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Role of USP4 in the Regulation of Gene Expression

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Role of USP4 in the Regulation of Gene Expression

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ABSTRACT

USP4 is a deubiquitinating enzyme whose levels have been shown to be elevated in certain human lung tumors. USP4 is thought to possess oncogenic properties due to its ability to promote tumors in nude mice assays. The lack of an overall effect on ubiquitin levels in overexpression studies has led to the hypothesis that USP4 may act on a few select substrates to edit their ubiquitination status. Although the structure/function relationship is more documented, the physiological substrates and role *in vivo* are not. In order to elucidate the mechanism by which USP4 could potentially exert its tumorigenic effect, an RNA knockdown approach was undertaken. The effect of changes in USP4 levels was investigated to determine if USP4 plays a role in transcription or mRNA stability. The data suggest that USP4 does not affect levels of mRNAs containing an ARE sequence in NIH-3T3 or Cos-7 cells. Although USP4 was not shown to have an mRNA stabilizing effect, USP4 was found to bind CBP *in vivo* using an immunoprecipitation experiment and to exert an effect on basal transcription levels. This data suggests that USP4 levels may affect global transcription, perhaps through binding with the transcriptional co-activator CBP.

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LIST OF ABBREVIATIONS

Arg	Arginine
BSA	Fetal bovine serum
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
cDNA	Complementary deoxyribonucleic acid
CR 1/2	Conserved regions1/2
CREB	Cyclic AMP response element binding protein
C-terminal	Carboxy-terminal
Cys	Cysteine
DNA	Deoxyribonucleic acid
DUB	Deubiquitinating enzyme
EDTA	Ethyl diaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
Gly	Glycine
His	Histidine
Kb	Kilobase
kDa	Kilodalton
Lys	Lysine
MEM	Minimal essential medium
mRNA	Messenger ribonucleic acid
mut	Mutant
NaCl	Sodium chloride
NAF	Sodium fluoride
NaPPi	Sodium pyrophosphate
NES	Nuclear export signal
NLS	Nuclear localization signal
N-terminal	Amino-terminal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
TBST	Tris buffered saline with Tween
TRIS	Tris (hydrozymethyl) aminomethane
Ub	Ubiquitin
UbC	Ubiquitin C
UCH	Ubiquitin C-terminal hydrolase
UBP	Ubiquitin specific protease
UPP	Ubiquitin proteasome pathway
USP	Ubiquitin specific protease
Wt	Wild type

