On the Role of Ras and BRF1 in the Regulation of ARE-Dependent mRNA-Turnover

Inauguraldissertation

Zur Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Sabrina Angélique Leuenberger

aus Wynigen, BE

Basel, 2004

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von Prof. Ch. Moroni und Prof. M. Hall

Basel, den 6.4.04

Prof. M. Tanner

TABLE OF CONTENTS

1. Summary

Stability of the mRNAs of cytokines, chemokines, growth-factors, proto-oncogenes and others are regulated via an AU-rich element (ARE) in their 3' untranslated region (UTR). Upon activation of signal transduction pathways, these mRNAs, which are short-lived under resting conditions, become stabilised. AU-rich element binding proteins (AUBPs) are directly involved in this process as they either promote decay (TTP, BRF1, AUF1, KSRP) or stabilisation (HuR) of the mRNA by binding to the ARE.

In this work, the influence of the small GTPase ras and three of its downstream signalling pathways on the stability of the IL-3 ARE-mRNA was investigated. Work in our laboratory has identified BRF1 as an AUBP responsible for IL-3 ARE-mRNA decay. Therefore the effects of these pathways on BRF1 activity was also studied.

Ras, as the most upstream signalling protein, not only stabilises the IL-3 ARE-reporter transcript but was also able to completely inhibit the activity of BRF1, indicating that ras activates one or several pathways that are able to inactivate BRF1. However, PI3-K and raf, two downstream targets of ras were not able to overcome the induction of ARE-mRNA decay by BRF1, although they were able to stabilise the IL-3 ARE-reporter on their own. Even in combination, PI3-K and raf were not able to inhibit BRF1.

Two downstream targets of PI3-K, PKB and rac, were able to stabilise the reporter but did not antagonise the decay-inducing activity of BRF1. An active p38 pathway was necessary for rac to stabilise the reporter as shown by experiments using a dominant negative form of p38. However the p38 pathway alone was not sufficient, as an activated form of MEK6 did not stabilise the IL-3 ARE-reporter, indicating that rac needs to activate at least two downstream pathways in order to stabilise ARE-mRNA.

BRF1 has been found to harbour consensus sequences for phosphorylation by PKB and MK2, a downstream target of p38. In a co-transfection experiment it was possible to demonstrate that MK2 and PKB together were able to inhibit BRF1 activity.

Mass-spectrometry on recombinant BRF1 in vitro phosphorylated by PKB revealed serine 92 as the PKB phosphorylation site. Therefore a mutant was constructed in which serine 92 was replaced with alanine, thereby preventing phosphorylation at this site, and tested alongside wild-type BRF1 in an in vitro decay assay. As expected the wild-type BRF1 protein induced a very fast decay, but surprisingly PKB phosphorylated BRF1 was completely inactivated, which is in contradiction to the cellular decay system. In addition the mutant $BRF1_{S92A}$, which was as active as the wild-type BRF1 in promoting decay, was not inactivated by PKB phosphorylation, proving the importance of serine 92 in regulation of BRF1 activity by PKB.

For future work it would be of interest to obtain additional in vivo data regarding

BRF1 regulation by PKB and to investigate the role of MK2, as it appears to participate in in vivo decay but not in the in vitro decay system.

2. Introduction

2.1. Post-transcriptional control of gene expression

The levels of gene expression are essential for the correct functioning of cells as well as for the development and maintenance of multicellular organisms. Therefore cells have developed specific mechanisms to regulate gene expression at transcriptional, post-transcriptional and translational levels. By influencing the stability of the mRNA, post-transcriptional mechanisms determine the steady state mRNA levels and thus the expression of specific genes. It has become increasingly clear in recent years that post-transcriptional regulation is of fundamental importance and takes place on different levels with different functions.

Highly important post-transcriptional functions are the surveillance mechanisms to eliminate errors in mRNA biogenesis. Two distinct mechanisms have been described thus far: the non-stop decay (Vasudevan et al., 2002) and the nonsense-mediated decay (Wormington, 2003). The non-stop decay targets mRNAs lacking a stop codon, which are mostly produced by either inappropriate cleavage and polyadenylation during nuclear processing or by incomplete degradation of an mRNA undergoing translation.

In contrast, nonsense-mediated decay is a feature that recognises transcripts harbouring premature stop codons due to mutations in the coding region, splicing errors which lead to retention of introns that contain stop codons, extended 5'UTRs with upstream open reading frames or extended 3'UTRs due to over-reading of the correct transcription termination signal (for review: McKendrick, 2003).

Another mechanism of post-transcriptional regulation is RNAi. This pathway has been implicated in silencing transposons and serves as a defense mechanism against invading viruses in plants by specifically recognising foreign or aberrantly over-expressed mRNAs. However, very little is known about the biological role of RNAi in mammalian systems (Denli and Hannon, 2003) (Carrington and Ambros, 2003).

MicroRNA, a fourth form of post-transcriptional regulation controls endogenous gene expression by small regulatory RNAs, the so called microRNAs (miRNAs). miRNAs do not affect the stability of the target mRNA but rather repress gene expression through an unknown tranlation repression mechanism. MicroRNAs are transcribed as a long RNA from endogenous genes, which is then processed to a ~70nt long pre-miRNA. This pre-mRNA in turn is shortened by Dicer to produce mature miRNA molecules of ~22nt. So far, two functional miRNAs have been identified in animals: Lin-4 and let-7. They partially base-pair to the 3'

UTR of target mRNA such as lin-14 and lin-41, respectively, thereby repressing the expression of these genes. Lin-4 and let-7 regulate in this way endogenous genes involved in developmental timing in C. elegans (Carrington and Ambros, 2003).
A further means of regulating gene expression at the post-transcriptional level is achieved via cis-elements present on particular mRNAs. These elements are anchoring sequences for RNA binding proteins, which regulates the expression of these genes. As one of these elements, the AU-rich element or ARE, is the subject of the work presented here, these elements will be discussed in greater detail later in this Introduction. Meanwhile the mechanism of mRNA degradation will be summarised in order to get a better understanding of the regulation of mRNA stability by the ARE and its binding proteins.

2.2. mRNA degradation

Post-transcriptional regulation of gene-expression is based on the regulation of the rate of mRNA decay, in contrast to transcriptional regulation where the rate of mRNA synthesis is the critical factor.
The first step to a functional mRNA is the synthesis of pre-mRNA by transcription. During the early steps of pre-mRNA processing, a 7-methyl-guanosine cap structure is added to the 5'end. 3' end formation involves the creation of a stretch of 3' adenosine residues, the so called poly(A)-tail, while intronic sequences are removed by splicing. After processing the nascent transcript, the mRNA is packaged with proteins to form a mature messenger ribonucleoprotein (mRNP), which is actively exported from the nucleus to the cytoplasm (Jensen et al., 2003). For efficient translation mRNAs are circularised by PABP binding to the poly(A) tail and eIF4G of the initiation complex leading to the "closed loop" mRNA translation structure (Mazumder et al., 2001).
Deadenylation of the poly(A) tail seems to be a critical first step in mRNA degradation in yeast as well as in mammalian cells. This initial step exposes the deadenylated transcript to 5'-3' degradation (mainly yeast) or 3'-5' degradation (mainly mammalian) of the mRNA body. The deadenylation step can be circumvented in some rare cases, where the mRNA body is cleaved endonucleolytically followed by 5'-3' or 3'-5' degradation of the resulting mRNA fragments.

Deadenylation

The first step in degradation of many yeast mRNAs is deadenylation, in which the poly(A) tail is shortened to ~10 nucleotides.
This also holds true for mammalian cells where the decay of most mRNAs is initiated by shortening of the poly(A) tail. Efficient deadenylation is mediated through an interaction between the deadenylase PARN and the 5'cap. Following

deadenylation, the body of the transcript decays rapidly (Tucker and Parker, 2000) (Paillard and Osborne, 2003). Two distinct decay pathways have been described to follow deadenylation: The 5'-3' pathway and the 3'-5' decay pathway.

5'-3' decay pathway

In Saccharomyces cerevisiae, the 5'-3' decay pathway is the major mRNA degradation pathway. Removal of the 5' cap structure by the Dcp1p decapping enzyme occurs after poly(A) shortening. Following decapping, the transcript is rapidly degraded by the Xrn1p 5'-3' exonuclease (Heikkinen et al., 2003).
Decapping of the 5' end is a prerequisite for 5'-3' degradation to occur. In mammalian cells however, only indirect evidence suggests that decapping follows poly(A) shortening. The isolation of a murine homologue of the yeast 5'-3' exoribonuclease, Xrn1p, provides some support that at least some mammalian decay pathways involve 5'-3' decay (Lejeune et al., 2003).

3'-5' decay pathway

In contrast to yeast, the 3'-5' pathway seems to be the major degradation pathway in mammalian cells. This pathway is driven by the exosome, which is present in both the nucleus and the cytoplasm. The exosome is composed of at least 10 subunits, all of which are putative 3'-5' exoribonucleases and/or RNA binding proteins and it is responsible for the exonucleolytic degradation of the RNA body in the cytoplasm. However, it does not appear to be required for poly(A) tail shortening (Chen et al., 2001) (van Hoof and Parker, 2002).
The nuclear exosome, which includes an additional component Rrp6p, is involved in processing of small nuclear and nucleolar RNAs, ribosomal RNAs and degradation of unspliced pre-mRNAs (for review: Butler, 2002).

Endonucleolytic cleavage

Degradation of mRNA by endonucleolytic cleavage is a specialised mRNA degradation pathway occurring in particular mRNAs like the transferrin receptor and insulin-like growth factor 2. Following endonucleolytic cleavage, the resulting 5'-fragment is targeted for 3'-5' degradation activity, while the 3'-fragment can then be a substrate for 5'-3' decay activity (reviewed from Schoenberg and Cunningham, 1999).

2.3. mRNA stability regulation determinants in cis (ARE, CRD and CDE)

The stability of many mRNAs is determined by specific cis-elements as a means of controlling the expression of these genes.
So far three such elements have been identified, the adenosine-uridine (AU)-rich element (ARE) (Chen and Shyu, 1995), the coding region determinant 1 and 2 (CRD-1 and –2) (Schiavi et al., 1994) and the constitutive decay element (CDE) (Stoecklin et al., 2003b). The ARE is the most important cis-determinant, and can be accompanied in some cases by the other two determinants.
AREs play a major role in the regulation of mRNA decay, by inducing decapping, deadenylation and degradation of the mRNA body (Gao et al., 2001) (Wilusz et al., 2001) (Chen and Shyu, 1995) (Chen et al., 2001). They are located in the 3'UTR of mRNAs coding for cytokines, growth factors, proto-oncogenes and others (Chen and Shyu, 1995). The ARE-database (ARED: http://rc.kfshrc.edu.sa/ared) suggests that 5-8% of all human mRNAs contain an ARE (Bakheet et al., 2003). They are involved in many transient biological processes such as cell growth and differentiation, signal transduction, transcriptional and translational control, hematopoesis, apoptosis and nutrient transport (Bakheet et al., 2001).
The ARE sequence requirements are only loosely conserved, but are grouped into three classes by Chen and colleagues according to sequence features and on functional criteria depending on the pattern of ARE-induced degradation of the poly(A) tail.

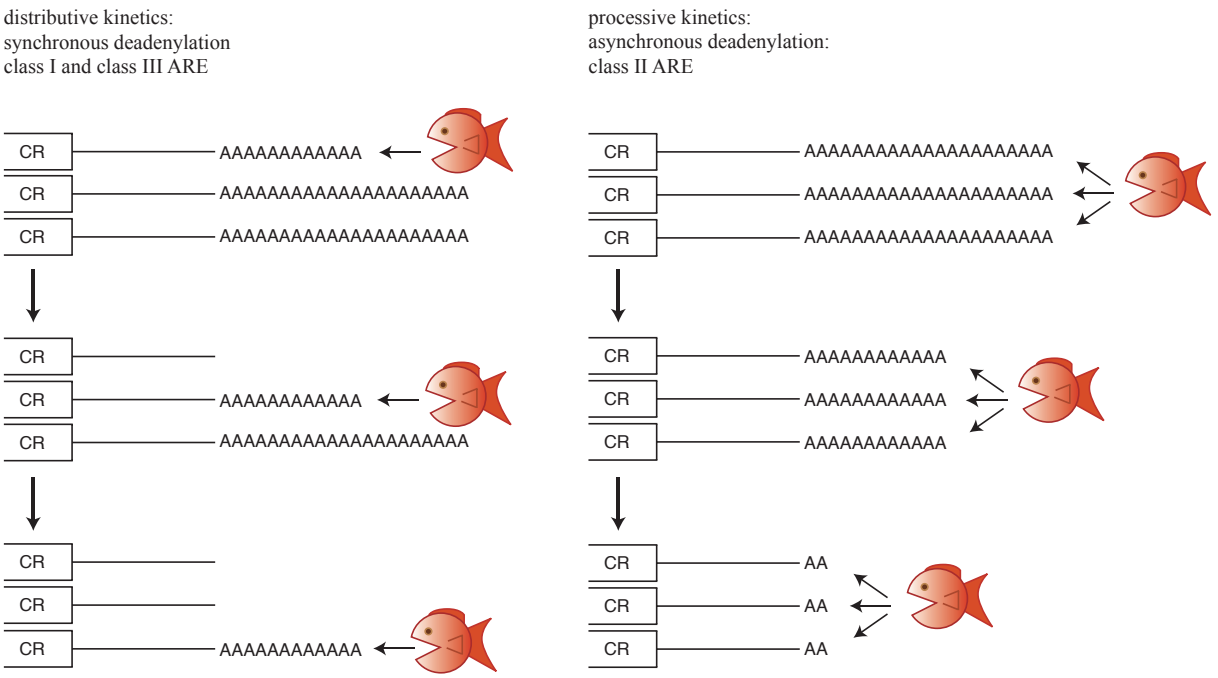


Figure 1: Kinetics of deadenylation, dependent on the ARE present in the 3'UTR, according to Xu et al., 1997.

The sequence features of class I AREs are 1-3 scattered AUUUA motifs in an U-rich context. Class II AREs are composed of multiple, partially overlapping AUUUA motifs and are flanked by an AU-rich 5' region. The UUAUUUA(U/A)(U/A) nonamer has been proposed to be the minimal motif responsible for the destabilising activity of class II AREs. Class III AREs are defined as non-AUUUA ARE, which is composed of an U-rich sequence (Chen and Shyu, 1995) (Peng et al., 1996).
Class I and III AREs show distributive deadenylation kinetics. The poly(A) shortening occurs synchronously, which can be interpreted as the activity of a distributive ribonuclease. Class II ARE in contrast, undergo asynchronous poly(A) shortening, which probably reflects the activity of a processive ribonuclease as shown in Figure 1 (Chen and Shyu, 1995) (Xu et al., 1997).
In Table 1, some examples of AREs are listed and classified according to the three groups.

class	gene	ARE	other examples
I	hu c-myc	cauaaaagaacuuuuuuaugcuuaccaucuuuuuuuuuucuu uaacagauuugu**auuua**agaauuguuuuuaaaaaauuuuaag **auuua**cacaauguuucucuguaaaauauugccauuaaaauguaa auaacuuuaau	c-fos
II	mu IL-3	auuuuauuccauuaaggcu**auuuauuua**ugu**auuua**ugu**a uuuauuuauuua**uu	TNFα, cox-2, IL-2, VEGF, GM-CSF
III	hu c-jun	uuucguuaacuguguauguacauauauauauuuuuuaauuug auuaaagcugauuacugugaauaaacagcuucaugccuuugua aguuauuucuuguuuguuuguuuggguauccugcccagugu uguuuguaaauaagagauuuggagca	Pitx2

Table 1: AU-rich elements classified according to Peng et al., (1996)

Another classification was proposed by Bakheet et al. A computationally derived 13-bp ARE motif (WWWUAUUUAUWWW; W=A or U) was the basis of the ARE-mRNA database (Bakheet et al., 2001) (Bakheet et al., 2003). In constructing this database, ARE containing mRNAs were clustered into five groups (see Table 2), thereby accounting only for class I and II AREs (according to the classification of Chen and Shyu). Group I (according to Bakheet et al.) contains many secreted proteins that affect the growth of haematopoietic and immune cells e.g. GM-CSF, IL-1, IL-11 and IL-12 but groups II-V contain functionally diverse gene families.

Group	Motif	Examples
I	AUUUAUUUAUUUAUUUAUUUA	TNFα, GM-CSF
II	AUUUAUUUAUUUAUUUA	Interferon-α
III	WAUUUAUUUAUUUAW	IL-3, cox-2, IL-2, VEGF
IV	WWAUUUAUUUAWW	FGF2
V	WWWWAUUUAWWWW	u-PA receptor

Table 2: AU-rich elements classified according to Bakheet et al., 2001.

AU-rich elements are not only regulators of the mRNA stability per se, but they also have been shown to be potent stimulators of the decapping-activity in HeLa cytoplasmic extracts (Gao et al., 2001). In yeast, ARE-containing transcripts were shown to undergo degradation through a rapid deadenylation-decapping mechanism, although it is not completely clear whether decapping is a direct consequence of the ARE or whether it is indirectly stimulated by deadenylation (Wilusz et al., 2001). Another function of the ARE is translational control (Zhang et al., 2002). In unstimulated macrophages, the class II ARE containing TNFα mRNA is translationally repressed, but upon cell activation efficient translation of TNFα mRNA is induced. This mechanism acts through 2 proteins binding the ARE: T-cell intracellular antigen-1 (TIA-1) and TIA-1 related protein (TIAR), which are claimed to act as TNFα mRNA translational silencers (compare also page 16) (Gueydan et al., 1999) (Piecyk et al., 2000). In addition the expression of cyclo-oxygenase-2 (COX-2) seems to be regulated by TIA-1, as colon cancer cells that over-express COX-2 through increased polysome association with COX-2 mRNA also showed defective TIA-1 binding. This result together with others indicate that TIA-1 functions as a translational silencer of COX-2 expression (Dixon et al., 2003). The ARE can also be combined with other cis-elements regulating mRNA stability, e.g. CRD-1 and CRD–2 on c-fos mRNA or CDE on TNFα mRNA (Grosset et al., 2000) (Stoecklin et al., 2003b).

CRD-1 and CRD–2 are two mRNA destabilising elements, of which CRD-1 is the major coding region determinant (mCRD). The mCRD, located in the coding region of the mRNA of fos, is recognised as a signal in the mRNA sequence during on-going translation as ribosome transit is required to activate this signal. For recognition of the mCRD it is important that there is a minimal distance of ~450nt between the mCRD and the poly(A) tail. The mCRD is not only involved in destabilising the mRNA but in addition it also directs accelerated deadenylation,

which precedes degradation of the RNA body (Grosset et al., 2000).
Very recently a novel mRNA decay determinant, the constitutive decay element (CDE), has been described by Stoecklin et al. In contrast to the two CRDs it is located like the ARE in the 3'UTR of the mRNA. The CDE has been found on the mRNA of TNFα, where it triggers constitutive rapid decay. The CDE is composed of a 80nt long fragment containing a highly conserved sequence of 15nt, which is necessary but not sufficient for CDE mediated decay (Stoecklin et al., 2003b).

2.4. Trans-acting regulating proteins of the ARE

Since the discovery of AREs, much work has focused on the identification and characterisation of cellular trans-acting factors or ARE-binding proteins (AUBPs) (Guhaniyogi and Brewer, 2001).
AUBPs have been shown to influence the decay and translation rate of ARE-mRNAs through binding to the ARE and are therefore very important in understanding the regulation of ARE-mRNA protein expression. AUBPs can target the ARE-mRNA to the exosome and even one of the exosomal proteins (PM-Scl75) is itself an AUBP, but they do not have any intrinsic endo- or exoribonuclease activity (Mukherjee et al., 2002). A balance model has been proposed by Ming et al., that suggests that the equilibrium between stabilising and destabilising AUBPs bound to the ARE will decide the fate of the ARE-mRNA (Ming et al., 2001).
In the following discussion AUBP families and individual AU-binding proteins will be presented with emphasis on BRF1 as this AUBP was the main topic of the work presented here. Table 3 at the end of this chapter gives a summary of the AUBPs.

2.4.1. hnRNP family

The primary protein-coding transcripts that are produced by RNA polymerase II are termed pre-mRNA (or, using the historical term that describes their size heterogeneity and cellular location, heterogeneous nuclear RNAs; hnRNAs), which are highly associated with proteins. The collective term for the proteins that bind hnRNAs, and which are not stable components of other classes of RNA binding protein such as snRNPs, are hnRNP proteins.
The most detailed picture of the protein composition of hnRNP complexes is for human cells. At least 20 major proteins have been identified and are designated from A0 (32 kDa) to U (120 kDa). Most if not all hnRNP proteins contain one or more RNA-binding motifs. The most common of these are the RRM (RNA-recognition motif), KH-domains and RGG (Arg-Gly-Gly) boxes (Myer and Steitz, 1995) (Dreyfuss et al., 2002).

2.4.1.1. hnRNP D (AUF1)

AUF1, first identified by its ability to bind c-myc ARE in vitro (Zhang et al., 1993) is one of the proteins encoded by the hnRNP D gene. There are four AUF1 isoforms with molecular masses of 37, 40, 42, and 45kDa, which are generated by alternative splicing. AUF1 proteins have been shown to stabilise or destabilise ARE-mRNAs depending on the cell-line investigated (Wilson and Brewer, 1999) (Chen et al., 2002). Recent work from our lab suggests, that in HT1080 cells the two isoforms p37 and p42 stabilise ARE-mRNA, while the p40 isoform leads to decay (Raineri et al., 2004). However in an in vitro decay system p37 AUF1 was found to induce degradation of ARE-mRNAs correlating with the binding-affinity of AUF1 to the ARE (DeMaria and Brewer, 1996).
ARE binding is mediated by two RNA Recognition Motifs (RRM), but AUF1 probably also interacts with other proteins via the glycine-rich C-terminus domain (Ehrenman et al., 1994).
Current evidence indicates that AUF1 binds and oligomerises on ARE substrates, and thereby locally remodels RNA structure. Interestingly, phosphorylation of p40 AUF1 does not change the binding affinity to the ARE, but inhibits the RNA structural transition (Wilson et al., 2003a) (Wilson et al., 2003b). In addition, there are indications that AUF1 may also be involved in general mRNA turnover. As a part of a complex binding the alpha-globin mRNA it mediates the unusual stability of this mRNA through a pyrimidine rich sequence in the 3'UTR (Kiledjian et al., 1997).
Cytoplasmic AUF1 is present in a multi-subunit complex containing other factors involved in the regulation of mRNA decay and translation, including the translation initiation factor eIF4G, poly(A)-binding protein, the heat shock proteins Hsp70 and Hsc70, and lactate dehydrogenase. In such a complex interplay AUF1 is targeted by ubiquitinylation and degraded, thereby linking turnover regulation of an mRNA containing the GM-CSF ARE to the ubiquitin-dependent proteolytic pathway (Laroia et al., 1999).

2.4.1.2. hnRNP A0

In human cells, hnRNP A0 is encoded by two mRNAs of 1.7 and 2.8kb that appear to differ only in usage of a polyadenylation site. The two forms are present at different levels in different tissue types, suggesting tissue-specific regulation of polyadenylation but it cannot be excluded that the two mRNAs are transcribed from separate genes encoding hnRNP A0 (Myer and Steitz, 1995). hnRNP A0 is characterised by a 1xRBD-Gly primary structure, which is an RNA-binding domain (RBD) flanked by a glycine-rich auxiliary sequence (Myer and Steitz, 1995). Rousseau et al., were able to show that TNFα, Cox-2 and MIP-2 mRNA,

which all contain an ARE, bind specifically to hnRNP A0. However they did not investigate if this ARE-dependent binding was involved in ARE-mRNA stability (Rousseau et al., 2002).

2.4.2. Tis11 Family

The typical features of members of the tetradecanoyl phorbol acetate (TPA)-inducible sequence 11 (Tis11) family are two very conserved CCCH zinc finger domains, which are characterised by a strict spacing of $CX_8CX_5CX_3$. The zinc-finger is preceded by a conserved YKTEL sequence and is responsible for ARE binding. The Tis11 family consists of TTP, BRF1, BRF2 and their homologues in different species. These proteins are nucleocytoplasmic shuttling proteins and rely on the nuclear export receptor CRM1 for their export from the nucleus (Phillips et al., 2002). All are induced rapidly and transiently in response to extracellular hormone and growth factor signals and promote destabilisation of the ARE-containing TNFα, GM-CSF and IL-3 mRNA in co-transfection experiments (Lai et al., 2000).

2.4.2.1. BRF1

BRF1 (Butyrate Response Factor 1), also called Tis11b, ZFP36L1, Berg36, cMG1 or ERF1, is the AUBP that was investigated in this study as an extension of work previously performed in the laboratory by Stoecklin et al. (Stoecklin et al., 2002). This work resulted in the identification of BRF1 via a functional cloning strategy as an ARE-mRNA decay promoting AUBP. The functional cloning strategy was based on the rationale of visualising changes in mRNA stability as a fluorescent signal to allow selection of mutants defective in mRNA decay and a subsequent gene library screen to identify ARE-mRNA decay promoting genes.
The cloning strategy is briefly summarised as follows: First, a GFP reporter fused to the IL-3 3'UTR containing the ARE was stably expressed in the diploid HT1080 cell line. The ARE in the reporter-construct promotes rapid reporter mRNA decay and therefore low GFP expression. In a second step, this reporter cell line, called HT1080-GFP-IL-3, was subjected to multiple rounds of mutagenesis using the frame-shift inducing compound ICR191 with the aim of knocking out putative ARE-mRNA decay promoting genes which would then result in an increase of GFP expression. Clones over-expressing the GFP reporter were identified, isolated by FACS (fluorescence activated cell sorting) and checked for stabilised reporter mRNA. Three mutant cell lines (slowA, slowB and slowC) were identified (Stoecklin et al., 2001), of which the phenotype of slowC was shown to be due to mutation of both alleles of BRF1. This was performed by rescuing the phenotype of slowC by transfecting a retroviral cDNA library and subsequently identifying the introduced cDNA. In further experiments Stoecklin et al., were

able to confirm the function of BRF1 as an ARE-mRNA decay promoting AUBP (Stoecklin et al., 2002). Subsequently it was shown by Lai et al., that BRF1 stimulates deadenylation in a cell free system, which is the first step leading to mRNA degradation (Lai et al., 2003).

BRF1 was originally cloned from an epithelial rat cell line. The human homologue is located on chromosome 14q22-24 (Maclean et al., 1995) and contains a single intron (~2.5kb) with a long 3'UTR of 1861bp. The 3' UTR contains a single UUAUUUAU motif, which may represent a weak ARE (Bustin et al., 1994), but the functionality of this ARE has yet to be tested. Human BRF1 has a length of 338 amino acids with a predicted weight of 36kDa (Bustin et al., 1994). It is predominantly cytoplasmic, but contains a NES (nuclear export signal) in its C-terminus. Therefore it is not surprising that BRF1 exhibits CRM1 (chromosome region maintenance 1) dependent nucleo-cytoplasmic shuttling (Phillips et al., 2002).

The physiological function of BRF1 is not known but the work of several groups show an involvement of BRF1 in diverse and even contradictory aspects of cell-biology. The most direct approach to assess the physiological role of BRF1 is to generate BRF1 knock-out mice. These knock-out mice are embryonic lethal pointing to the importance of BRF1 (Blackshear, personal communication).

BRF1 has been shown to induce apoptosis via the mitochondrial/caspase9-pathway (Johnson et al., 2000). Apoptosis due to continuous expression of BRF1 at physiological levels has no synergistic effect on TNFα induced apoptosis, in contrast to the related TTP where such a synergistic effect has been observed (Johnson and Blackwell, 2002). Furthermore there are also indications that BRF1 is required for apoptosis in response to treatment with calcium ionophore in a human B-cell line, as shown by an antisense inhibition of BRF1 (Ning et al., 1996).

In contradiction to its apoptosis inducing function, BRF1 has been proposed to support cell transformation as the work of Shimada et al. show. The AML1-MTG8 fusion transcription factor generated by a leukemogenic t(8:21) translocation is thought to deregulate a set of genes that are crucial for normal differentiation and proliferation of haematopoietic progenitors to cause acute myelogenous leukaemia (AML). One of these genes turned out to be BRF1 which was highly expressed in t(8:21) leukaemic cells, and the over-expression of BRF1 induced myeloid cell proliferation in response to granulocyte colony-stimulating factor. These results suggest that high-level expression of BRF1 contributes to AML1-MTG8-mediated leukaemogenesis (Shimada et al., 2000).

In another cancer model system for human colorectal cancer, the regulation of BRF1 expression was tested. Butyrate, a fermentation product of dietary fibres that affects colorectal cancer proliferation, has different effects on the transcription of the three human TIS11 family members in T84 and HT-29 human colorectal cancer cell lines. Butyrate response factor 1 (BRF1) transcription was repressed, with altered mRNA levels detectable within 15 minutes of butyrate addition

(Maclean et al., 1998).
In contrast to butyrate, ACTH, a trophic hormone from the adrenal cortex strongly and rapidly induces BRF1 expression in adenocortical glandular cells. Shortly after induction of vascular endothelial growth factor (VEGF) expression by ACTH, BRF1 was induced and remained elevated when VEGF mRNA levels started to drop probably due to a functional interaction between BRF1 and the 3'UTR of VEGF which contains an ARE (Chinn et al., 2002).
Insulin-like growth factor 1 (IGF-1) and insulin are two other compounds inducing BRF1 expression as has been shown in RIE-1 cells (rat intestinal epithelial-1 cells). The PI3-K pathway appears to be involved in the regulation of BRF1 expression by insulin and IGF-1 as incubation of RIE-1 cells with wortmannin, a specific PI3-K inhibitor, prevented the insulin/IGF-1 induced elevation of BRF1 mRNA (Corps and Brown, 1995).
The work of two different research groups show an interaction between BRF1 and 14-3-3. 14-3-3 is a scaffold protein that participates in diverse regulatory processes by binding to phosphorylated amino acids, suggesting that 14-3-3 might be involved in BRF1 regulation. In a co-immunoprecipitation with lysates of 293T cells co-expressing HA-14-3-3β along with GFP-BRF1, it was shown that 14-3-3β binds to GFP-BRF1 (Johnson et al., 2002), and Bustin et al. showed that 14-3-3β and τ strongly bind BRF1 in a yeast two hybrid system (Bustin and McKay, 1999).
In summary, BRF1 has been shown to be an AUBP inducing decay. Various stimuli such as butyrate, ACTH and Insulin regulate BRF1 expression and it seems to be involved in certain types of cancer and also in apoptosis. So far it is still not clear if BRF1 is directly involved in these different functions, or if these functions are all due to the activity of BRF1 to induce decay of different sets of mRNAs generating these diverse responses.

