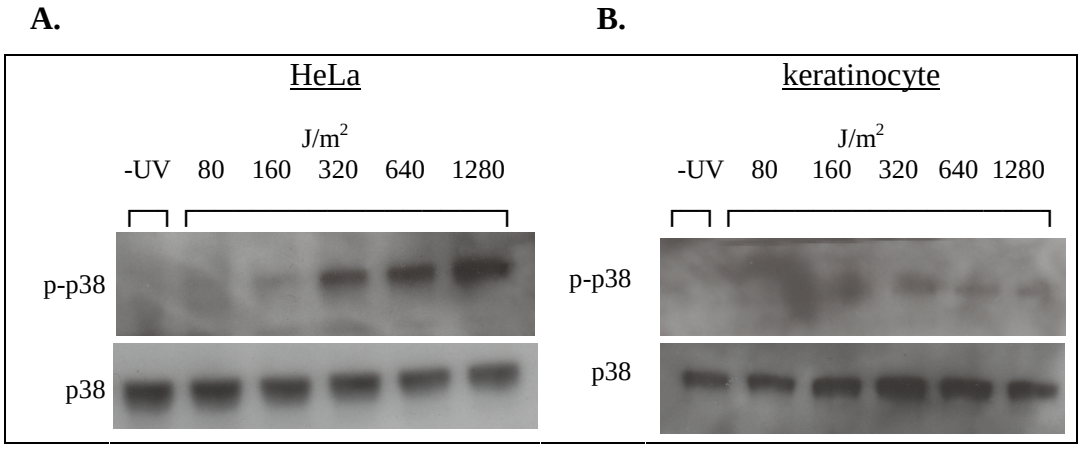


Fig.15 Western blot analysis for the detection of p38 and phospho-p38 MAP kinase. HeLa cells (A) and keratinocytes (B) were exposed to the indicated doses of UV light. After 30 min of incubation the cells were lysed and a western blot analysis was performed as described in section 3.3.4. Phospho-p38 MAPK could be detected by phospho-p38 MAPK (Thr180/Tyr182) antibody (upper panel). p38 MAP kinase was detected using a p38 MAP kinase antibody as loading control (lower panel).

4.5 Role of HuR in stabilization of IL-8 mRNA in HeLa cells

HuR is a ubiquitously expressed member of embryonically lethal abnormal vision (ELAV) family of RNA binding proteins (Ma et al., 1996). It is a 36-kDa RNA binding protein, which has been observed to bind to a large number of mRNAs with AU rich elements and stabilize them (Fan and Steitz, 1998, Peng et al., 1998, Levy et al., 1998). It was shown that lowering of endogenous HuR levels through expression of antisense RNA or small interfering RNA (siRNA) inhibited stabilization induced by UVC light (Wang et al., 2000). The interaction of IL-8 ARE with HuR was described before from our group (Winzen et al., 2004). Here we studied the effect of HuR on the stability of IL-8 mRNA by siRNA knock-down experiments and influence of UV-B light as a stress factor in HuR depleted cells.

4.5.1 Effect of siRNA against HuR on basal IL-8 mRNA degradation

To investigate the impact of HuR on the stability of IL-8 mRNA, we knocked down HuR in HeLa cells with two siRNAs ($siHuR_{278}$ and $siHuR_{649}$), which were chosen from two different regions of HuR mRNA. Knock down efficiency of HuR protein is presented in Fig.16A. Both siRNAs suppress the expression of HuR protein; however this suppression was more efficient with $siHuR_{649}$.

Knock down of HuR with $siHuR_{278}$ appears to have no effect on the stability of IL-8 mRNA, since in both control (siGFP) and knocked-down cells IL-8 mRNA degrades with a half life of 30 min. The cells knocked-down with $siHuR_{649}$ showed increased stability of IL-8 mRNA (Fig.16B). Both results argue against a stabilizing role of HuR in this setting.

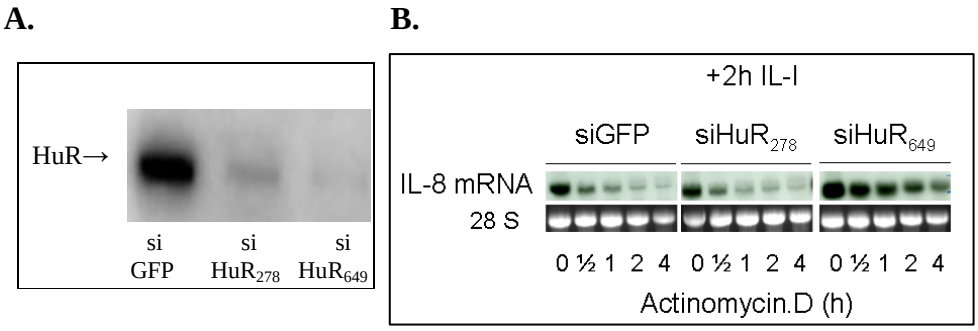


Fig.16 Effect of HuR knock down on the degradation of IL-8 mRNA. HeLa cells were transfected with two siRNAs directed against HuR (siHuR$_{278}$ and siHuR$_{649}$) and with a siRNA directed against GFP as the negative control (see chapter 3.1.5, siRNA transfection). (A) Western blot with antibody recognising HuR (1:2000) to control knock down efficiency. (B) Endogenous IL-8 mRNA was induced by incubating the cells for 2 h with IL-1α (2ng/ml). mRNA half-life was determined after stopping the transcription with Act-D (5µg/ml) at the indicated times. Northern blots were hybridized to an IL-8 antisense probe. 28S rRNA is shown to allow comparison of RNA amounts loaded.

4.5.2 Effect of siRNA against HuR on mRNA stabilization

In this assay we studied effect of HuR knock down on the stability of endogenous IL-8 mRNA treated with UV-B and IL-1α. The cells were stimulated with IL-1 for 40 min or 2h and then exposed to 200J/m^2 of UV-B light. HuR knock down was accomplished as shown in Fig.17A & C for siHuR$_{278}$ and siHuR$_{649}$ respectively.

Here HuR knock down with both siRNA sequences (siHuR$_{278}$ and siHuR$_{649}$) did not affect the stability of IL-8 mRNA mediated by UV light, since the UV-induced stabilization for siHuR transcripts remained similar to the control siGFP (Fig.17 B & D right panels). We can conclude that here HuR does not play any significant role in UV-induced stabilization of IL-8 mRNA.

Regarding IL-1-mediated stabilization, as it can be observed in Fig.17 B & D, this effect is transient. Stabilization decreases when the cells are stimulated longer with IL-1α. After 2 hours of IL-1 incubation we can see a more rapid degradation of IL-8 mRNA than after 40 min incubation, which is another evidence for the short half life of IL-8 mRNA. This rapid degradation of IL-8 mRNA was decreased by knock down with siHuR$_{649}$ (Fig.17.D, IL-1 2h).

The apparent increase of IL-8 mRNA amount in Fig.17.D/siGFP for the last three Act-D time points (IL-1 40 min) is only due to the differences in RNA loading amount in agarose gel, when compared to its 28S rRNA loading control.

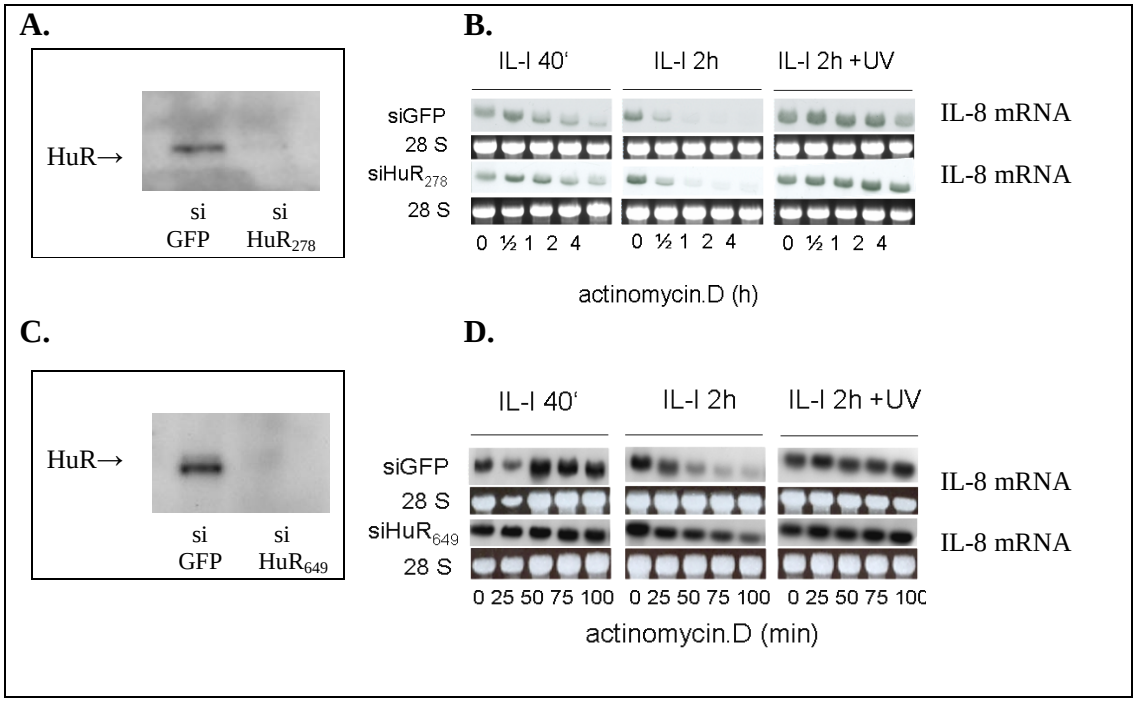


Fig.17 Effect of HuR knock down on stabilization of IL-8 mRNA in response to IL-1 or UV light. HeLa cells were transfected with siRNAs specific for HuR, $siHuR_{278}$ (B) and $siHuR_{649}$ (D), or GFP. Endogenous IL-8 mRNA was induced by incubating the cells for 40 min or 2 h with IL-1α (2ng/ml). 200J/m^2 of UV-B light was exposed to the cells after 2h of IL-1 induction. Transcription was stopped by addition of Act-D. The mRNA half-life was determined with Northern blot assay for IL-8 mRNA. 28S rRNA is shown to allow comparison of RNA amounts loaded. (A and C) Western blot analysis with anti-HuR antibody to control the knock down efficiency.

4.6 Control of mRNA Translation by IL-1

Translation of mRNA into protein represents the final step in the gene-expression pathway. Molecular mechanisms of translational control act either in a global manner, in which the translation of most mRNAs in the cell is regulated or it is mRNA-specific, whereby the translation of a defined group of mRNAs is modulated without affecting general protein biosynthesis (Gebauer et al., 2004). Among all the cytokine families, the IL-1 family is closely associated with inflammatory and immune responses (Dinarello, 2009). In our group the effect of IL-1α on the mRNA stability is well studied. Since translation and degradation are known to be linked (Hosoda et al., 2003; Kobayashi et al., 2004), we intended to investigate the effect of IL-1α at the translational level, which is very important in the post-transcriptional gene regulation.

Polysome fractionation is the classical technique employed to study the translational state of mRNAs in cells. Ribosomes are high density ribo-nucleoprotein complexes, which further assembles as polysomes or poly-ribosomes on an actively translated mRNA. The poly and mono- ribosomes are separated into fractions based on their difference in density by subjecting to sucrose - density gradient centrifugation. These fractions are analyzed by blotting or PCR for specific mRNAs to look for their translational state in the cell. Translational state analysis with an oligonucleotide microarray that covers ca. 31000 human genes (carried out in the lab in cooperation with O. Dittrich-Breiholz) indicated an increase in polysome association of a group of mRNAs from HeLa cells after stimulation with IL-1α (data not shown, Dhamija et al., 2010). Polysome association or active translation state was calculated as the ratio of signals obtained by microarray analysis of total RNA from "translated" over "untranslated" pools of fractions after density gradient centrifugation of cytoplasmic extracts (Fig.18).

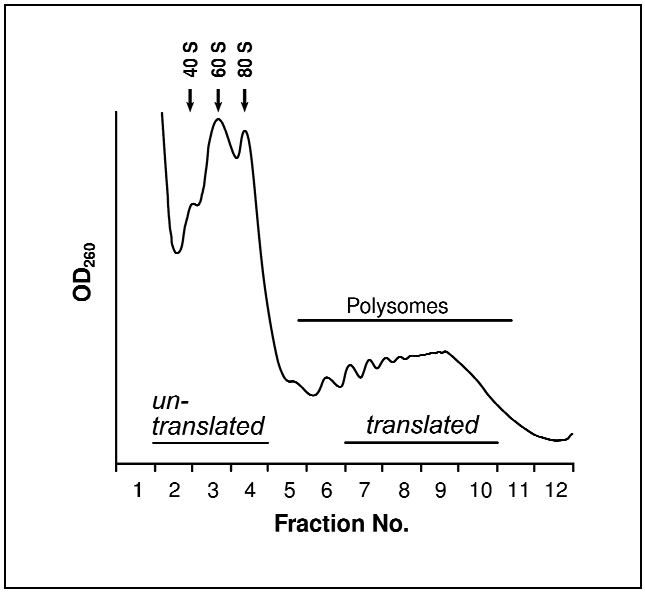


Fig.18 Sucrose gradient fractions and polysomes distribution. To prepare polysome gradient cytoplasmic extracts of HeLa cells were fractioned on sucrose gradients. The optical density of the fractions was read at 260 nm. Fractions 2 to 4 contain free ribosomes and untranslated mRNAs, whereas fractions 7 to 10 contain polysomes with mRNAs (From Dhamija et al., 2010).

4.6.1 IL-1 increases polysome association of IκBζ mRNA

Next we investigated in detail the translational regulation of different mRNAs which are induced and stabilized by IL-1.