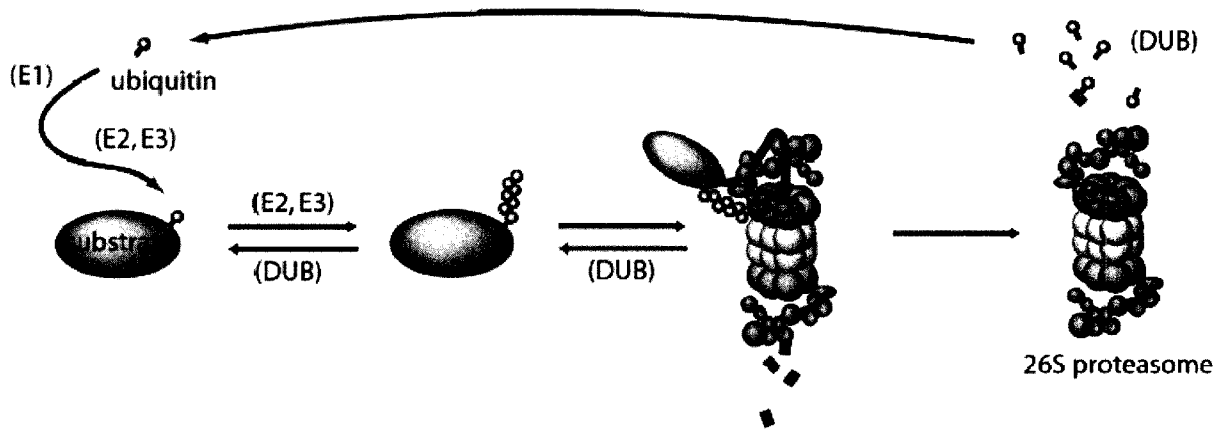
INTRODUCTION

Proteolysis

Cellular proteins are in a dynamic state of synthesis and degradation. Proteolysis is accomplished by proteases which hydrolyze the peptide bonds between amino acids and releases energy. An important role of proteolysis is the removal of damaged proteins or proteins involved in cell cycle or signaling cascades that need to be degraded in a timely manner. Proteolysis is also responsible for recovering molecules that can serve in protein synthesis. Two different systems carry out proteolysis in the cell: lysosomal degradation and the ubiquitin proteasome pathway (UPP) (Ciechanover, 2005). Lysosomal degradation is accomplished via the fusion of endosomes or phagosomes, derived from the plasma membrane, to lysosomes which contain proteases. The lysosomal system is responsive to environmental changes, enhancing lysosomal degradation of certain proteins following nutrient deprivation (Dice et al., 1986). A degree of substrate specificity is observed in the lysosomal system, proteins require a KFERQ-like sequence in order to be transported into the lysosome and are the preferred ones targeted for degradation under stress conditions (reviewed in (Dice et al., 1990)). Proteolysis via the UPP is a highly specific process occurring in both the cytoplasm and nucleus. The protease activity of the UPP is found in the core of the 26S proteasome. Since substrates need to be unfolded to be degraded by the proteasome, proteins that have adopted their appropriate tertiary conformation are not good substrates of the proteasomes. Figure 1 represents a schematic diagram of the UPP (Gray et al., 2003).

Figure 1. Schematic representation of the ubiquitin proteasome pathway (UPP)

The covalent attachment of ubiquitin to a protein substrate occurs through a three-step cascade. First the C-terminal G76 of ubiquitin is activated by an ubiquitin activating enzyme E1. This is followed by substrate recognition and addition of an ubiquitin moiety to the substrate, a step mediated by E2 conjugases and E3 ligases. Subsequent addition of ubiquitin moieties through the formation of an isopeptide bond between K48-G76 forms a polyubiquitin chain. Recognition of threshold polyubiquitin chains by the 26S proteasome leads to degradation of the ubiquitin-tagged substrate and the release of small peptides. Deubiquitinating enzymes (DUBs) recycle ubiquitin by releasing the chains from protein remnants and by cleaving the polyubiquitin chains into free ubiquitin monomers.



Gray et al, SAGEKE, 2003

Ubiquitin-proteasome pathway

Our current understanding of the ubiquitin-proteasome pathway supports the importance of a complex and tightly regulated mechanism for proteolysis of cellular proteins. This pathway has a variety of specific substrates including mutated or post-translational abnormal proteins, cell cycle and growth regulators, signal transduction components as well as housekeeping and metabolic enzymes (DiAntonio et al., 2001; Hershko, 1997; Hershko and Ciechanover, 1998; Wilkinson, 1995). The extensive list of substrates supports the fact that the UPP plays a role in many biological processes including cell cycle progression, signal transduction, transcriptional activation, morphology of neuronal networks, processing of major histocompatibility complex (MHC) antigens, receptor internalization, vesicle trafficking, DNA repair and apoptosis (reviewed in (Ciechanover, 1998)).

Ubiquitin is a 76 amino acid protein that is highly conserved with only 3 amino acids that differ between yeast and human ubiquitin (Ozkaynak et al., 1984). The role of ubiquitin in the UPP is to serve as a tag in order to identify proteins that are destined to be degraded. Proteolysis through the ubiquitin system occurs primarily in the cytoplasm (Glickman and Ciechanover, 2002) and in the nucleus (Cressman and Taub, 1994; von Mikecz, 2006) and proceeds in two successive steps: (i) covalent attachment of multiple ubiquitin moieties to the targeted substrate (ii) degradation of the tagged substrate by the 26S proteasome. Ubiquitin moieties need to be activated at the C-terminal glycine residue in order to bind to substrates. This ATP-dependent activation is accomplished by an ubiquitin activating enzyme E1 and results in the conversion of ubiquitin into a high energy thioester intermediate. This intermediate is then transferred to an ubiquitin conjugating enzyme E2 (UBC). An ubiquitin E3 ligase attaches the activated Gly76 residue of ubiquitin to an

internal lysine of a substrate via an isopeptide bond. Polyubiquitin chains can be formed by subsequent attachments of Gly76 of one ubiquitin moiety to Lys 48 of the preceding ubiquitin. Once the ubiquitin chain reaches a certain threshold level, four ubiquitin moieties (Thrower et al., 2000), the protein substrate is targeted to the proteasome for degradation (reviewed in (Glickman and Ciechanover, 2002)).