2.4.2.2. Tristetraprolin (TTP)

TTP (also named ZFP36, NuP475, Tis11, or Gos24) was originally identified through genetic screens designed to find immediate early response genes (Gomperts et al., 1990). The name tristetraprolin or TTP derives from the presence of three tetraproline amino acid repeats that are conserved across species. TTP, which is unusually proline-rich and has a predicted molecular weight of 33.6kDa, binds to the ARE of TNFα, thereby destabilising the TNFα message (Lai et al., 1999) (Blackshear et al., 2003). Other studies have also suggested an interaction between TTP and both IL-3 and GM-CSF via the ARE in their mRNA (Lai et al., 2002) (Stoecklin et al., 2003a). Binding of TTP to the ARE-mRNA is dependent on the integrity of both zinc-fingers. Mutation of a single cystein residue to arginine in either of the zinc-finger mutants strongly reduced binding to the TNFα-ARE. By binding to the ARE, TTP initiates deadenylation and degradation of its target as could be shown by Lai et al. (Lai et al., 1999). TTP knock-out mice show a

pronounced pro-inflammatory phenotype, which include cachexia, dermatitis, medullary and extramedullary myeloid hyperplasia, erosive polyarthritis and autoimmunity with auto-antibodies. Mice that over-express TNFα show a similar phenotype (Taylor et al., 1996), leading to the finding that TTP deficient mice were over-producing TNFα, as the TNFα mRNA was no longer rapidly degraded. The pro-inflammatory phenotype could be reversed by weekly injections of a neutralising TNFα antibody. In addition, mice with the double deletion of TTP and TNF receptor (TNFR)-1 do not manifest the aggressive inflammation observed in TTP-deficient mice (Carballo and Blackshear, 2001).
TTP is a nuclear-cytoplasmic shuttling protein. Binding of TTP to 14-3-3 favours the cytoplasmic localisation of TTP. 14-3-3 is a scaffold protein that has previously been shown to promote cytoplasmic localisation of proteins, e.g. FKHRL1. Binding to 14-3-3 is phosphorylation-dependent and in TTP it has been shown to be dependent on phosphorylation at serine 178. This site is also conserved in the other two members of the TIS11-family (Johnson et al., 2002).

2.4.3. TIA-1, TIAR

TIA-1 (T-cell intracellular antigen-1) and/or TIAR (TIA-1-related protein) bind to class II AREs such as TNFα and GM-CSF. The importance of TIA-1 binding to TNFα ARE was revealed in macrophages derived from TIA1$^{-/-}$ mice. Significantly more TNFα protein was produced in the TIA$^{-/-}$ macrophages compared to wt controls due to translational suppression of TNFα. TIA-1 translational silencing seems to act specifically on TNFα mRNA as the translation of GM-CSF and Interferon-γ mRNAs does not appear to be influenced by TIA-1. This could be explained by the possibility that only TIAR binds to the GM-CSF ARE. The role of TIAR in the regulation of TNFα mRNA translation has yet to be clarified.
TIA-1 and TIAR are closely related members of the RNA-recognition motif (RRM) family. They have three RRM domains in their N-termini which bind to uridine-rich motifs of the RNA (Tian et al,. 1991; Kawakami et al., 1992, 1994; Beck et al., 1996; Dember et al., 1996). TIA-1 and TIAR are expressed as two isoforms that result from alternative splicing of their pre-mRNAs. They are multifunctional regulatory proteins that shuttle between the nucleus and the cytoplasm. TIA-1 and TIAR are not only involved in translational silencing of TNFα but also in alternative splicing, for instance of their own mRNA. Moreover they regulate general translational arrest by inducing stress granules in the cytoplasm under environmental stress conditions (for review: Zhang et al., 2002; Kedersha and Anderson, 2002).

2.4.4. CUGBP2

CUGBP2 (CUG binding Protein 2) is an RNA binding protein that contains three nonidentical RNA recognition motifs (RRM) and which binds to the ARE of cox-2 (Mukhopadhyay et al., 2003). When subjected to radiation-mediated apoptosis, CUGBP2 expression is induced in epithelial cells. At the same time cox-2 mRNA levels are increased, but the message is not translated. Recently, Mukhopadhyay et al., provided some evidence that CUGBP2 binding to cox-2 stabilised cox-2-mRNA but paradoxically inhibited its translation. Upon antisense-mediated downregulation of CUGBP2 expression, cox-2 expression increased and apoptosis decreased. These findings suggest a function for CUGBP2 as a translational inhibitor and an ARE-mRNA stabiliser, thereby contolling apoptosis after exposure to radiation (Mukhopadhyay et al., 2003).

2.4.5. KSRP

The K-homology splicing regulatory protein (KSRP) also called FUSE binding protein 2 (FBP2) is a 78kDa AUBP with a K-type RNA binding protein motif that consists of 4 KH-domains. It was discovered as a splicing regulator of the c-src N1 exon (Min et al., 1997).
Consistent with its strong ARE-mRNA decay inducing activity, KSRP has further been shown to interact with the ARE and the exosome in vivo and in vitro, suggesting an important role in targeting ARE-mRNA to the exosome for degradation. The binding activity of KSRP to Pitx2 mRNA is crucial for the induction of Pitx2 degradation. Upon induction of the Wnt-pathway the in vivo and in vitro binding to Pitx2 mRNA is strongly decreased leading to stabilisation of Pitx2 mRNA. The changes in AUBPs/mRNA interaction reflect Wnt activation dependent changes in the cytoplasmic levels of the same AUBPs (Briata et al., 2003).

2.4.6. Hu-family

HuB, HuC, HuD and HuR (alternatively named HuA) are the four members of the Hu-family. The Elav-like proteins, as the Hu-family members are also called, exhibit strong sequence and structural similarities and contain three RRMs, allowing specific binding to AREs. It has been shown by several laboratories that Hu-proteins exert a stabilising influence on ARE-containing mRNAs, thereby upregulating their protein expression. In contrast to the other members, which are neuron-specific, HuR is ubiquituosly expressed (for review: Keene, 1999; Brennan and Steitz, 2001).

2.4.6.1. HuR

HuR has been identified as a trans-acting factor that stabilises ARE-containing mRNA, which is consistent with the finding that expression of antisense RNA to HuR tends to increase the decay of ARE-mRNAs (Levy et al., 1998). Several studies demonstrate the physiological significance of HuR in cell growth and differentiation (Van Der Giessen et al., 2003) (Brennan and Steitz, 2001). HuR contains a nuclear-cytoplasmic shuttling sequence which allows this mostly nuclear protein to redistribute to the cytoplasm (Atasoy et al., 1998). De Silanes et al. propose that HuR-regulated target mRNA expression contributes to colon cancer growth (de Silanes et al., 2003). So far it is the only AUBP which is generally accepted to have an ARE stabilising function (Brennan and Steitz, 2001), although indirect evidence suggests hnRNP A0 may also possess stabilising properties (Rousseau et al., 2002).

2.4.7. PM-Scl75

PM-Scl75 is one of the components of the human exosome and shows homology to the RNase PH exonuclease of Escherichia coli. Mukherjee et al. were able to show that PM-Scl75 is an ARE-binding protein, although it does not contain any homology to known RNA-binding domains that would explain its sequence-specific binding with AREs. PM-Scl75 is not only found in the nucleus but also in the cytoplasm, consistent with a role for the protein in cytoplasmic mRNA turnover. While PM-Scl75 protein can target itself directly to specific RNA substrates that contain AREs, its activity is possibly regulated by additional protein-protein interactions, since TTP and KSRP are crucial for the exosomal activity on ARE-mRNAs (Mukherjee et al., 2002).

Family	AUBP	Synonyms	Function on ARE-mRNA
hnRNP family	AUF1	hnRNP D	stabilisation/decay
	hnRNA A0		?
Tis11 family	TTP	NuP475, Tis11, ZFP36, Gos24	decay
	BRF1	Tis11b, ZFP36L1, Berg36, ERF1	decay
	BRF2	Tis11d, ZFP36L2	decay
	TIA-1		translational inhibition
	TIAR		translational inhibition
	CUGBP2		stabilisation, translational inhibition
	KSRP	FBP2	decay
Hu family	HuR	HuA	stabilisation
	HuB		stabilisation
	HuC		stabilisation
	HuD		stabilisation
	PM-Scl75		loading the exosome onto ARE-mRNA?

Table 3: Summary of the AUBPs

2.5. Regulation of ARE-mRNA stability by signal transduction pathways

Post-transcriptional regulation of gene expression is a very rapid process as only the steady state levels of the concerned mRNA needs to be changed either by stabilisation or degradation of the mRNA in response to external stimuli. The intrinsic rapid decay of ARE-mRNA in resting cells allows for stabilisation under appropriate conditions and thereby regulation of the protein expression. Stabilisation, e.g. in activated cells, or more generally post-transcriptional regulation of ARE-mRNA turnover is regulated by signal transduction pathways. Upon an incoming signal, signalling cascades are activated by sequential phosphorylation of one pathway member to the next. This gives the possibility of modifying the signalling cascade at every step, allowing the integration of a variety of signals to a final coordinated response. For example stabilisation of GM-CSF is controlled by two pathways induced by PMA and a Ca^{2+}-ionophore, whereas, in the same cell line post-transcriptional regulation of IL-3 is regulated by the Ca^{2+}-ionophore induced signalling alone, showing a specific and diverse mode of regulation for different ARE-mRNAs (Wodnar-Filipowicz and Moroni, 1990).
In the next chapter, the signalling pathways which are relevant for this work will be presented. The following chapter expands then on the link between the signalling pathways and the regulation of ARE-mRNA stability.

2.5.1. Cellular signalling pathways

Figure 2 gives an overview of the signalling proteins presented below and show how they are interconnected and what cellular functions the pathways are involved in.

2.5.1.1. Ras

Ras is the prototype of a large superfamily of small, membrane bound, monomeric GTPases also known as small G-proteins that contain 2 subfamilies: (1) the rho and rac proteins, involved in relaying signals from cell-surface receptors to the actin cytoskeleton and (2) the rab family, involved in regulating the traffic of intracellular transport vesicles. The upstream activators of ras are the receptor tyrosine kinases (RTK) and G-protein coupled receptors. Ras is coupled to RTKs by a short series of linking proteins, a SH2-containing adaptor protein (Grb2) that is activated by the RTK, and a ras-activating protein mSos (the mammalian equivalent of ‘son of sevenless’, a protein involved in photoreceptor development in Drosophila) that is activated and localised to the membrane by

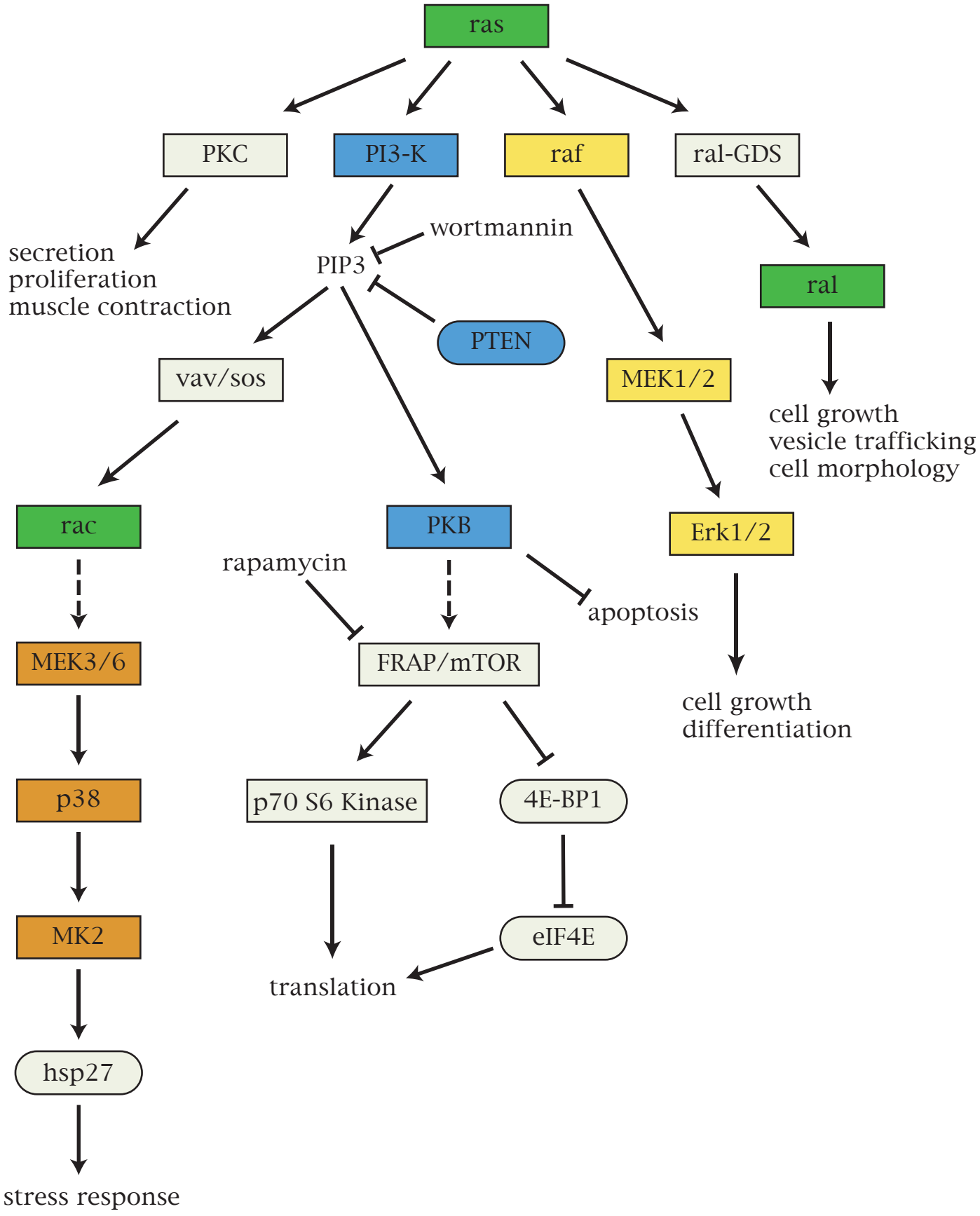


Figure 2: Scheme of the ras downstream pathways which are important for the work presented here. The small G-proteins are represented in green, the PKB pathway in blue, the p38 pathway in orange and the raf pathway in yellow.

adaptor proteins such as Grb2 and Shc.
The active form of ras is bound to GTP and is inactivated by the hydrolysis of GTP to GDP. This process can be accelerated by GTPase activating proteins (GAPs) and therefore these inhibit ras activity. In contrast, guanine nucleotide dissociation inhibitors (GDIs) inhibit ras by blocking the replacement of GDP by GTP. On the other hand, ras activation can be promoted by guanine nucleotide exchange factors (GEFs). As the name implies, they accelerate the exchange of GDP with GTP leading directly to ras-activation. Ras activates either other small G-proteins via their GEFs (e.g. ral) or it directly activates kinases (e.g. PI3-K, raf or PKC).
Ras can stimulate very diverse and even contradictory biological responses like cell proliferation, growth arrest, senescence, differentiation, apoptosis and survival. Despite these diverse responses, overall it is considered to be an important oncogene. Mutations at the amino acids 12, 13, 59 and/or 61 are crucial for its oncogenicity as these sites are involved in the GTPase activity of ras. Mutations at these sites disable ras from hydrolising GTP and therefore force it into a constantly active state. Three distinct proto-oncogenes were identified: Ha-ras, K-ras and N-ras. Data from several groups suggest that the different ras isoforms might have preferential targets and possess distinct cellular functions. For reviews see (Oxford and Theodorescu, 2003a) (Oxford and Theodorescu, 2003b) and (Adjei, 2001) (Liebmann, 2001).

2.5.1.2. PKC

A variety of hormones, neurotransmitters and growth factors express their biological activity by stimulating phospholipase C-mediated hydrolysis of phosphoinositides, which in turn activate protein kinase C (PKC) in the presence of phospholipids and Ca^{2+}. PKCs are a family of serine/threonine kinases responsible for signal transduction in a multitude of intracellular responses arising from G-protein coupled receptors, receptors with tyrosine kinase activity and non-receptor tyrosine kinases. Activation of PKC is one of the earliest events in the cascade leading to numerous cellular processes such as secretion, gene expression, proliferation and muscle contraction. All PKCs contain an amino-terminal regulatory domain and a carboxylterminal catalytic domain. The PKC family comprises at least 10 isoforms with distinct means of regulation and tissue distribution patterns (for review: Gutcher et al., 2003).

2.5.1.3. PI3-K/PKB pathway

PI3-K

Phosphoinositide 3-kinases (PI3-Ks) are a family of lipid kinases that are involved in a variety of cellular responses, including cell growth, survival, metabolism, differentiation, cytoskeletal organisation and membrane trafficking (Leevers et al., 1999). PI-3K is activated by tyrosine kinase- and G-protein-coupled receptors, for example after insulin treatment of cells, and activates the PKB pathway (Figure 3), which leads to induction of translation and suppression of apoptosis.
PI3-K consists of two subunits, a catalytic p110 subunit, and a regulatory and localising p85 subunit. p110 catalyses phosphorylation of inositol phospholipids (PIP2: phosphatidylinositol 4,5 bis-phosphate) in the plasma membrane on the 3-OH group generating the second messenger phosphatidylinositol-3, 4, 5-tris-phosphate. Some studies have suggested that in cells expressing mutant oncogenic H-ras the p110 subunit of PI3-K can directly bind to Ras-GTP leading to catalytic activation of the kinase (Cantrell, 2001).
PI3-K activates PKB, a protein kinase inducing translation and inhibiting apoptosis, via PIP3. PIP3 levels themselves are tightly regulated by the action of phosphatases such as PTEN and SHIP (Djordjevic and Driscoll, 2002).

PKB/Akt

PKB was cloned in 1991 as the cellular homolog to the retroviral oncogene viral akt (v-Akt) (Bellacosa et al., 1991) and encodes a protein kinase with some similarities to protein kinase C (PKC) and protein kinase A (PKA). Its relatedness to PKA and PKC led to it being named PKB. To date, three members of the family have been isolated and are referred to as PKBα (or Akt1), PKBβ (Akt2), and PKBγ (Akt3). Each isoform possesses an N-terminal pleckstrin homology (PH) domain of approximately 100 amino acids. Through binding of the PH domain to PIP3, PKB is targeted to the membrane to alter its conformation to allow subsequent phosphorylation by the phosphoinositide dependent kinase-1 (PDK1). PDK1 is a 63 kDa serine/threonine kinase containing a C-terminal PH domain that binds with high affinity to PIP3. The PH domain of PKB is followed by the kinase catalytic domain. This region contains a threonine residue (T308 in PKB/Akt1) whose phosphorylation by PDK1 is necessary for activation of PKB/Akt. Following the kinase domain, there is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site (S473 in PKB/Akt1). Although phosphorylation at T308 partially activates PKB/Akt, full activation of PKB/Akt requires phosphorylation on this second site (S473 in PKB/Akt1). The mechanism mediating S473 phosphorylation remains controversial. Modification of S473 has been shown to occur through autophosphorylation, but other findings

Substrate	Effect
Prosurvival	
ASK1	Inhibition of stress-activated kinases
BAD	Association of BAD with 14-3-3 proteins Suppression of BAD-induced cell death
CREB	Increased transcription of CREB-regulated survival genes
Forkhead family	Promotes nuclear exclusion Association with 14-3-3 proteins Prevention of transcription of pro-apoptotic genes
Iκ-B kinase	Induction of NF-κB transcriptional activity
Procaspase-9	Suppression of caspase-9-induced cell death
Cell cycle progression	
GSK-3-α, -β	Inhibition of GSK-3 catalytic activity
mTOR/FRAP	Modulation of mRNA translation?
p21 WAF1	Cell cycle progression
Others	
AR	Decreased transcription of AR-regulated genes Modulation of AR-mediated apoptosis
BRCA1	Nucleocytoplasmic localisation?
ER-α	Increased transcription of ER-regulated genes
eNOS	Activation of eNOS Production of nitric oxide
Nur77	Reduced transcriptional activity
c-Raf	Inhibition of c-Raf signalling
B-Raf	Inhibition of B-Raf activity
Telomerase reverse transcriptase subunit	Enhanced telomerase activity

Table 4: PKB substrates according to the review from Nicholson and Anderson, 2001 (Nicholson and Anderson, 2002).

suggest that S473 is modified by a distinct kinase activity that was named PDK2 but remains to be cloned (Nicholson and Anderson, 2002).
Artificial membrane targeting of PKB/Akt (accomplished, for example, by the fusion of an amino-terminal myristylation signal) results in constitutive kinase activation. PKB/Akt has been implicated in a variety of cellular processes, including cell growth and proliferation, protection from apoptosis, regulation of gene expression by translational control via FRAP/mTOR (Brazil and Hemmings, 2001), and, as shown in this work, regulation of ARE-mRNA stability. Often phosphorylation by PKB results in binding of 14-3-3 to the PKB target, leading to its regulation (Tzivion and Avruch, 2002). To promote these multiple functions, PKB targets numerous downstream-proteins as shown in Table 4:

FRAP/mTOR

FRAP/mTOR (FKBP and Rapamycin-associated protein / mammalian target of rapamycin) has a mass of 289kDa and shares 45% identity with the yeast TOR proteins. Only one homologue is present in mammals and the human, rat and mouse FRAP/mTOR proteins share >95% identity at the amino acid level.
TOR appears to regulate growth by altering gene transcription and translation in response to nutrient changes. In the presence of amino acids TOR induces an upregulation of translation. In contrast, yeast and mammalian TOR responses to lack of amino acids leads to autophagy, a process of protein degradation in a

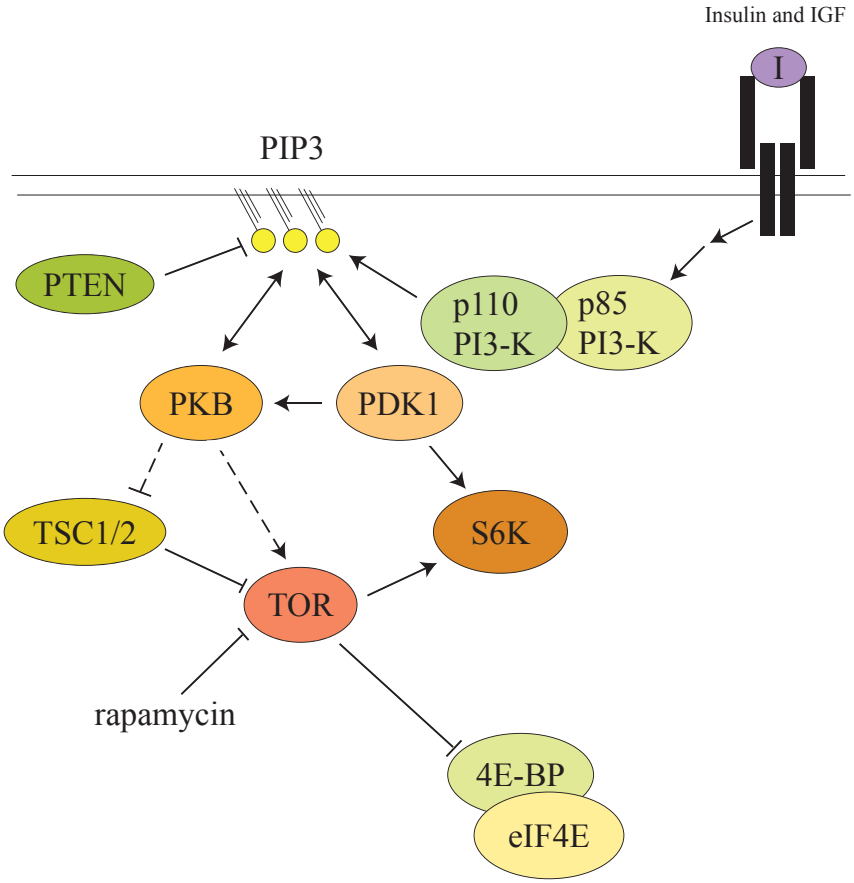


Figure 3: Scheme of the Insulin-PKB-TOR pathway according to Oldham and Hafen, 2003.

compartment called the autophagosome. In poor nitrogen and carbon conditions, yeast TOR also alters the metabolic state by partially regulating the activity of nutrient-dependent transcription factors and by changing the selectivity of amino acid transporters. Intracellular concentration of ATP has further been shown to influence the FRAP/mTOR pathway. The mechanism by which ATP acts on TOR is unknown, but it seems to be different from that used by amino acids as ATP and amino acids are required to activate FRAP/mTOR.
FRAP/mTOR is apparently activated by PKB, although more recent studies suggest that this activation occurs indirectly by inactivating TSC (tuberous sclerosis complex), which is an inhibitor of FRAP/mTOR. However, other studies indicate that FRAP/mTOR activation is independent of PKB (Radimerski et al., 2002) (Oldham et al., 2000). FRAP/mTOR on the other hand signals directly to 4E-BP and to S6K, two proteins involved in the regulation of translation, and is specifically inhibited by rapamycin (Figure 3) (Oldham and Hafen, 2003).
Translation of many mRNAs that respond to hormone and growth factor stimulation are regulated by FRAP/mTOR and are therefore extremely sensitive to rapamycin treatment. The best-studied example of rapamycin-sensitive mRNAs is the class possessing a 5'TOP (terminal oligo pyrimidine tract). Most 5'TOP-containing mRNAs code for components of the translation machinery, such as ribosomal proteins, elongation factors and the poly(A) binding protein.
Another category of rapamycin sensitive mRNAs are those presumed to require high amounts of eIF4F helicase activity, due to the presence of long structured 5'UTRs. Indeed the translation of c-myc, one such mRNA, is strongly inhibited by rapamycin.
Several other mRNAs whose translation is specifically affected by rapamycin (e.g. Bcl3 and Cyclin D1) have also been identified, but the mechanism mediating the rapamycin-sensitivity of these transcripts remains to be established (for reviews: (Gingras et al., 2001), (Crespo and Hall, 2002), (Oldham and Hafen, 2003)).

2.5.1.4. MAPK pathways

Following activation, ras stimulates a cascade of serine/threonine protein kinases, called the mitogen-activated protein kinases (MAPKs). These include mitogen-activated protein kinase kinase kinase (MAPKKK or MKKK; also called raf), which phosphorylates MAPK kinase (MAPKK or MKK; also called MEK), which in turn phosphorylates MAPK (Figure 4). MAPK then phosphorylates various gene regulatory proteins. This MAPK cascade is highly conserved amongst eukaryotic species (Liebmann, 2001).

The Erk-pathway

The Erk or p44/42 MAP kinase pathway is activated by ras, which in turn activates c-raf, MEK1/2 and Erk1/2.

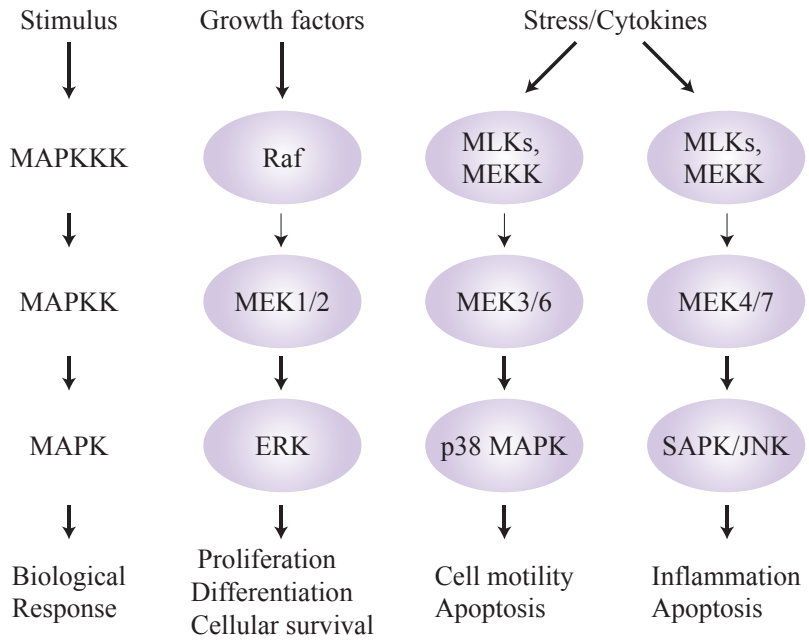


Figure 4: Scheme of the MAP kinase pathways according to Cowan and Storey, 2003.

The importance of this pathway in both growth control and development has been demonstrated via the transforming properties of various mutant forms of ras, raf, MEK and by their effects on development. The potential for cross talk and signal amplification appear to be important features for this regulatory pathway (Chang et al., 2003).
c-raf, a mitogen-activated protein kinase, is the main effector recruited by GTP-bound ras in order to activate the MAP kinase pathway. Binding of raf to 14-3-3 is required for c-raf kinase activity (Tzivion et al., 1998). PKB mediated phosphorylation has been shown in vivo to inhibit raf kinase activity (Zimmermann and Moelling, 1999).
MEK1 and MEK2, also called MAP or Erk kinases, are dual specificity protein kinases controlling cell growth and differentiation. Activation of MEK1 and MEK2 occurs through phosphorylation by raf. MEK1/2 is activated by a wide variety of growth factors, cytokines and also by membrane depolarisation and calcium influx. Constitutively active forms of MEK1/2 are sufficient for the transformation of NIH-3T3 cells. MEK activates p44 and p42 MAP kinase by phosphorylating both threonine and tyrosine residues (for review: Johnson and Lapadat, 2002).

The SAP/JNK pathway

The stress-activated protein kinase/Jun-terminal kinase SAPK/JNK is activated by a variety of environmental stresses including UV and γ radiation, ceramides and inflammatory cytokines mediating the stimulation of MEKKs (for review: Dent et al., 2003).