On the basis of microarray experiment mentioned above we analyzed the distribution of IκBζ mRNA for individual fractions by reverse transcription and quantitative PCR (density gradient fractionation carried out by Anneke Doerrie). We could see a distinctive shift from untranslated to translated fractions after IL-1stimulation for IκBζ mRNA, which indicated that IL-1α induced an increase in translation of IκBζ mRNA (B). GAPDH was analyzed as a housekeeping mRNA control and showed a slight shift to non-translated fractions. This was observed in some but not all subsequent assays (A). In the case of IL-8 mRNA (C) and IκBα mRNA (D) there was no shift in the polysome gradient fractions, even though IL-8 mRNA is well known to be stabilized by IL-1 stimulation (Winzen et al., 1999, Winzen et al., 2004) and IκBα mRNA is usually rapidly induced by IL-1. This indicates that IL-1 induced stabilization does not necessarily lead to a translational activation.

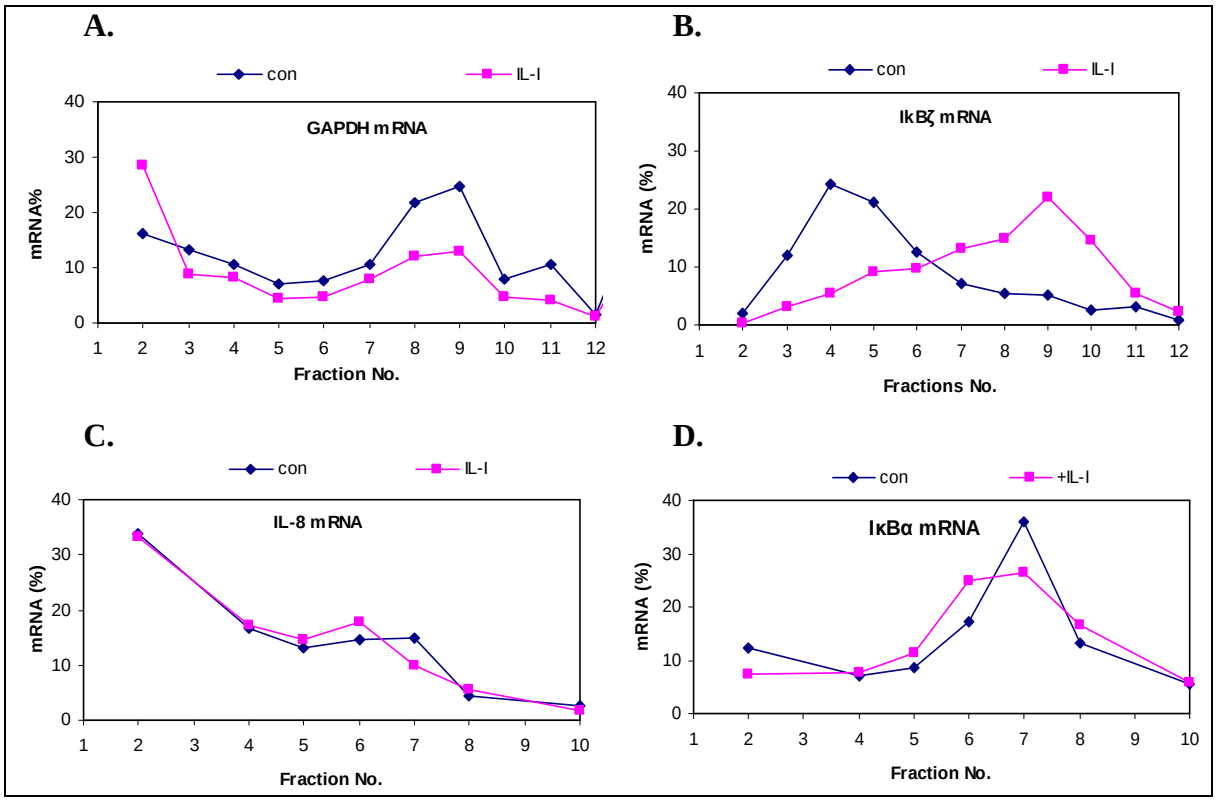


Fig.19 Increased polysome association of IκBζ mRNA after stimulation with IL-1. Cytoplasmic lysates from unstimulated or IL-1α stimulated HeLa cells (2ng/ml, 1 hour) were fractioned in density gradients and polysome profiles of the indicated endogenous mRNAs was obtained by RT-qPCR analysis.

4.6.2 IκBζ 3' UTR contains translational silencing activity

For further studies, a luciferase reporter assay system was used to confirm the regulation of translation by IL-1. Since a shift in the distribution after IL-1 treatment was also observed with a reporter mRNA that contained only the 3' UTR of IκBζ (Dhamija et al., 2010), the IκBζ 3' UTR was cloned 3' of the firefly luciferase coding region in pMir-Report.

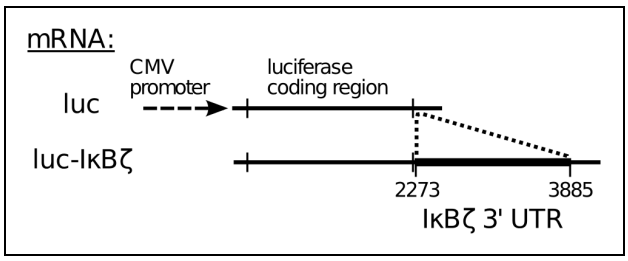


Fig.20 Luc-IκBζ construct. 3' UTR of IκBζ mRNA (nt 2273-3885) was inserted 3' of the firefly luciferase coding region in a plasmid expressing luciferase under control of the CMV promoter (From Dhamija et al., 2010).

To obtain information about the translational activity, the luciferase mRNA amount is measured by reverse transcription and quantitative PCR and the mRNA units were calculated as described in section 3.2.18. The luciferase protein amount is determined by measuring the firefly luciferase activity as described in section 3.3.6. Dividing the luciferase activity by RNA units gives the luciferase protein/mRNA ratio.

Next we checked the luciferase activity and its mRNA amount in HeLa cells transfected with the vector lacking or containing the 3' UTR of IκBζ mRNA. The cells were stimulated with IL-1α or left untreated. They were then lysed and firefly luciferase activity and the amount of mRNA were determined as mentioned above.

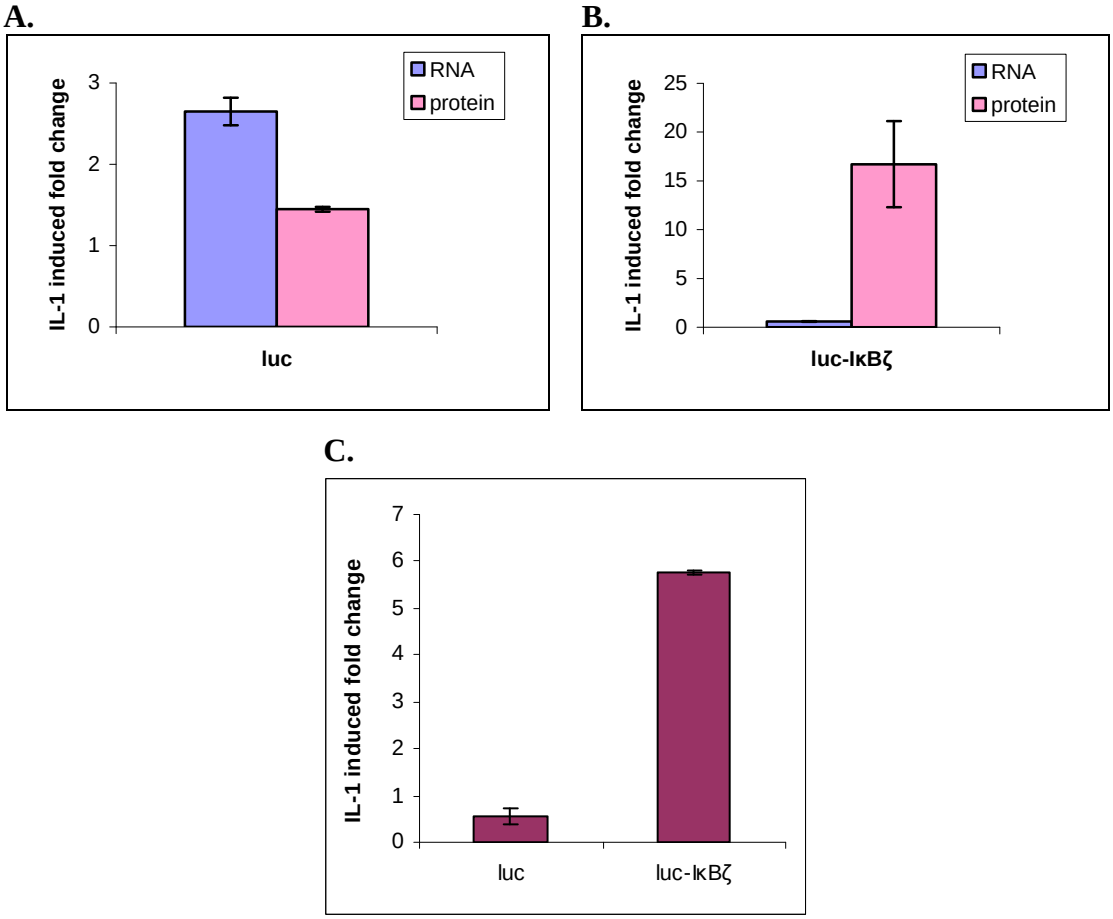


Fig.21 IL-1 induced fold increase of luciferase mRNA & protein. HeLa cells were transfected with empty luciferase expression vector or luciferase expression vector containing 3' UTR of IκBζ mRNA. The cells were stimulated with IL-1α, incubated for 4 hours and lysed thereafter. The firefly luciferase activity was determined as explained in section 3.3.6. The mRNA amount was analyzed by RT-q PCR using SYBR Green based sense and antisense luciferase primers. The expression of each gene was analyzed in duplicates. Values with standard deviations > 0.2−0.25 cycles were excluded from the analysis. The values were normalized to endogenous GAPDH mRNA and the mRNA units were calculated as described in section 3.2.18. In response to IL-1 treatment, A & B show the fold change in mRNA and protein level, whereas C shows the fold change in the translational activation of luciferase reporter constructs.

Transcripts containing 3' UTR of IκBζ (Fig.21 B) showed a strong induction in the luciferase protein expression, whereas there was less change seen in the mRNA value (16.7 versus 0.6 fold). In the case of the transcripts containing the empty luciferase expression vector (Fig.21 A) there was approximately 2 fold induction of RNA but there was hardly any induction at the protein level (2.6 versus 1.4 fold). Comparing the general translational activation induced by IL-1, we see a fold change of 10.5 in the luciferase activity/RNA of transcripts containing the 3' UTR of IκBζ mRNA (Fig.21 C).

We can conclude from these results that IκBζ 3' UTR contains translational silencing activity and its function is affected by IL-1 treatment. This translational activation can occur even in the absence of reporter mRNA stabilization.

4.6.3 IL-1 does not affect translation of a luciferase reporter construct containing IL-8 ARE

IL-8 is a well known target of IL-1-induced stabilization. But in contrast to IκBζ we could not see an IL-1-induced shift for IL-8 mRNA in the polysome gradient fractions (Fig.19C), which suggested absence of translational activation. To observe this interplay between stabilization and the translational activation we investigated the effect of IL-1 on the translation of luciferase mRNA containing IκBζ 3' UTR (luc-IκBζ), ARE of IL-8 mRNA (luc-IL-8 ARE) or only the reporter (luc). The transfected HeLa cells were stimulated with IL-1α for 4 hours or left untreated.

As described earlier, the AU rich elements are able to repress the translational activity (see section 1.4). Here in Fig.22A, we observe that the level of translational activation in the luciferase reporter construct containing ARE of IL-8 mRNA (luc-IL-8 ARE) is much lower compare to that of the empty luciferase reporter (luc). This indicates a translational silencing executed by the IL-8 ARE, which - unlike that of IκBζ translational silencing element - is not influenced by IL-1. Since in our previous experiments we have observed IL-8 and IκBζ mRNA stabilization induced by UV light, it was interesting to observe the UV influence on the translation of these reporter mRNAs as well. Therefore, the cells were additionally exposed to low and high dose of UV-B and the changes in the luciferase mRNA and protein level was monitored.

In Fig.22B Stimulating the cells with IL-1 induced a fold increase of 5.8 in the luciferase activity of the reporter mRNA containing luc-IκBζ 3' UTR, whereas there was no increase in the luciferase activity with the reporter construct containing the IL-8 ARE or no insertion. Treating the cells with UV-B in turn reduced the translation of these reporter mRNA, which is presumably due to the stress caused by UV-B.