The 26S proteasome is a large multicatalytic protease that degrades polyubiquitinated proteins into small peptides (reviewed in (Bochtler et al., 1999)). It is composed of two major subcomplexes: the 20S core particle (CP) and a regulatory 19S regulatory particle. The 20S carries the catalytic activity and is composed of four heptameric rings, two identical outer alpha rings and two identical inner beta rings. The two beta rings face inward into the barrel-shaped proteolytic chamber and contain the proteolytic active sites while the alpha rings are required for structural reasons (Groll et al., 1997). Each end of the 20S can be capped by a 19S regulatory particle which is divided into the base and the lid. The base contains six ATPase subunits and three non ATPase subunits (Rpn1,2 and 10). The lid is made up of eight non-ATPase subunits (Rpn3, 5, 6, 7, 8, 9, 11, 12) (reviewed in (Hilt and Wolf, 1995)). The 19S complex is responsible for the recognition of ubiquitinated proteins and other potential substrates of the proteasome (Braun et al., 1999). Through its ATPase activity, it is in charge of opening the alpha ring of the 20S CP in order to allow the substrates to enter the proteolytic chamber. In addition, it has been suggested that the 19S complex unfolds the substrates in order to insert them into the 20S CP (Braun et al., 1999).

The proteasome recognizes polyubiquitin chains linked by Glycine76-Lysine48 isopeptide bonds and degrades those substrates. However, ubiquitin has been found to be involved in non-proteolytic cellular processes (Aguilar and Wendland, 2003; Sun and Chen, 2004). Cases of polyubiquitin chains linked by Glycine76-Lysine63 bonds also occur.

These chains do not lead to degradation but are thought to participate in DNA repair (Spence et al., 1995), endocytosis of cell surface receptor targeted for lysosomal degradation, stress response and ribosomal function (Glickman and Ciechanover, 2002). Monoubiquitination of proteins can also be observed and is proposed to play a role in processes including transcriptional control through monoubiquitination of histone proteins (Kao et al., 2004; Pavri et al., 2006), endocytocis (Mosesson and Yarden, 2006) and internalization of membrane bound receptors (Haglund et al., 2003).

Deubiquitinating Enzymes

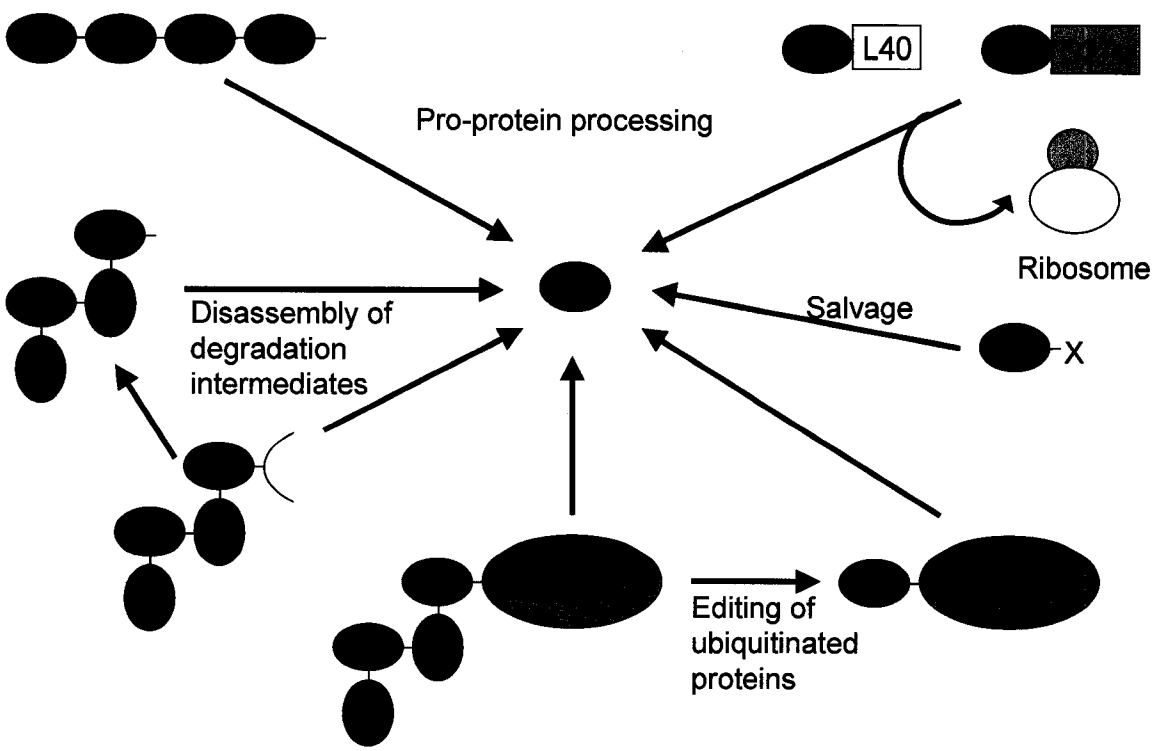
The enzymes responsible for forming ubiquitin conjugates as well as proteasome degradation have received much attention in recent years. However, less is known about the reversible process of ubiquitination, a process performed by deubiquitinating enzymes (DUBs), a family of enzymes with opposing effects. Although their catalytic activity has been tested on artificial substrates (Amerik et al., 2000; Baker et al., 1992; Layfield et al., 1999), little is known about their *in vivo* function and their physiological substrates. DUBs are specific cysteine proteases that can hydrolyze ester, thiol ester and amide bonds of the G76 carboxyl group of ubiquitin (Wilkinson, 1997). DUBs can function by direct or indirect association with the proteasome and provide key regulation of ubiquitin-mediated pathways (Guterman and Glickman, 2004; Hu et al., 2005; Lam et al., 1997; Papa et al., 1999; Stone et al., 2004). Their activity has been implicated in many important biological pathways including cell growth and differentiation, development, oncogenesis, transcriptional regulation and neuronal disease (Amerik and Hochstrasser, 2004; Fischer, 2003; Kim et al., 2003; Soboleva and Baker, 2004).