The p38 pathway

p38 MAPK is a member of the MAPK family of kinases that is activated by a variety of environmental stresses including ionising radiation, heat shock, oxidative stress, osmotic shock, inflammatory cytokines and receptor systems of the TNF family. As with other MAPKs, the core signalling units is composed of an MAPKKK, typically an MEKK (e.g. MEKK1/4) that would phosphorylate and activate the p38 MAPK kinase by activating MEK3/6.
Stress signals are delivered to this cascade by members of small GTPases of the rho family (racs and rhos and cdc42s). Both rac1 and cdc42 mediate the stimulation of MEKKs. Four p38 genes have been identified in mammalian cells, the α and β isoforms are associated with activation of MAPKAP-2 (MK2) and the heat shock proteins (Hsp25, Hsp27) (for review: Johnson and Lapadat, 2002; Dent et al., 2003).

2.5.1.5. Rac

The PI3-K pathway and the p38 pathway have been shown to be linked by the small GTPase protein rac1. However, so far it is not clear what signalling molecules are all involved in this pathway (Zhang et al., 1995) (Xu et al., 2003). Rac1 belongs to the rho subfamiliy of ras proteins and is activated by PI3-K. Rac can activate the p38 and the jun kinase pathways via pak1.
Like all GTPases, rac is activated by switching from a GDP to a GTP-loaded form. PIP3, a product of PI3-K, has been shown to activate rac via some GEFs, namely vav and sos, by binding to the pleckstrin homology (PH) domain of the exchange factor.
Dominant negative forms of rho proteins are created by a single amino acid substitution, most frequently from threonine to asparagine at amino acid 17 in rac or its equivalent in other proteins. Constitutively activated rho proteins are obtained by a single amino acid mutation from Gly to Val at position 12 or from Glu to Leu at position 61 (rac numbering). These proteins have reduced GTPase activity and remain therefore permanently activated (Bishop and Hall, 2000) (Welch et al., 2003).

2.5.1.6. Ral

Two highly similar ral proteins, ralA and ralB, constitute a family of proteins within the ras branch of small GTPases. As such they are activated and inactivated specifically by GEFs and GAPs respectively (compare also chapter 2.5.1.1., page 20).
Ral is activated by ras via ral guanine exchange factors like ral-GDP dissociation stimulator (ral-GDS), rgl1, rgl2 and rlf. However, ras is not sufficient for full

activation of ral. The ras target PI3-K needs to be activated in order to activate PDK1, which subsequently binds to the N-terminus of ral-GDS. Without involving the protein kinase activity of PDK1, this binding relieves an inhibitory effect of the N-terminal region of ral-GDS on its catalytic domain. Other studies show that ral proteins can also be activated by Ca^{2+} either through a Ca^{2+}-sensitive ral-GEF that has not been identified, or by the direct binding of Ca^{2+}/calmodulin to the C-terminus of ral.
On the other hand, ral-GDS activity can be suppressed through PKC-induced phosphorylation of its inhibitory N-terminal domain which presumably prevents its dissociation from the catalytic domain. This event seems to limit the duration of Ral activation by ligands.
Ral proteins are involved in different cellular functions such as its regulatory activity in vesicle sorting that influences endocytosis. It also influences the basolateral delivery of membranes in polarised cells and neurosecretion. Through effects on the actin cytoskeleton, recent studies suggest a function of ral proteins in cell morphology. Moreover, its function in signal transduction alter gene expression and cellular growth control. It has been shown that ral guanine nucleotide exchange factors contribute to cellular transformation induced by oncogenic ras (for review: Feig, 2003).

2.5.2. Involvement of signal transduction pathways in ARE-mRNA turnover

Stabilisation of ARE-containing mRNA can be achieved by different stimuli applied to the cells such as bacterial lipopolysaccharide, Ca^{2+}-ionophores, immune stimulation and phorbol esters (Raabe et al., 1998) (Lindstein et al., 1989) (Wodnar-Filipowicz and Moroni, 1990). In addition β-adrenergic receptors, stress and tumourigenesis also influences the stability of ARE-mRNA (Pende et al., 1996) (Wang et al., 2000) (Nair et al., 1994) (Dixon et al., 2001), suggesting that the stability of ARE-mRNA might be regulated by signal transduction pathways. Indeed, several pathways have been shown to stabilise ARE-containing mRNAs. The main approach to elucidate the function of each pathway is by adding specific kinase inhibitors or transfecting activated or dominant-negative forms of specific signalling molecules and observing the resulting effect on ARE-mRNA stability.
Most prominently, the p38 pathway seems to play a very crucial role (for review see Clark et al., 2003). The expression of cyclooxygenase 2 (cox-2) was blocked at protein and steady-state mRNA levels by p38 inhibitors. A p38 inhibitor strongly destabilised cox-2 mRNA in LPS stimulated primary human monocytes and in IL-1 stimulated HeLa cells. The expression of TNFα was also blocked in primary monocytes at protein and mRNA levels (Dean et al., 1999). In further experiments from different groups, ARE-dependent stabilisation of cox-2, IL-6,

IL-8, uPA and TNFα mRNAs has been shown to be mediated by MK2 (Brook et al., 2000), (Neininger et al., 2002), (Winzen et al., 1999), (Lasa et al., 2000), (Han et al., 2002), (Tran et al., 2003), (Subbaramaiah et al., 2003). Using a transient transfection system with a subsequent actinomycin D chase assay Ming et al. were able to show parallel and independent stabilisation of IL-3 ARE mRNA by the PI3-K and the p38 pathway, where the p38 pathway seemed to collaborate with HuR (Ming et al., 2001).

The c-Jun NH2 terminal kinase (JNK) pathway is also a stabilising kinase pathway as shown in PB-3c mast cells. This pathway mediates the ARE-dependent stabilisation of IL-3 mRNA by Ca^{2+}-ionophores (Ming et al., 1998). In T-cells, IL-2 is also stabilised via JNK, but in this case a JNK-responsive element (JRE) in the 5' UTR is involved. Two proteins, nucleolin and YB-1, have been shown to bind to the JRE and mediate the JNK stabilisation in an indirect way. For full stabilisation, the JRE is not sufficient; an interaction between the 5' UTR and the 3' UTR of IL-2 is required (Chen et al., 2000).

In addition the MEK1/p42/44 MAP kinase signalling pathway has been shown to be involved in cox-2 mRNA stability by using PD98059, a specific inhibitor of this pathway (Zhang et al., 2000).

Work from different groups have shown that these signalling pathways can also target AUBPs. Studies by Mahtani et al. (2001) and Chrestensen et al. (2003), suggest that TTP is phosphorylated by MK2, whereas others pointed to phosphorylation of TTP by MAPK p38 itself (Carballo et al., 2001) (Zhu et al., 2001). Cao et al. (2003) show phosphorylation of recombinant TTP by p42, p38 and JNK, confirming previous results of in vitro phosphorylation of TTP by p42 at serine 220 (Taylor et al., 1995).

Another AUBP that has been found to be phosphorylated was hnRNP A0. It is phosphorylated at serine 84 by MK2 in vitro and in vivo and upon phosphorylation its affinity to TNFα, cox-2 and MIP-2 ARE is increased (Rousseau et al., 2002).

The third ARE-binding protein which is known to be phosphorylated is AUF1. The p40 isoform recovered from polysomes was phosphorylated at serine 83 and serine 87 in untreated cells, but loses these modifications following ARE-mRNA stabilisation by TPA treatment (Wilson et al., 2003a) (Wilson et al., 2003b).

HuR, the only well characterised stabilising AUBP, is also regulated by phosphorylation. It is involved in AMP-activated kinase (AMPK)-mediated suppression of cell cycle regulatory genes. This kinase is directly activated by elevated levels of 5'-AMP and inhibited by high concentrations of ATP. AMPK phosphorylated HuR is mainly localised in the nucleus and the remaining cytoplasmic HuR shows a decreased affinity to AREs (Wang et al., 2002) (Wang et al., 2003).

Concerning the regulation of BRF1 by cell signalling, nothing is known thus far, leading us to the aim of this work.

2.6. Aim of the work

The goal of this work was to systematically investigate which signal transduction pathways are involved in stabilising mRNAs bearing AU-rich elements (ARE), in our case a reporter mRNA containing the ARE of the IL-3 gene. Pathways that are identified to stabilise ARE-mRNA would then be tested next for potential regulation of BRF1, a known destabilising AUBP, in co-transfection assays. A BRF1 regulating candidate would be one that could stabilise ARE-mRNA, and that this stabilisation could over-ride the decay promoting activity of BRF1 in a co-transfection assay. Such assays would permit a deeper insight into the regulation of both ARE-mRNA stability and AUBP function, here BRF1, which would give a better understanding on the rapid regulation of gene expression of ARE bearing mRNAs.

Similar experiments have been performed in our laboratory before by Ming et al. (Ming et al., 2001). Various kinases were co-transfected with TTP, a decay inducing AUBP related to BRF1, in a cellular decay system based on NIH-3T3 fibroblast cells. Decay of a reporter construct containing an ARE was measured by stopping transcription using actinomycin D and extracting the RNA at precise time-points, which was then analysed by northern blotting. However, in the present work the following aspects were modified:

1. Use of BRF1 in place of TTP. BRF1 has been identified in our laboratory as being an AUBP inducing decay of the target mRNA. Very little is known about the regulation of BRF1 activity and it was of interest to study its behaviour in a similar experimental set-up.
2. Testing signalling molecules in a more systematic way. Beginning with PI3-K, which has been shown by Ming et al. (2001) to stabilise ARE-mRNA and proceeding to PI3-K downstream pathways. The upstream effector ras was also studied as it was shown to be important for IL-3 stabilisation in oncogenically transformed mast-cells (Nair et al., 1994).
3. Changing the actinomycin D-dependent cellular decay assay to an assay independent of actinomycin D. It was shown by Shyu et al. (Shyu et al., 1989), that actinomycin D can have an mRNA stabilising effect. To circumvent this problem they developed a tetracycline dependent decay assay (Xu et al., 1998), which we wanted to establish in our laboratory in order to perform the experiments mentioned above.

3. Materials & Methods

3.1. Materials

3.1.1. Chemicals

Unless indicated otherwise, all chemicals used were purchased from Merck, Fluka, Sigma or BioRAD in the highest available grade of purity.

3.1.2. Plasmids

pSRL: The Tet-Off β-globin reporter construct pSRL was generated from pTet-BBB-ARE^{GMCSF} (Xu et al., 1998) by exchanging the BamHI-BstXI (blunted with T4 DNA polymerase) fragment for the BamHI-SphI (blunted with T4 DNA polymerase) fragment of pMXh-β-IL3(UTR)wt (Ming et al., 2001), thereby replacing the ARE of GM-CSF with the 3'UTR of IL-3 (for the map, see Appendix).

pSRL-ΔAU: In pSRL-ΔAU, the ARE was removed from the IL-3-3'UTR on pSRL by excision of a 220nt NcoI-StyI fragment (Stoecklin et al., 2002).

bsd-HisBRF1$_{wt}$, bsd-HisBRF1$_{S90A}$, bsd-HisBRF1$_{S92A}$ and bsd-HisBRF1$_{AA}$: The plasmids bsd-HisBRF1$_{S90A}$ and bsd-HisBRF1$_{S92A}$ were generated from bsd-HisBRF1$_{wt}$ (Stoecklin et al., 2002) by site directed mutagenesis using as upstream primers TV43 (5'-CCG CGCCTTCTCGGAAGGGGGCGAG-3') and M2521 (5'-GGAGCGGTCTCGGAAGCGGC-3'), respectively, and as downstream primers TV44 (5'-TCTCGGAAGCGGCTGTC TCGCGAGC-3') and M2518 (5'-TTCGCCGAAGGGGGCGAGCG-3'), respectively. bsd-HisBRF1$_{S90A}$ served as template to construct bsd-HisBRF1$_{AA}$ using M2518 and M2522 (5'-GGCGCGGTCTCGGAAGCGGC), which contained the S90A and the S92A mutations.

bsd-HisBRF1$_{C120R}$ and bsd-HisBRF1$_{C158R}$: These plasmids have been described previously (Stoecklin et al., 2002).

pQE-30-BRF1$_{wt}$, pQE-30-BRF1$_{S90A}$, pQE-30-BRF1$_{S92A}$ and pQE-30-BRF1$_{AA}$: pQE-30-BRF1$_{S90A}$, pQE-30-BRF1$_{S92A}$ and pQE-30-BRF1$_{AA}$ were generated by insertion of the BamHI(blunt)-XhoI fragment from the corresponding bsd-HisBRF1$_{S90A}$, bsd-HisBRF1$_{S92A}$ and bsd-HisBRF1$_{AA}$ plasmids into the SmaI and SalI sites of pQE-30 (Qiagen). For the map, see Appendix.
pQE-30-BRF1$_{wt}$ has been described previously (Stoecklin et al., 2002).

pQE-30-N-term.: To generate pQE-30-N-term., pQE-30-BRF1$_{wt}$ was digested with

HindIII and HincII, blunt-ended by a fill in reaction with T4 DNA polymerase (NEB) and religated (Mallaun, 2002).

pcDNA-mTTP-myc/His.A: has been described previously (Stoecklin et al., 2000).

pSP73-β-globin: has been described previously (Ming et al., 2001).

pGEM-T-actin: has been described previously (Stoecklin et al., 2001).

SP6-ARE: For plasmid SP6-ARE, a 59nt fragment spanning the ARE of IL-3 including 6 AUUUA pentamers was amplified from murine IL-3 cDNA by PCR using primers M1977 (5'-ATGGATCCTTCCATTAAGGC-3') and M1978 (5'-ATAGATCTTCACAGAAGGC-3'). The amplicon was digested with BamHI and BglII, and ligated into the BglII-site of pSP73 (Promega).

T7-ARE$^-$: T7-ARE$^-$ has been described previously (Stoecklin et al., 2001).

Ras: pDCR-HA-rasV12 (Verheijen et al., 1999) was kindly provided by G. Radziwill and J.L. Bos.

PI3-K (p110): pEF-Bos-rCD2-p110 was generously provided by D. A. Cantrell (Reif et al., 1996) (Reif et al., 1997).

m/pPKB: pECE.m/p-HA-PKBα (Andjelkovic et al., 1997) was generously provided by B. A. Hemmings.

Ral and rac: pRK5myc-L61-Ral and pRK5myc-L61-Rac1 (Driessens et al., 2001) were generously provided by Alan Hall.

MEK6DD and p38-AGF: SRα3-MEK6DD (Stein et al., 1996) was generously provided by M. Karin and pCMV-p38-AGF (Raingeaud et al., 1995) were provided by R. J. Davis.

Raf: pcDNA3-Flag-rafCT (Heinrich et al., 2000) was kindly provided by K. Moelling.

PKC-δ: pCO2-PKC-δ was kindly provided by P. J. Parker (Schonwasser et al., 1998).

p70S6K: pRK5myc-p70s6k (Ming et al., 1994) was kindly provided by G. Thomas.

pcDNA3.1/Myc-HisA: was purchased from Invitrogen.

3.1.3. Primers

All primers were purchased from Microsynth:

mTTP
TV21: 5'-TGACTCGAAGAGACCCTAACC-3'
TV22: 5'-CAGATCCTCTTCTGAGATGAG-3'

huBRF1
M2241: 5'-CACTATAGGGAGACCCAAGCT-3'
M2243: 5'-TTCATCCACAACGCTGAAGAGC-3'
M2244: 5'-CCTCACATGTTTGACTCTC-3'
M2245: 5'-GGCTTAGTCATCTGAGATGG-3'
M2246: 5'-GCAAAGCTAAAGCTATGCTGG-3'
M2247: 5'-CTTGTCCCCGTACTTACAGG-3'

huBRF1 for mutation
M2518: 5'-TTCGCCGAAGGGGGCGAGCG-3'
M2521 (5'-GGAGCGGTCTCGGAAGCGGC-3')
M2522: 5'-GGCGCGGTCTCGGAAGCGGC-3'
TV 43: 5'-CCGCGCCTTCTCGGAAGGGGGCGAG-3'
TV 44: 5'-TCTCGGAAGCGGCTGTCTCGCGAGC-3'

IL-3 ARE
M1977 (5'-ATGGATCCTTCCATTAAGGC-3')
M1978 (5'-ATAGATCTTCACAGAAGGC-3')

Actin
M1170: 5'-ACTGTGTTGGCATAGAGGTC-3'
M1171: 5'-ACATCAAAGAGAAGCTGTGC-3'

3.1.4. Antibodies

Phospho-Akt (Ser 473) 4E2 Monoclonal Antibody (Cell Signaling Technology)
Phospho-(Ser/Thr) Akt Substrate Antibody; polyclonal (Cell Signaling Technology)
Phospho-p70 S6 Kinase (Thr389) Polyclonal Antibody (Cell Signaling Technology)
p70 S6 Kinase Polyclonal Antibody (Cell Signaling Technology)
Anti-c-myc 9E10 monoclonal antibody (Roche Molecular Biochemicals)

Anti-His antibody: Tetra-His Antibody (Qiagen)
Anti-Flag antibody: Anti-Flag® M2 antibody (Stratagene)
Anti-Mouse IgG (H&L) AP Conjugate (Promega)
Anti-Rabbit IgG (Fc) AP Conjugate (Promega)

3.1.5. Recombinant Proteins

$rBRF1_{wt}$, $rBRF1_{S90A}$, $rBRF1_{S92A}$, $rBRF1_{AA}$, rBRF1-N-term. were made as described under Methods; activated PKB was a kind gift of Brian Hemmings (Yang et al., 2002a).

3.1.6. Cell-lines

3.1.6.1. Bacteria

HB101: an amber suppressing strain that can be transformed efficiently by plasmids and used for large-scale plasmid production (Sambrook and Russell, 2001).

XL2-Blue ultracompetent cells (Stratagene): are high-efficiency derivatives of XL1-Blue supercompetent cells. They contain the $lacI^qZ\Delta M15$ gene on the F' episome, which allows blue-white color screening of recombinant plasmids.

M15 competent E. coli (Qiagen Cloning System): provide a host for optimal propagation of pQE30 plasmid vectors (Qiagen) and high yield expression of recombinant proteins. The strain allows resistance selection with the antibiotics ampicillin (200μg/ml) and kanamycin (25μg/ml).

3.1.6.2. Eukaryotic Cells

NIH-3T3 (Swiss mouse embryo): fibroblasts, growing as adherent monolayer. Cultured in M2-medium (Iscove's modified Dulbecco medium (IMDM) from Invitrogen supplemented with 10% fetal calf serum (Invitrogen), 50μM 2-mercaptoethanol (Invitrogen), 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin (Sigma)) at 37°C in an atmosphere of 7% CO_2. Split every 3-4 days 1:10 using Trypsin/EDTA (Invitrogen).

B_2A_2: Clone of NIH-3T3 cells containing tTA, a Tet-Off transactivator (Xu et al., 1998).

B_2A_2-23: Subclone of B_2A_2 having a good tetracycline regulation. Cultured in M2-medium containing 200ng/ml tetracycline.

B_2A_2-Tet-off-$BRF1_{wt}$: B_2A_2-23 cell-line expressing exogenous BRF1 wt under the control of tetracycline (Stoecklin et al., 2002).

SlowC: Mutant clone of HT-1080 stably expressing a GFP-IL3 reporter construct and carrying a frameshift mutation in each BRF1 allele. Cells were split every 3 days 1:10 using Trypsin/EDTA and incubated at 37°C in an atmosphere of 7% CO_2 (Stoecklin et al., 2001).

3.2. Methods

All molecular biology techniques described below are according to Sambrook and Russell (Sambrook and Russell, 2001) unless indicated otherwise.

3.2.1. Isolation of plasmid DNA

Small-scale plasmid preparations were done from a 2ml bacterial overnight culture in LB (Luria-Bertani Medium, Invitrogen). The cells were pelleted by centrifugation for 10min at 4°C and 4000g and plasmid DNA was isolated from the bacterial pellets by the alkaline lysis method using a plasmid miniprep kit (QIAGEN Inc.). The DNA pellets were dissolved in 20ml TE (10mM Tris-HCl, 1mM EDTA, pH 8.0) or water. Large-scale plasmid preparations were made from 250ml culture, similarly as described above, using a plasmid maxiprep kit (QIAGEN Inc.). The purified DNA was dissolved in TE or water. The DNA concentration was determined photometrically by measuring absorbance at 260nm (note: $1OD_{260}$-unit = 50mg/ml DNA). Purity was estimated by the ratio of OD_{260}/OD_{280}, which was ~1.6. Plasmid DNA samples were stored at –20°C.

3.2.2. DNA cloning procedures

Restriction digests of plasmid DNA were carried out using the Roche restriction buffer system and either NEB, Promega or Roche enzymes according to the manufacturer's recommendations. The digested plasmid DNA was separated on a 0.8% (w/v) agarose/1xTAE (40mM Tris-acetate, 1mM EDTA, pH 8) gel supplemented with 0.05% ethidium bromide. The DNA Molecular Weight Markers IV or XIV (Roche) were used as size standards. The sample was mixed with $1/5^{th}$ volume of agarose gel loading buffer (15% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol FF) prior to loading. The gel running buffer was 1xTAE. Ethidium-bromide stained DNA fragments were visualised using a MultiImage

Light Cabinet/AlphaImager system (Witec ag), and, if needed, DNA bands were excised from the gel under UV light. The DNA was purified from gel slices using a QIAquick gel extraction kit (QIAGEN Inc.), according to the manufacturer's instructions.
Cloning of DNA fragments was carried out overnight at 16°C using the T4 DNA ligase with the provided buffer (Promega) and vector/insert-ratios of approximately 1/4. When necessary, the vector was dephosphorylated using calf intestinal alkaline phosphatase (Roche) at 37 °C for 30min in the buffer supplied with the enzyme. The ligation-products were directly used for transformation of $CaCl_2$-competent bacteria by heat-shock.
$CaCl_2$-competent E. coli were prepared from a 5ml culture inoculated with a single colony overnight. 250ml of LB medium were inoculated with 2.5ml of the overnight culture and grown until an OD_{600nm} of 0.5 was reached. The culture was centrifuged for 10min at 4°C and 2500g and the pellet resuspended in 60ml sterile 100mM $MgCl_2$. After a centrifugation step of 10min and 2500g at 4°C the pellet was taken up in 250ml 100mM $CaCl_2$ and incubated at 4°C for 20min. After centrifugation (2500g, 10min, 4°C) the pellet was resuspended in 2.5 ml of 85% 100mM $CaCl_2$ + 15% Glycerol and the aliquots were flash frozen in liquid nitrogen.
Up to 100ng plasmid were added to 50µl of $CaCl_2$-competent cells and incubated for 30min on ice followed by an incubation of 2min at 40°C and put back on ice immediately. To allow the cells to express plasmid derived antibiotic resistance, 1ml LB was added and the cells were incubated for 1h at 37°C with shaking before plating on selective LB medium.
If XL2-Blue cells were used, β-mercaptoethanol was added to the cells and the heat-shock was performed for only 0.5min instead of 2min according to the manufacturer's recommendations.
Putative recombinants were picked with a sterile toothpick and grown in 3ml LB medium for small-scale plasmid isolation. Occasionally, colony-PCR was performed by briefly dipping the toothpick into a premixed PCR reaction prior to inoculating a culture.

3.2.3. Reverse Transcription

Before starting the Reverse Transcription reaction, a DNase digest of the RNA using RQ1 DNase (Promega) was carried out according to the manufacturer's protocol to remove any possible DNA contamination of the RNA.
Reverse Transcription (RT) using 2µg of total cytoplasmic RNA was performed with 1µM of each dNTP (Roche), 2µM dT oligos (Microsynth), 10mM DTT (Promega), 1U/µl RNasin (Promega), 1xM-MLV-buffer and 10U/µl M-MLV-RT (Promega). After 45min of incubation at 37°C the reaction was stopped by heating the samples for 10min at 72°C. For a subsequent PCR reaction 1µl of the synthesised cDNA was used.

3.2.4. Polymerase Chain Reaction

Polymerase chain reaction (PCR) using 1 to 50ng of plasmid or cDNA as template was performed with 0.3mM of each dNTP, 0.4mM forward and reverse primers (Microsynth) and 0.5U Taq DNA polymerase (QIAGEN) in the buffer supplied with the enzyme by the manufacturer. Standard cycling parameters were 1min denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 45sec, annealing at the appropriate temperature for 1min, and elongation at 72°C for 1min. When PCR-products were to be cloned, native Pfx DNA polymerase (Stratagene) and the supplied buffer were used instead. The number of cycles was reduced to 12, and the elongation time was increased to 1min/kb. The annealing temperature was chosen to be 2 to 5°C below the calculated melting temperature, which is T (in °C) = 69.3 + 0.41 x %GC – 650 / length of the oligonucleotide (Mazars et al., 1991). The PCR-products were analysed by agarose gel electrophoresis or purified from gel slices as described below.

3.2.5. RT-PCR of BRF1 and actin

A reverse transcription followed by a polymerase chain reaction was performed for BRF1 and actin as described before. The annealing temperature was 55°C for both reactions and the following primers were used:
BRF1: M2243 and M2245
actin: M1170 and M1171
For BRF1 the primers were designed in a way that only the exogenous cDNA was amplified.

3.2.6. Mutations

Mutations of BRF1 were introduced according to the protocol of ExSite PCR-Based Site-Directed Mutagenesis (Stratagene).
To mutate the serine 90 site of BRF1 to alanine a PCR was performed using 0.4μM TV43 and TV44 as primers, 2ng/μl bsd-HisBRF1$_{wt}$ as template, 0.3mM dNTP's, 1mM $MgSO_4$, 1xPfx-amplification-buffer and 0.05U/μl Platinum® Pfx DNA Polymerase (Invitrogen) under the following program:

1. 94°C 5min
2. 58°C 30sec
3. 68°C 7min
4. 94°C 15sec
5. 68°C 8min steps 4 and 5 were repeated for 12 times
6. 68°C 10min
7. 4°C pause

After purification of the PCR products with the Qiaquick PCR fragment purification kit (QIAGEN), they were digested with Dpn1 and the provided buffer (Promega) for 2h at 37°C. The fragments were ligated after a second purification step and transformed into XL2-blue cells as described above.
To obtain the $BRF1_{S90A/S92A}$ double mutant ($BRF1_{AA}$), mutagenesis was performed as previously described with the following changes: $BRF1_{S90A}$ was used as template with the M2518 and M2522 primers and the PCR reaction mix was supplemented with 10xdiluted PCRxEnhancer Solution (Invitrogen).

3.2.7. Sequencing

For sequencing reactions, 2µl Big Dye Terminator RR Mix (Applied Biosystems), 3µl 5xBuffer (Applied Biosystems), 0.4µg of the DNA to be sequenced and 3.2pmol of primer were mixed to a final volume of 20µl. For the reaction, the following program was used: 25 cycles with 30sec denaturation at 96°C, 15sec annealing at 50°C and 4min elongation at 60°C. The samples were stored at 4°C. Prior to running the samples, 2µl 5M ammonium acetate pH 4.6 and 50µl ethanol were added to the reactions. After 10min incubation on ice, they were centrifuged for 20min at 20'000g, the supernatant was discarded and the pellet washed with 250µl 70% ethanol. The pellet was dried in a speed vac (UniEquip), dissolved in 25µl Template Suppression Reagent (Applied Biosystems) and denatured by heating for 2min at 95°C. Finally the samples were sequenced with a ABI Prism 310 Genetic Analyzer (Applied Biosystems).

3.2.8. Cell culture

Mouse NIH-3T3 B_2A_2 cells (Xu et al., 1998) were kindly provided by A.-B. Shyu. The cells were maintained in M2, which is complete Iscove's modified Dulbecco medium (IMDM) from Invitrogen supplemented with 10% fetal calf serum (Invitrogen), 50µM 2-mercaptoethanol (Invitrogen), 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Sigma) at 37°C in an atmosphere of 7% CO_2. When the cells reached confluency, they were washed and passaged using Trypsin/EDTA (Invitrogen). For subcloning, the cells were seeded at a density of 1 cell per well in a 6x12 Microwell plate (Nunc, Brand Products) using IMDM supplemented with 20% FCS (fetal calf serum).

3.2.9. Northern blot analysis

^{32}P-labeled RNA probes
For preparation of templates for in vitro transcription, 20µg plasmid was linearised downstream of the probe sequence using an appropriate restriction

enzyme. Complete digestion was verified by agarose gel electrophoresis.
The following templates were used for in vitro transcription:
β-globin: SP6 probe generated from the 86bp BglII-EcoRI fragment of rabbit β-globin. EcoRI was used to linearise the plasmid.
Actin: SP6 probe from a 260bp fragment of murine β-actin generated by RT-PCR with the primers M1170 and M1171. ApaI or NcoI were used to linearise the plasmid.
The SP6 in vitro transcription was performed mixing 1.5 mM ATP, CTP and UTP, 1.25 μM GTP, 2.5 mM DTT, 0.05 μg/μl BSA (NEB), 1x transcription buffer (Promega), 0.025 μg/μl linearised DNA template (usually 100ng template/3kb length of template, for a final volume of 20μl), 1U/μl RNasin, 1U/μl SP6 Polymerase (Promega) and 10μCi α^{32}P-GTP with a specific activity of 3000Ci/mmol (Hartmann Analytic) in a total reaction volume of 20μl. The reaction mix was incubated for 1h at 37°C and purified with a Sephadex G-25 DNA Grade column (NAP5; Amersham Pharmacia Biotech) according to the manufacturer's recommendations. The probe was denatured for 5min at 95°C before using.

RNA isolation

Total cytoplasmic RNA was isolated by the method of Gough, 1988: 400μl of lysis buffer (150mM NaCl, 1.5mM $MgCl_2$, 0.65% NP-40, 10mM Tris-HCl pH 7.5) were added to the cells, collected with the cells and centrifuged at 20'000g for 1min at 4°C. 400μl extraction buffer (350mM NaCl, 7M Urea, 1% SDS, 10mM EDTA pH 8, 10mM Tris-HCl pH 7.5) and 400μl of phenol/chloroform/isoamylalcohol (25:24:1) were added to the supernatant, vortexed and centrifuged for 15min at 20'000g at room temperature. 700μl of the aqueous phase was recovered and added to the same amount of isopropanol, then the RNA was precipitated for at least 1h at -20°C. The samples were centrifuged for 25min at 20'000g at room temperature and the pellet was washed with 70% ethanol and dissolved by heating the samples at 65°C for 30min in either 20μl of formamide or water. The RNA concentration was determined photometrically by measuring absorbance at 260nm (note: 1OD_{260}-unit = 40μg/ml RNA).