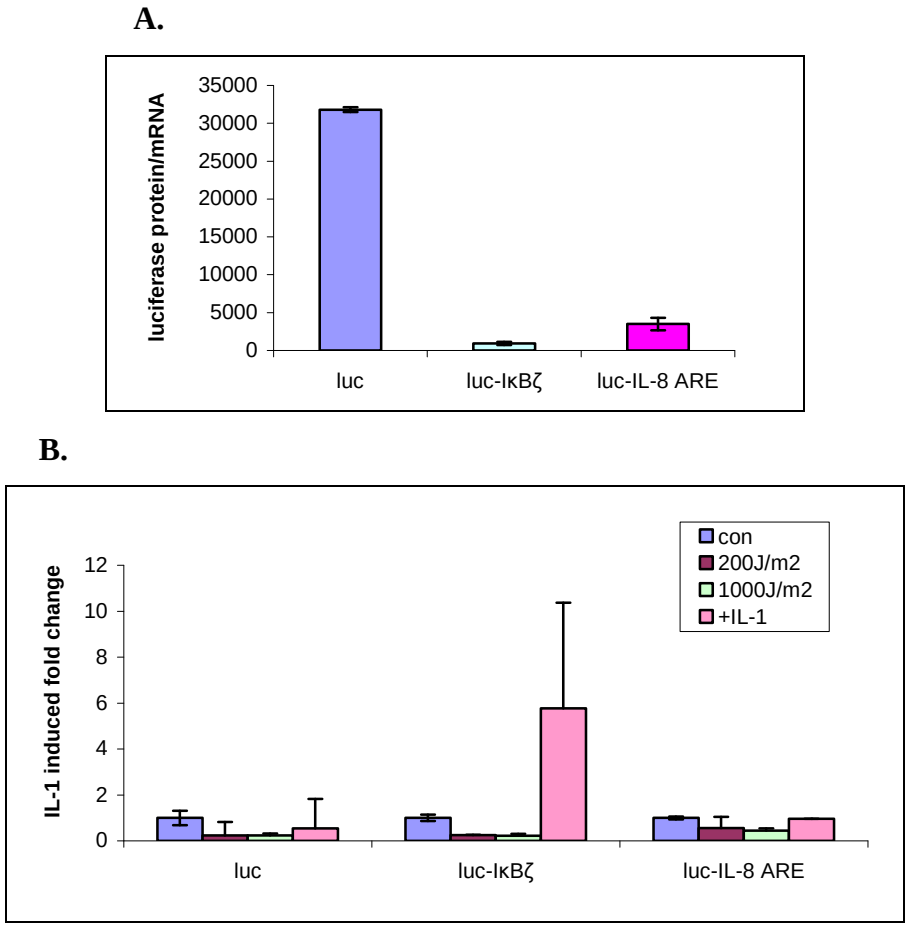


Fig.22 Lack of translational activation of reporter mRNA containing luc-IL-8 ARE by IL-1. HeLa cells were transfected with pMir-luc; pMir-IκBζ$_{2273-3885}$ and pMir-IL-8 ARE separately. Stimulation with IL-1α followed on the next day. The cells were lysed after 4 hours later. The firefly luciferase activity and the luciferase mRNA amount were analyzed as described in the legend to Fig.21. The luciferase protein activity/mRNA is shown as fold change in Fig.B and the ratios in unstimulated cells were set as 1.

4.6.4 Role of KSRP in the translational regulation of IκBζ 3' UTR mRNA induced by IL-1

KSRP is an ARE-binding protein, which promotes ARE-directed mRNA turnover by recruiting the degradation machinery (Gherzi et al., 2004). p38 MAP kinase activation results in phosphorylation of KSRP, which in turn attenuates its mRNA destabilization function (Briata et al., 2005). With the help of microarray analysis done by our group, it was shown that KSRP also interacts with non-ARE mRNA (Winzen et al., 2007).

In order to analyse the effect of KSRP in the translational regulation of IκBζ mRNA, which lacks an AU rich element, we knocked down KSRP in HeLa cells with siRNA and co-transfected them with the luc-IκBζ 3' UTR reporter plasmid. Firefly luciferase activity (protein amount) and the mRNA amount were determined as described before (see 4.6.2). In

response to IL-1 the luciferase activity/mRNA ratio was increased 3.2 fold after KSRP knock-down as compared to 1.4 fold in the control knock-down. A similar experiment could be reproduced by J. Klöhn in our group (not shown). However it should be noticed that IL-1 induction in the control knock-down was low. Therefore to make an exact statement more experiments should be carried out to reproduce this result with higher IL-1 induction.

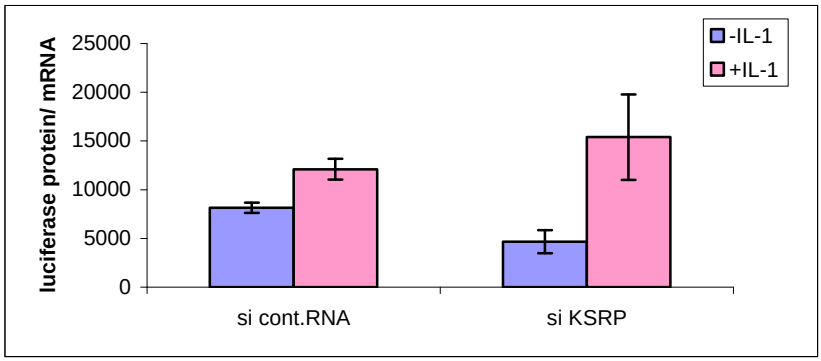


Fig.23 Increased translational activation of luc-IκBζ 3' UTR mRNA induced by IL-1 in KSRP knock-down cells. HeLa cells were transfected with siKSRP$_{479}$ and co-transfected with pMir luc-IκBζ 3' UTR (see chapter 3.1.5 siRNA transfection). The cells were stimulated with IL-1α on the next day and lysed after 4 hours. The firefly luciferase activity and the luciferase mRNA amount were analyzed as described in the legend to Fig.21.

4.6.5 Effect of other pro-inflammatory cytokines on the translation regulation of IκBζ 3' UTR mRNA

Next we applied two other pro-inflammatory cytokines, IL-17 (IL-17A) and TNF-α, in addition to IL-1α, to examine their effect on the IκBζ 3' UTR-dependent translational induction. The results from two independent experiments are shown in Fig.24. HeLa cells transfected with luciferase expression vector containing the 3' UTR of IκBζ, showed an increase in the luciferase protein value induced by IL-17. In contrast TNF-α could not induce any increase in the translational level of this reporter.

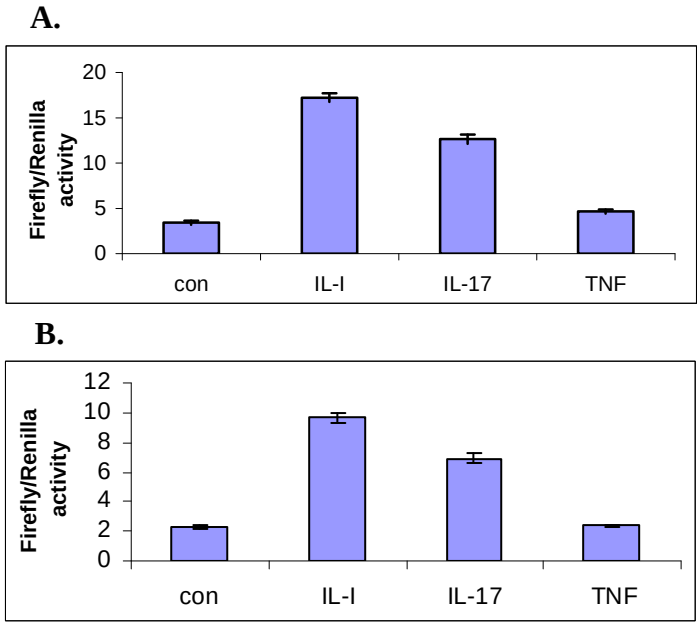


Fig.24 Effect of IL-17 and TNF-α on translation of mRNA containing IκBζ 3' UTR. HeLa cells transfected with luciferase expression vector containing 3' UTR of IκBζ were stimulated with IL-1α (2ng/ml), human IL-17 (25ng/ml) or TNF-α (100ng/ml) separately. After 4 hours of incubation the cells were lysed. Firefly luciferase activity was analyzed and normalized to co-expressed *Renilla* luciferase activity. A & B are two independent experiments error bars show the standard deviations.

In a subsequent experiment we stimulated HeLa cells transfected with luc-IκBζ 3' UTR only with IL-1α and IL-17 separately to analyze the kinetics of translational activation for these two pro-inflammatory cytokines for different time periods. As it is shown in Fig.25 IL-17, like IL-1 increased the translational activation of luc-IκBζ, also till 6 hours after addition of the stimulant. Only after 6 hours this increase diminished, whereas with IL-1 this effect was prolonged till 8 hours of incubation.

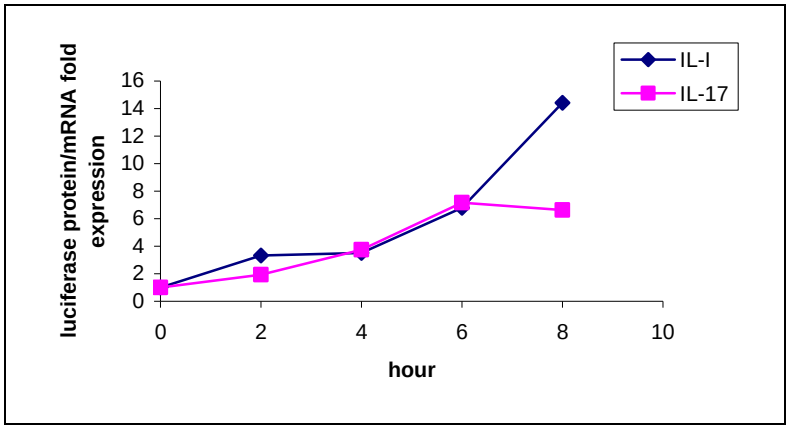


Fig.25 Increased translation of luc-IκBζ 3' UTR mRNA with IL-1 and IL-17 for different time periods. HeLa cells transfected with luciferase expression vector containing the 3' UTR of IκBζ were stimulated with IL-1α (2ng/ml) or human IL-17 (25ng/ml). Cells were lysed after the indicated times and firefly luciferase activity and luciferase mRNA amount were analyzed as described in the legend to Fig.21.

4.6.6 Role of p38 MAPK pathway in the translational activation of mRNA containing IκBζ 3' UTR induced by IL-1

Work from our group showed that IL-1 can stabilize mRNAs through activation of p38 MAPK/MK2 signalling cascade (Winzen et al., 1999). To study the role of p38 MAPK/MK2 pathway in the translational activation of IκBζ 3' UTR induced by IL-1, we co-transfected HeLa cells without (con) or with plasmids for dominant-negative p38 MAP kinase, or for $MKK6_{2E}$. The dominant-negative mutant of p38 MAPK ($p38_{AGF}$) can reverse the IL-1 induced stabilization of mRNAs (Winzen et al., 1999) and $MKK6_{2E}$ is a constitutively active mutant of the p38 MAP kinase-selective upstream kinase MKK6 and stabilizes IL-8 mRNA (Winzen et al., 1999, Winzen et al., 2004). The cells without or with dominant-negative p38 MAP kinase were stimulated with IL-1, whereas the transfected cells with $MKK6_{2E}$ left untreated. The cells were lysed after 4 hours.

An increase in the luciferase activity over mRNA ratio induced by IL-1 (IL-1/con) is observed (ratio: 3.6). dn-p38 MAPK did not change the IL-1 induced effect clearly. In response to IL-1only a slight decrease was seen in the transcripts containing the dn-p38 MAPK (0.7 fold). Similarly active MKK6 did not cause any change in the translational activity of the reporter-IκBζ 3' UTR mRNA. From these observations we can conclude that the 3' UTR dependent translational effect of IL-1 on IκBζ mRNA is independent of the p38 MAP kinase pathway.

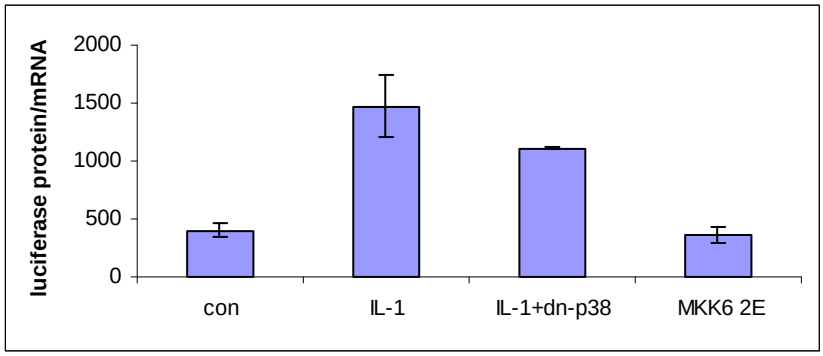


Fig.26 Lack of p38 MAP kinase involvement in IL-1-induced translational activation of IκBζ 3' UTR reporter. HeLa cells were all transfected with luc-IκBζ 3' UTR. Some were additionally co-transfected with pCMV flag dn-p38 or with pCDNA3-MKK6$_{2E}$. On the next day, cells treated without or with IL-1α for 4 hours were lysed. The firefly luciferase activity and its mRNA amount were analyzed as described in the legend to Fig.21.

4.6.7 Role of PI3 kinases in the translational activation of IκBζ 3' UTR mRNA induced by IL-17

Phosphoinositide 3-kinases (PI3K) have been reported to lead to stabilization of mRNAs (Gherzi et al., 2006). Wortmannin is a broad spectrum inhibitor of the PI3 kinases. In this experiment we applied two different concentration of wortmanin to HeLa cells.

Blocking the PI3 kinases by wortmannin did not inhibit the IL-1-induced translational activation of luciferase expression vector containing IκBζ 3' UTR. As it is shown in Fig.27, there is still an increase in the luciferase activity for mRNAs, which were stimulated with IL-1. In the case of the cells containing empty reporter there was no effect of wortmannin in the level of luciferase protein/mRNA (data not shown). Since wortmannin is toxic under higher concentration and there are small changes observed in the luciferase values with low concentration wortmannin, more experiments are needed to make an exact statement about the role of PI3 kinases.

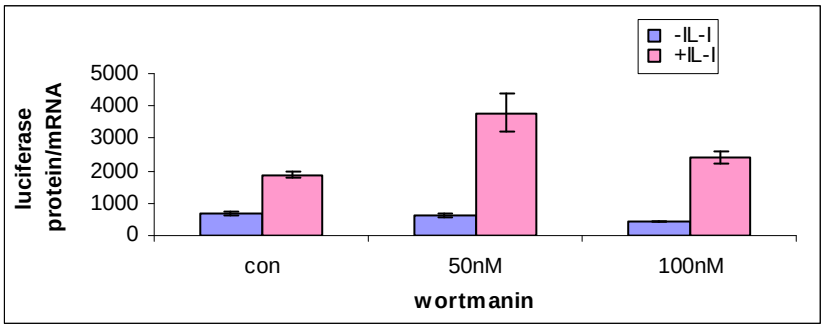


Fig.27 Effect of blocking PI3 kinases on the translation of luc-IκBζ 3' UTR mRNA induced by IL-1. HeLa cells transfected with luc-IκBζ 3' UTR were left untreated or were treated with 50nM and 100nM of wortmannin for 30 min. The cells were then stimulated with IL-1 or left alone and lysed after 4 hours. The firefly luciferase activity and the luciferase mRNA amount were analyzed as described in the legend to Fig.21.

4.6.8 Involvement of IRAK Proteins in translational activation of IκBζ 3' UTR mRNA

Interleukin-1 receptor-associated kinases (IRAKs) are serine/threonine protein kinases, involved in the IL-1 and Toll-like-receptor (TLR) activated signal pathways. They interact downstream with TRAF6 (tumour necrosis factor receptor-associated factor 6) and cause mRNA stabilization and NFκB activation. However Hartupee et al showed that signals leading to mRNA stabilization and NFκB activation can diverge at IRAK1. This adaptor generates a distinct signal that links to a signaling pathway, independent of TRAF6/p38, which is responsible for IL-1α-induced stabilization of certain mRNAs (Hartupee et al., 2008).

Our next question was whether IRAK1 and IRAK2, which are receptor-proximal downstream components in IL-1 signalling, can reproduce the translational effect of IL-1 on IκBζ mRNA (3' UTR). Therefore we co-transfected HeLa cells with expression vectors for IRAK1 or -2 and compared their effect with IL-1. It was seen that over expression of IRAK1 & -2 can up regulate the luciferase activity.

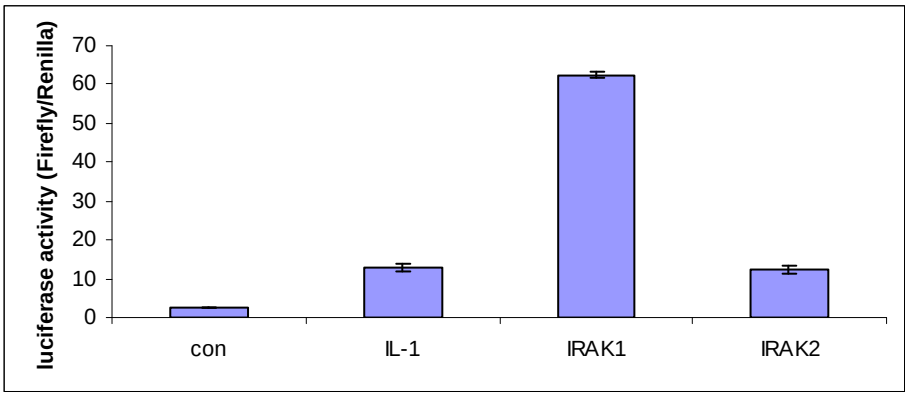


Fig.28. Overexpression of IRAK1 or -2 increase the translation of the reporter mRNA containing IκBζ 3' UTR. HeLa cells were transfected with luciferase expression vector containing 3' UTR of IκBζ. They were either stimulated with IL-1α for 4 hours or co-transfected with expression vectors for IRAK1 or -2. Firefly Luciferase activity was normalized to co-expressed *Renilla* luciferase.

In a subsequent experiment we co-transfected HeLa cells with a truncated form of IRAK1 consisting of the death domain alone which acts in a dominant-negative manner. The death domain of IRAK1 contains highly conserved threonine at position 66 (T66), which is the regulatory phosphorylation site controlling recruitment of IRAK to the receptor associated signalling complex e.g., the adaptor MyD88, the silencer Tollip, or the activator kinase IRAK-4 (Neumann et al., 2007; Neumann et al., 2008).Thus this deletion mutant of IRAK is recruited to IL-1 receptor but fails to initiate down stream signalling and acts as a dominat negative variant.

As it can be observed in Fig.29 there was no induction of luciferase activity by IL-1 when cells were co-transfected with plasmid for dn-IRAK1, which again approves the involvement of IRAK proteins in translational activation of IκBζ 3' UTR-containing mRNA.

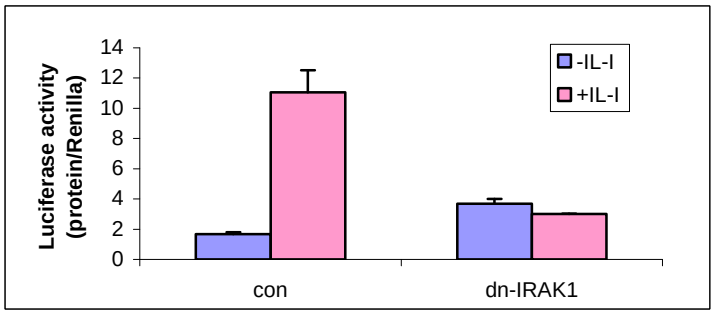


Fig.29 Suppression of IκBζ 3' UTR-dependent translational activation induced by IL-1 by the death domain of IRAK1. HeLa cells were either transfected exclusively with luc-IκBζ 3' UTR (con) or co-transfected with pFL-IRAK1-DD (dn-IRAK1). Luciferase activity was determined following a 4 hour-incubation without or with IL-1α. Firefly Luciferase activity was normalized to co-expressed *Renilla* luciferase activity.

5 DISCUSSION

Post-transcriptional mechanisms contribute to the changes in gene expression induced by cell stress and inflammatory stimuli. The pro-inflammatory cytokine IL-1α and UV-B radiation are two well known stimuli and inducers of inflammation, which activate various signalling pathways affecting multiple levels of gene regulation. These were the main stimuli used in this study, where their effect on the stability and translation of selected mRNAs differing in their regulatory elements has been examined. The short lived-mRNAs analyzed include ARE containing IL-8 mRNA and the mRNAs of IκBζ and IκBα which lack classical AREs.

5.1 UV light and mRNA stability and signalling involved

In the first part of this work the effect of low and high dose of UV-B radiation on stabilization of these three representative mRNAs has been investigated. As reported previously, UV exhibits a generalized effect on mRNA stability as it stabilizes both ARE and non-ARE containing mRNAs. It was seen that activation of the p38 MAPK pathway alone or in combination with the JNK and NF-κB pathways was not sufficient for UV-B mediated stabilization of the non-ARE reporters (Bollig et al., 2002). Earlier works from our group reported UV-B effect on IL-8 mRNA by inhibition of its deadenylation and degradation (Gowrishankar et al., 2005). Similarly, the stabilization of IκBα mRNA in response to UV-B light has been shown by Microarray data (Gowrishankar et al., 2006) and by Northern blot analysis (PhD thesis of Meera Shah 2007).

According to the initial experiments in HeLa cells, endogenous IL-8 and IκBζ mRNAs showed stabilization as a result of UV exposure (high dose) (Fig.6). Furthermore we analyed the UV-induced stabilization in a UV dose-dependent manner and investigated the involvement of p38 MAPK at different UV doses for our selected mRNAs by using pyridinyl imidazole SB203580, a selective p38 MAPK inhibitor. Regarding endogenous and reporter IL-8-ARE mRNA (Fig.7), we observe UV-induced stabilization with both low (160 J/m^2) and high dose of UV light (1280 J/m^2). p38 MAPK is apparently involved in the stabilization caused by low dosed-UV light, since blocking of this pathway could reverse the UV-induced stabilization of both endogenous IL-8 and reporter IL-8 ARE mRNAs (Fig.7). Their stabilization induced by high dosed-UV was maintained even after SB203580 treatment,

which excludes a role for the p38 MAPK pathway. This is reminiscent of the observations from Blattner et al who could not prevent UV-C mediated mRNA stabilization using SB203580 and various other protein kinase inhibitors (Blattner et al., 2000).

The two non-ARE endogenous IκBζ and IκBα mRNAs in turn do not show any stabilization with low dose of UV; however, high doses stabilize these mRNAs slightly and this effect was not inhibited by SB203580 (Fig.8 & Fig.10), which again fortifies the hypothesis that the stabilization induced by high dosed-UV is independent of p38 MAPK. Taken together, these observations indicate that the stabilization of specific mRNAs (e.g. IL-8 mRNA here) by low doses of UV-B is through p38 MAPK pathway. In response to high dose of UV-B there is the possibility of involvement of other mechanisms in the mRNA stability. This regulation must be through another signalling pathway or a combined activation of several signalling pathways, which is yet unidentified.

The influence of low and high dose of UV-B light on the regulation of mRNA stability was further analyzed in the epidermal keratinocytes. It was noticeable that in keratinocytes all the selected mRNAs were comparatively stable without UV exposure (Fig.12, Fig.13 & Fig.14). One speculation is that the keratinocytes are already pre-activated and the exposure to UV light leads only to their further stabilization, which can not be blocked by SB203580. Therefore the involvement of p38 MAPK still remains open here. The special nutrient and growth factor rich medium requirements for maintaining primary keratinocytes in could contribute to this basal stabilization. It should be noticed that the expression of these mRNAs in keratinocytes was much lower than in HeLa cells. It is well established in the literature and was observed here that these transcripts are strongly induced by IL-1 in HeLa cells. However, according to our observation, IL-1 does not induce the expression of these genes in keratinocytes (data not shown). This can be due to cell differences and characteristics. This unresponsiveness to IL-1 stimulation could also be due to the already pre-activated condition of keratinocytes. This cytokine is reported to be constitutively produced in keratinocytes (Yu et al., 1996). On the other hand, other groups have reported on IL-1β responses in cultured human keratinocytes (Otkjae et al., 2010). It is likely that these contradictory observations are due to different cultural conditions.

The data obtained from microarray analysis from an independent experiment in keratinocytes showed less or no stabilization of IL-8, IκBζ and IκBα mRNAs by low dose of UV light

(Table 6), which correlates to some extent with the results obtained from RT-qPCR experiments, explained above (Fig.12, Fig.13 & Fig.14). According to the microarray there are several other genes whose mRNA is stabilized more strongly by UV-B (data not shown), which indicates the general responsiveness of the keratinocytes to UV-B. These genes belong partly to the immediate early response genes and are functionally involved in signal transduction, apoptosis cell division and tissue differentiation, which make them interesting for future studies.

5.2 p38 MAPK activation in response to different dose of UV-B light

Stress signals, such as LPS, heat shock and ultraviolet light or pro-inflammatory cytokines like IL-1 can induce phosphorylation and activation of p38 MAPK (see section 1.5.4). Investigation on the effect of different UV-doses on the phosphorylation of p38 MAPK in HeLa cells and in keratinocytes revealed that in both the cell types there is activation of p38 MAPK by 160J/m^2 (Fig.15). Functional consequence of activation of p38 MAPK by low dose UV could also be detected by Northern blot analysis of IL-8 mRNA (see 5.2 for IL-8). In HeLa cells higher doses of UV-B induce enhanced activation of p38 MAPK, as shown by higher levels of phospho-p38 MAPK. This effect could not be seen in the keratinocytes. This can be either due to the pre-activated condition of keratinocytes resulting from the specific experimental conditions chosen for culturing and handling of primary keratinocytes, which was discussed before (see above), or due to cell characteristics.

There is a considerable amount of data generated on UV mediated post-transcriptional gene expression in HeLa cells. This study hints towards possible differences between keratinocytes and HeLa cells in response to UV irradiation. Since UV mediated signalling would have physiologically relevant outcomes in keratinocytes, additional studies in primary keratinocytes are required to shed light on the mechanisms involved in UV-induced mRNA stabilization and its role in stress response in the skin.

5.3 Role of HuR in UV-mediated stabilization of IL-8 mRNA

Our group could previously show the interaction of mRNA stabilizing protein HuR with IL-8-ARE in gel shift assays (Bollig et al., 2002; Winzen et al., 2004). However it was also shown that destabilization of IL-8 ARE was not counteracted by HuR over expression (Winzen et al., 2004). HuR was also reported to mediate UV-induced mRNA stabilization (Wang et al., 2000). Therefore, in the present work we further studied the role of HuR in IL-1- and UV-induced stabilization for IL-8 mRNA by the siRNA mediated knock down of HuR.

We observed that knock down of HuR by two different siRNAs did not affect the stability of IL-8 mRNA mediated by UV light. Despite a successful knock-down as confirmed by Western blots, since the UV-induced stabilization for the samples transfected with siHuR was similar to that of the control (siGFP) (Fig.16 B & D). Hence HuR did not reveal any role in UV-mediated stabilization of IL-8 mRNA.