Electrophoresis and blotting

18-20μg of RNA was mixed with 5xloading buffer (for 15ml 5x loading buffer add: 7.2ml formamide, 1.6ml 10xMOPS buffer, 2.6ml formaldehyde (37%), 1.8ml H_2O, 1ml 80% Glycerol, 0.25% bromophenol blue) to the final concentration of 1x, denatured for 10min at 65°C and fractionated on a 1.2% agarose gel containing 0.66M formaldehyde in a 1xMOPS buffer (20mM MOPS, pH 7.0, 8mM Sodium Acetate, 1mM EDTA, pH 8) and blotted overnight onto Highbond N+ membranes by capillary force using 10xSSC as transfer solution (1xSSC is 150mM NaCl, 15mM Na-citrate). The membrane was washed briefly in 2xSSC and then UV crosslinked using a Stratalinker®.

Hybridisation and visualisation

Hybridisation was performed using a Techne hybridiser (HB-1D, Brouwer AG). The membrane was prehybridised for 1h at 55°C in 20ml hybridisation solution containing 50% (v/v) deionised Formamide (Intergen Company), 5xSSC, 5xDenhardt's reagent (50xDenhardt's reagent contains: 1% (w/v) Ficoll 400, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (Sigma, Fraction V)), 10mM Pipes pH 6.4, 0.4mg/ml yeast RNA, 0.5% SDS and 5mM EDTA, pH 8.0.

The hybridisation solution was exchanged with 20ml fresh hybridisation solution containing the probe, and hybridisation was carried out overnight under the same conditions.

The membranes were washed at 65°C with 2xSSC/0.2% SDS, followed by 0.2xSSC/0.2% SDS and 0.1xSSC/0.2% SDS washes for 30min each, sealed and exposed to a Phosphor-Imager screen (BioRad). Northern blot signals were visualised using a Phosphor-Imager (Molecular Dynamics) and the intensities were quantified with Quantity One software (BioRad).

3.2.10. Western blot analysis

Cell lysates were prepared from confluent tissue culture dishes (145/20mm) by addition of 250µl RIPA buffer (120mM NaCl, 50mM Tris-HCl pH 8.0, 1% Triton X-100, 0.5% deoxycholic acid) complemented with 1 tablet of Complete Mini Protease Inhibitors (Roche) or by addition of extraction buffer (50mM Tris, 120mM NaCl, 5mM EGTA, 1mM EDTA, 5mM NaPPi, 10mM NaF, 30mM paran itrophenylphosphate, 1mM benzamidine, 0.2mM PMSF, 0.1% NP-40, adjusted to pH 8), if phosphorylation sites were to be detected. Protein concentration of the extracts was determined using a Bradford protein assay kit (BioRad). A series of dilutions of 20ml of each extract in PBS including a dilution series of BSA as standard was measured. Optical density was measured at 595nm and the concentrations were calculated by intrapolation of the standard values. Equal amounts of protein were used for protein analysis.

60µg of proteins were resolved on a 12% polyacrylamide gel, blotted onto Immobilon-P (Millipore) membrane, briefly washed in PBS and blocked for 1h with PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4) containing 5% skimmed milk powder and 0.1% Tween-20 or, for detection of phosphorylated sites, TBS (150mM NaCl, 50mM Tris-HCl, pH 7.5) containing 3% BSA and 0.1% Tween-20 was used instead. The membrane was incubated overnight at 4°C with the appropriate antibody and washed twice for 15min with either PBS/0.1% Tween-20 or TBS/0.1% Tween-20. Incubation with the secondary, alkaline-phosphatase-coupled anti-rabbit or anti-mouse antibody (Promega) was performed for 1h at room temperature, followed by washing 2 times for 15min with 0.1% Tween-20 in PBS or TBS. The proteins were visualised with CDPstar (Roche) according the manufacturer's protocol and subsequent autoradiography

using the Hyperfilm ECL films (Amersham Pharmacia Biotech).

3.2.11. Coomassie blue staining

The polyacrylamide gel (see Western blot) was placed in a plastic box and soaked in fixing solution (50% methanol and 10% acetic acid in H_2O) for 30min with gentle agitation. For staining, the gel was placed into Coomassie blue solution (50% methanol, 0.05% Coomassie Brilliant Blue R and 10% acetic acid in H_2O; the Coomassie Brilliant Blue R has first to be dissolved in methanol before adding acetic acid and water) and incubated for 30min. Thereafter the gel was incubated from 4h up to overnight in a destaining solution (5% methanol and 7% acetic acid in H_2O) at room temperature. To maintain a permanent gel record, the gel was rinsed for 10min in 10% glycerol before drying it under vacuum. The gel was then placed between a sheet of 3MM Whatmann paper and a cellophane membrane previously soaked in 10% glycerol. The membrane was dried at 80°C for 60min.

3.2.12 Recombinant Protein

Recombinant proteins were made using the "QIAexpress Expression System" from Qiagen according to the manufacturer's protocol:
M15 bacteria were transformed by heat shock with pQE-30-$BRF1_{wt}$, pQE-30-$BRF1_{S90A}$, pQE-30-$BRF1_{S92A}$, pQE-30-$BRF1_{AA}$ or pQE-30-BRF1-N-term. and plated on LB plates containing 25μg/ml kanamycin plus 200μg/ml ampicillin. For small scale protein production, a single bacteria colony was grown in 2ml selective LB overnight. 2ml fresh medium were inoculated with the overnight culture to an $OD_{(600nm)}$ of 0.1 and incubated until an OD of 0.6 was reached. To 1ml of the culture, 1μl of 1M IPTG was added and incubated for 4h at 37°C. 5μl of the induced and the non-induced cultures were run on a SDS-PAGE and a coomassie blue staining or a western blot was performed to detect the induced recombinant protein.
For large scale preparation, a single colony was picked and cultured overnight in 20ml selective LB medium. 1l fresh LB medium was inoculated with an amount of the overnight culture to obtain an $OD_{(600nm)}$ of 0.1 and incubated until an OD of 0.6 was reached. A sample was saved as a control, 1ml 1M IPTG was added to the culture to a final concentration of 1mM and the culture was incubated for a further 4h. Another control sample was saved and the culture was centrifuged at 2000g for 20min at 4°C. The supernatant was discarded and the cell pellet frozen at –80°C.
The cell pellet was thawed and resuspended in 40ml lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8) with protease inhibitors. Lysozyme (Sigma) was added to a final concentration of 1mg/ml to disrupt the

bacterial cell wall and cells were incubated for 30min on ice. To complete this reaction, 1min ultrasound sonication at 200W (Soniprep150, N.Zivy & Cie S.A.) was applied 5 times, with cells always kept on ice. DNaseI (Qiagen) was added to 1μg/ml and incubated for 15min on ice. The bacterial lysate was centrifuged at 10'000g for 30min at 4°C. The pellet was resuspended overnight at 4°C in the purification buffer (100mM NaH_2PO_4, 20mM Tris-HCl pH7.5, 300mM NaCl, 20mM Imidazole, 10% Glycerol, adjusted to pH 8, stored at 4°C) containing 6M urea. To the supernatant, glycerol was added to a final concentration of 10%. Both fractions were stored at –80°C, while aliquots were tested for the presence of the recombinant protein. Recombinant BRF1 protein was determined to be in the pellet fraction.
Purification of the pellet fraction over Ni-beads column:
3ml of 50% Ni-NTA agarose beads (Qiagen) were washed two times in purification buffer containing 6M urea for 15min each. Beads were added to the dissolved pellet fraction and rotated for 3h at 4°C. The suspension was centrifuged for 5min at 1000g. The supernatant was frozen at –80°C, while the beads were packed onto a column. The column was washed with purification buffer containing 6M, 3M, 2M, 1M, 0.5M 0.2M 0.1M and 0M urea for 0.5h each. Protease inhibitors were added to buffer containing less than 2M urea. The proteins were eluted into 1ml fractions with purification buffer containing 200mM imidazole and protease inhibitors. The fractions were tested on a gel by coomassie blue staining.

3.2.13. In vitro kinase assay

40ng of recombinant BRF1 and 100ng activated PKB (Andjelkovic et al., 1999; Yang et al., 2002a) were incubated at 30°C for 30min in kinase buffer (30mM Tris-HCl, 5mM $MgCl_2$, 1mM DTT and 0.2mM ATP) in a total volume of 20μl. For visualisation of phosphorylation, 0.02mM γ-^{32}P-ATP and 0.18mM cold ATP was used in the kinase buffer instead of 0.2mM cold ATP and the reaction was run on a 12%-SDS-PAGE. Radioactivity was visualised on the vacuum dried gel (2h at 80°C in a vacuum gel drying apparatus; BioRad) using a Phosphor-Imager (Molecular Dynamics). The intensities were quantified with ImageQuant software.

3.2.14. Analysis of the NBRF-phosphorylation site (from Daniel Hess, FMI)

The NBrf1 band was excised from the gel, reduced with 10mM DTT, alkylated with 55mM iodoacetamide and cleaved with 0.5μg LysC (Achromobacter, Wako BioProducts, Richmond, VA) at 37°C overnight in 25mM ammonium bicarbonate buffer (pH 8.0). After LysC cleavage the gel was dried and 0.5μg of Asp-N (Roche Molecular Biochemicals, sequencing grade) was added in 40μl of 50mM

sodium phosphate buffer (pH 8.0) at 37°C for 6h. The extracted peptides were analysed by capillary liquid chromatography tandem mass spectrometry (LC-MSMS) using a Magic C18 100μmx10cm HPLC column (Spectronex) connected on line to an iontrap Finnigan DecaXP (ThermoFinnigan, CA). A linear gradient from 5% to 50% B (0.1% Formic Acid, 80% Acetonitril in H_2O) in A (0.1% Formic Acid, 2% Acetonitril in H_2O) was delivered with a Rheos 2000 HPLC system (Flux, Switzerland) at 100μl/min. A precolumn flow splitter reduced the flow to approximately 300nl and the peptides were manually loaded with a 10μl Hamilton syringe on a peptide captrap (Michrom BioResources, Inc. CA) mounted in the injection loop of the MS. The eluting peptides were ionised by electrospray ionisation, detected, and the peptide ions were automatically selected and fragmented in the iontrap.
Phosphopeptides were identified with the neutral loss function, searching for fragment ions formed by the loss of phosphoric acid, 32.3, 48.5 or 97 Da from the (M+3H)3+, (M+2H)2+, and (M+H)+ ions, respectively.
The phosphopeptide was isolated by LC-MS followed by cleavage with 0.1μg trypsin (Promega, sequencing grade) in 50mM ammonium bicarbonate (ph 8.0) at 37°C for 1h. The peptide mixture was then analysed by LC-MSMS as described above and the phosphopeptide was additionally sequenced by a MS3 experiment. In such an experiment first the peptide ion is isolated and fragmented in the iontrap, the main ion formed is the precursor ion minus the phosphoric acid. In this reaction dehydroalanine is formed from phosphoserine and the phosphorylation site can unambiguously be identified by isolation and fragmentation of this ion.

3.2.15. NIH-3T3 B_2A_2-23 decay assay

The NIH-3T3 B_2A_2-23 cells were seeded at a density of $6x10^6$ per cell culture dish 145/20mm (Greiner bio-one) in 10ml M2 supplemented with 200ng/ml tetracycline 18 to 20h before transfection by the calcium phosphate method (Shyu et al., 1989). Transfection mixtures for each plate contained 9 to 18μg of pSRL, or 9μg of pSRLΔAU, 0.5 to 6μg of test plasmid(s) and pcDNA3.1/Myc-HisA to a final amount of 20μg, diluted into 1.5ml 0.25M $CaCl_2$ and added to 1.5ml of 2xBBS (50mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethansulfonic acid), 280mM NaCl, 1.5mM $Na_2HPO_4 \cdot 2H_2O$, pH 6.95). This mixture was incubated for 10min at room temperature and added to the cells. After exposure to the plasmid precipitates for 12 to 16h in an atmosphere of 3% CO_2, the cells were washed twice with Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (PBS contains 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4), split into 3 tissue-culture dishes 94/16mm (Greiner bio-one) and cultured in M2 containing 0.5% Tet System Approved FCS (CLONTECH Laboratories) for 22 to 24h before the addition of 1μg/ml tetracycline (final concentration) to stop transcription of the reporter mRNA. If rapamycin (Sigma) was used, 20ng/ml was added to the cells 1h prior

to tetracycline treatment. Actinomycin D (Calbiochem) was added, if needed, at the same time as tetracycline to a final concentration of 1μg/ml. RNA was extracted at various time intervals and a northern blot performed as described previously. Note that different amounts of reporter plasmids were used in order to give a comparable reporter signal on the northern blot.

3.2.16. In vitro decay analysis

Synthesis of ^{32}P-UTP labeled reporter RNA:
SP6-ARE and T7-ARE^{-} plasmids linearised with BglII or SpeI, respectively, served as template for in vitro transcription. To prevent the RNA from degradation the cap analogue m^{7}G(5')ppp(5')G (Ambion) was incorporated during transcription. The transcription reaction (1μg DNA template, 1x transcription buffer, each 1mM of ATP, GTP and CTP, 0.1mM of UTP (Roche), 0.05mCi ^{32}P-UTP (Hartmann Analytic), 1mM cap analogues, 15U SP6 RNA polymerase or 15U T7 RNA polymerase (Promega)) was incubated for 45min at 37°C. To digest the template, 1U RQ1 DNase (Promega) was added and incubated for 15min at 37°C. The reaction was stopped by adding 30μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the RNA was purified using CHROMA spin columns (Clontech).

Preparation of S100 cell extracts from slowC cells:
Cells from 50 confluent cell culture dishes 145/20mm (Greiner bio-one) were washed with PBS, scraped off the plates, collected in PBS and centrifuged at 1000g for 5min. The cell pellet was dissolved in 2.5ml buffer A (10mM Tris-HCl pH7.5, 1.5mM KOAc, 1mM MgOAc and 2mM DTT) containing 10μM leupeptin (Sigma), 1μM pepstatinA (Sigma) and 1mM PMSF (Sigma). The cells were carefully homogenised for about 1min using a homogeniser with a teflon coated piston (Bellco).
To check for good homogenisation, a trypan blue staining was performed by mixing equal amounts of Trypan blue stain 0.4% (Life Technologies) and lysate, which was then anaysed under the microscope. The lysate was centrifuged at 500g for 15min. 2M KCl was added to the supernatant to a final concentration of 0.15M KCl under constant stirring. After centrifugation at 100'000g for 1h using a Beckman L7-65 ultracentrifuge with swing buckets (rotor SW55TI), the protein concentration of the supernatant was determined by Bradford assay, glycerol was added to the supernatant to a final concentration of 10% and the protein concentration was recalculated. The lysate was aliquoted and immediately snap frozen using liquid nitrogen and stored at –80°C.

In vitro decay:
Recombinant proteins were added to the in vitro decay mix and the volumes of

the samples were equalised to 25µl with buffer A.
In vitro decay mix for 1 sample:

500mM Potassium acetate	2.5 µl
200mM Magnesium acetate	0.5 µl
500mM Tris-HCl pH 7.5	0.5 µl
100mM DTT	0.5 µl
2.5mM Spermine	1 µl
1M Creatine phosphate kinase	1 µl
500mM Creatine phosphate	0.5 µl
50mM ATP	0.5 µl
20mM GTP	0.5 µl
500mM poly(A)	1 µl
RNAsin (2U/µl; Promega)	0.1 µl
Cytoplasmic cell lysate (see above)	10µg
^{32}P-labeled ARE RNA	50'000cpm
^{32}P-labeled ΔARE RNA	50'000cpm

If phosphorylated BRF1 was used, 0.1µM of okadaic acid (final concentration) was added to the in vitro decay reaction.
The incubation was performed at 37°C from 0min to 45min. Thereafter the reactions were stopped by addition of 100µl stop buffer (25mM Tris-HCl pH7.5, 400mM NaCl, 0,1% SDS in MilliQ H_20) and 125µl of phenol/chloroform (1:5). The samples were vortexed and centrifuged at 10'000g for 3min.
120µl of the aqueous phase of each sample was added to previously prepared 12,5µl 3M NaOAc (pH 5.1) and 1µl of 10mg/ml yeast tRNA (BioRad). 325µl ethanol was added to each sample and incubated for a minimum of 2h at –20°C to precipitate the RNA. After centrifugation at 10'000g for 20min at room temperature, ethanol was carefully removed and residual ethanol allowed to evaporate by air-drying the tubes for 20min. The RNA pellets were dissolved in 5µl of IVD loading buffer (95% formamide, 20mM EDTA, 5mg Bromophenol blue and 50mg Xylene cyanol for 10ml), vortexed and incubated for 20min at 65°C and afterwards 5min at 95°C before loading onto a denaturing 13% polyacrylamide gel containing urea (for 10ml: 4,6g urea, 3.35ml 40% (w/v) acrylamide/bis-acrylamide (29:1), 1.2ml 5x TBE). The urea was dissolved by constant shaking. 33µl 10% APS and 8µl TEMED were added and the gels cast immediately and allowed to polymerise for 30min. Gels were pre-run for 10min at 25mA in 0.5x TBE buffer. Then the samples were loaded onto the gel and electrophoresis was performed for 90min at 25mA (Chen et al., 2001). Fixing of the gel was carried out for 2h at 80°C in a vacuum gel drying apparatus (BioRad). RNA bands were visualised using a Phosphor-Imager (BioRad). Dried gels were exposed to a ^{32}P-sensitive screen for 2h followed by scanning and computer assisted signal conversion (Quantity One/BioRad).

4. Results

4.1. Signalling pathways involved in stabilising ARE-mRNA and regulating BRF1

4.1.1. The ARE-mRNA in vivo decay assay

In order to investigate the influence of signalling molecules on the stability of ARE-mRNA, a suitable screen was required that could measure the decay rate of a reporter mRNA. Thus far mRNA decay was measured in our laboratory using an actinomycin D chase experiment. In this work, the tet-off system was preferred as it provides a reporter-specific inhibition of transcription in contrast to actinomycin D based experiments that blocks all cellular transcription leading to the generation of possible non-specific effects. This is significant as Shyu and co-workers found an mRNA decay impeding effect in transcriptional inhibitors such as actinomycin D and DRB (Shyu et al., 1989) (Chen et al., 1995).
A tet-off mRNA decay system has been developed by A.-B. Shyu in NIH-3T3 cells stably transfected with the tetracycline transactivator (tTA), henceforth referred to as B_2A_2 cells (Xu et al., 1998). A reporter mRNA under the control of a tetracycline-sensitive promoter was introduced into B_2A_2 cells. This enabled transcription of the reporter gene to be blocked by addition of tetracycline and the decay of the transcript could be followed over a time-course by northern blot hybridisation (Xu et al., 1998).
As a first step, this assay was established in our laboratory. A reporter construct (pSRL) was made by fusing the β-globin gene to the IL-3 3'UTR containing the ARE (Figure 5A). Transcription of this reporter is controlled by a minimal CMV promoter with a tetracycline responsive element (TRE) enabling the inhibition of transcription upon tetracycline addition to the cells (tet-off) (Baron et al., 1997). The construct was introduced into B_2A_2 cells (obtained from A.-B. Shyu). As the reporter pSRL was only poorly expressed in mass transfected B_2A_2 cells, the cells were subcloned. Out of 24 subclones, B_2A_2-23 was selected for optimal reporter gene expression and tetracycline-dependent regulation (data not shown). In Figure 5B the inhibition of expression of the reporter by addition of 1μg/ml tetracycline is documented by northern blotting. The reporter was detected using a β-globin specific probe. As a loading control the blot was rehybridised with an actin probe. To confirm the expected rapid decay of an ARE containing mRNA in this system, two decay experiments were performed. In the first experiment, $1x10^7$ cells were transfected with 1.2μg/ml of reporter plasmid (pSRL). After 24 hours the cells were trypsinised and plated in separate tissue culture dishes. Cytoplasmic RNA was isolated at various time-points following addition of tetracycline and northern blotting was performed. As shown in Figure 5C, the

reporter signal decreased steadily over 2 hours. To confirm the ARE dependency of the decay, B_2A_2-23 cells were transfected with a β-globin-IL-3-3'UTR reporter lacking the ARE (pSRL-ΔAU). After 16 to 24 hours, the transfected cells were split into 3 tissue culture dishes and a decay assay was performed over 2h as indicated in Figure 5D. The reporter mRNA remained stable, showing that the decay in Figure 5C is dependent on the ARE of the IL-3 3'UTR present on the reporter.

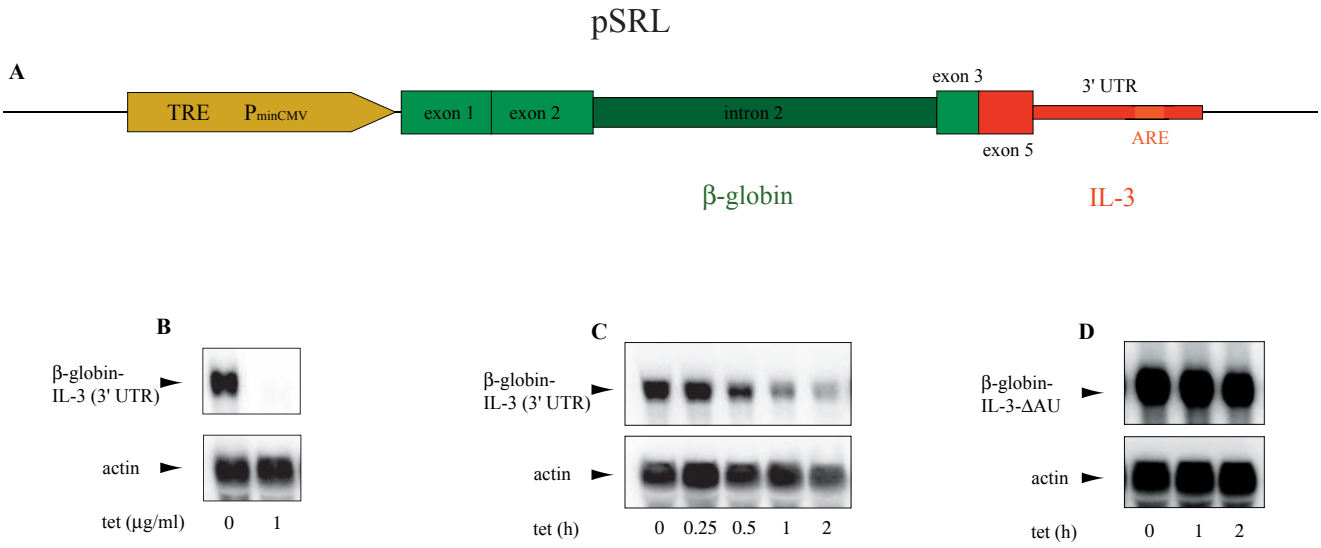


Figure 5: The NIH-3T3 Tet-off decay system (NIH-3T3 B2A2-23). (A) Scheme of the tetracycline responsive Tet-β-globin-IL3-3'UTR reporter (pSRL). (B) Tetracycline inhibition of pSRL: pSRL was transfected into NIH-3T3 B2A2-23 cells. After 36h transcription of the reporter was stopped for 12h using 1μg/ml of tetracycline and cytoplasmic RNA was isolated. Northern blotting analysis was performed using a β-globin specific probe for detection of the reporter mRNA and a β-actin probe for the loading control. (C) Time course: NIH-3T3 B2A2-23 cells were transiently transfected with pSRL. After 48h, transcription of the reporter gene was blocked using tetracycline (1μg/ml), and RNA was isolated at the time points indicated. Northern blotting analysis was performed as above. (D) The same transfection as in (C) was carried out with a β-globin-IL-3 reporter plasmid lacking the ARE (β-globin-IL-3-ΔAU).

Based on these results, the following protocol was established for all subsequent decay assays (see Figure 6):
NIH-3T3 B_2A_2-23 cells were seeded at a density of $6x10^6$ cells per 145/20mm tissue culture dish in IMDM medium supplemented with 10% fetal calf serum (FCS). The following day, the cells were transfected using the $CaPO_4$-method with 0.7μg/ml of plasmids. After 12-16h the cells were washed and split into 3 tissue culture dishes (94/16mm) of IMDM medium containing 0.5% Tet System Approved FCS (CLONTECH Laboratories). After 22-24h transcription was stopped with 1μg/ml tetracycline and the cytoplasmic RNA was isolated after 0, 1 or 2 hours post-tetracycline treatment.

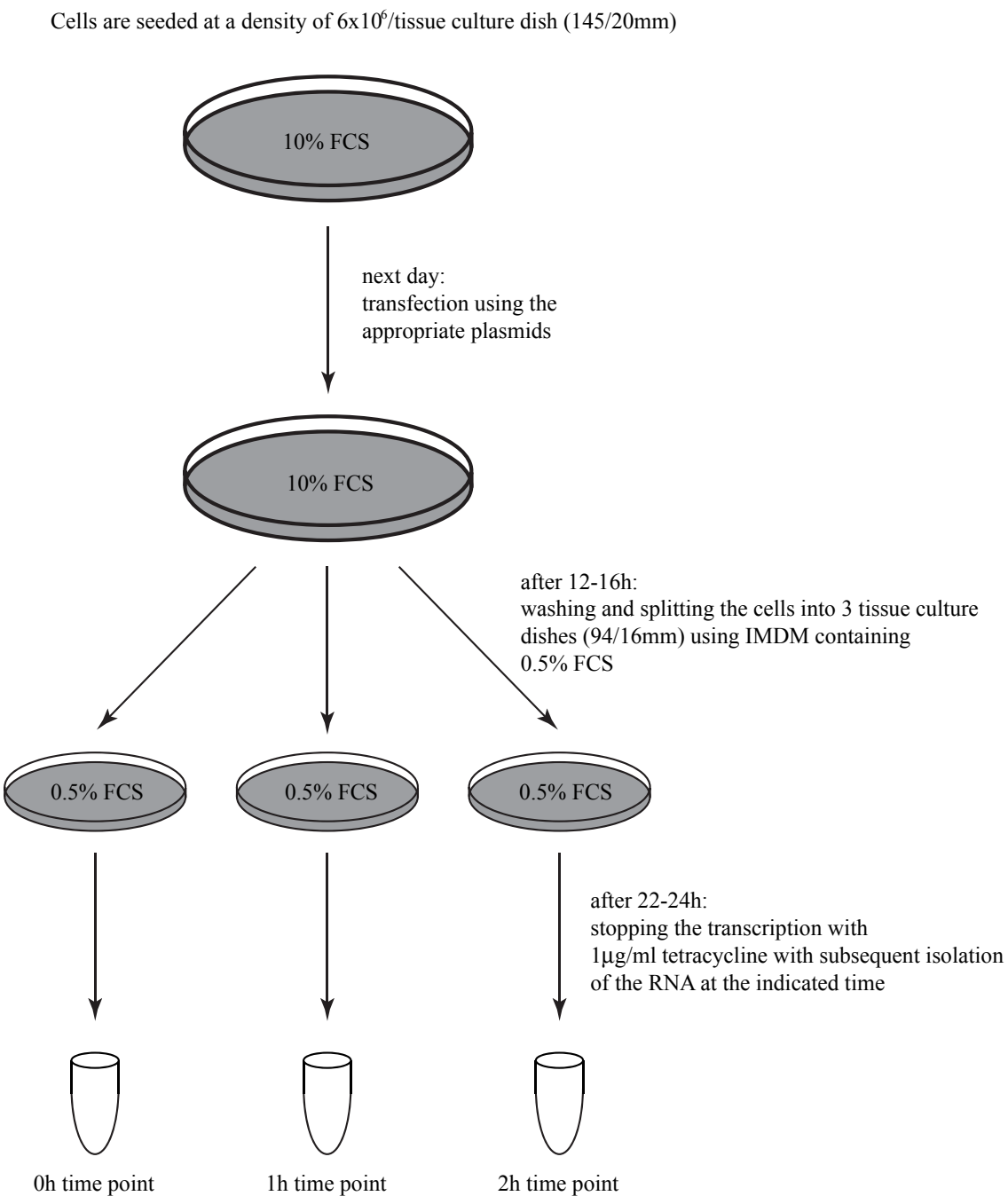


Figure 6: Flow chart showing the procedure for the in vivo decay assay.

4.1.2. Involvement of PI3-K and its downstream targets in ARE-mRNA stabilisation

Previous work done in our laboratory showed a stabilising effect of PI3-K on a reporter containing the IL-3 ARE by co-transfection and a subsequent actinomycin D chase experiment. Ming et al. further demonstrated that the stabilising effect of PI3-K could be antagonised by co-transfecting TTP (Ming et al., 2001), suggesting that the stabilising effect of PI3-K does not function directly via inactivation of TTP.

BRF1 is a destabilising AUBP that has been identified as such in our laboratory, therefore we wanted to investigate the regulation of BRF1 activity by PI3-K. Consequently the experiments of Ming et al. mentioned above were repeated using BRF1 in place of TTP. Co-transfection of $BRF1_{wt}$ with the reporter appeared to enhance the decay of the mRNA (Figure 7A, lanes 4-6) compared to the reporter alone (Figure 7A, lanes 1-3). However, the effect was minimal.