Regarding IL-1-mediated stabilization, stimulating the cells 40 min with IL-1 leads to the stabilization of IL-8 mRNA (Fig 4.13 B & D). This is in accordance with the known stabilization effect induced by IL-1. In this effect of IL-1 HuR is apparently not involved, since suppression of HuR with two different siRNAs did not impact this effect. By 2 hours post-IL-1 addition, at a time when the IL-8 mRNA is degraded rapidly again, divergent results regarding knock down of HuR, were obtained. The cells knocked down with $siHuR_{278}$ showed no change in degradation of IL-8 mRNA, which is consistent with a lack of effect of HuR on this mRNA. On the other hand, using $siHuR_{649}$ for knock down, we could even observe stabilization of IL-8 mRNA compared to the control, the GFP siRNA- transfected cells (Fig.16, Fig.17 B & D). This argues against a stabilizing effect of HuR. There have been reports, in which HuR deviates from its established stabilizing function; for example it was reported that Hu proteins can block the polyadenylation (Zhu et al., 2007) or decrease the protein expression by inhibiting translation (Kullmann et al., 2002). However, this effect observed here requires more evidences. Another possible explanation is a lesser specificity of this siRNA ($siHuR_{649}$). It is possible that there are other target molecules which possess sequence homology to this siRNA, the so-called off-target effect (Jackson et al., 2003). It was reported that even siRNAs without a physiological target, which are often used as negative controls for siRNA experiments, can exhibit sequence-specific off-target effects in mammalian cells (Tschuch et al., 2008). General guidelines have been discussed to insure experimental approaches to maximize siRNA specificity (Cullen et al., 2006; Echeverri et al., 2006). To sum up, HuR does not play any role as a stabilizing factor in the basal half-life and IL-1- or UV-induced stabilization of IL-8 mRNA. In the case of $siHuR_{649}$, the data indicate a converse impact, which we speculate to be due to the off-target effect of this siRNA. For elucidation additional experiments using different siRNAs directed against HuR are required.

5.4 IL-1 increases ribosome occupancy of certain mRNAs

The interleukin-1 (IL-1) family of cytokines comprises 11 proteins, which function mainly in the control of pro-inflammatory gene expression in the innate immune response and can mediate mRNA stability by inducing various signalling pathways. Translation is the final

process in the flow of the genetic information, which regulates the amount of protein synthesis. Microarray analysis of changes in ribosomal occupancy in HeLa cells led to the identification of mRNA targets including IκBζ which are translationally activated by IL-1 (data not shown, Dhamija et al., 2010). Based on this microarray analysis, the distribution of IκBζ mRNA for individual fractions was studied. A shift in the ribosomal gradients from untranslated to translated fractions suggested for a higher translational level of endogenous IκBζ mRNA on IL-1 stimulation (Fig.19 B). This effect was further analyzed for IκBα and IL-8 mRNAs, which are known to be rapidly induced by IL-1. Unlike IκBζ mRNA, the ribosomal distribution of IκBα and IL-8 mRNAs was not affected by IL-1 (Fig.19 C & D).
Since the 3' UTR region of IκBζ mRNA exhibited a shift to the polysomal fractions in reporter mRNA experiments in response to IL-1 treatment (Dhamija et al., 2010), a luciferase reporter assay system containing this region was established to further study the regulation of translation by IL-1.

5.5 Translational silencing effect mediated by 3' UTR of IκBζ mRNA

The Luciferase activity and mRNA were determined in parallel in HeLa cells by expressing the vector lacking or containing the IκBζ 3' UTR. After comparing the level of protein and mRNA amounts in response to IL-1 and the general IL-1 induced translational activation for both reporter constructs, we came to two important conclusions. One is that the IκBζ 3' UTR contains translational silencing activity and its function is affected by IL-1 treatment (Fig.21 C). This observation correlates with the study on murine IκBζ mRNA, which is stabilized by IL-1. It was reported that the first 165 nucleotides at the 3´UTR of this mRNA are responsible for this response and can mediate post-transcriptional control (Watanabe et al., 2007). The second conclusion is that the translational activation can occur even in the absence of reporter mRNA stabilization (comparing the low mRNA amount to the protein amount in IκBζ mRNA, Fig.21 B).

5.6 Lack of IL-1-induced translational activation of luciferase reporter mRNA containing IL-8 ARE

Previous studies reported stabilization of many short-lived mRNAs by IL-1 stimulation (Winzen et al., 1999, Tebo et al, 2003). Though IL-8 is a well known target of IL-1-induced stabilization, we could not detect any change in the level of translational activation of luciferase reporter construct containing ARE of IL-8 mRNA (Fig.22 B). This confirms the earlier result obtained for endogenous IL-8 mRNA in the ribosomal gradients, where

distribution of IL-8 mRNAs was not affected by IL-1 (Fig.19 C). Interestingly, in the absence of IL-1 stimulation the level of expression of the luciferase reporter construct containing ARE of IL-8 mRNA is much lower compare to that of the empty luciferase reporter (Fig.22 A). These data suggest a translational silencing executed by the IL-8 ARE, which - unlike that of IκBζ translational silencing element - is not influenced by IL-1. However it is possible that other stimuli are capable of affecting the translational activation of IL-8 ARE mRNA, which requires more investigations by further experiments.

5.7 Effect of UV-B radiations on the luciferase translational activity

UV light, a known stress stimulus, mediates stabilization of many short-lived mRNAs (see section 1.6). Exposing the cells to low and high doses of UV-B light decreased the level of translational activation of luciferase reporter activity irrespective of the presence of 3' UTR of IκBζ mRNA or ARE of IL-8 mRNA. Even empty luciferase reporter indicated this reduction (Fig.22). Since both IL-8 and IκBζ mRNAs are stabilized by UV-B light (discussed in 5.1) this brings us again to the conclusion that mRNA stabilization does not automatically result in translational activation. Presumably the protein synthesis is inhibited as a consequence of UV-induced stress, which probably requires phosphorylation of eukaryotic initiation factor 2α (eIF2α), and the untranslated RNAs are accumulated in cytoplasmic aggregates termed stress granules (Kedersha and Anderson et al., 2002).

5.8 Signal transduction pathways involved in IL-1 induced translational activation

The p38 MAP kinase pathway, which is known to stabilize numerous ARE-containing mRNAs (Frevel et al., 2003) is apparently not involved in the translational activation luciferase reporter containing IκBζ 3' UTR, since expressing a dominant negative mutant of p38 MAP kinase ($p38_{AGF}$), did not significantly alter the IL-1 induced effect. Similarly expressing a constitutively active mutant of the p38 MAP kinase-selective upstream kinase, MKK6 ($MKK6_{2E}$), did not cause any change in the translational activity of the IκBζ 3' UTR-luc reporter (Fig.26). Blocking the PI3 kinases by wortmannin did not significantly affect the translational activation by IL-1 (Fig.27). To make a clear statement about their role further experiments should be carried out using a positive control for PI3 kinase inhibition. Over expression of IRAK1 and -2 proteins, which are recognized to function upstream of TRAF6 and are receptor-proximal downstream components in IL-1 signaling, can reproduce the translational effect of IL-1 on IκBζ mRNA (Fig.28), whereas expressing the truncated

dominant negative form of IRAK1 interfered with this effect (Fig.29). According to these results we can assume that an uncharacterized signalling pathway(s) inducing translational activation of IκBζ mRNA probably segregate downstream of IRAK from p38 MAP kinase pathway. IL-17, like IL-1 decreases suppression of luciferase activity executed by 3' UTR of IκBζ mRNA, whereas TNF-α did not alter the translational activity of this reporter mRNA at all. As mentioned in the literature TNF activates p38 MAPK (Saklatvala et al., 1996; Freshney et al., 1994). Lack of the translational regulation by TNF argues against the role of p38 MAPK again.

Taken together, analyzing three selected mRNAs reveals that one same stimulus can affect different mRNAs variably (e.g.: Stabilization of IL-8 mRNA by low and high doses of UV-B; no or slight stabilization of IκBζ and IκBα mRNAs by low and high doses of UV-B; different IL-1 induced translational activation of these mRNAs) and diverse signaling pathways contribute to the same effect (e.g.: low dosed UV-mediated stabilization through p38 MAPK, high dosed UV-mediated stabilization through other yet unidentified pathway(s)). This study disclose that, depending on the stimulus, different RNA cis-elements and activity of divers signaling pathways, post-transcriptional mechanisms can be executed which selectively influence the expression of specific genes.

REFERENCES

Abdelmohsen K, Pullmann R Jr, Lal A, Kim HH, Galban S, Yang X, Blethrow JD, Walker M, Shubert J, Gillespie DA, Furneaux H, Gorospe M. (2007) Phosphorylation of HuR by Chk2 regulates SIRT1 expression. *Mol. Cell.* **25**, 543-557.

Adinolfi, S, Bagni C, Castiglione Morelli MA, Fraternali F, Musco G, Pastore A. (1999) Novel RNA-binding motif: the KH module. *Biopolymers.* **51**, 153–164.

Alkalay I, Yaron A, Hatzubai A, Orian A, Ciechanover A. Ben-Neriah Y. (1995) Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A.* **92**, 10599-603.

Anderson P (2008) Post-transcriptional control of cytokine production. *Nat Immunol* **9**, 353-359.

Anderson P, Kedersha N. (2009) Stress granules. *Curr Biol.* **19**, R397-8.

Aström J, Aström A,Virtanen A. (1992) Properties of a HeLa cell 3′ exonuclease specific for degrading poly(A) tails of mammalian mRNA. *J. Biol. Chem.* **267**, 18154–18159.

Atasoy U, Curry SL, Lopez de Silanes I, Shyu AB, Casolaro V, Gorospe M, Stellato C. (2003) Regulation of eotaxin gene expression by TNF-alpha and IL-4 through mRNA stabilization: involvement of the RNA-binding protein HuR. *J Immunol*. **171**, 4369-4378.

Baeuerle P, Henkel T. (1994) Function and activation of NF- κB in the immune system. *Annu. Rev. Immunol.* **12**, 141–79.

Baeuerle, PA, Baltimore D. (1996) NF-κB: ten years after. *Cell.* **87**, 13– 20.

Bakheet T, Freve, M, Williams BR, Greer W, Khabar KS. (2001) ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res*. **29**, 246-254.

Bakheet T, Williams BR, Khabar KS. (2003) ARED 2.0: an update of AU-rich element mRNA database. *Nucleic Acids Res*. **31**, 421–423.

Baldwin AS Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol*. **14**, 649-683.

Barreau C, Paillard L, Osborne HB. (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res*. **33**, 7138-7150.

Bartel DP. (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell.* **116**, 281–297.

Beg A, Baldwin A. (1993) The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. *Genes Dev.* **7**, 2064–70.

Bernstein P, Ross J. (1989) Poly(A), poly(A) binding protein and the regulation of mRNA stability. *Trends Biochem. Sci.* **14**, 373-377.

Bernstein P, Peltz SW, Ross J. (1989) The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. *Mol. Cell. Biol*. **9**, 659–670.

Bernstein PL, Herrick DJ, Prokipcak RD, Ross J. (1992) Control of c-myc mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant
Genes Dev. **4**, 642-54.

Bhattacharya A, Czaplinski K, Trifillis P, He F, Jacobson A, Peltz SW. (2000) Characterization of the biochemical properties of the human Upf1 gene product that is involved in nonsense-mediated mRNA decay. *RNA* **6**, 1226–1235.

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. (2006) Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell.* **125**, 1111–1124.

Binder R, Horowitz JA, Basilion JP, Koeller DM, Klausner RD, Harford JB. (1994) Evidence that the pathway of transferring receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. *EMBO J*. **13**, 1969-1980.

Blattner C, Kannouche P, Litfin M, Bender K, Rahmsdorf HJ, Angulo JF, Herrlich P. (2000) UV-Induced stabilization of c-fos and other short-lived mRNAs. *Mol Cell Biol*. **20**, 3616-3625.

Bode AM, Z Dong (2006) Molecular and cellular targets. *Mol. Carcinog*. **45**, 422–430.

Boeck R, Tarun S Jr, Rieger M, Deardorff JA, Müller-Auer S, Sachs AB. (1996) The yeast Pan2 protein is required for poly(A)-binding protein-stimulated poly(A)-nuclease activity. *J. Biol. Chem.* **271**, 432–438.

Bollig F, Winzen R, Kracht M, Ghebremedhin B, Ritter B, Wilhelm A, Resch K, Holtmann H. (2002) Evidence for general stabilization of mRNAs in response to UV light. *Eur J Biochem*. **269**, 5830-5839.

Brennan CM, Gallouzi IE, Steitz JA. (2000) Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J. Cell Biol*. **151**, 1-14.

Briata P, Forcales SV, Ponassi M, Corte G, Chen CY, Karin M, Puri PL, Gherzi R. (2005) p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP controls the stability of select myogenic transcripts. *Mol. Cell.* **20**, 891-903.

Brook M, Sully G, Clark AR, Saklatvala J. (2000) Regulation of tumour necrosis factor alpha mRNA stability by the mitogenactivated protein kinase p38 signalling cascade. *FEBS Lett.* **483**, 57–61.

Brown CE, Tarun SZ Jr, Boeck R, Sachs AB. (1996) PAN3 encodes a subunit of the Pab1p-dependent poly(A) nuclease in Saccharomyces cerevisiae. *Mol Cell Biol*. **16**, 5744-53.

Caput,D, Beutler B., Hartog K, Thayer R, Brown-Shimer S, Cerami A. (1986) Identification of a common nucleotide sequence in the 3' untranslated region of mRNA molecules specifying inflammatory mediators. *Proc. Natl Acad. Sci. U S A.* **83**, 1670–1674.

Carballo E, Lai WS, Blackshear PJ. (1998) Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science.* **281**, 1001–1005.

Carballo E, Lai WS, Blackshear PJ. (2000) Evidence that tristetraprolin is a physiological regulator of granulocytemacrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood.* **95**, 1891–1899.

Chen CY, Shyu AB. (1994) Selective degradation of earlyresponse-gene mRNAs: Functional analysis of sequence features of the AU-rich elements. *Mol. Cell Biol*. **14**, 8471–8482.

Chen CY, Shyu AB. (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci*. **20**, 465-470.

Chen CY, Del Gatto-Konczak F, Wu Z, Karin M. (1998) Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. *Science*. **280**, 1945-1949.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**, 451-464.

Chen W, Q Tang, Gonzales MS, Bowden GT. (2001) Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. *Oncogene.* **20**, 3921–3926.

C**houinard** N, Valerie K, Rouabhia M, Huot J. (2002) UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. *Biochem J.* **365**, 133–145.

Conne B, Stutz A, Vassali J. (2000) The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? *Nat Med.* 6, 637-41.