Next, p110 was co-transfected with BRF1 (Figure 7A, lanes 10-12) or without (Figure 7A, lanes 7-9). p110 stabilised the reporter mRNA, and this stabilisation was antagonised when co-transfected with BRF1. The experiment was repeated using the ARE minus reporter (pSRL-ΔAU). The ΔAU mRNA was intrinsically

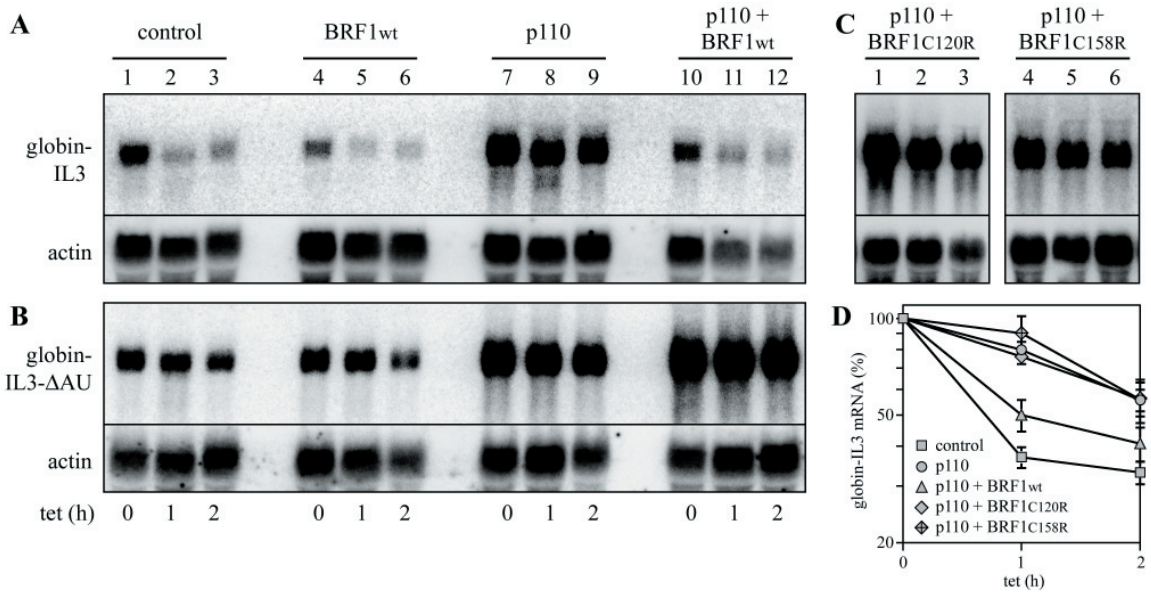


Figure 7: Influence of BRF1 on mRNA decay. (A) NIH 3T3 B2A2-23 cells were transiently transfected with pSRL (lanes 1-3) or in combination, bsd-HisBRF1wt (lanes 4-6), rCD2-p110 (lanes 7-9) or rCD2-p110 and bsd-HisBRF1wt together (lanes 10-12). After 48h, reporter gene transcription was blocked with tetracycline (1μg/ml), and RNA was isolated at the time points indicated. Northern blotting analysis was performed as explained in Figure 2. (B) The same co-transfections as in (A) were carried out using the β-globin-IL3-ΔARE reporter. (C) Zinc-finger mutants of BRF1 were analysed by co-transfection of pSRL together with bsd-HisBRF1C120R (lanes 1-3) or bsd-HisBRF1C158R (lanes 4-6). (D) For quantification of globin-IL3 mRNA decay, signal intensities from (A) and (C), as well as from at least 3 further independent repeat transfections were measured by PhosphoImager, normalised to actin, and plotted as mean values +/- SE against time.

stable, and even co-transfection of BRF1 could not induce decay of the ΔAU mRNA (Figure 7B). This result confirmed that BRF1 acts specifically on ARE-containing mRNAs. Furthermore, two BRF1 zinc-finger mutants were tested for ARE-mRNA decay inducing activity. Both mutants (C120R and C158R) were co-transfected with p110 and a decay assay was performed (Figure 7C). Neither of the mutant BRF1 constructs were able to accelerate degradation of the ARE-mRNA (Figure 7C), indicating that integrity of both zinc-finger domains were required for BRF1 activity.

As PI3-K activates PKB (Djordjevic and Driscoll, 2002), this kinase was transfected next and a decay assay performed. As shown in Figure 8A, lanes 4-6, PKB was able to stabilise the reporter mRNA. When cells were pre-treated with rapamycin, an inhibitor of FRAP/mTOR (Gingras et al., 2001) prior to stopping transcription, no change was observed in the pattern of decay. This indicated that FRAP/mTOR was not necessary for PKB to stabilise the ARE- containing mRNA (Figure 8A, lanes 7-9).

To confirm the efficacy of rapamycin treatment, a western blot using an antibody against the T-389 phosphorylation site of p70 S6-kinase was performed. In the presence of rapamycin, no phosphorylated p70 S6-kinase could be detected, showing that rapamycin is indeed inhibiting FRAP/mTOR. A second antibody was used to detect total amounts of p70 S6-kinase as a loading control (Figure 8D).

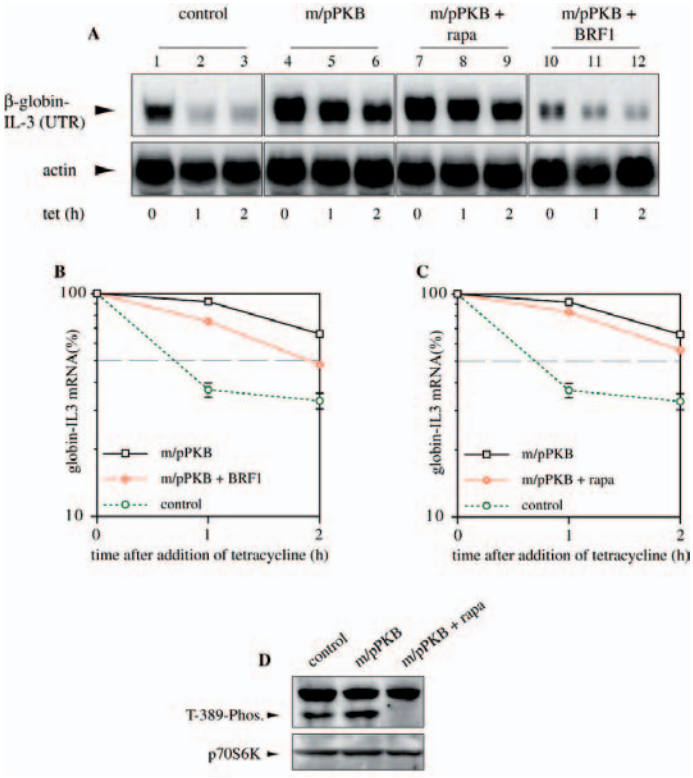


Figure 8: Rapamycin-insensitive, BRF1 sensitive stabilisation of reporter mRNA by PKB. (A) pSRL was transfected alone (lanes 1-3) or in presence of pECE.m/p-HA-PKBα with (lanes 7-9) or without rapamycin (lanes 4-6) or together with bsd-HisBRF1wt (lanes 10-12) into NIH-3T3 B2A2-23 cells. After 24h, transcription was stopped by adding tetracycline and the RNA was isolated at the indicated times and processed for northern blotting. (B) The graph shows the quantifications of at least 5 independent decay assays normalised to the actin signal. Standard errors are shown, unless too small to be represented. (C) Rapamycin inhibits PKB-dependent phosphorylation of p70S6K. Cells treated as in panel (A) were examined for phosphorylated p70S6K by western blotting using phospho (T389)-specific antibodies and anti-p70S6K for loading control.

PKB was next co-transfected with BRF1 as shown in Figure 8A, lanes 10-12. The stabilisation of the ARE-mRNA by PKB was abrogated by BRF1, showing that PKB as well as PI3-K could not overcome the decay inducing activity of over-expressed BRF1.

To determine whether rac, another downstream target of PI3-K, is involved in the regulation of ARE-mRNA stabilisation, a constitutively activated rac mutant was transfected into NIH-3T3 B_2A_2-23 cells and a decay assay was performed. Figure 9, lanes 4-6, show that rac also stabilised the reporter RNA.

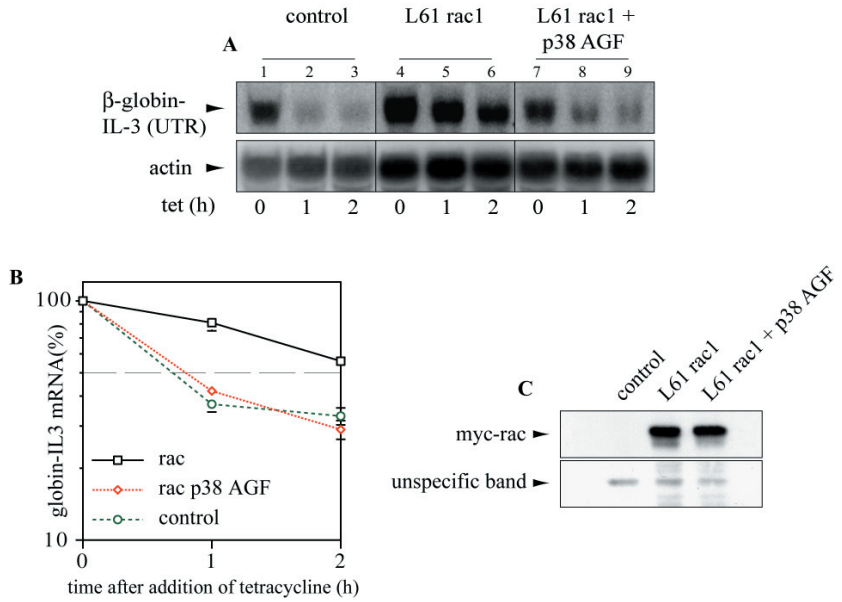


Figure 9: The p38 MAP-kinase pathway is necessary to stabilise reporter mRNA by rac. (A) pSRL alone (lanes 1-3) or together with an activated form of rac (lanes 4-6) or with rac and a dominant-negative form of p38 (lanes 7-9) were co-transfected into NIH-3T3 B2A2-23 cells. The decay experiment and northern blotting were performed as described in Figure 2. (B) For quantification of globin-IL3 mRNA decay, signal intensities from (A), as well as from at least 2 further independent repeat transfections were measured by PhosphoImager, normalised to actin, and plotted as mean values +/- SE against time. (C) To control the expression of rac, a western blot was performed from cells cotransfected in the same way as in (A). rac expression was detected using the anti-c-myc 9E10 monoclonal antibody, an unspecific band served as loading control.

Rac activates the p38 MAPK stress pathways via MEK6 (Xu et al., 2003). To investigate the possibility that rac exerts its stabilising function via the p38 pathway, rac and a dominant negative p38 mutant (p38-AGF) were co-transfected. The result showed that the stabilising activity of rac was blocked (Figure 9A, lanes 7-9), indicating that rac has to signal via p38 in order to perform its stabilising role. The expression of rac was determined by western blotting as described in Figure 9C. No difference in rac expression was seen in co-transfections with p38-AGF or without, situations where the reporter was unstable or stable respectively (Figure 9B). This suggests that the induction of ARE-mRNA decay in the rac/p38-AGF co-transfections was not due to down-

regulation of rac expression, but rather to a direct effect of p38-AGF. To test the p38 pathway directly, MEK6DD, an activated mutant of MEK6, was transfected (Figure 10A, lanes 4-6). Surprisingly and in contrast to the results published by Ming et al. (Ming et al., 2001), the ARE-reporter was still subject to decay in the MEK6DD transfected cells (Figure 10A, lanes 4-6), which suggested that the p38 pathway is necessary but not sufficient for rac to stabilise the reporter mRNA.

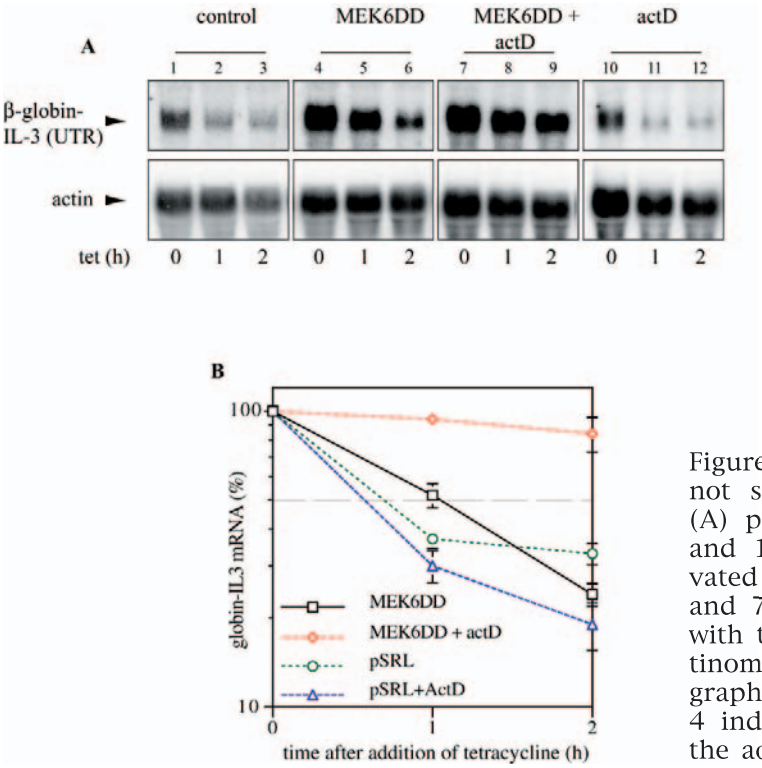


Figure 10: The p38 MAP-kinase pathway is not sufficient to stabilise reporter mRNA. (A) pSRL was transfected alone (lanes 1-3 and 10-11) or with an constitutively activated form of MEK6: MEK6DD (lanes 4-6 and 7-9). Transcription was either stopped with tetracycline (lanes 1-3 and 4-6) or actinomycin D (lanes 7-9 and 10-12). (B) The graph shows the quantifications of at least 4 independent decay assays normalised to the actin signal. Standard errors are shown, except where too small to be represented.

The main difference between the experiments of Ming et al. and those performed here was the use of actinomycin D instead of tetracycline to block transcription. Therefore the same MEK6DD experiment was repeated with the use of actinomycin D. As Figure 10A, lanes 7-9, show, the reporter ARE-mRNA now appeared to be stable. To rule out the possibility that actinomycin D could stabilise the ARE-mRNA on its own, actinomycin D was added to cells transfected only with the reporter and a control vector (pcDNA3.1, see Materials), which resulted again in the rapid degradation of the ARE-mRNA reporter (Figure 10A, lanes 10-12). This indicated an interaction between a non-specific actinomycin D induced effect and MEK6 activity in stabilising ARE mRNAs.
Returning to the p38 pathway, we wanted next to investigate MK2, a downstream target of p38 (Johnson and Lapadat, 2002). The result in Figure 14A, lanes 4-6, showed that MK2 was not able to stabilise the mRNA of the reporter, confirming that the p38 pathway did not directly stabilise mRNAs containing the IL-3 ARE, although it was necessary for the stabilising effect of rac.

4.1.3. Involvement of ras and raf in ARE-mRNA stabilisation

The upstream effector of PI3-K is ras (see Figure 2) (Rodriguez-Viciana et al., 1994), which was tested next. As shown in Figure 11A, lanes 4-5, activated ras stabilised the reporter. When ras was co-transfected with BRF1 (Figure 11A, lanes 7-8), the reporter was still stabilised. To rule out a dose-dependent effect, the experiment was repeated by transfecting 10μg instead of 1.5μg BRF1 plasmid (Figure 11A, lanes 10-12) and even this increased amount of BRF1 was not able to induce decay. To check for BRF1 expression levels, an RT-PCR was performed

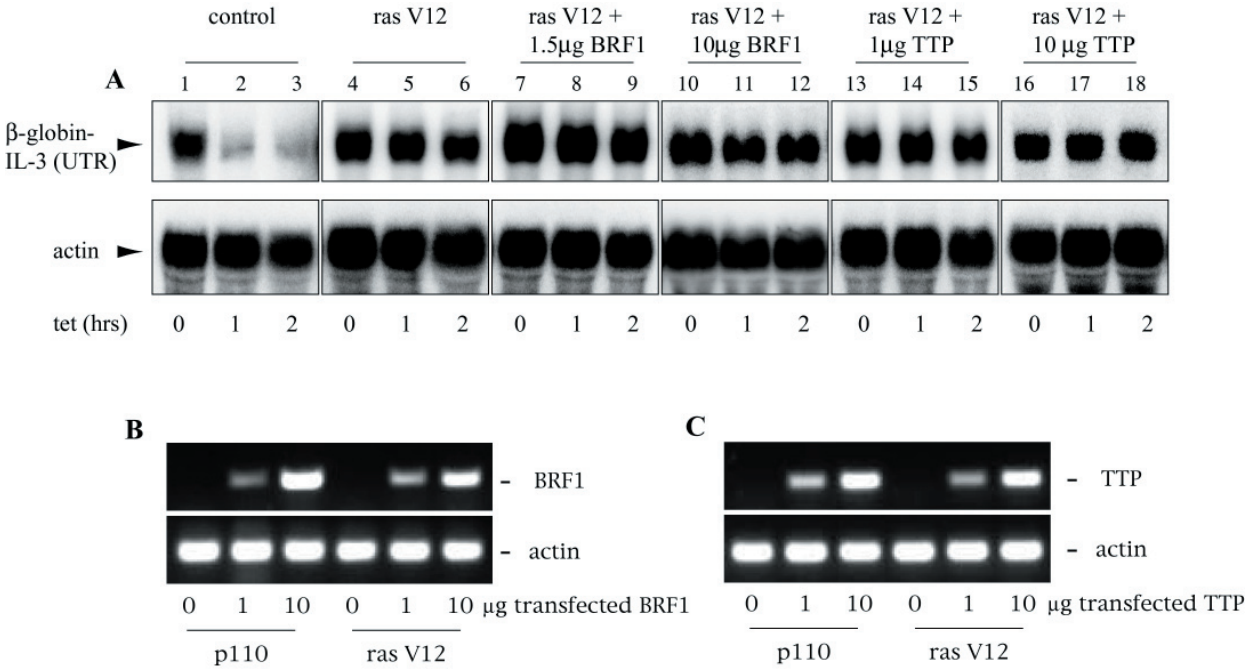


Figure 11: Ras stabilises the reporter mRNA even in the presence of BRF1. (A) NIH 3T3 B2A2-23 cells were transiently transfected with pSRL alone (lanes 1-3) or with pDCR-Ha-rasV12 (lanes 4-6) or pDCR-Ha-rasV12 together with bsd-HisBRF1wt (amounts as indicated, lanes 7-12). After 48h, reporter gene transcription was blocked with tetracycline (1μg/ml), and mRNA was isolated at the time points indicated. Northern blotting analysis was performed as explained in Figure 2. (B) NIH-3T3 B2A2-23 cells were transiently transfected with pSRL, rCD2-p110 or pDCR-Ha-rasV12, and different amounts of bsd-HisBRF1wt, as indicated. After 48h, mRNA was isolated and tested for the exogenous BRF1wt expression and as control for actin expression by RT-PCR. (C) NIH-3T3 B2A2-23 cells were transiently transfected with pSRL, rCD2-p110 or pDCR-Ha-rasV12, and different amounts of pcDNA-mTTP, as indicated. After 48h, mRNA was isolated and tested for the exogenous TTP expression and as control for actin expression by RT-PCR.

to detect exogenous BRF1 mRNA. No difference in BRF1 expression was seen in co-transfections with PI3-K or with ras, situations where BRF1 was active or inactive, respectively (Figure 11B). This suggests that the lack of BRF1 activity in the ras co-transfections was not due to down-regulation of BRF1 expression, but rather to a direct effect of ras. Furthermore, TTP was also tested in a co-

transfection experiment with ras using two different amounts of TTP plasmid as indicated (Figure 11A, lanes 13-18). As seen for BRF1, TTP was also incapable of antagonising ras mediated ARE-mRNA stabilisation. The TTP expression levels were shown by RT-PCR (Figure 11C).
The BRF1-insensitive stabilisation of the reporter by ras might conceivably be due to the effect of a ras downstream pathway other than PI3-K (Chang et al., 2003). Therefore the raf pathway was tested next. A constitutively activated

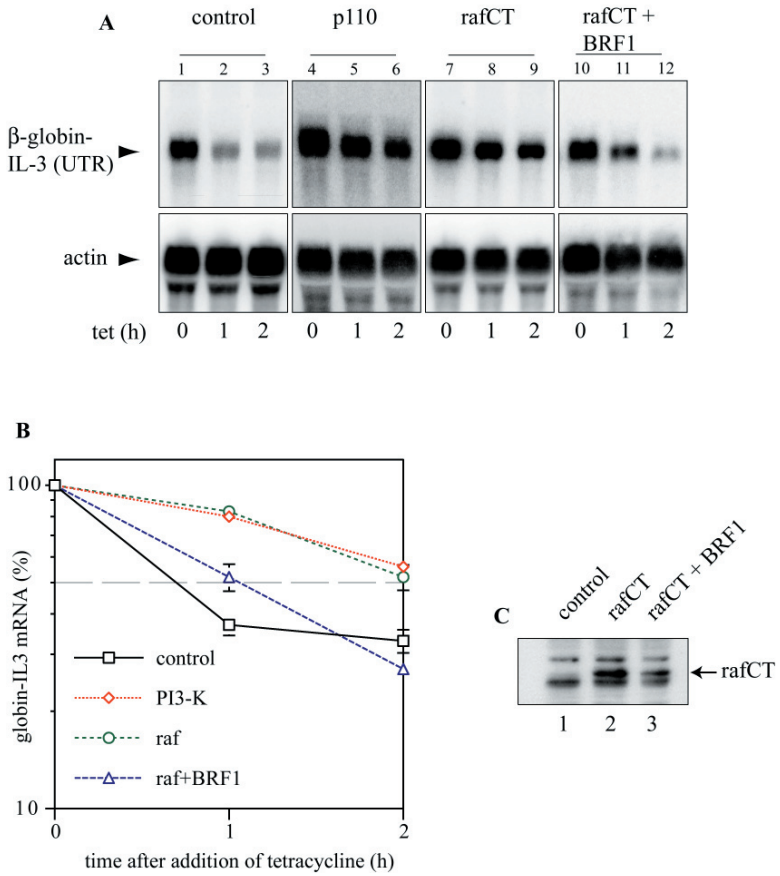


Figure 12: Raf stabilises the reporter in a similar way as PI3-K. (A) pSRL alone (lanes 1-3) or together with activated PI3-K (lanes 4-6) or an active form of raf without BRF1 (lanes 7-9) or with BRF1 (lanes 10-12) were co-transfected into NIH-3T3 B2A2-23 cells. The decay experiment and northern blotting were performed as described in Figure 2. (B) For quantification of globin-IL3 mRNA decay, signal intensities from (A), as well as from independent repeat transfections were measured by PhosphoImager, normalised to actin, and plotted as mean values +/- SE against time. All experiments were repeated at least 4 times. (C) pSRL alone (lane 1) or together with an active form of raf without BRF1 (lane 2) or with BRF1 (lanes 3) were co-transfected into NIH-3T3 B2A2-23 cells. A western blot was performed to detect the transfected raf using an anti-flag antibody.

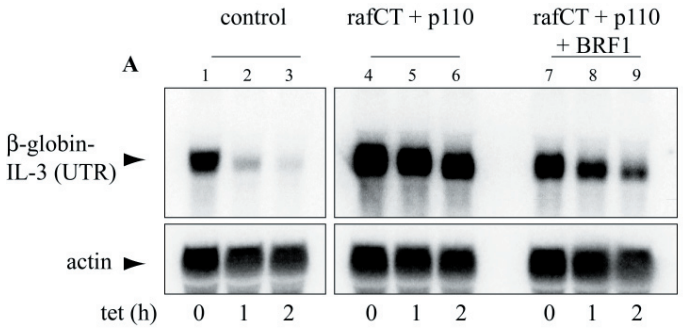
form of raf consisting of the raf C-terminus (rafCT) was transfected with (Figure 12A, lanes 10-12) or without BRF1 (Figure 12A, lanes 7-9) in the NIH-3T3 B_2A_2-23 decay system. By performing a decay assay, it was determined that raf stabilised the reporter to the same extent as PI3-K (see Figure 12B). When raf was co-transfected with BRF1, BRF1 was able to antagonise the stabilisation of the

reporter by raf. To check for raf-expression a western blot was performed from cells transfected with the reporter alone (Figure 12C, lane 1), with raf (Figure 12C, lane 2) or with raf and BRF1 (Figure 12C, lane 3). Raf was expressed, when co-transfected with or without BRF1, but no quantitative statement can be made, as the transfection efficiency was not determined.
Two other downstream targets of ras, ral (Feig, 2003) and PKC (Gutcher et al., 2003), did not yield consistent results in the decay assay. Therefore it was not possible to draw any conclusions or to proceed with further investigations on these signalling proteins (data not shown).

4.1.4. Function of combined pathways in ARE-mRNA stabilisation

4.1.4.1. Cooperation between PI3-K and raf

As ras activates several different downstream pathways (see Figure 2), it is possible that the observed effect of ras, i.e. BRF1 insensitive stabilisation, could be achieved by the collaboration of two pathways, namely the PI3-K and the raf pathway. Therefore we co-transfected raf and PI3-K, which stabilised the reporter as expected (Figure 13A, lanes 4-6). However, co-transfection of BRF1



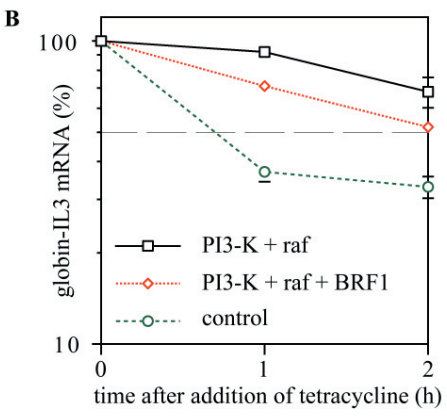


Figure 13: Raf and PI3-K together are not sufficient to antagonise BRF1. (A) pSRL alone (lanes 1-3), together with activated forms of PI3-K and raf together (lanes 4-6) or both kinases with BRF1-wt (lanes 7-9) were co-transfected into NIH-3T3 B2A2-23 cells. The decay experiment and northern blotting were performed as described in Figure 2. (B) For quantification of globin-IL3 mRNA decay, signal intensities from (A), as well as from at least 2 further independent repeat transfections were measured by phosphoImager, normalised to actin, and plotted as mean values +/- SE against time.

with both kinase genes, revealed that the decay promoting activity of BRF1 could not be completely inhibited by the combined effect of both kinases (Figure 13A, lanes 7-9). Nevertheless, the decay curve of the raf/PI3-K/BRF1 co-transfection (Figure 13B) was less pronounced compared to the raf/BRF1- and the PI3-K/BRF1 co-transfections (Figures 9B and 4D, respectively), suggesting some additivity in stabilisation by these kinases.
Taken together, this suggests that the BRF1-insensitive stabilisation effect of ras cannot be explained by simple co-activation of the PI3-K and raf pathways.

4.1.4.2. Cooperation between PKB and MK2

Potential cooperation between MK2 and PKB was studied next. MK2 did not have any stabilising effect on the reporter by itself (Figure 14A, lanes 4-6), but it might possibly assist PKB to overcome the effect of BRF1. Therefore PKB and

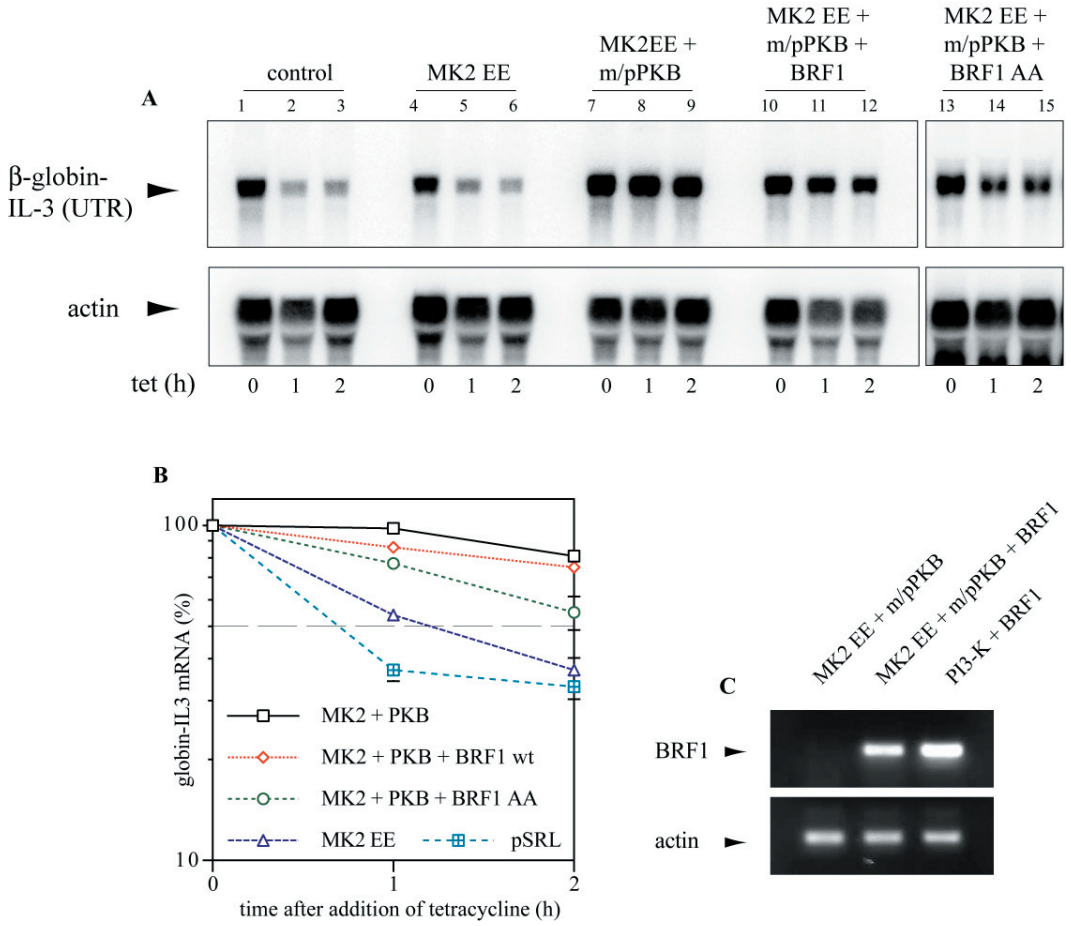


Figure 14: MK2 and PKB together inactivate BRF1. (A) pSRL was transfected alone (lanes 1-3), or in different combinations with activated MK2, activated PKB, BRF1wt and BRF1AA (lanes 4-15) as indicated. (B) For quantification of globin-IL3 mRNA decay, signal intensities from (A), as well as from at least 4 further independent repeat transfections were measured by PhosphoImager, normalised to actin, and plotted as mean values +/- SE against time. (C) RT-PCR was performed from RNA of cells that were transfected with pSRL and plasmids coding for MK2EE, m/pPKB, PI3-K and BRF1-wt, as indicated.