Cougot, N, Babajko S, Seraphin B. 2004. Cytoplasmic foci are sites of mRNA decay in human cells. *J. Cell Biol.* **165**, 31–40.

Cullen BR. (2006) Enhancing and confirming the specificity of RNAi experiments. *Nat Methods*. **3**, 677-81.

Davis N, Ghosh S, Simmons D, Tempst P, Liou HC, Baltimore D, Bose H. (1991) Rel-associated pp40: an inhibitor of Rel family of transcription factors. *Science*. 253, 1268–71.

Decker CJ. Parker RA. (1993) turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. *Genes Dev*. 8, 1632–1643.

Decker CJ, Parker R. (2002) mRNA decay enzymes: decappers conserved between yeast and mammals. *Proc NatI Acad Sci U S A.* 99, 12512-4.

Deleault KM., Skinner SJ, Brooks SA. (2008) Tristetraprolin regulates TNF alpha mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. *Mol. Immunol.* ***45***, 13-24.

de Nadal E, Alepuz PM, Posas F (2002) Dealing with osmostress through MAP kinase activation. *EMBO Rep.* **8**, 735-740.

Dhamija S, Doerrie A, Winzen R, Dittrich-Breiholz O, Taghipour A, Kuehne N, Kracht M, Holtmann H. (2010) IL-1-induced post-transcriptional mechanisms target overlapping translational silencing and destabilizing elements in IκBζ mRNA. *J Biol Chem.* [Epub ahead of print].

Dinarello CA. (2009) Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* **27**, 519-550.

Dixon DA, Balch GC, Kedersha N, Anderson P, Zimmerman GA, Beauchamp RD, Prescott SM. (2003) Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1. *J. Exp. Med*. **198**, 475–481.

Dixon DA, Tolley ND, Bemis-Standoli K, Martinez ML, Weyrich AS, Morrow JD, Prescott SM, Zimmerman GA (2006) Expression of COX-2 in platelet-monocyte interactions occurs via combinatorial regulation involving adhesion and cytokine signaling. *J Clin Invest.* **116**, 2727–2738.

Doller A, Huwiler A, Muller R, Radeke HH, Pfeilschifter J, Eberhardt W. (2007) Protein kinase C alpha-dependent phosphorylation of the mRNA-stabilizing factor HuR: implications for posttranscriptional regulation of cyclooxygenase-2. *Mol. Biol. Cell*. **18**, 2137-2148.

Dompenciel RE, Garnepudi VR, Schoenberg DR. (1995) Purification and characterization of an estrogen-regulated *Xenopus* liver polysomal nuclease involved in the selective destabilization of albumin mRNA. *J. Biol. Chem.* **270**, 6108-6118.

Draper MP, Salvadore C, Denis CL. (1995) Identification of a mouse protein whose homolog in Saccharomyces cerevisiae is a component of the CCR4 transcriptional regulatory complex. *Mol. Cell. Biol.* **15**, 3487–3495.

Dupressoir A, Barbot W, Loireau MP, Heidmann T. (1999) Characterization of a mammalian gene related to the yeast CCR4 general transcription factor and revealed by transposon insertion. *J. Biol. Chem.* 274, 31068–31075.

Dupressoir A, Morel AP, Barbot W, Loireau MP, Corbo L, Heidmann T. (2001) Identification of four families of yCCR4- and Mg2+-dependent endonuclease-related proteins in higher eukaryotes, and characterization of orthologs of yCCR4 with a conserved leucine-rich repeat essential for hCAF1/hPOP2 binding. *BMC Genom.* **2**, 9–22.

Echeverri CJ, Perrimon N. (2006) High-throughput RNAi screening in cultured cells: a user's guide. *Nat Rev Genet.* **7**, 373-84.

Ernst M, Dunn L, Rice N. (1995) The PEST-like sequence of IκBα is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers. *Mol. Cell. Biol.* **15**, 872–82.

Esnault S, Malter JS. (2002) Extracellular signal-regulated kinase mediates granulocyte-macrophage colony-stimulating factor messenger RNA stabilization in tumor necrosis factor-alpha plus fibronectin-activated peripheral blood eosinophils. *Blood*. **99**, 4048-4052.

Eto A, Muta T, Yamazaki S, Takeshige K. (2003) Essential roles for NF-kappa B and a Toll/IL-1 receptor domain-specific signal(s) in the induction of I kappa B-zeta. *Biochem Biophys Res Commun*. **301**, 495-501.

Fan XC, Steitz JA. (1998a). HNS, a nuclear-cytoplasmic shuttling sequence in HuR. *Proc. Natl. Acad. Sci. U. S. A* **95**, 15293-15298.

Fan XC, Steitz JA. (1998b) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J*. **17**, 3448-3460.

Filipowicz, W. (2005) RNAi: The nuts and bolts of the RISC machine. *Cell.* **122**, 17–20.

Fiorini E, Schmitz I, Marissen WE, Osborn SL, Touma M, Sasada T. (2002) Peptide-induced negative selection of thymocytes activates transcription of an NF-kappa B inhibitor. *Mol Cell.* **9**, 637–48.

Ford LP, Wilusz J. (1999a) An in vitro system using HeLa cytoplasmic extracts that reproduces regulated mRNA stability. *Methods*. **17**, 21-27.

Ford LP, Watson J, Keene JD, Wilusz J. (1999b) ELAV proteins stabilize deadenylated intermediates in a novel *in vitro* mRNA deadenylation/degradation system. *Genes Dev*. **13**, 188−201.

Franzoso G, Bours V, Azarenko V, Park S, Tomita-Yamaguchi M, Kanno T, Brown K, Siebenlist U. (1993) The oncoprotein Bcl-3 can facilitate NF-kappa B-mediated transactivation by removing inhibiting p50 homodimers from select kappa B sites. *EMBO J*. **12**, 3893-3901.

Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, Saklatvala J. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell.* **6,** 1039-49.

Frevel MA, Bakheet T, Silva AM, Hissong JG, Khabar KS, Williams BR. (2003) p38 Mitogen-activated protein kinasedependent and -independent signaling of mRNA stability of AUrich element-containing transcripts. *Mol Cell Biol.* **23**, 425–436.

Gao M, Fritz DT, Ford LP, Wilusz J. (2000) Interaction between a poly(A)- specific ribonuclease and the 5' cap influences mRNA deadenylation rates in vitro. *Mol Cell*, **5**, 479-488.

Gao M, Wilusz CJ, Peltz SW, Wilusz J. (2001) A novel mRNA-decapping activity in HeLa cytoplasmic extracts is regulated by AU-rich elements. *EMBO J.* **20**, 1134–1143.

Garcia-Sanz JA, Lenig D. (1996) Translational control of interleukin 2 messenger RNA as a molecular mechanism of T cell anergy. *J. Exp. Med*. **184**, 159-164.

Garcia-Mayoral MF, Diaz-Moreno I, Hollingworth D, Ramos A. (2008) The sequence selectivity of KSRP explains its flexibility in the recognition of the RNA targets. *Nucleic Acids Res*. **36**, 5290-6.

Ge B, Gram H, Di Padova F, Huang B, New L, Ulevitch RJ, Luo Y, Han J. (2002) MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. *Science.* **295**, 1291-1294.

Gebauer F, Hentze MW. (2004) Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol.* **5**, 827-835.

Gherzi R, Lee KY, Briata P, Wegmuller D, Moroni C, Karin M, Chen CY. (2004) A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. *Mol Cell*. **14**, 571-583.

Gherzi R, Trabucchi M, Ponassi M, Ruggiero T, Corte G, Moroni C, Chen CY, Khabar KS, Andersen JS, Briata P. (2006) The RNA-binding protein KSRP promotes decay of betacatenin mRNA and is inactivated by PI3K-AKT signaling. *PLoS Biol*. **1**, e5.

Ghosh M, Aguila HL, Michaud J, Ai Y, Wu MT, Hemmes A, Ristimaki A, Guo C, Furneaux H, Hla T (2009) Essential role of the RNA-binding protein HuR in progenitor cell survival in mice. *J Clin Invest.* **119**, 3530–3543.

Gingras AC, Raught B, Sonenberg N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem.* **68**, 913-963.

Gorospe M, Kumar S, Baglioni C. (1993) Tumor necrosis factor increases stability of interleukin-1 mRNA by activating protein kinase C. *J. Biol. Chem.* **268**, 6214-6220.

Gossen M, Bujard H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A.* **89**, 5547-5551.

Gowrishankar G, Winzen R, Bollig F, Ghebremedhin B, Redich N, Ritter B, Resch K, Kracht M, Holtmann H. (2005) Inhibition of mRNA deadenylation and degradation by ultraviolet light. *Biol Chem*. **386**, 1287-1293.

Gowrishankar G, Winzen R, Dittrich-Breiholz O, Redich N, Kracht M, Holtmann H. (2006) Inhibition of mRNA deadenylation and degradation by different types of cell stress. *Biol Chem*. **387**, 323-7.

Halees AS, El-Badrawi R, Khabar KS. (2008) ARED organism: expansion of ARED reveals AU-rich element cluster variations between human and mouse. *Nucleic Acids Res.* **36**, D137–D140.

Han J, Brown T, Beutler B. (1990) Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. J. *Exp. Med.*. 171, 465-475.

Han J, Lee J, Bibbs L, Ulevitch RJ. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science.* **5173**, 808-11.

Hartupee J, Li X, Hamilton T. (2008) Interleukin 1alpha-induced NFkappaB activation and chemokine mRNA stabilization diverge at IRAK1. *J Biol Chem* **283**, 15689-15693.

Haruta H, Kato A, Todokoro K. (2001) Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. *J Biol Chem.* **276**, 12485-12488.

Haskill S, Beg AA, Tompkins SM, Morris JS, Yurochko AD, Sampson-Johannes A, Mondal K, Ralph P, Baldwin AS Jr. (1991) Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. *Cell*. **65**, 1281-1289.

Hau HH, Walsh RJ, Ogilvie RL, Williams DA, Reilly CS, Bohjanen PR. (2007) Tristetraprolin recruits functional mRNA decay complexes to ARE sequences. *J. Cell Biochem.* **100**, 1477-1492.

Hayden MS, Ghosh, S. (2004) Signaling to NF-kappaB. *Genes Dev*. **18**, 2195-2224.

Herrlich, P, Blattner C, Knebel A, Bender K, Rahmsdorf HJ. (1997) Nuclear and non-nuclear targets of genotoxic agents in the induction of gene expression. Shared principles in yeast, rodents, man and plants. *Biol Chem*. **378**, 1217-1229.

Holtmann H, Winzen R, Holland P, Eickemeier S, Hoffmann E, Wallach D, Malinin NL, Cooper JA, Resch K, Kracht M. (1999) Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol Cell Biol*. **19**, 6742–6753.

Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M. (2002). Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* **72**, 847-855.

Hosoda N, Kobayashi T, Uchida N, Funakoshi Y, Kikuchi Y, Hoshino S, Katada T. (2003) Translation termination factor eRF3 mediates mRNA decay through the regulation of deadenylation. *J Biol Chem*. **278**, 38287-38291.

Houseley J, Tollervey D. (2009) The many pathways of RNA degradation. *Cell.* **136**, 763-76.

Huang C, Ma WY, Maxiner A, Sun Y, Dong Z. (1999) p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *J Biol Chem.* **274**, 12229-12235.

Inoue J, Kerr LD, Kakizuka A, Verma IM. (1992) I kappa B gamma, a 70 kd protein identical to the C-terminal half of p110 NF-kappa B: a new member of the I kappa B family. *Cell*. **68**, 1109-1120.

Iordanov M, Bender K, Ade T, Schmid W, Sachsenmaier C, Engel K, Gaestel M, Rahmsdorf HJ, Herrlich P. (1997) CREB is activated by UVC through a p38/HOG-1-dependent protein kinase. *EMBO J*. **16**, 1009–1022.

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotech.* **21**, 635–637.

Jacobs-Anderson JS, Parker R. (1998) The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. *EMBOJ.* **17**, 1497–1506.

Jakymiw A, Lian S, Eystathioy T, Li S, Satoh M, Hamel JC, Fritzler MJ, Chan EK. (2005) Disruption of GW bodies impairs mammalian RNA interference. *Nat. Cell Biol.* **7**, 1167–1174.

Johnson TM, Dolan OM, Hamilton TA, Lu MC, Swanson NA, Lowe L. (1998) Clinical and histologic trends of melanoma. *J. Am. Acad. Dermatol*. **38**, 681–686.

Kao CY, Kim C, Huang F, Wu R. (2008) Requirements for two proximal NF-kappaB binding sites and IkappaB-zeta in IL-17A-induced human beta-defensin 2 expression by conducting airway epithelium. *J Biol Chem.* **283**, 15309-18.

Karin M, Liu ZG, Zandi E. (1997) AP-1 function and regulation. *Curr. Opin. Cell. Biol.* **9**, 240–246.

Katsanou V, Milatos S, Yiakouvaki A, Sgantzis N, Kotsoni A, Alexiou M, Harokopos V, Aidinis V, Hemberger M, Kontoyiannis DL. (2009) The RNA-binding protein Elavl1/HuR is essential for placental branching morphogenesis and embryonic development. *Mol Cell Biol*. **29**, 2762–2776.

Kayama H, Ramirez-Carrozzi VR, Yamamoto M, Mizutani T, Kuwata H, Iba H, Matsumoto M, Honda K, Smale ST, Takeda K. (2008) Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IkappaBzeta. *J Biol Chem.* **283**, 12468-12477.

Kedersha N, Anderson P. (2002) Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. *Biochem Soc Trans*. **30**, 963-969.

Kim HH, Abdelmohsen K, Lal A, Pullmann R Jr, Yang X, Galban S, Srikantan S, Martindale JL, Blethrow J, Shokat KM, Gorospe M. (2008) Nuclear HuR accumulation through phosphorylation by Cdk1. *Genes Dev*. **22**, 1804-1815.