MK2 were co-transfected without (Figure 14A, lanes 7-9), or with BRF1 (Figure 14A, lanes 10-12). Simultaneous co-transfection of PKB and MK2 resulted in strong stabilisation of the reporter mRNA that could not be overcome by BRF1, indicating that the activity of BRF1 might be directly regulated by MK2 and PKB. To control for BRF1 expression, an RT-PCR was performed as described for Figure 11, showing BRF1 expression as indicated (Figure 14C).
Since both kinases are necessary to antagonise BRF1 activity, and as serine 90 and 92 of BRF1 are putative PKB phosphorylation sites (see also next chapter), a preliminary experiment was performed to investigate the importance of serine 90 and/or 92. Therefore a BRF1 mutant containing alanine at positions 90 and 92, which abolished phosphorylation of these sites, was co-transfected with MK2 and PKB. In this experiment BRF1 retained some of its decay activity (Figure 14A, lanes 13-15), which points to a possible function of either serine 90 or 92 in the regulation of BRF1 activity.

```
  1   M  T  T  T  L  V  S  A  T  I  F  D  L  S  E  V  L  C  K  G   20-
 21   N  K  M  L  N  Y  S  A  P  S  A  G  G  C  L  L  D  R  K  A   40-
 41   V  G  T  P  A  G  G  G  F  P  R  R  H  S  V  T  L  P  S  S   60-
 61   K  F  H  Q  N  Q  L  L  S  S  L  K  G  E  P  A  P  A  L  S   80-
 81   S  R  D  S  R  F  R  D  R  S  F  S  E  G  G  E  R  L  L  P  100-
101   T  Q  K  Q  P  G  G  G  Q  V  N  S  S  R  Y  K  T  E  L  C  120-
121   R  P  F  E  E  N  G  A  C  K  Y  G  D  K  C  Q  F  A  H  G  140-
141   I  H  E  L  R  S  L  T  R  H  P  K  Y  K  T  E  L  C  R  T  160-
161   F  H  T  I  G  F  C  P  Y  G  P  R  C  H  F  I  H  N  A  E  180-
181   E  R  R  A  L  A  G  A  R  D  L  S  A  D  R  P  R  L  Q  H  200-
201   S  F  S  F  A  G  F  P  S  A  A  A  T  A  A  A  T  G  L  L  220-
221   D  S  P  T  S  I  T  P  P  P  I  L  S  A  D  D  L  L  G  S  240-
241   P  T  L  P  D  G  T  N  N  P  F  A  F  S  S  Q  E  L  A  S  260-
261   L  F  A  P  S  M  G  L  P  G  G  G  S  P  T  T  F  L  F  R  280-
281   P  M  S  E  S  P  H  M  F  D  S  P  P  S  P  Q  D  S  L  S  300-
301   D  Q  E  G  Y  L  S  S  S  S  S  S  H  S  G  S  D  S  P  T  320-
321   L  D  N  S  R  R  L  P  I  F  S  R  L  S  I  S  D  D  338
```

Figure 15: Amino acid sequence of human BRF1. Serines 90 and 92, the putative PKB phosphorylation sites, are indicated in red, whereas serines 54 and 283, the putative MK2 phosphorylation sites, are indicated in green. For the corresponding nucleic acid sequence see Appendix.

4.2. In vitro regulation of BRF1 activity by PKB

The work in this section was performed in collaboration with the group of Brian Hemmings (Friedrich Miescher Institute, Basel). Phosphorylation of recombinant (r)BRF1 by PKB in an in vitro kinase assay was tested and it was confirmed that PKB indeed phosphorylates rBRF1 (unpublished data of Brian Hemmings). Analysis of the BRF1 sequence indicates two putative PKB phosphorylation sites at serine 90 and 92 shown on Figure 15 (Yang et al., 2002b). This was consistent with the finding that the N-terminal-fragment (aa 3-110), which contains serine 90 and 92, was phosphorylated in an in vitro PKB kinase assay, whereas the zinc-finger- (aa 111-179) and the C-terminal fragments (aa 180-338) were unphosphorylated (unpublished data of Min Lu and diploma thesis of Michel Mallaun (Mallaun, 2002)). By mass-spectrometry on the phosphorylated N-terminal fragment, serine 92 was determined to be the actual PKB phosphorylation site (unpublished data of Daniel Hess and Brian Hemmings).
Meanwhile, a BRF1-dependent in vitro decay assay was established in our laboratory by Min Lu according to Chen et al. (Chen et al., 2001), which was performed in the following way:
S100 extracts were prepared from slowC cells, a cell line lacking native BRF1 expression as the result of frame shift mutations in both alleles (Stoecklin et al., 2001). Radioactive-labelled probes of the IL-3 ARE (ARE_{IL3}, 59nt) and a control

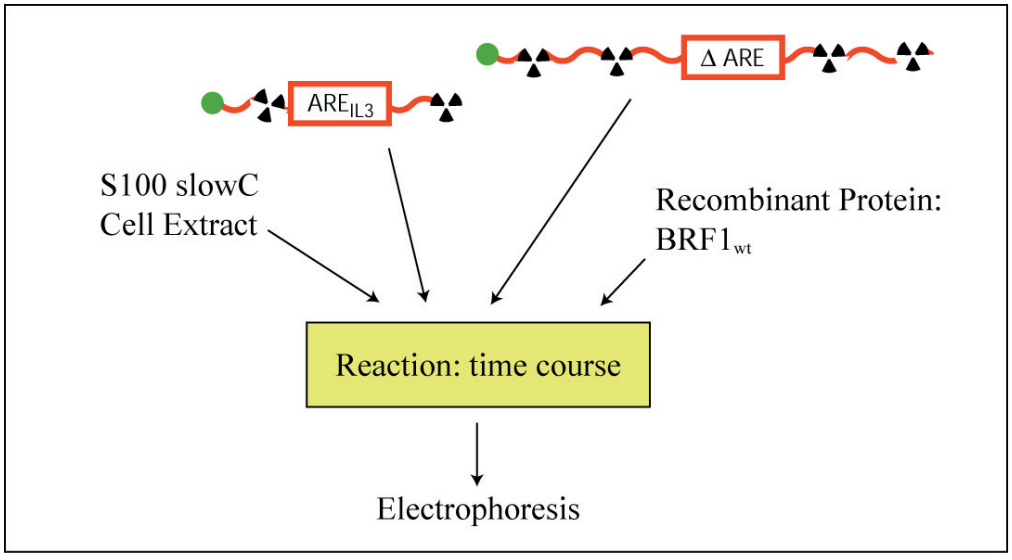


Figure 16: Scheme of the in vitro decay assay.

RNA consisting of the IL-3 3'UTR with an ARE deletion (ΔARE, 270nt) were incubated with the slowC cell extract and BRF1 (Figure 16). Addition of rBRF1 functioned in promoting decay of the ARE-RNA as shown in Figure 17: ARE_{IL3} was stable in the slowC extract over a 45 minute period, but the rate of decay increased as 5ng or more recombinant BRF1 were added. The ΔARE probe served

as an internal control for ARE-specificity of the decay reaction and for equal loading.
This BRF1-dependent in vitro decay system was ideal for investigating the role of PKB phosphorylation on the activity of BRF1. In order to perform the

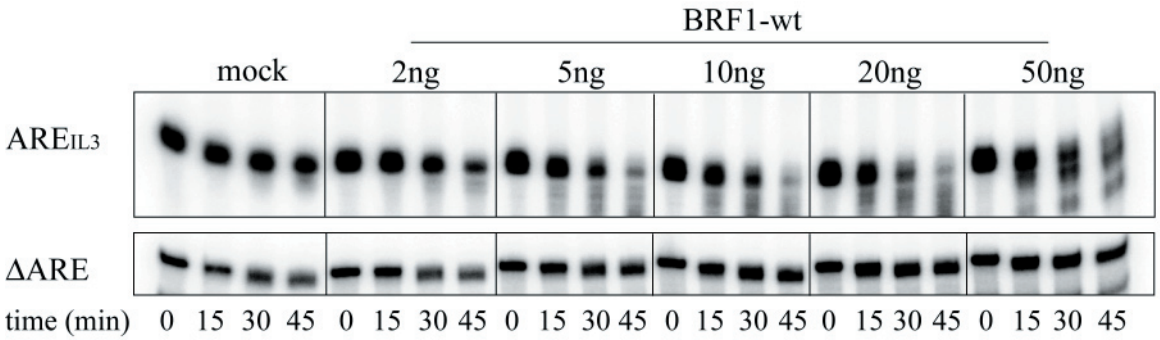


Figure 17: Radioactively labelled ARE_{IL3} and ΔARE were incubated alone (lanes 1-4) or in presence of increasing amounts of BRF1-wt. The decay reaction was stopped at the indicated times and run on a 13% urea-polyacrylamide gel. ΔARE transcripts served as loading and specificity control.

intended experiments it was first necessary to produce recombinant BRF1 proteins that harboured mutations at the sites of interest, namely serine 90 and 92, to abrogate a possible regulatory effect of PKB. Therefore, the serines at positions 90 and 92 were changed either individually or in combination to alanine residues to prevent PKB from phosphorylating these sites. To construct these mutant proteins, the BRF1 gene on the bsd-HisBRF1 plasmid was subjected to site-directed mutagenesis (see Methods) and the mutated BRF1 genes were re-cloned in the pQE-30 vector, which is suitable for production of recombinant proteins (see Methods).

4.2.1. Recombinant BRF1 S90A and S92A

The QIAexpress Expression System from Qiagen was used to construct plasmids carrying the mutated BRF1 gene. M15 bacteria were transformed with these plasmids and several colonies were picked for testing. Figure 18 shows a screen of M15 bacterial clones to select for BRF1 S90A and S92A high expressors. The $BRF1_{S90A}$ clone 3 and the $BRF1_{S92A}$ clone 1 express the recombinant protein (indicated by arrows on Figure 18) after IPTG induction. These clones were selected for large-scale preparation of recombinant protein. After M15 cell lysis and centrifugation, BRF1 was mainly present in the pellet fraction, which was dissolved in a 6M urea buffer (Figure 19). The pellet fraction was purified over a Ni-column, the protein refolded using a urea gradient ranging from 6M to 0M urea and eluted in 1ml fractions. In Figure 20 the fractions 3-7 were run on

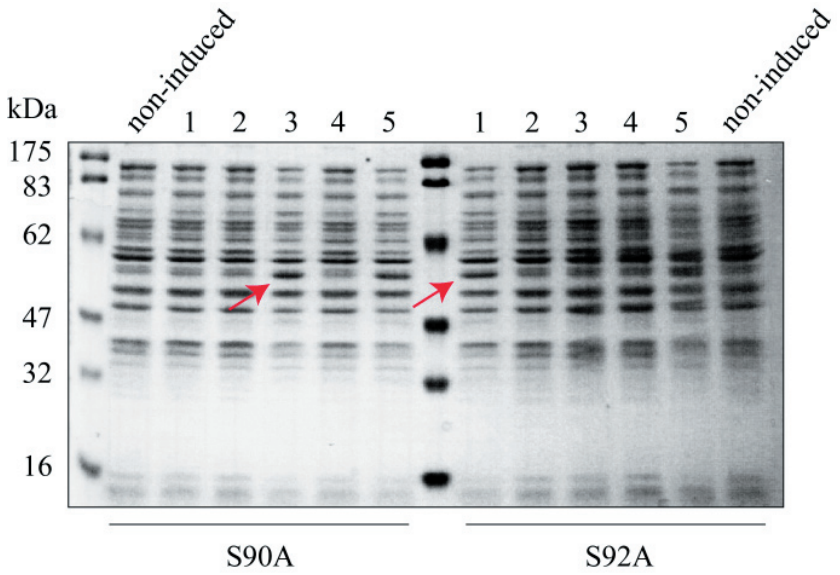


Figure 18: M15 clones that highly express recombinant BRF1.
Ten clones of M15 bacteria transfected each with either pQE-30-BRF1S90A or pQE-30-BRF1S92A were picked, cultured and induced with IPTG for BRF1 expression. 5µl of cell lysates were subjected to SDS-PAGE and stained with coomassie blue to test for recombinant BRF1 high expressing clones. As control, lysates of non-induced cells from the respective clone 1 were used. Arrows indicate induced recombinant BRF1.

a SDS-PAGE. A distinct recombinant BRF1 band could be observed, however it was accompanied by some degradation products of the protein and non-specific contaminants. The same procedure was used to produce recombinant $BRF1_{AA}$, carrying the serine 90 and serine 92 mutations.

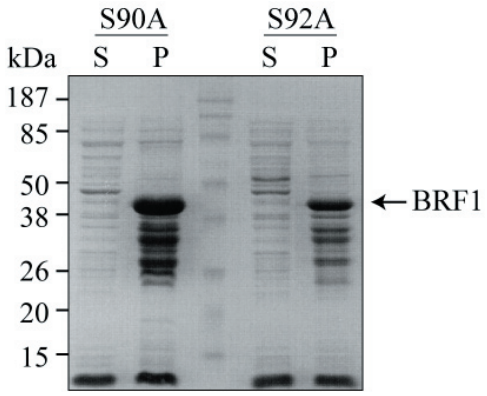


Figure 19: Large scale preparation of BRF1S90A and BRF1S92A recombinant protein. 2µl of the soluble fraction (S) and the pellet fraction (P) of two clones expressing either BRF1S90A or BRF1S92A recombinant protein were tested using a SDS-PAGE to determine in which fraction BRF1 was present.

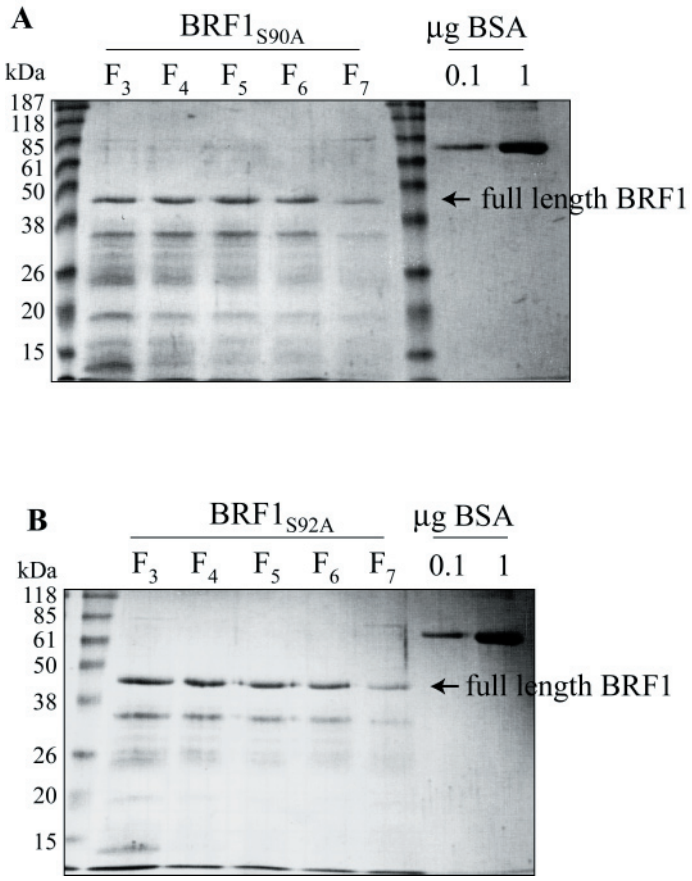


Figure 20: Purified recombinant BRF1 protein. After purification and refolding of the recombinant protein, fractions were eluted from the Ni-beads column and tested on SDS-PAGE for the content and purity of recombinant BRF1.
(A) Fractions of BRF1S90A recombinant protein
(B) Fractions of BRF1S92A recombinant protein

4.2.2. In vitro phosphorylation of BRF1

To confirm the mass-spectrometry result which designated serine 92 as the PKB phosphorylation site, the mutated recombinant proteins were tested in collaboration with Min Lu in a PKB in vitro kinase assay over a time course from 0 to 30 minutes as shown in Figure 21. $BRF1_{wt}$ was strongly phosphorylated by PKB. Phosphorylation of the S92A and the AA double mutant, but not the S90A mutant, was heavily impaired indicating that serine 92 is indeed the preferred PKB phosphorylation site. Residual phosphorylation of $BRF1_{AA}$ may point to an additional minor site.

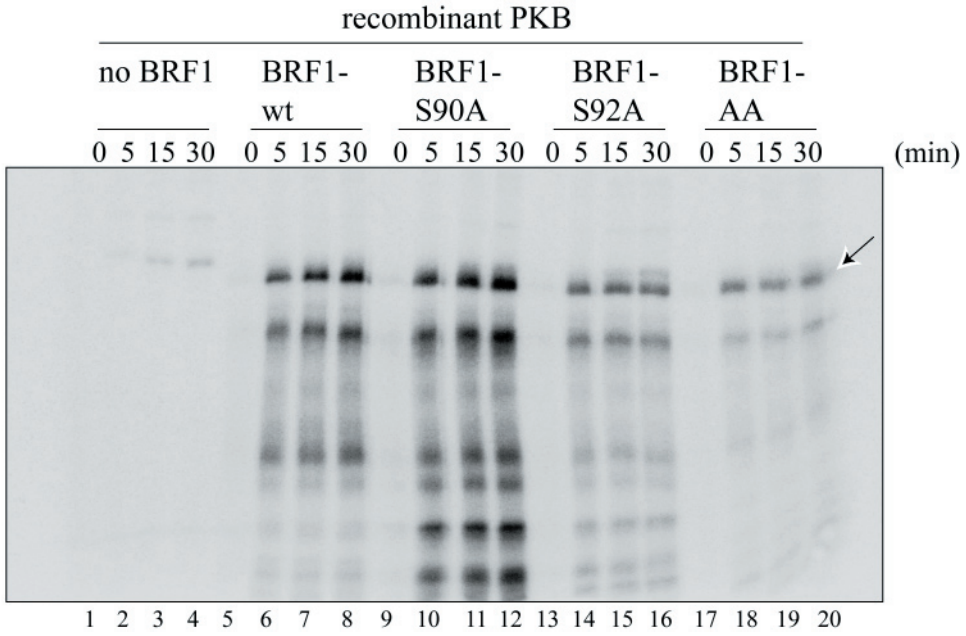


Figure 21: In vitro phosphorylation reactions were performed using 40ng of recombinant BRF1wt, BRF1S90A, BRF1S92A or BRF1AA and 100ng of activated recombinant PKB. The reactions were for 5, 15, 30min as indicated. Arrow indicates full length BRF1.

4.2.3. In vitro regulation of BRF1 by PKB (collaborative experiments with Min Lu)

To determine if PKB phosphorylation regulates the decay-promoting activity of BRF1, the previously described in vitro decay system was used (page 59). This allows control to be exercised over the added BRF1 protein with respect to phosphorylation status and the particular mutation to be tested.

To investigate whether phosphorylation of BRF1 by PKB would alter its activity in vitro, unphosphorylated $rBRF1_{wt}$ was compared to $rBRF1_{wt}$ that was previously incubated with active PKB for 30 minutes (Figure 22A, lanes 5-12). A striking reduction of ARE_{IL3} decay was observed with phosphorylated $rBRF1_{wt}$, showing a loss of decay promoting activity. The experiment was repeated with $rBRF1_{S92A}$ which revealed that the mutation did not affect the basal decay-promoting activity as compared to wt. However, upon pre-incubation with PKB, $BRF1_{S92A}$ could no longer be inactivated in contrast to $BRF1_{wt}$ (Figure 22C). This indicates that regulation of BRF1 activity by PKB is due to phosphorylation at serine 92. The inability to inactivate $BRF1_{S92A}$ after phosphorylation by PKB (Figure 22C, lanes 5-8), also served as an important control to exclude the possibility that

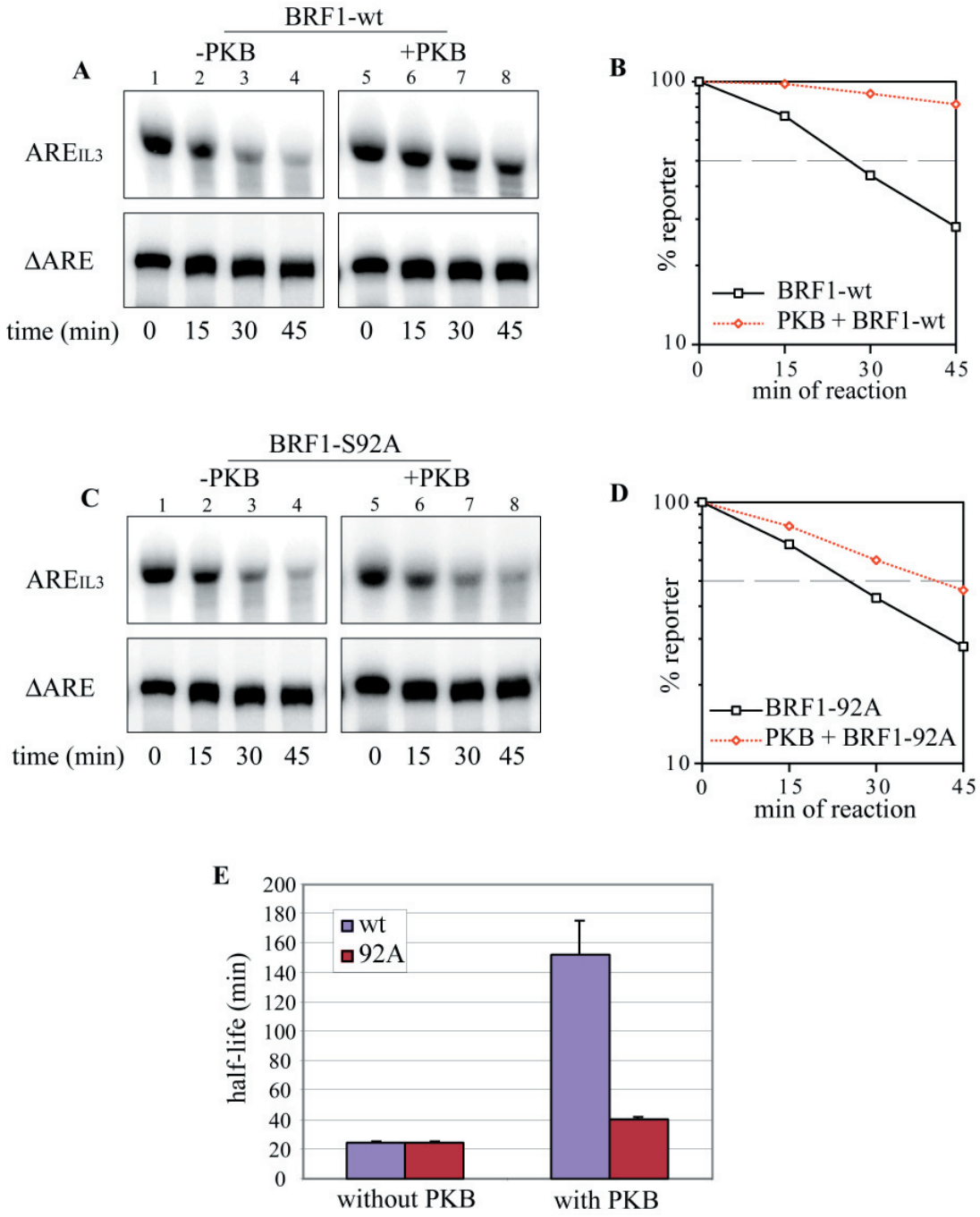


Figure 22: (A) Radioactively labelled ARE_{IL3} and ΔARE were incubated in presence of 20ng unphosphorylated (lanes 1-4) or phosphorylated BRF1-wt (lanes 5-8). The decay reaction was performed as described in Figure 18.
(B) The graph shows the quantifications of at least 4 independent experiments normalised to the ΔARE signal. Standard error bars were too small to be represented.

(C) Radioactively labelled ARE_{IL3} and ΔARE were incubated in presence of BRF1-S92A pre-treated with PKB (lanes 5-8) or buffer (lanes 1-4). Reaction and controls were as in panel A.
(D) The graph shows the quantifications of at least 4 independent experiments normalised to the ΔARE signal. Standard error bars were too small to be represented.
(E) Graph showing the half-lives of in vitro decay reactions from panels B and D.

the stable ARE probe in the presence of phosphorylated BRF1 and PKB was a secondary effect due to addition of PKB to the cell lysate. The quantifications of the experiments shown in Figure 22A and 22C and their repeats were plotted on the graphs in Figure 22B and 22D, respectively. The half–lives are represented on the graph in Figure 22E, showing an identical basal activity of $BRF1_{wt}$ and $BRF1_{S92A}$ and the inactivation of $BRF1_{wt}$ but not of $BRF1_{S92A}$.

4.2.4. Preliminary experiments to investigate PKB regulation in vivo

Insulin is a known activator of the PKB/Akt kinase (Burgering and Coffer, 1995). This could be exploited in order to investigate the regulation of BRF1 ARE-mRNA decay promoting activity by PKB in vivo. In particular the slowC cell line (Stoecklin et al., 2002) was of interest, as wild-type and mutant BRF1 could be stably transfected in this $BRF1^{-/-}$ cellular background with the goal of assaying the BRF1 decay promoting activity of the wild-type and mutant forms after induction of PKB. However, it was necessary to first confirm that PKB could be activated by insulin in slowC cells and also in NIH-3T3 B_2A_2-Tet-off-$BRF1_{wt}$ cells as an additional cell line. Therefore insulin treated slowC cells and B_2A_2–Tet-off-

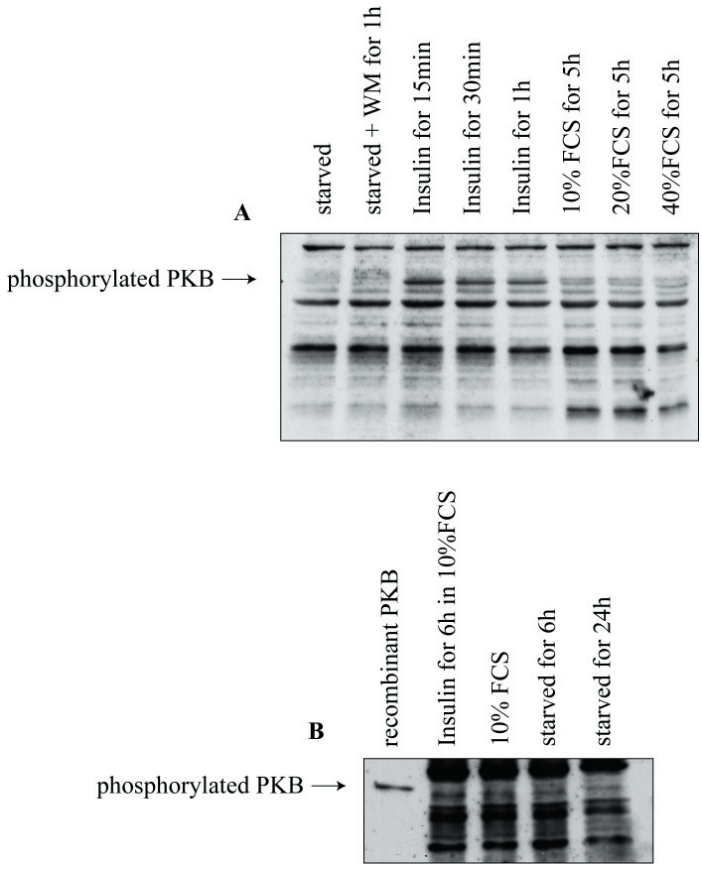


Figure 23: Western blot of NIH-3T3 (A) and of slowC cells (B), that were starved and treated with insulin (0.1mg/ml), wortmannin (200nM) or FCS as indicated. Activation of PKB was detected using an anti phospho-PKB antibody.