Kitamura H, Kanehira K, Okita K, Morimatsu M, Saito M (2000) MAIL, a novel nuclear I kappa B protein that potentiates LPS-induced IL-6 production. *FEBS Lett.* **485**, 53-56.

Kobayashi T, Funakoshi Y, Hoshino S, Katada T. (2004) The GTP-binding release factor eRF3 as a key mediator coupling translation termination to mRNA decay. *J Biol Chem*. **279**, 45693-45700.

Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. (1999) Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity. 10, 387-398.

Korhonen R, Linker K, Pautz A, Forstermann U, Moilanen E, Kleinert H. (2007) Post-transcriptional regulation of human inducible nitric-oxide synthase expression by the Jun N-terminal kinase. *Mol Pharmacol.* **71**, 1427–1434.

Körner CG, Wahle E. (1997) Poly(A) tail shortening by a mammalian poly(A)-specific 3′-exoribonuclease. *J. Biol. Chem.* **272**, 10448–10456.

Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD, Gaestel M. (1999) MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nat. Cell Biol*. **1**, 94-97.

Kruys V, Marinx O, Shaw G, Deschamps J, Huez G. (1989) Translational blockade imposed by cytokine-derived UA-rich sequences. *Science.* **245**, 852-855.

Kullmann M, Göpfert U, Siewe B, Hengst L. (2002) ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. *Genes Dev.* **16**, 3087-99.

Kuma Y, Sabio G, Bain J, Shpiro N, Marquez R, Cuenda A. (2005) BIRB796 inhibits all p38 MAPK isoforms *in vitro* and *in vivo*. *J Biol Chem.* **280**, 19472-19479.

Lafarga V, Cuadrado A, Lopez de Silanes I, Bengoechea R, Fernandez-Capetillo O, Nebreda AR. (2009) p38 Mitogen-activated protein kinase- and HuR-dependent stabilization of p21Cip1 mRNA mediates the G1/S checkpoint. *Mol Cell Biol.* **29**, 4341–4351.

Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, Blackshear PJ. (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor-α mRNA. *Mol. Cell.Biol.* **19**, 4311–4323.

Lai WS, Kennington EA, Blackshear PJ. (2003) Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. *Mol Cell Biol*. **23**, 3798-3812.

Lal A, Mazan-Mamczarz K, Kawai T, Yang X, Martindale JL, Gorospe M. (2004) Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. *EMBO J*. **23**, 3092-3102.

Lal A, Kawai T, Yang X, Mazan-Mamczarz K, Gorospe M. (2005) Antiapoptotic function of RNA-binding protein HuR effected through prothymosin alpha. *EMBO J*. **24**, 1852-62.

Lee RC, Feinbaum RL, Ambros V. (1993) The C. elegans heterochronic gene lin- 4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. **75**, 843-854.

Lee JC, Laydon J, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*. **372**, 739-46.

Lee HK, Jeong S. (2006) Beta-catenin stabilizes cyclooxygenase- 2 mRNA by interacting with AU-rich elements of 3'-UTR. *Nucleic Acids Res.* **34**, 5705–5714.

Levy NS, Chung S, Furneaux H, Levy AP. **(**1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J. Biol. Chem*. **273,** 6417–6423.

Lewis T, Gueydan C, Huez G, Toulme JJ, Kruys V. (1998) Mapping of a minimal AU-rich sequence required for lipopolysaccharide-induced binding of a 55- kDa protein on tumor necrosis factor-alpha mRNA. *J Biol Chem*. **273**, 13781-13786.

Lindstein T, June CH, Ledbetter JA, Stella G, Thompson CB. (1989) Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science*. **244**, 339-343.

Liu H, Rodgers ND, Jiao X, Kiledjian M. (2002) The scavenger mRNA decapping enzyme DcpS is a member of the HIT family of pyrophosphatases. *EMBO J*. **21**, 4699–4708.

Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. (2005a) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat. Cell Biol.* **7**, 719–723.

Liu J, Rivas FV, Wohlschlegel J, Yates JR, Parker R, Hannon GJ. (2005b) A role for the P-body component GW182 in microRNA function. *Nat. Cell Biol.* **7**, 1261–1266.

Loflin P, Chen CY, Shyu AB. (1999) Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element. *Genes Dev*. **13**, 1884-1897.

Lopez CB, Rao TD, Feiner H, Shapiro R, Marks JR, Frey AB. (1998) Repression of interleukin-2 mRNA translation in primary human breast carcinoma tumor-infiltrating lymphocytes. *Cell. Immunol.* **190**, 141-155.

Luo X, Puig O, Hyun J, Bohmann D, Jasper H. (2007) Foxo and Fos regulate the decision between cell death and survival in response to UV irradiation. *EMBO J*. **26**, 380-390.

Lykke-Andersen J. (2002) Identification of a human decapping complex associated with hUpf proteins in nonsense-mediated decay. *Mol Cell Biol*. **22**, 8114-8121.

Lynne E. Maquat (2006) Nonsense-Mediated mRNA Decay. Landes Bioscience.

Ma WJ, Cheng S, Campbell C, Wright A, Furneaux, H. (1996) Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J Biol Chem*. **271**, 8144-8151.

Madronich S, Mc Kenzie R, Bjorn LO, Caldwell MM. (1998) Changes in biologically active ultraviolet radiation reaching the Earth's surface. *J Photochem Photobiol B*. **46**, 5-19.

Matsuo S, Yamazaki S, Takeshige K, Muta T. (2007) Crucial roles of binding sites for NFkappaB and C/EBPs in IkappaB-zeta-mediated transcriptional activation. *Biochem J*. **405**, 605-615.

May MJ, Ghosh S. (1998) Signal transduction through NF-kappa B. *Immunol. Today*. **19**, 80–88.

Mazan-Mamczarz K, Galban S, Lopez de Silanes I, Martindale JL, Atasoy U, Keene JD, Gorospe M. (2003) RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. *Proc Natl Acad Sci USA* **100**, 8354-9.

Mazan-Mamczarz K, Lal A, Martindale JL, Kawai T, Gorospe M. (2006) Translational repression by RNA-binding protein TIAR. *Mol Cell Biol.* **26**, 2716-27.

Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Luhrmann R, Tuschl T. (2005) Identification of novel argonaute-associated proteins. *Curr. Biol.* **15**, 2149–2155.

Mendell JT, Medghalchi SM, Lake RG, Noensie EN, Dietz HC. (2000) Novel upf2p orthologues suggest a functional link between translation initiation and nonsense surveillance complexes. *Mol. Cell. Biol.* **20**, 8944–8957.

Min H, Turck CW, Nikolic JM, Black DL. (1997) A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. *Genes Dev*, **11**, 1023-1036.

Ming XF, Kaiser M, Moroni C. (1998) c-jun N-terminal kinase is involved in AUUUA-mediated interleukin- 3 mRNA turnover in mast cells. *EMBO J*. **17**, 6039-6048.

Ming XF, Stoecklin G, Lu M, Looser R, Moroni C. (2001) Parallel and independent regulation of interleukin-3 mRNA turnover by phosphatidylinositol 3-kinase and p38 mitogenactivated protein kinase. *Mol Cell Biol.* **21**, 5778–5789.

Moriguchi T, Kuroyanagi N, Yamaguchi K. (1996) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J Biol Chem*. **271**, 13675-9.

Muhlrad D, Decker CJ, Parker R. (1994) Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5'→3' digestion of the transcript. *Genes Dev*, **8**, 855-866.

Muhlrad D, Parker R. Premature translational termination triggers mRNA decapping. *Nature* **370**, 578–581 (1994).

Muhlrad D, Decker CJ, Parker R. (1995) Turnover mechanisms of the stable yeast PGK1 mRNA. *Mol Cell Biol*, **15**, 2145-2156.

Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS, Wilusz J. (2002) The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J*. **21**, 165–174.

Nair AP, Hirsch HH, Colombi M, Moroni C. (1999) Cyclosporin A promoters translational silencing of autocrine interleukin-3 via ribosome-associated deadenylation. *Mol. Cell. Biol.* **19**, 889-898.

Neininger A, Kontoyiannis D, Kotlyarov A, Winzen R, Eckert R, Volk HD, Holtmann H, Kollias G, Gaestel M. (2002) MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different posttranscriptional levels. *J Biol Chem.* **277**, 3065–3068.

Neumann D, Kollewe C, Resch K, Martin MU. (2007) The death domain of IRAK-1: an oligomerization domain mediating interactions with MyD88, Tollip, IRAK-1, and IRAK-4. *Biochem Biophys Res Commun* **354**, 1089-1094.

Neumann D, Kollewe C, Pich A, Cao P, Resch K, Martin MU. (2008) Threonine 66 in the death domain of IRAK-1 is critical for interaction with signaling molecules but is not a target site for autophosphorylation. *J Leukoc Biol.* **84**, 807-813.

Nickoloff BJ, Qin JZ, Chaturvedi V. (2002) Life and death signalling pathways contributing to skin cancer. *J Invest Dermatol Symp Proc.* **7**, 27–35.

Nielson FC, Christiansen J. (1992). Endonucleolysis in the turnover of insulin-like growth factor II mRNA. *J. Biol. Chem*. **267**, 19404-19411.

Papadaki O, Milatos S, Grammenoudi S, Mukherjee N, Keene JD, Kontoyiannis DL. (2009) Control of thymic T cell maturation, deletion and egress by the RNA-binding protein HuR. *J Immunol.* **182**, 6779–6788.

Park JW, Jang M, Lee YH, Passaniti A, Kwon TK. (2001) p53-independent elevation of p21 expression by PMA results from PKC-mediated mRNA stabilization. *Biochem Biophys Res Commun.* **280**, 244-8.

Peng SS, Chen CY, Shyu AB. (1996) Functional characterization of a non-AUUUA AU-rich element from the c-jun proto-oncogene mRNA: evidence for a novel class of AU-rich elements. *Mol. Cell Biol.* **16**, 1490–1499.

Peng SS, Chen CY, Xu N, Shyu AB. (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J*. **17**, 3461-3470.

Piccirillo C, Khanna R, Kiledjian M. (2003) Functional characterization of the mammalian mRNA decapping enzyme hDcp2. *RNA*. **9**, 1138-1147.

Piecyk M, Wax S , Beck AR, Kedersha N, Gupta M, Maritim B, Chen S, Gueydan C, Kruys V, Streuli M, Anderson P. (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNF-α. *EMBO J*. **19**, 4154–4163.

Pulak R, Anderson P. (1993) mRNA surveillance by the Caenorhabditis elegans smg genes. *Genes Dev*. **7**, 1885–1897.

Pullmann R Jr, Kim HH, Abdelmohsen K, Lal A, Martindale JL, Yang X, Gorospe M. (2007) Analysis of turnover and translation regulatory RNA-binding protein expression through binding to cognate mRNAs. *Mol Cell Biol*. **27**, 6265-78.

Raghavan A, Bohjanen PR. (2004) Microarray based analyses of mRNA decay in the regulation of mammalian gene expression, *Brief Funct. Genomic Proteomic.* **3**, 112–124.

Raineri I, Wegmueller D, Gross B, Certa U, Moroni C. (2004) Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference. *Nucleic Acids Res*. **32**, 1279-1288.

Raymond V, Atwater JA, Verma IM. (1989) Removal of mRNA destabilizing element correlates with the increased oncogenicity of proto-oncogene fos. *Oncogene Res*. **5**, 1–12.

Rice NR, MacKichan ML, Israel A. (1992) The precursor of NF-kappa B p50 has I kappa B-like functions. *Cell*. **71**, 243-253.

Ross J, Kobs G, Brewer G, Peltz SW. (1987) Properties of the exonuclease activity that degrades H4 histone mRNA. *J. Biol. Chem.* **262**, 9374–9381.

Saklatvala J, Davis W, Guesdon F. (1996) Interleukin 1 (IL1) and tumour necrosis factor (TNF) signal transduction. *Philos Trans R Soc Lond B Biol Sci*. **351**, 151-7.

Scheinman RI, Beg AA, Baldwin AS Jr. (1993) NF-kappa B p100 (Lyt-10) is a component of H2TF1 and can function as an I kappa B-like molecule. *Mol Cell Biol*. **13**, 6089-6101.

Schiavi SC, Belasco JG, Greenberg ME. (1992) Regulation of proto-oncogene mRNA stability. *Biochim. Biophys. Acta.* **1114**, 95–106.

Schuler GD, Cole MD. (1988) GM-CSF and oncogene mRNA stabilities are independently regulated in trans in a mouse monocytic tumor. *Cell*. **55**, 1115–1122.

Sen R, Baltimore D. (1986a) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*. **46**, 705–16.

Sen R, Baltimore D. (1986b) Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a post-translational mechanism. *Cell.* **47**, 921–8.

Sen GL, Blau HM. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat. Cell Biol.* **7**, 633–636.

Serin G, Gersappe A, Black JD, Aronoff R, Maquat LE. (2001) Identification and characterization of human orthologues to *Saccharomyces cerevisiae* upf2 protein and upf3 protein (*Caenorhabditis elegans* SMG-4) *Mol. Cell. Biol.* **21**, 209–223.

Shah M. (2007) Control of IκBα mRNA degradation. PhD thesis. Institute of Biochemistry, Medical School Hannover.

Shatkin AJ. (1976). Capping of eucaryotic mRNAs. *Cell.* **9**, 645-653.

Shaw G, Kamen R. (1986) A conserved AU sequence from the 3' untranslated region of GMCSF mRNA mediates selective mRNA degradation. *Cell*. **46**, 659-667.

Sheth U, Parker R. (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science.* **5620**, 805-8.

Shyu AB, Belasco JG, Greenberg ME. (1991) Two distinct destabilizing elements in the *c fos* message trigger deadenylation as a first step in rapid mRNA decay. *Genes Dev.* **5**, 221–231.

Siebenlist U, Franzoso G, Brown K. (1994) Structure, regulation and function of NF-κB. *Annu. Rev. Cell Biol.* **10**, 405–55.

Simeonidis S, Liang S, Chen G, Thanos D. (1997) Cloning and functional characterization of mouse IkappaBepsilon. *Proc. Natl. Acad. Sci. U S A.* **94**, 14372–14377.

Siomi H, Matunis MJ, Michael WM, Dreyfuss G. (1993) The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. *Nucleic Acids Res.* **21**, 1193-8.

Siomi H, Choi M, Siomi MC, Nussbaum RL, Dreyfuss G. (1994) Essential role for KH domains in RNA binding: Impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. *Cell.* **77**, 33-39.

Stoecklin G, Ming XF, Looser R, Moroni C. (2000) Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. *Mol Cell Biol*. **20**, 3753-3763.

Stoecklin G, Stoeckle P, Lu M, Muehlemann O, Moroni C. (2001) Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. *RNA.* **7**, 1578–1588.

Stoecklin G, Stubbs T, Kedersha N, Wax S, Rigby WF, Blackwell TK, Anderson P. (2004) MK2-induced tristetraprolin: 14–3-3 complexes prevent stress granule association and ARE-mRNA decay. *EMBO J.* **23**, 1313–1324.

Stoecklin G, Mayo T, Anderson P. (2006) ARE-mRNA degradation requires the 5'-3' decay pathway. *EMBO Rep*. **7**, 72-7.

Suswam E, Li Y, Zhang X, Gillespie GY, Li X, Shacka JJ, Lu L, Zheng L, King PH. (2008) Tristetraprolin down-regulates interleukin-8 and vascular endothelial growth factor in malignant glioma cells. *Cancer Res*. **68**, 674-682.

Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel DD, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF, Blackshear PJ. (1996) A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity*. **4**, 445–454.

Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P, Ghosh S. (1995) I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell*. **80**, 573-582.

Thompson MJ, Lai WS, Taylor GA, Blackshear PJ. (1996) Cloning and characterization of two yeast genes encoding members of the CCCH class of zinc finger proteins: zinc finger-mediated impairment of cell growth. *Gene*. **174**, 225-233.

Totzke G, Essmann F, Pohlmann S, Lindenblatt C, Janicke RU, Schulze-Osthoff K. (2006) A novel member of the IkappaB family, human IkappaB-zeta, inhibits transactivation of p65 and its DNA binding. *J Biol Chem.* **281**, 12645-12654.

Tschuch C, Schulz A, Pscherer A, Werft W, Benner A, Hotz-Wagenblatt A, Barrionuevo LS, Lichter P, Mertens D. (2008) Off-target effects of siRNA specific for GFP. *BMC Mol Biol.* **24**, 9-60.

Tucker M, Valencia-Sanchez MA, Staples RR, Chen J, Denis CL, Parker R. (2001) The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in *Saccharomyces cerevisiae*. *Cell.* **104**, 377–386.

Tucker M, Staples R, Valencia-Sanchez M.A, Muhlrad D, Parker R. (2002) Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in Saccharomyces cerevisiae. *EMBOJ*. **6**, 1427-36.

Tyrrell RM. (1996) Activation of mammalian gene expression by the UV component of sunlight--from models to reality. *Bioessays*. **18**, 139-148.

Vile GF, Tyrrell RM. (1993) Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenasedependent increase in ferritin. *J Biol Chem.* **268**,14678–81.

Wang W, Furneaux H, Cheng H, Caldwell MC, Hutter D, Liu Y, Holbrook N, Gorospe M. (2000) HuR regulates p21 mRNA stabilization by UV light. *Mol Cell Biol*. **20**, 760-769.

Wang W, Czaplinski K, Rao Y, Peltz SW. (2001) The role of Upf proteins in modulating the translation read-through of nonsense-containing transcripts. *EMBO J.* **20**, 880–890.

Wang W, Fan J, Yang X, Furer-Galban S, Lopez de Silanes I, von Kobbe C, Guo J, Georas SN, Foufelle F, Hardie DG, Carling D, Gorospe M. (2002) AMP-activated kinase regulates cytoplasmic HuR. *Mol Cell Biol*. **22**, 3425-3436.

Wang Z, Day N, Trifillis P, Kiledjian M. (1999) An mRNA stability complex functions with poly(A)-binding protein to stabilize mRNA in vitro. *Mol. Cell. Biol.* 19, 4552–4560.

Wang Z, Jiao X, Carr-Schmid A, Kiledjian M. (2002) The hDcp2 protein is a mammalian mRNA decapping enzyme. *Proc Natl Acad Sci U S A*. **99**, 12663-12668.

Wells SE, Hillner PE, Vale RD, Sachs AB. (1998) Circularization of mRNA by eukaryotic translation initiation factors. *Mol Cell*. **2**, 135-140.

Weng Y, Czaplinski K, Peltz SW. (1996) Identification and characterization of mutations in the UPF1 gene that affect nonsense suppression and the formation of the Upf protein complex but not mRNA turnover. *Mol. Cell. Biol.* **16**, 5491–5506.

White FC, Benehacene A, Scheele JS, Kamps M. (1997) VEGF mRNA is stabilized by *ras* and tyrosine kinase oncogenes, as well as by UV radiation – evidence for divergent stabilization pathways. *Growth Factors.* **14**, 199–212.

Whiteside ST, Epinat JC, Rice NR, Israel A. (1997) I kappa B epsilon, a novel member of the I kappa B family, controls RelA and cRel NF-kappa B activity. *EMBO J*. **16**, 1413 1426.

Whitmarsh AJ, Davis RJ. (1996) Transcription factor AP-1 regulation by mitogenactivated protein kinase signal transduction pathways. *J Mol Med.* **74**, 589–607.

Widmann C, Gibson S, Jarpe MB, Johnson GL.(1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev*. **79**, 143.

Wilson T, Treisman R. (1988) Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3' AU-rich sequences. *Nature*. **336**, 396-399.

Wilusz CJ, Wormington M, Peltz SW. (2001) The cap-to-tail guide to mRNA turnover. *Nat Rev Mol Cell Biol*. **2**, 237-246.

Winzen R, Kracht M, Ritter B, Wilhelm A, Chen CY, Shyu AB, Muller M, Gaestel M, Resch K, Holtmann H. (1999) The p38 MAP kinase pathway signals for cytokine induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *Embo J*. **18**, 4969-4980.

Winzen R, Gowrishankar G, Bollig F, Redich N, Resch K, Holtmann H. (2004) Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR. *Mol. Cell Biol*. **24**, 4835-4847.

Winzen R, Thakur BK, Dittrich-Breiholz O, Shah M, Redich N, Dhamija S, Kracht M, Holtmann H. (2007) Functional analysis of KSRP interaction with the AU-rich element of interleukin-8 and identification of inflammatory mRNA targets. *Mol. Cell Biol*. **27**, 8388-8400.

Wu Z, Zhang X, Yang J, Wu G, Zhang Y, Yuan Y, Jin C, Chang Z, Wang J, Yang X, He F. (2009) Nuclear protein IkappaB-zeta inhibits the activity of STAT3. *Biochem Biophys Res Commun.* **387**, 348-52.

Xu N, Loflin P, Chen CY, Shyu AB. (1998) A broader role for AU-rich element-mediated mRNA turnover revealed by a new transcriptional pulse strategy. *Nucleic Acids Res*. **26**, 558–565.

Xu N, Chen CY, Shyu AB. (2001) Versatile role for hnRNPD isoforms in the differential regulation of cytoplasmic mRNA turnover. *Mol. Cell Biol.* **21**, 6960–6971.

Yamamoto M, Yamazaki S, Uematsu S, Sato S, Hemmi H, Hoshino K, Kaisho T, Kuwata H, Takeuchi O, Takeshige K, Saitoh T, Yamaoka S, Yamamoto N, Yamamoto S, Muta T, Takeda K, Akira S. (2004) Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature.* **430**, 218-222.

Yamamoto M, Takeda K. (2008) Role of nuclear IkappaB proteins in the regulation of host immune responses. *J Infect Chemother.* **14**, 265-9.

Yamazaki S, Muta T, Takeshige K. (2001) A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J Biol Chem.* **276**, 27657-27662.

Yamazaki S, Matsuo S, Muta T, Yamamoto M, Akira S, Takeshige K. (2008) Gene-specific requirement of a nuclear protein, IkappaB-zeta, for promoter association of inflammatory transcription regulators. *J Biol Chem.* **283**, 32404-32411.

Yang X, Wang W., Fan J, Lal A, Yang D, Cheng H, Gorospe M. (2004) Prostaglandin A2 mediated stabilization of p21 mRNA through an ERK-dependent pathway requiring the RNA-binding protein HuR. *J Biol Chem*. **279**, 49298-306.

Yarovinsky TO, Butler NS, Monick MM, Hunninghake GW. (2006) Early exposure to IL-4 stabilizes IL-4 mRNA in CD4+ T cells via RNA-binding protein HuR. *J. Immunol.* **177**, 4426–4435.

Yekta S, Shih IH, Bartel DP. (2004) MicroRNA-directed cleavage of HOXB8 mRNA. *Science*. **304**, 594-596.

Yu HS., Chang KL, Yu CL, Chen JW, Chen GS. (1996) Low-energy helium-neon laser irradiation stimulates interleukin-1 alpha and interleukin-8 release from cultured human keratinocytes. *J. Invest. Dermatol.* **107**, 593–596.

Zarubin T, Han J. (2005) Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* **15**, 11-8.

Zhang W, Wagner B J, Ehrenman K, Schaefer AW, DeMaria CT, Crater D, DeHaven K, Long L, Brewer G. (1993) Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. *Mol. Cell. Biol*. 13, 7652-7665.

Zhang Q, Didonato JA, Karin M, Mckeithan TW. (1994) BCL3 encodes a nuclear protein which can alter the subcellular location of NF-*κ*B proteins. *Molecular and Cellular Biology*. **14**, 3915–3926.

Zhao Z, Chang FC, Furneaux HM. (2000) The identification of an endonuclease that cleaves within an HuR binding site in mRNA. *Nucleic Acids Res.* **28**, 2695−2701.

Zhu H., Zhou HL., Hasman RA, Lou H. (2007) Hu proteins regulate polyadenylation by blocking sites containing U-rich sequences. *J. Biol. Chem*. **282**, 2203−2210.

Zuo Y, Deutscher MP. (2001) Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res.* **29**, 1017–1026.

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ABBREVIATIONS

%	percent
Act-D	actinomycin D
APS	ammonium peroxide sulfate
ARE	AU-rich element
ARE-BP	ARE-binding protein
ATP	adenosine-5'-triphosphape
BCL-3	B-cell leukemia/lymphoma 3
BSA	bovine serum albumin
°C	degrees celsius
cDNA	complementary DNA
CIAP	Calf Intestine Alkaline Phosphatase
cm	centimeter
COX-2	cyclooxygenase-2
CSPD	chloro-5-substituted adamantyl-1,2-dioxetane phosphate
C-terminal	carboxy-terminal
ddH_2O	double distilled water
Dcp	decapping protein
DIG	cigoxygenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphate mix
DTT	cithiothreitol
E.coli	*E.coli Escherichia coli*
EDTA	ethylenediaminetetracetic acid
EGTA	ethylene glycol tetraacetic acid
eIF	eukaryotic initiation factor
ELAV	*embryonic lethal abnormal vision*
ERK	extracellular signal-regulated kinase
EtBr	Ethidium Bromide
et al	and others
FCS	fetal calf serum
Fig	figure
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GMCSF	granulocyte/macrophage colony stimulating factor
h	hour
HEBS	Hepes buffered saline
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hn	human
hnRNP	hetrogenous nuclear ribonucleoprotein
HuR	*human antigen* R
IL	interleukin
IκB	inhibitor of NF-κB
IKK	IκB kinase
JNK	*c jun* N-terminal kinase
Kb	kilobase (pair)
kDA	kilodalton

KSRP	*KH-type splicing regulatory protein*
LB	luria-bertoni
LPS	lipopolysaccharide
µ	micro
m	milli
M	molar
mA	milliampere
MAPK	mitogen activated protein kinase
min	minute
MKK	MAPK kinase
ml	milliliter
mM	millimolar
MMLV	mouse mammary leukemia virus
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor κB
NLS	nuclear localisation signal
NMD	nonsense-mediated mRNA decay
nt	nucleotide
N-terminal	amino-terminal
OD	optical density
PABP1	poly (A) binding protein 1
PARN	Poly(A)-specific Ribonuclease
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	pondus Hydrogenii, -log10 c(H+)
PI3K	Phosphatidylinositol 3-Kinase
pmol	pico molar
PVDF	polyvinylidene difluoride membrane
RCF (g)	relative centrifugation force
RISC	*RNA-induced silencing complex*
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SAPK	stress activated protein kinase
SDS	Sodium dodecyl sulphate
siRNA	small interfering RNA
TAE	Tris-Acetate-EDTA
TEMED	N, N, N', N'- tetramethlenediamine
TNF	tumour necrosis factor
Triton X-100	alkylpehnylpolyethylenglycol
Tris	Tris (hydroxymethyl)-aminoethane
tRNA	Transfer ribonucleic acid
TTP	Tristetraprolin
Tween 20	Polyoxyethylene-sorbitan-monolaurate
UTR	untranslated region
U	uridine
UV	ultraviolet light
V	volt
VEGF	vascular endothelial growth factor

v/v	volume/volume
w/v	weight/volume
X	times
XRN	5'-3' exoribonuclease

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If we knew what it was we were doing,
it would not be called research, would it?
(Albert Einstein)

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