$BRF1_{wt}$ cells were tested for PKB activation by western blotting using a phospho-PKB-specific antibody. In NIH-3T3 cells, PKB was strongly activated by insulin after only 15 minutes. This activation decreased slowly over the 1 hour time course of the experiment. FCS, which switches the cells from a quiescent to a growing state and thereby activates growth-inducing pathways, did not have any effect on activation of PKB (Figure 23A).
In contrast, in slowC cells PKB could not be activated either by insulin or FCS (Figure 23B).
As a result it was not possible to establish a system to investigate the effect of in vivo phosphorylation on exogenously introduced wild-type and mutant BRF1 on ARE-mRNA decay in slowC as PKB activity could not be induced. In NIH-3T3 cells at least two distinct phosphorylation signals have to be present in order to inactivate BRF1 (compare Figure 15) and therefore it was not possible to investigate the effect of PKB in isolation. However, the 3T3 cells might still be useful to confirm the phosphorylation of BRF1 by PKB in an actual cellular environment, by using an antibody directed against a phospho-PKB-substrate

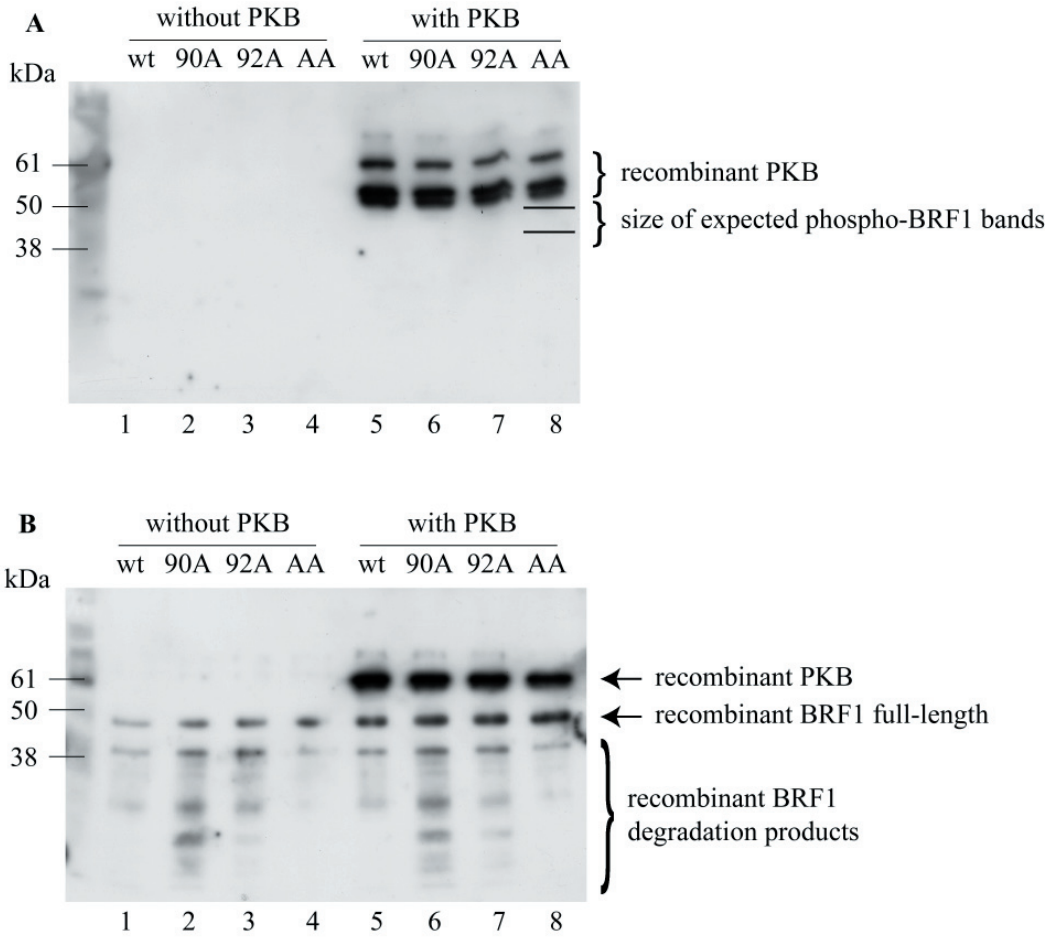


Figure 24: Testing of a PKB substrate antibody: 40ng of recombinant BRF1wt, BRF1S90A, BRF1S92A and BRF1AA were each loaded on a gel either with or without PKB treatment. In panel (A) the proteins were detected using a phospho-PKB substrate antibody, whereas in panel (B) a tetra-His antibody was used.

motif that was designed to recognise any protein phosphorylated by PKB. To first test if the antibody could be used to detect BRF1 phosphorylated by PKB, recombinant $BRF1_{wt}$, $BRF1_{S90A}$, $BRF1_{S92A}$ and $BRF1_{AA}$ were loaded as either PKB-phosphorylated or unphosphorylated forms on separate SDS-polyacrylamide gels. The proteins were detected with either the phospho-PKB-substrate antibody (Figure 24A) or the tetra-His antibody (Figure 24B). The phospho-PKB-substrate antibody was not able to detect any BRF1 regardless of phosphorylation status. The three bands detected by this antibody are PKB derived as was confirmed on other gels by Min Lu (data not shown). As a control for the presence and amounts of BRF1, a tetra-His antibody was used on the second gel and as expected recombinant His-tagged BRF1 was detected with this antibody. As the phospho-PKB-substrate antibody was incapable of detecting BRF1 phosphorylated in vitro by PKB it was unlikely that it would be able to detect the presence of in vivo phosphorylated BRF1 in NIH-3T3 cell extracts.
Therefore alternative systems need to be explored for in vivo confirmation of the in vitro data presented in this work.

5. Discussion

5.1. Multiple signalling pathways regulate the ARE_{IL3}-reporter

In the first part, ras and three of its downstream signalling pathways were investigated for their effect on the stability of an IL-3 ARE reporter mRNA, and for their ability to inactivate BRF1 in co-transfections.

Co-transfection of ras with wild-type BRF1 stabilised the ARE-mRNA reporter and completely inhibited the BRF1 decay inducing activity (see Figure 11). As ras activates a number of downstream pathways, e.g. the PI3-K and the raf pathways (for review: Shields et al., 2000), it was of interest to test which of these pathways were responsible for promoting the effects observed in the ras co-transfections.

Both PI3-K and raf are capable of stabilising the reporter ARE-mRNA by themselves. However when co-transfected with BRF1, BRF1 was able to overcome the stabilising effect of either of these kinases (Figures 7 and 12). Even a simultaneous co-transfection of PI3-K and raf was not able to overcome the decay inducing activity of BRF1 (Figure 13). PI3-K activates two crucial downstream pathways: the PKB and the rac/p38 pathway (Djordjevic and Driscoll, 2002). The PKB pathway is involved in cell growth by activating translation and inhibiting apoptosis (Brazil and Hemmings, 2001), while the rac/p38 pathway is a stress activated signalling pathway that is involved in cytoskeletal reorganisation (Bishop and Hall, 2000). Numerous studies have attributed to the p38/MK2 pathway a central role in regulating ARE-mRNA stabilisation and phosphorylation of different AUBP's (Clark et al., 2003).

In our NIH-3T3 B_2A_2–23 decay system, PKB was able to stabilise the reporter (Figure 8) and when the BRF1 sequence was searched for the PKB phosphorylation consensus, two putative phosphorylation sites were found: at serine 90 and serine 92 (Figure 15, serines shown in red). Mass spectrometry and in vitro kinase assays on $BRF1_{wt}$ and BRF1 mutated at serine 90 and/or serine 92 showed that serine 92 was the PKB phosphorylation site (Figure 21). However, when PKB was co-transfected with BRF1, PKB was unable to antagonise BRF1, although BRF1 activity appeared to be reduced (Figure 8).

Rac was also able to stabilise ARE-mRNA in our system. When co-transfected with p38-AGF, a dominant negative mutant of p38, the stabilisation was blocked, showing the necessity for p38 pathway activation (Figure 9).

Rac activates p38 via signalling through the Pak1/MEK3/6 pathway (Raingeaud et al., 1996) (Xu et al., 2003). Therefore MEK6DD (Stein et al., 1996), a constitutively activated form of MEK6, was tested next in the NIH-3T3 B2A2-23 decay system.

Surprisingly, MEK6DD was unable to stabilise the ARE-reporter (Figure 10),

indicating that the p38 pathway is necessary but not sufficient for rac to stabilise. This was especially surprising as previously published work by Ming et al. (Ming et al., 2001) have indicated that MEK6 stabilises ARE-mRNA. A technical difference between the two approaches was that their experiment was performed using actinomycin D to block transcription while this was accomplished by addition of tetracycline in the present system. It has been shown by Shyu et al., that actinomycin D itself is capable of stabilising mRNA (Shyu et al., 1989). Indeed, when the experiments were repeated using actinomycin D, then the reporter was stabilised (Figure 10). Therefore actinomycin D and MEK6 can somehow interact to achieve ARE-mRNA stabilisation. The other pathway or pathways that are necessary for rac to stabilise are unknown. One possibility might be the PI3-K pathway, as it seems that rac can activate the PI3-K pathway through an unknown mechanism (for review: Welch et al., 2003).

5.2. Cooperation between signalling pathways

The observation that ras inactivates BRF1 indicates that multiple pathways may be involved in BRF1 stabilisation. PKB has been shown to phosphorylate BRF1. However, there is also the possibility that MK2, the downstream kinase of p38 (Engel et al., 1995), might phosphorylate BRF1, as it also has a MK2 consensus phosphorylation site. By comparing the MK2 consensus sequence (X-X-Hyd-X-R-X-X-S-X-X, where Hyd is a bulky hydrophobic residue: Phe>Leu>Val>>Ala; (Huang et al., 1997) (Stokoe et al., 1993)) with the BRF1 sequence, BRF1 was found to contain two putative MK2 phosphorylation sites: S54 and S283, where S54 is the more likely since it has a phenylalanine as Hyd, whereas leucine is at the Hyd-position for the putative MK2 phosphorylation site at position S283 (Figure 12, serines shown in green).
These data indicate that MK2 (which does not stabilise the reporter on its own) and PKB (Figure 14) might co-regulate BRF1. Therefore PKB and MK2 were co-transfected into the NIH-3T3 B_2A_2-23 cell line. Together they were not only able to stabilise the ARE-reporter, but when co-transfected with BRF1 they were also able to overcome the effect of over-expressed BRF1 from initiating decay (Figure 14). This result was in contrast to that observed in the in vitro decay assay.
In the in vitro decay assay, both recombinant $BRF1_{wt}$ and $BRF1_{S92A}$ were able to induce degradation of the ARE_{IL-3}. By subjecting these recombinant proteins to phosphorylation by PKB before they were added to the in vitro decay assay, $BRF1_{wt}$ activity could be inhibited, but not $BRF1_{S92A}$ activity (Figure 22). This shows firstly that phosphorylation of BRF1 at serine 92 by PKB is crucial for inactivation of BRF1 activity in the in vitro decay system, and secondly that PKB alone is sufficient to completely inactivate BRF1, whereas in the NIH-3T3 decay system PKB alone was not sufficient for in vivo inhibition of BRF1 (Figure 8).
The difference between the two systems could be explained by the following.

The mode of regulation of BRF1 is cell-type specific, so that while in NIH-3T3 cells two signals are required, in contrast only one signal is sufficient in HT1080 derived slowC extracts (Stoecklin et al., 2001) to antagonise BRF1. Alternatively, it could be due to the experimental system employed. BRF1 may require a two-step inactivation with a hypothetical first-step, which is PKB independent. By using the slowC S100 cell extract, the first step could be skipped without compromising the second PKB-dependent step. That would suggest that the hypothetical first step may involve changes in localisation within the cell either into cell organelles or at the membrane as these structures are no longer present in S100 extracts. A third possibility is that BRF1 is also phosphorylated at two sites in the in vitro decay assay and that both sites are necessary to inactivate BRF1. In vivo, this second site might be the MK2 site or another adjacent site with the same function as the MK2 site, namely to help PKB inactivate BRF1.
A further intriguing point is that PI3-K does not inactivate BRF1 in the NIH-3T3 cellular system although both PKB and MK2 (as a component of the p38 pathway) are downstream of PI3-K as shown in Figure 2 (Brazil and Hemmings, 2001) (Xu et al., 2003).
However, as these two kinases are only expressed at physiological levels, they are probably not sufficiently active to overcome over-expressed BRF1. Ras, which in contrast to PI3-K is able to inactivate BRF1, activates multiple pathways such as the raf pathway and the PI3-K pathway (for review: Shields et al., 2000). This could enhance the total phosphorylation state of BRF1 as compared to when only the PI3-K pathway is activated, especially when one takes into consideration that different pathways might converge on one particular phosphorylation site on BRF1.

5.3. AUBPs are regulated by multiple phosphorylation

Several other groups have also investigated regulation of AUBP's. As the following paragraphs will show, they are all regulated by several signal transduction pathways and are most probably multiply phosphorylated, which argues for a similar regulation of BRF1 by multiple pathways.
TTP and AUF1 have been shown to be multiply phosphorylated. In the p40 isoform of AUF1 two phosphorylation sites were identified: Serine 83 and serine 87, which were phosphorylated by GSK3β and PKA respectively. The phosphorylated and unphosphorylated proteins could bind equally well to ARE-mRNA, however binding of unphosphorylated p40 AUF1 induces a condensed RNA conformation. In contrast, phosphorylation of p40 AUF1 at serine 83 and serine 87 inhibits this RNA structural transition. TPA leads to stabilisation of the RNA and dephosphorylation of p40 AUF1 (Wilson et al., 2003a) (Wilson et al., 2003b).

TTP was also shown to be phosphorylated at multiple sites by p38, MK2, JNK and p42 MAPK. Serine 187 and Serine 220 were identified as the MK2 and p42 MAPK phosphorylation sites respectively, but the sites phosphorylated by p38 and JNK remain unknown (Chrestensen et al., 2003) (Cao et al., 2003) (Taylor et al., 1995).
In an in vivo system, Rousseau et al. (Rousseau et al., 2002), were able to show that two signalling pathways, the p38 and the raf pathways, are both necessary to promote stabilisation of the ARE-containing chemokine MIP-2 in LPS stimulated cells. By blocking either of the pathways only partial restoration of MIP-2 mRNA decay was achieved, in contrast to complete reinduction of mRNA decay by inhibition of both pathways together. Although they did not show which AUBP was responsible for stabilisation, their result indicates a finely tuned regulation of ARE-mRNA stabilisation and also supports the model of different collaborating pathways in order to achieve full stabilisation. These two pathways could either influence one AUBP or change the balance between different AUBPs.

5.4. Involvement of 14-3-3

14-3-3 proteins are a family of very abundant scaffold proteins involved in the regulation of a variety of cellular processes. Mammals encode seven closely related 14-3-3 isoforms, each of ~31 kDa. They bind to phosphorylated proteins, generally as dimers, and often bind to multiple sites in the same protein. 14-3-3 binding influences the activity of several proteins, and localises others to the cytoplasm in response to external signals. 14-3-3 proteins also act as signal-responsive anti-apoptotic factors by binding to phosphorylated forms of proteins such as the Forkhead-related transcription factors, the apoptosis-inducing kinase ASK1, and the protein BAD (Tzivion and Avruch, 2002).
14-3-3 has been shown to bind to TTP at the phosphorylated serine 187 in an MK2 dependent manner (Johnson et al., 2002) (Chrestensen et al., 2003) and BRF1 has also been shown to bind to 14-3-3 when serine 92 was phosphorylated by PKB (unpublished data by Min Lu and Georg Stoecklin). In BRF1 the binding to the ARE is unchanged by serine 92 phosphorylation, but binding to 14-3-3 is induced which results in antagonisation of BRF1 activity as shown by Min Lu (unpublished data): When the 14-3-3/BRF1 complex was disrupted by a trisdekapeptide containing phosphorylated serine 92, BRF1 was reactivated strongly suggesting that 14-3-3 binding to BRF1 is responsible for BRF1 inactivation. However, it remains a formal possibility that the trisdekapeptide may also interfere with other as yet unknown proteins binding to BRF1. Mechanistically it is still unclear how the 14-3-3/BRF1 interaction regulates the turnover of ARE-mRNA. Two distinct possibilities could be either the inability of the 14-3-3/BRF1 complex to bind to the ARE or that the 14-3-3/BRF1 complex is no longer able to target the exosome to the ARE-mRNA.

5.5. A model of BRF1 regulation

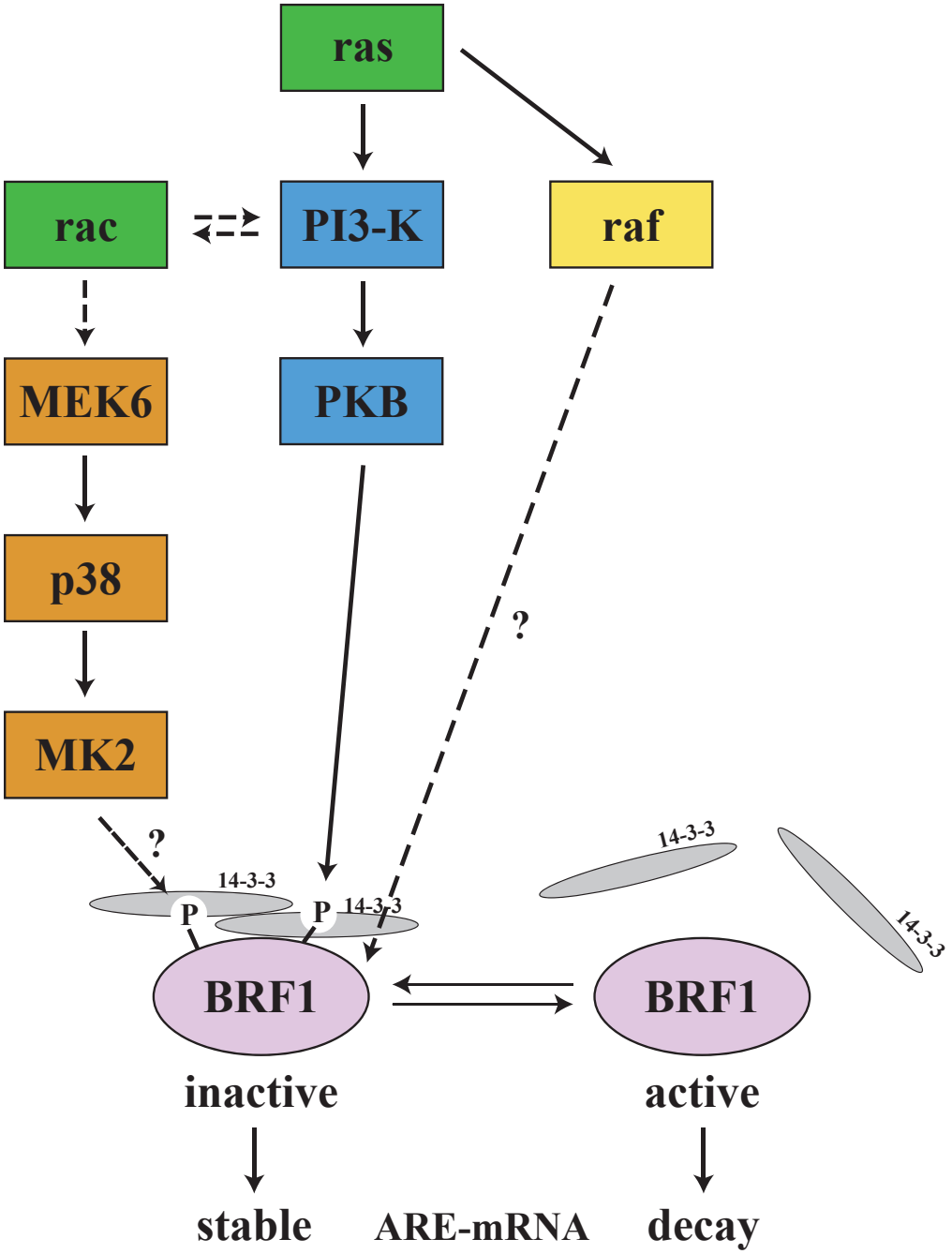


Figure 25: A model showing pathways regulating BRF1 activity. PKB and MK2 phosphorylate BRF1, which might allow binding of 14-3-3 to BRF1 and its subsequent inactivation. The small G-proteins are represented in green, the PKB pathway in blue, the p38 pathway in orange and the raf pathway in yellow.

From the results presented in this work and by others, the following model can be postulated (Figure 22):
Ras, as the most upstream signalling protein stabilises the IL-3 ARE-reporter transcript and was also able to completely inhibit the activity of BRF1 by activating multiple downstream pathways. However none of the three downstream pathways tested, the PI3-K/PKB, the raf and the rac/p38 pathways were able on their own to inhibit BRF1, despite being able to stabilise the ARE-mRNA. In contrast, when PKB and MK2 were co-transfected, they inhibited BRF1, most probably by direct phosphorylation, which might result in binding to 14-3-3.
This model considers only one AUBP, BRF1. In vivo, the fate of a particular mRNA is probably determined by several AUBPs, some having a stabilising or destabilising effect. According to a model by Ming et al. the final result is dependent on the net outcome of the levels and affinities of the various AUBPs that bind to the ARE. Several of these AUBPs have been shown to be phosphorylated by MK2 (see chapter 2.5.2., page 29), which might point to a general role of MK2 in influencing this balance-system of determining mRNA stability.

5.6. Conclusions

The main achievement of this work was to identify signalling molecules regulating BRF1 activity. Serine 92 of BRF1 was shown to be a PKB phosphorylation site of key regulatory importance for the inactivation of BRF1.
It also became clear in the course of the work that the regulation of ARE-mRNA stability is highly complex involving signalling networks with multiple collaborating pathways allowing a very stringent and possibly fine tuned regulation.
Thus far, we have only just begun to get an insight into the fascinating topics of ARE-mRNA stability and signalling, which are functionally linked through the regulation of the respective AUBPs by phosphorylation. Only by a thorough understanding of all the players alone or in combination will we be able to fully understand the regulation of ARE-mRNA stabilisation.

5.7. Outlook

The primary interest would be on BRF1 regulation in vivo, whether BRF1 is phosphorylated by other pathways at additional sites and what their resulting effects are. At present the most interesting candidate for further investigation is MK2 as it is necessary for antagonising BRF1 in the NIH-3T3 B_2A_2-23 decay system. Mutation of the putative phosphorylation sites, serine 54 and serine 283, will allow identification of the actual site and antibodies could be raised against the confirmed phosphorylation site to allow in vivo studies. A PKB/MK2 double

mutant could help to resolve the still open question about the discrepancy between the in vitro system, where PKB alone was sufficient to inhibit BRF1, and the cellular system, in which PKB and MK2 were both required in order to inhibit BRF1. By purifying endo- or exogenous BRF1 using a pull-down strategy or the TAP-tag method in the presence of phosphatase inhibitors followed by mass-spectrometry, physiologically relevant phosphorylation sites could be identified in unstimulated or stimulated cells. This procedure would give a more general overview of the phosphorylation status of BRF1. At every step the binding capacity of BRF1 to the ARE could be tested by band shifts with and without 14-3-3, as this protein also appears to play a role in the regulation of BRF1, as well as the binding capacity between BRF1 and 14-3-3, as this binding is dependent on phosphorylation.

6. Acknowledgements

I want to thank Christoph Moroni for giving me the opportunity to carry out my PhD work in his laboratory and for supporting me during all this time. I'm also especially grateful to Xiu-Fen Ming who introduced me into the subject of signal transduction and who was always ready to advice me even after she moved to Fribourg, but also to Georg Stoecklin for all his support, the challenging discussions and for providing the pSRL-ΔAU and the SP6-ARE plasmids. I further want to thank all the members of the lab. Special thanks to Min Lu, who performed the in vitro kinase assays and the in vitro decay assays and helped in getting the BRF1 AA recombinant protein, to Michel Mallaun and Brigitte Gross for the recombinant N-term. BRF1 fragment and their help in making the recombinant BRF1 mutants and to Don Benjamin for his great support and for all his valuable help on this manuscript. Many thanks also to Brian Hemmings for the exciting collaboration and all the help and advice for the investigations concerning BRF1 phosphorylation by PKB and to Daniel Hess for performing the mass-spectrometry.

For kindly providing plasmids, I want to thank to G. Thomas, P. J. Parker, K. Moelling, A. Hall, B. A. Hemmings, D. A. Cantrell, G. Radziwill and J. L. Bos. For providing the recombinant PKB protein, I want to thank Brian Hemmings.

Last but not least, I also want to thank my parents and Florence Botteri for all their moral support during this time.

7. References

Adjei, A.A. (2001) Blocking oncogenic Ras signaling for cancer therapy. J Natl Cancer Inst, 93, 1062-74.

Andjelkovic, M., Alessi, D.R., Meier, R., Fernandez, A., Lamb, N.J., Frech, M., Cron, P., Cohen, P., Lucocq, J.M. and Hemmings, B.A. (1997) Role of translocation in the activation and function of protein kinase B. J Biol Chem, 272, 31515-24.

Andjelkovic, M., Maira, S.M., Cron, P., Parker, P.J. and Hemmings, B.A. (1999) Domain swapping used to investigate the mechanism of protein kinase B regulation by 3-phosphoinositide-dependent protein kinase 1 and Ser473 kinase. Mol Cell Biol, 19, 5061-72.

Atasoy, U., Watson, J., Patel, D. and Keene, J.D. (1998) ELAV protein HuA (HuR) can redistribute between nucleus and cytoplasm and is upregulated during serum stimulation and T cell activation. J Cell Sci, 111, 3145-56.

Bakheet, T., Frevel, M., Williams, B.R., Greer, W. and Khabar, K.S. (2001) ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. Nucleic Acids Res, 29, 246-54.

Bakheet, T., Williams, B.R. and Khabar, K.S. (2003) ARED 2.0: an update of AU-rich element mRNA database. Nucleic Acids Res, 31, 421-3.

Baron, U., Gossen, M. and Bujard, H. (1997) Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. Nucleic Acids Res, 25, 2723-9.

Bellacosa, A., Testa, J.R., Staal, S.P. and Tsichlis, P.N. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science, 254, 274-7.

Bishop, A.L. and Hall, A. (2000) Rho GTPases and their effector proteins. Biochem J, 348, 241-55.

Blackshear, P.J., Lai, W.S., Kennington, E.A., Brewer, G., Wilson, G.M., Guan, X. and Zhou, P. (2003) Characteristics of the interaction of a synthetic human tristetraprolin tandem zinc finger peptide with AU-rich element-containing RNA substrates. J Biol Chem, 278, 19947-55.

Brazil, D.P. and Hemmings, B.A. (2001) Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci, 26, 657-64.

Brennan, C.M. and Steitz, J.A. (2001) HuR and mRNA stability. Cell Mol Life Sci, 58, 266-77.

Briata, P., Ilengo, C., Corte, G., Moroni, C., Rosenfeld, M.G., Chen, C.Y. and Gherzi, R. (2003) The Wnt/beta-catenin-->Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs. Mol Cell, 12, 1201-11.

Brook, M., Sully, G., Clark, A.R. and Saklatvala, J. (2000) Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. FEBS Lett, 483, 57-61.

Burgering, B.M. and Coffer, P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature, 376, 599-602.

Bustin, S.A. and McKay, I.A. (1999) The product of the primary response gene BRF1 inhibits the interaction between 14-3-3 proteins and cRaf-1 in the yeast trihybrid system. DNA Cell Biol, 18, 653-61.

Bustin, S.A., Nie, X.F., Barnard, R.C., Kumar, V., Pascall, J.C., Brown, K.D., Leigh, I.M., Williams, N.S. and McKay, I.A. (1994) Cloning and characterization of ERF-1, a human member of the Tis11 family of early-response genes. DNA Cell Biol, 13, 449-59.

Butler, J.S. (2002) The yin and yang of the exosome. Trends Cell Biol, 12, 90-6.

Cantrell, D.A. (2001) Phosphoinositide 3-kinase signalling pathways. J Cell Sci, 114, 1439-45.

Cao, H., Dzineku, F. and Blackshear, P.J. (2003) Expression and purification of recombinant tristetraprolin that can bind to tumor necrosis factor-alpha mRNA and serve as a substrate for mitogen-activated protein kinases. Arch Biochem Biophys, 412, 106-20.

Carballo, E. and Blackshear, P.J. (2001) Roles of tumor necrosis factor-alpha receptor subtypes in the pathogenesis of the tristetraprolin-deficiency syndrome. Blood, 98, 2389-95.

Carballo, E., Cao, H., Lai, W.S., Kennington, E.A., Campbell, D. and Blackshear, P.J. (2001) Decreased sensitivity of tristetraprolin-deficient cells to p38 inhibitors suggests the involvement of tristetraprolin in the p38 signaling pathway. J Biol Chem, 276, 42580-7.

Carrington, J.C. and Ambros, V. (2003) Role of microRNAs in plant and animal development. Science, 301, 336-8.

Chang, F., Steelman, L.S., Lee, J.T., Shelton, J.G., Navolanic, P.M., Blalock, W.L., Franklin, R.A. and McCubrey, J.A. (2003) Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia, 17, 1263-93.

Chen, C.Y., Gherzi, R., Andersen, J.S., Gaietta, G., Jurchott, K., Royer, H.D., Mann, M. and Karin, M. (2000) Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. Genes Dev, 14, 1236-48.

Chen, C.Y., Gherzi, R., Ong, S.E., Chan, E.L., Raijmakers, R., Pruijn, G.J., Stoecklin, G., Moroni, C., Mann, M. and Karin, M. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell, 107, 451-64.
Chen, C.Y. and Shyu, A.B. (1995) AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci, 20, 465-70.
Chen, C.Y., Xu, N. and Shyu, A.B. (1995) mRNA decay mediated by two distinct AU-rich elements from c-fos and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation. Mol Cell Biol, 15, 5777-88.
Chen, C.Y., Xu, N. and Shyu, A.B. (2002) Highly selective actions of HuR in antagonizing AU-rich element-mediated mRNA destabilization. Mol Cell Biol, 22, 7268-78.
Chinn, A.M., Ciais, D., Bailly, S., Chambaz, E., LaMarre, J. and Feige, J.J. (2002) Identification of two novel ACTH-responsive genes encoding manganese-dependent superoxide dismutase (SOD2) and the zinc finger protein TIS11b [tetradecanoyl phorbol acetate (TPA)-inducible sequence 11b]. Mol Endocrinol, 16, 1417-27.
Chrestensen, C.A., Schroeder, M.J., Shabanowitz, J., Hunt, D.F., Pelo, J.W., Worthington, M.T. and Sturgill, T.W. (2003) MK2 phosphorylates tristetraprolin on in vivo sites including S178, a site required for 14-3-3 binding. J Biol Chem, 19, 19.
Clark, A.R., Dean, J.L. and Saklatvala, J. (2003) Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. FEBS Lett, 546, 37-44.
Corps, A.N. and Brown, K.D. (1995) Insulin and insulin-like growth factor I stimulate expression of the primary response gene cMG1/TIS11b by a wortmannin-sensitive pathway in RIE-1 cells. FEBS Lett, 368, 160-4.
Crespo, J.L. and Hall, M.N. (2002) Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae. Microbiol Mol Biol Rev, 66, 579-91, table of contents.
de Silanes, I.L., Fan, J., Yang, X., Zonderman, A.B., Potapova, O., Pizer, E.S. and Gorospe, M. (2003) Role of the RNA-binding protein HuR in colon carcinogenesis. Oncogene, 22, 7146-54.
Dean, J.L., Brook, M., Clark, A.R. and Saklatvala, J. (1999) p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. J Biol Chem, 274, 264-9.
DeMaria, C.T. and Brewer, G. (1996) AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. J Biol Chem, 271, 12179-84.
Denli, A.M. and Hannon, G.J. (2003) RNAi: an ever-growing puzzle. Trends Biochem Sci, 28, 196-201.
Dent, P., Yacoub, A., Fisher, P.B., Hagan, M.P. and Grant, S. (2003) MAPK pathways in radiation responses. Oncogene, 22, 5885-96.
Dixon, D.A., Balch, G.C., Kedersha, N., Anderson, P., Zimmerman, G.A., Beauchamp, R.D. and Prescott, S.M. (2003) Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1. J Exp Med, 198, 475-81.
Dixon, D.A., Tolley, N.D., King, P.H., Nabors, L.B., McIntyre, T.M., Zimmerman, G.A. and Prescott, S.M. (2001) Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. J Clin Invest, 108, 1657-65.
Djordjevic, S. and Driscoll, P.C. (2002) Structural insight into substrate specificity and regulatory mechanisms of phosphoinositide 3-kinases. Trends Biochem Sci, 27, 426-32.
Dreyfuss, G., Kim, V.N. and Kataoka, N. (2002) Messenger-RNA-binding proteins and the messages they carry. Nat Rev Mol Cell Biol, 3, 195-205.
Driessens, M.H., Hu, H., Nobes, C.D., Self, A., Jordens, I., Goodman, C.S. and Hall, A. (2001) Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho. Curr Biol, 11, 339-44.
Ehrenman, K., Long, L., Wagner, B.J. and Brewer, G. (1994) Characterization of cDNAs encoding the murine A+U-rich RNA-binding protein AUF1. Gene, 149, 315-9.
Engel, K., Schultz, H., Martin, F., Kotlyarov, A., Plath, K., Hahn, M., Heinemann, U. and Gaestel, M. (1995) Constitutive activation of mitogen-activated protein kinase-activated protein kinase 2 by mutation of phosphorylation sites and an A-helix motif. J Biol Chem, 270, 27213-21.
Feig, L.A. (2003) Ral-GTPases: approaching their 15 minutes of fame. Trends Cell Biol, 13, 419-25.
Gao, M., Wilusz, C.J., Peltz, S.W. and Wilusz, J. (2001) A novel mRNA-decapping activity in HeLa cytoplasmic extracts is regulated by AU-rich elements. Embo J, 20, 1134-43.
Gingras, A.C., Raught, B. and Sonenberg, N. (2001) Regulation of translation initiation by FRAP/mTOR. Genes Dev, 15, 807-26.
Gomperts, M., Pascall, J.C. and Brown, K.D. (1990) The nucleotide sequence of a cDNA encoding an EGF-inducible gene indicates the existence of a new family of mitogen-induced genes. Oncogene, 5, 1081-3.
Grosset, C., Chen, C.Y., Xu, N., Sonenberg, N., Jacquemin-Sablon, H. and Shyu, A.B. (2000) A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a c-fos RNA coding determinant via a protein complex. Cell, 103, 29-40.

Gueydan, C., Droogmans, L., Chalon, P., Huez, G., Caput, D. and Kruys, V. (1999) Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor alpha mRNA. J Biol Chem, 274, 2322-6.
Guhaniyogi, J. and Brewer, G. (2001) Regulation of mRNA stability in mammalian cells. Gene, 265, 11-23.
Gutcher, I., Webb, P.R. and Anderson, N.G. (2003) The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol Life Sci, 60, 1061-70.
Han, Q., Leng, J., Bian, D., Mahanivong, C., Carpenter, K.A., Pan, Z.K., Han, J. and Huang, S. (2002) Rac1-MKK3-p38-MAPKAPK2 pathway promotes urokinase plasminogen activator mRNA stability in invasive breast cancer cells. J Biol Chem, 277, 48379-85.
Heikkinen, H.L., Llewellyn, S.A. and Barnes, C.A. (2003) Initiation-mediated mRNA decay in yeast affects heat-shock mRNAs, and works through decapping and 5'-to-3' hydrolysis. Nucleic Acids Res, 31, 4006-16.
Heinrich, J., Bosse, M., Eickhoff, H., Nietfeld, W., Reinhardt, R., Lehrach, H. and Moelling, K. (2000) Induction of putative tumor-suppressing genes in Rat-1 fibroblasts by oncogenic Raf-1 as evidenced by robot-assisted complex hybridization. J Mol Med, 78, 380-8.
Huang, C.K., Zhan, L., Ai, Y. and Jongstra, J. (1997) LSP1 is the major substrate for mitogen-activated protein kinase-activated protein kinase 2 in human neutrophils. J Biol Chem, 272, 17-9.
Jensen, T.H., Dower, K., Libri, D. and Rosbash, M. (2003) Early formation of mRNP: license for export or quality control? Mol Cell, 11, 1129-38.
Johnson, B.A. and Blackwell, T.K. (2002) Multiple tristetraprolin sequence domains required to induce apoptosis and modulate responses to TNFalpha through distinct pathways. Oncogene, 21, 4237-46.
Johnson, B.A., Geha, M. and Blackwell, T.K. (2000) Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival. Oncogene, 19, 1657-64.
Johnson, B.A., Stehn, J.R., Yaffe, M.B. and Blackwell, T.K. (2002) Cytoplasmic localization of tristetraprolin involves 14-3-3-dependent and -independent mechanisms. J Biol Chem, 277, 18029-36.
Johnson, G.L. and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science, 298, 1911-2.
Kedersha, N. and Anderson, P. (2002) Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. Biochem Soc Trans, 30, 963-9.
Keene, J.D. (1999) Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. Proc Natl Acad Sci U S A, 96, 5-7.
Kiledjian, M., DeMaria, C.T., Brewer, G. and Novick, K. (1997) Identification of AUF1 (heterogeneous nuclear ribonucleoprotein D) as a component of the alpha-globin mRNA stability complex. Mol Cell Biol, 17, 4870-6.
Lai, W.S., Carballo, E., Strum, J.R., Kennington, E.A., Phillips, R.S. and Blackshear, P.J. (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol, 19, 4311-23.
Lai, W.S., Carballo, E., Thorn, J.M., Kennington, E.A. and Blackshear, P.J. (2000) Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to Au-rich elements and destabilization of mRNA. J Biol Chem, 275, 17827-37.
Lai, W.S., Kennington, E.A. and Blackshear, P.J. (2002) Interactions of CCCH zinc finger proteins with mRNA: non-binding tristetraprolin mutants exert an inhibitory effect on degradation of AU-rich element-containing mRNAs. J Biol Chem, 277, 9606-13.
Lai, W.S., Kennington, E.A. and Blackshear, P.J. (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, 3798-812.
Laroia, G., Cuesta, R., Brewer, G. and Schneider, R.J. (1999) Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. Science, 284, 499-502.
Lasa, M., Mahtani, K.R., Finch, A., Brewer, G., Saklatvala, J. and Clark, A.R. (2000) Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. Mol Cell Biol, 20, 4265-74.
Leevers, S.J., Vanhaesebroeck, B. and Waterfield, M.D. (1999) Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Curr Opin Cell Biol, 11, 219-25.
Lejeune, F., Li, X. and Maquat, L.E. (2003) Nonsense-mediated mRNA decay in mammalian cells involves decapping, deadenylating, and exonucleolytic activities. Mol Cell, 12, 675-87.
Levy, N.S., Chung, S., Furneaux, H. and Levy, A.P. (1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J Biol Chem, 273, 6417-23.
Liebmann, C. (2001) Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. Cell Signal, 13, 777-85.
Lindstein, T., June, C.H., Ledbetter, J.A., Stella, G. and Thompson, C.B. (1989) Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway.

Science, 244, 339-43.
Maclean, K.N., McKay, I.A. and Bustin, S.A. (1998) Differential effects of sodium butyrate on the transcription of the human TIS11 family of early-response genes in colorectal cancer cells. Br J Biomed Sci, 55, 184-91.
Maclean, K.N., See, C.G., McKay, I.A. and Bustin, S.A. (1995) The human immediate early gene BRF1 maps to chromosome 14q22-q24. Genomics, 30, 89-90.
Mahtani, K.R., Brook, M., Dean, J.L., Sully, G., Saklatvala, J. and Clark, A.R. (2001) Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. Mol Cell Biol, 21, 6461-9.
Mallaun, M. (2002) Characterisation of the ARE-Binding protein BRF1 (ZFP36L1). Institute of Medical Microbiology. University of Basel, Basel, p. 66.
Mazars, G.R., Moyret, C., Jeanteur, P. and Theillet, C.G. (1991) Direct sequencing by thermal asymmetric PCR. Nucleic Acids Res, 19, 4783.
Mazumder, B., Seshadri, V., Imataka, H., Sonenberg, N. and Fox, P.L. (2001) Translational silencing of ceruloplasmin requires the essential elements of mRNA circularization: poly(A) tail, poly(A)-binding protein, and eukaryotic translation initiation factor 4G. Mol Cell Biol, 21, 6440-9.
McKendrick, L. (2003) What's new in translation initiation? The first translation determines the fate of mRNA. Cell Mol Life Sci, 60, 639-47.
Min, H., Turck, C.W., Nikolic, J.M. and Black, D.L. (1997) A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. Genes Dev, 11, 1023-36.
Ming, X.F., Burgering, B.M., Wennstrom, S., Claesson-Welsh, L., Heldin, C.H., Bos, J.L., Kozma, S.C. and Thomas, G. (1994) Activation of p70/p85 S6 kinase by a pathway independent of p21ras. Nature, 371, 426-9.
Ming, X.F., Kaiser, M. and Moroni, C. (1998) c-jun N-terminal kinase is involved in AUUUA-mediated interleukin-3 mRNA turnover in mast cells. Embo J, 17, 6039-48.
Ming, X.F., Stoecklin, G., Lu, M., Looser, R. and Moroni, C. (2001) Parallel and independent regulation of interleukin-3 mRNA turnover by phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase. Mol Cell Biol, 21, 5778-89.
Mukherjee, D., Gao, M., O'Connor, J.P., Raijmakers, R., Pruijn, G., Lutz, C.S. and Wilusz, J. (2002) The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. Embo J, 21, 165-74.
Mukhopadhyay, D., Houchen, C.W., Kennedy, S., Dieckgraefe, B.K. and Anant, S. (2003) Coupled mRNA stabilization and translational silencing of cyclooxygenase-2 by a novel RNA binding protein, CUGBP2. Mol Cell, 11, 113-26.
Myer, V.E. and Steitz, J.A. (1995) Isolation and characterization of a novel, low abundance hnRNP protein: A0. Rna, 1, 171-82.
Nair, A.P., Hahn, S., Banholzer, R., Hirsch, H.H. and Moroni, C. (1994) Cyclosporin A inhibits growth of autocrine tumour cell lines by destabilizing interleukin-3 mRNA. Nature, 369, 239-42.
Neininger, A., Kontoyiannis, D., Kotlyarov, A., Winzen, R., Eckert, R., Volk, H.D., Holtmann, H., Kollias, G. and Gaestel, M. (2002) MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. J Biol Chem, 277, 3065-8.
Nicholson, K.M. and Anderson, N.G. (2002) The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal, 14, 381-95.
Ning, Z.Q., Norton, J.D., Li, J. and Murphy, J.J. (1996) Distinct mechanisms for rescue from apoptosis in Ramos human B cells by signaling through CD40 and interleukin-4 receptor: role for inhibition of an early response gene, Berg36. Eur J Immunol, 26, 2356-63.
Oldham, S. and Hafen, E. (2003) Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. Trends Cell Biol, 13, 79-85.
Oldham, S., Montagne, J., Radimerski, T., Thomas, G. and Hafen, E. (2000) Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes Dev, 14, 2689-94.
Oxford, G. and Theodorescu, D. (2003a) Ras superfamily monomeric G proteins in carcinoma cell motility. Cancer Lett, 189, 117-28.
Oxford, G. and Theodorescu, D. (2003b) The role of Ras superfamily proteins in bladder cancer progression. J Urol, 170, 1987-93.
Paillard, L. and Osborne, H.B. (2003) East of EDEN was a poly(A) tail. Biol Cell, 95, 211-9.
Pende, A., Tremmel, K.D., DeMaria, C.T., Blaxall, B.C., Minobe, W.A., Sherman, J.A., Bisognano, J.D., Bristow, M.R., Brewer, G. and Port, J. (1996) Regulation of the mRNA-binding protein AUF1 by activation of the beta-adrenergic receptor signal transduction pathway. J Biol Chem, 271, 8493-501.
Peng, S.S., Chen, C.Y. and Shyu, A.B. (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

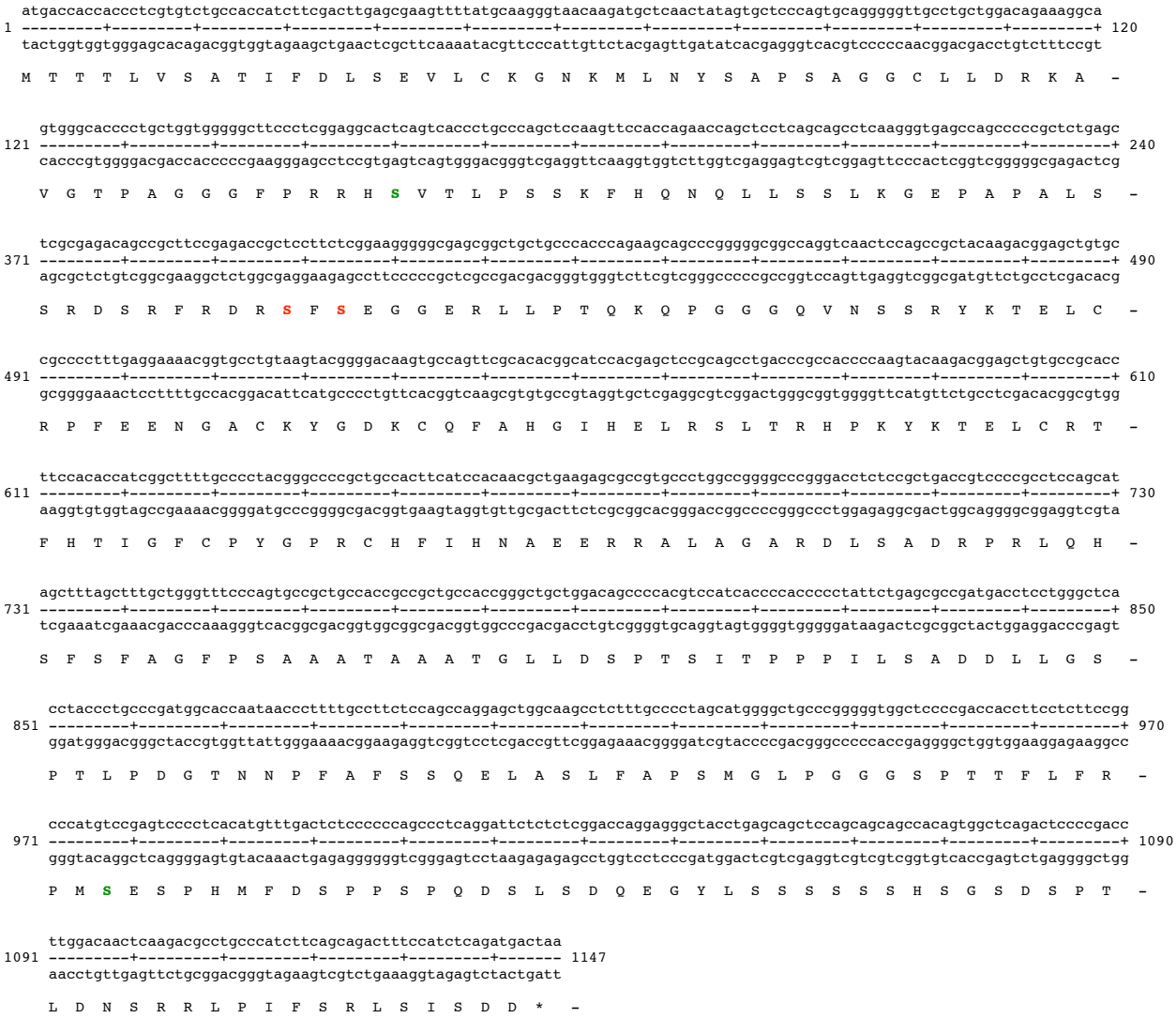
elements. Mol Cell Biol, 16, 1490-9.
Phillips, R.S., Ramos, S.B. and Blackshear, P.J. (2002) Members of the tristetraprolin family of tandem CCCH zinc finger proteins exhibit CRM1-dependent nucleocytoplasmic shuttling. J Biol Chem, 277, 11606-13.
Piecyk, M., Wax, S., Beck, A.R., Kedersha, N., Gupta, M., Maritim, B., Chen, S., Gueydan, C., Kruys, V., Streuli, M. and Anderson, P. (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. Embo J, 19, 4154-63.
Raabe, T., Bukrinsky, M. and Currie, R.A. (1998) Relative contribution of transcription and translation to the induction of tumor necrosis factor-alpha by lipopolysaccharide. J Biol Chem, 273, 974-80.
Radimerski, T., Montagne, J., Rintelen, F., Stocker, H., van der Kaay, J., Downes, C.P., Hafen, E. and Thomas, G. (2002) dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1. Nat Cell Biol, 4, 251-5.
Raineri, I., Wegmueller, D., Gross, B., Certa, U. and 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 Print 2004.
Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J. and Davis, R.J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen- activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem, 270, 7420-6.
Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. and Davis, R.J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol Cell Biol, 16, 1247-55.
Reif, K., Lucas, S. and Cantrell, D. (1997) A negative role for phosphoinositide 3-kinase in T-cell antigen receptor function. Curr Biol, 7, 285-93.
Reif, K., Nobes, C.D., Thomas, G., Hall, A. and Cantrell, D.A. (1996) Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. Curr Biol, 6, 1445-55.
Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D. and Downward, J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature, 370, 527-32.
Rousseau, S., Morrice, N., Peggie, M., Campbell, D.G., Gaestel, M. and Cohen, P. (2002) Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs. Embo J, 21, 6505-14.
Sambrook, J. and Russell, D.W. (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Schiavi, S.C., Wellington, C.L., Shyu, A.B., Chen, C.Y., Greenberg, M.E. and Belasco, J.G. (1994) Multiple elements in the c-fos protein-coding region facilitate mRNA deadenylation and decay by a mechanism coupled to translation. J Biol Chem, 269, 3441-8.
Schoenberg, D.R. and Cunningham, K.S. (1999) Characterization of mRNA endonucleases. Methods, 17, 60-73.
Schonwasser, D.C., Marais, R.M., Marshall, C.J. and Parker, P.J. (1998) Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol Cell Biol, 18, 790-8.
Shields, J.M., Pruitt, K., McFall, A., Shaub, A. and Der, C.J. (2000) Understanding Ras: 'it ain't over 'til it's over'. Trends Cell Biol, 10, 147-54.
Shimada, H., Ichikawa, H., Nakamura, S., Katsu, R., Iwasa, M., Kitabayashi, I. and Ohki, M. (2000) Analysis of genes under the downstream control of the t(8;21) fusion protein AML1-MTG8: overexpression of the TIS11b (ERF-1, cMG1) gene induces myeloid cell proliferation in response to G-CSF. Blood, 96, 655-63.
Shyu, A.B., Greenberg, M.E. and Belasco, J.G. (1989) The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev, 3, 60-72.
Stein, B., Brady, H., Yang, M.X., Young, D.B. and Barbosa, M.S. (1996) Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. J Biol Chem, 271, 11427-33.
Stoecklin, G., Colombi, M., Raineri, I., Leuenberger, S., Mallaun, M., Schmidlin, M., Gross, B., Lu, M., Kitamura, T. and Moroni, C. (2002) Functional cloning of BRF1, a regulator of ARE-dependent mRNA turnover. Embo J, 21, 4709-18.
Stoecklin, G., Gross, B., Ming, X.F. and Moroni, C. (2003a) A novel mechanism of tumor suppression by destabilizing AU-rich growth factor mRNA. Oncogene, 22, 3554-61.
Stoecklin, G., Lu, M., Rattenbacher, B. and Moroni, C. (2003b) A constitutive decay element promotes tumor necrosis factor alpha mRNA degradation via an AU-rich element-independent pathway. Mol Cell Biol, 23, 3506-15.
Stoecklin, G., Ming, X.F., Looser, R. and Moroni, C. (2000) Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. Mol Cell Biol, 20, 3753-63.

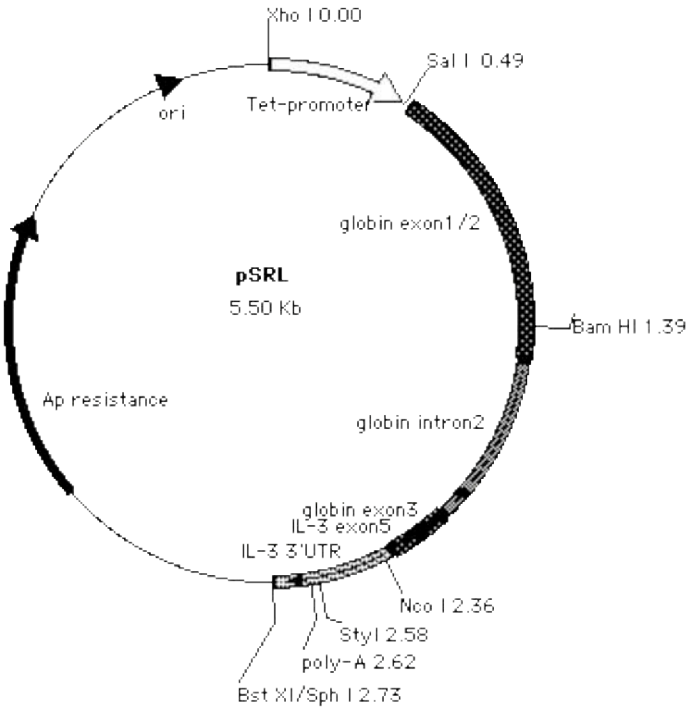
Stoecklin, G., Stoeckle, P., Lu, M., Muehlemann, O. and Moroni, C. (2001) Cellular mutants define a common mRNA degradation pathway targeting cytokine AU-rich elements. Rna, 7, 1578-88.
Stokoe, D., Caudwell, B., Cohen, P.T. and Cohen, P. (1993) The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2. Biochem J, 296, 843-9.
Subbaramaiah, K., Marmo, T.P., Dixon, D.A. and Dannenberg, A.J. (2003) Regulation of cyclooxgenase-2 mRNA stability by taxanes: evidence for involvement of p38, MAPKAPK-2, and HuR. J Biol Chem, 278, 37637-47.
Taylor, G.A., Carballo, E., Lee, D.M., Lai, W.S., Thompson, M.J., Patel, D.D., Schenkman, D.I., Gilkeson, G.S., Broxmeyer, H.E., Haynes, B.F. and Blackshear, P.J. (1996) A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity, 4, 445-54.
Taylor, G.A., Thompson, M.J., Lai, W.S. and Blackshear, P.J. (1995) Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by mitogen-activated protein kinase in vitro. J Biol Chem, 270, 13341-7.
Tran, H., Maurer, F. and Nagamine, Y. (2003) Stabilization of urokinase and urokinase receptor mRNAs by HuR is linked to its cytoplasmic accumulation induced by activated mitogen-activated protein kinase-activated protein kinase 2. Mol Cell Biol, 23, 7177-88.
Tucker, M. and Parker, R. (2000) Mechanisms and control of mRNA decapping in Saccharomyces cerevisiae. Annu Rev Biochem, 69, 571-95.
Tzivion, G. and Avruch, J. (2002) 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem, 277, 3061-4.
Tzivion, G., Luo, Z. and Avruch, J. (1998) A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. Nature, 394, 88-92.
Van Der Giessen, K., DiMarco, S., Clair, E. and Gallouzi, I. (2003) RNAi-Mediated HuR depletion leads to the inhibition of muscle cell differentiation 9. J Biol Chem, 27, 27.
van Hoof, A. and Parker, R. (2002) Messenger RNA degradation: beginning at the end. Curr Biol, 12, R285-7.
Vasudevan, S., Peltz, S.W. and Wilusz, C.J. (2002) Non-stop decay--a new mRNA surveillance pathway. Bioessays, 24, 785-8.
Verheijen, M.H., Wolthuis, R.M., Defize, L.H., den Hertog, J. and Bos, J.L. (1999) Interdependent action of RalGEF and Erk in Ras-induced primitive endoderm differentiation of F9 embryonal carcinoma cells. Oncogene, 18, 4435-9.
Wang, W., Fan, J., Yang, X., Furer-Galban, S., Lopez de Silanes, I., von Kobbe, C., Guo, J., Georas, S.N., Foufelle, F., Hardie, D.G., Carling, D. and Gorospe, M. (2002) AMP-activated kinase regulates cytoplasmic HuR. Mol Cell Biol, 22, 3425-36.
Wang, W., Furneaux, H., Cheng, H., Caldwell, M.C., Hutter, D., Liu, Y., Holbrook, N. and Gorospe, M. (2000) HuR regulates p21 mRNA stabilization by UV light. Mol Cell Biol, 20, 760-9.
Wang, W., Yang, X., Lopez de Silanes, I., Carling, D. and Gorospe, M. (2003) Increased AMP:ATP ratio and AMP-activated protein kinase activity during cellular senescence linked to reduced HuR function. J Biol Chem, 278, 27016-23.
Welch, H.C., Coadwell, W.J., Stephens, L.R. and Hawkins, P.T. (2003) Phosphoinositide 3-kinase-dependent activation of Rac. FEBS Lett, 546, 93-7.
Wilson, G.M. and Brewer, G. (1999) The search for trans-acting factors controlling messenger RNA decay. Prog Nucleic Acid Res Mol Biol, 62, 257-91.
Wilson, G.M., Lu, J., Sutphen, K., Suarez, Y., Sinha, S., Brewer, B., Villanueva-Feliciano, E.C., Ysla, R.M., Charles, S. and Brewer, G. (2003a) Phosphorylation of p40AUF1 regulates binding to A + U-rich mRNA-destabilizing elements and protein-induced changes in ribonucleoprotein structure. J Biol Chem, 278, 33039-48.
Wilson, G.M., Lu, J., Sutphen, K., Sun, Y., Huynh, Y. and Brewer, G. (2003b) Regulation of A + U-rich element-directed mRNA turnover involving reversible phosphorylation of AUF1. J Biol Chem, 278, 33029-38.
Wilusz, C.J., Gao, M., Jones, C.L., Wilusz, J. and Peltz, S.W. (2001) Poly(A)-binding proteins regulate both mRNA deadenylation and decapping in yeast cytoplasmic extracts. Rna, 7, 1416-24.
Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C.Y., Shyu, A.B., Muller, M., Gaestel, M., Resch, K. and 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, 4969-80.
Wodnar-Filipowicz, A. and Moroni, C. (1990) Regulation of interleukin 3 mRNA expression in mast cells occurs at the posttranscriptional level and is mediated by calcium ions. Proc Natl Acad Sci U S A, 87, 777-81.
Wormington, M. (2003) Zero tolerance for nonsense: nonsense-mediated mRNA decay uses multiple degradation pathways. Mol Cell, 12, 536-8.
Xu, N., Chen, C.Y. and Shyu, A.B. (1997) Modulation of the fate of cytoplasmic mRNA by AU-rich

elements: key sequence features controlling mRNA deadenylation and decay. Mol Cell Biol, 17, 4611-21.
Xu, N., Loflin, P., Chen, C.Y. and Shyu, A.B. (1998) A broader role for AU-rich element-mediated mRNA turnover revealed by a new transcriptional pulse strategy. Nucleic Acids Res, 26, 558-65.
Xu, Q., Karouji, Y., Kobayashi, M., Ihara, S., Konishi, H. and Fukui, Y. (2003) The PI 3-kinase-Rac-p38 MAP kinase pathway is involved in the formation of signet-ring cell carcinoma. Oncogene, 22, 5537-44.
Yang, J., Cron, P., Good, V.M., Thompson, V., Hemmings, B.A. and Barford, D. (2002a) Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. Nat Struct Biol, 9, 940-4.
Yang, J., Cron, P., Thompson, V., Good, V.M., Hess, D., Hemmings, B.A. and Barford, D. (2002b) Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. Mol Cell, 9, 1227-40.
Zhang, S., Han, J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R.J. and Bokoch, G.M. (1995) Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J Biol Chem, 270, 23934-6.
Zhang, T., Kruys, V., Huez, G. and Gueydan, C. (2002) AU-rich element-mediated translational control: complexity and multiple activities of trans-activating factors. Biochem Soc Trans, 30, 952-8.
Zhang, W., Wagner, B.J., Ehrenman, K., Schaefer, A.W., DeMaria, C.T., Crater, D., DeHaven, K., Long, L. and Brewer, G. (1993) Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol Cell Biol, 13, 7652-65.
Zhang, Z., Sheng, H., Shao, J., Beauchamp, R.D. and DuBois, R.N. (2000) Posttranscriptional regulation of cyclooxygenase-2 in rat intestinal epithelial cells. Neoplasia, 2, 523-30.
Zhu, W., Brauchle, M.A., Di Padova, F., Gram, H., New, L., Ono, K., Downey, J.S. and Han, J. (2001) Gene suppression by tristetraprolin and release by the p38 pathway. Am J Physiol Lung Cell Mol Physiol, 281, L499-508.
Zimmermann, S. and Moelling, K. (1999) Phosphorylation and regulation of Raf by Akt (protein kinase B). Science, 286, 1741-4.

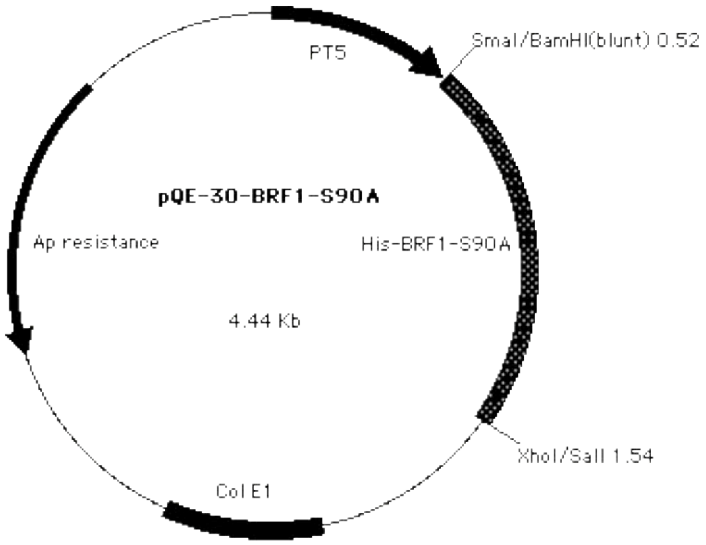
8. Appendix

Nucleic and amino acid sequences of human BRF1





Plasmid name : pSRL
Plasmid size : 5.50 kb
Constructed by : Sabrina Leuenberger
Construction date : 2.2.01
Comments/References : The Tet-Off b-globin reporter construct pSRL was generated from pTet-BBB-AREGMCSF (Xu et al., 1998) by exchanging the BamHI-BstXI (blunted with T4 DNA polymerase) fragment for the BamHI-SphI(blunted with T4 DNA polymerase) fragment of pMXh-b-IL3(UTR)wt (



Plasmid name: pQE-30-BRF1-S90A
Plasmid size: 4.44 kb
Constructed by: Sabrina Leuenberger
Construction date: 8.6.02
Comments/References: pQE-30-BRF1S90A, pQE-30-BRF1S92A and pQE-30-BRF1AA were constructed in the same way as pQE-30-BRF1wt (Stoecklin et al., 2002) using bsd-HisBRF1S90A, bsd-HisBRF1S92A and bsd-HisBRF1AA, respectively.