Deubiquitinating enzymes have traditionally been classified into two major groups on the basis of sequence homology: ubiquitin carboxy-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs, USPs in mammals). This classification does not imply distinct functions to each group and needs to expand following the identification of novel DUB sequences. UCHs are relatively small enzymes (20-30 kDa) that possess 4 conserved motifs spanning a domain of approximately 200 amino acids (Chung and Baek, 1999; D'Andrea and Pellman, 1998). UBPs/USPs vary in size (50-300 kDa) and contain 6 conserved motifs spanning a catalytic domain of at least 400 residues. The highly conserved cysteine and histidine motifs are essential for the catalytic activity of these enzymes (reviewed in (D'Andrea and Pellman, 1998)). Many DUBS, especially UBPs/USPs possess extensions on both or one side of the core region. These extensions can appear at the N-terminal, the C-terminal or as insertions in the catalytic domain. They are thought to be important in substrate recognition, subcellular localization and in protein-protein interactions. In *Saccharomyces cerevisiae*, 1 UCH and 16 UBPs have been identified (Amerik et al., 2000) whereas mammalian genomes encode around 4-5 UCHs and 50-60 USPs (Soboleva and Baker, 2004). The great number of deubiquitinating enzymes supports the idea that they possess highly specific functions and targets.

There are many steps in the ubiquitin pathway in which DUBs can intervene as depicted in Figure 2 (Wilkinson, 1997). Ubiquitin is synthesized as a polyubiquitin fusion arranged in tandem or as a fusion with ribosomal subunits L40 or S27a (Finley and Chau, 1991; Redman and Rechsteiner, 1988; Redman and Rechsteiner, 1989). DUBs cleave these precursor proteins at the C-terminal glycine into their mature forms in order to yield mono-ubiquitin and/or free ribosomal subunits (Wing, 2003). Many UBPs/USPs demonstrate the ability to process these fusions, *in vitro* and *in vivo,* which appears to be a co-translational

Figure 2. Recycling of ubiquitin by deubiquitinating enzymes

DUBs can act to co-translationally process ubiquitin multimers or fusions of ubiquitin with ribosomal subunits. They also disassemble degradation intermediates that arise post-proteasomally and serve in editing polyubiquitin chains to rescue substrates from destruction. They also have a role in salvaging ubiquitin that is trapped by reactions with cellular thiols or amines to replenish ubiquitin pools.



adapted from Wilkinson, 1997

activity (Turner and Varshavsky, 2000). DUBs, mostly UCHs, are also responsible for rescuing ubiquitin from small peptide remnants following proteasomal degradation of proteins (Pickart and Rose, 1985) and are also involved in more target specific roles such as preventing binding of ubiquitin ligases to protein substrates or cleaving ubiquitin once it has been conjugated (reviewed in (Amerik and Hochstrasser, 2004; Soboleva and Baker, 2004)). The editing of polyubiquitin chains can result in the prevention of proteolysis by shortening the ubiquitin chains (van der Horst et al., 2006). DUBs can also bind ligases and prevent ubiquitination through substrate-specific binding competition (Soboleva and Baker, 2004). Although many UBPs/USPs have been recognized for some time, not much is known about their specific substrates.

Some DUBs have been shown to be positive regulators of proteolysis and therefore, promote protein degradation. Yeast Doa4 (Ubp4) is responsible for releasing ubiquitin chains from substrate remnants following proteasome degradation. *doa4* null yeast cells display an accumulation of ubiquitinated proteins which may result in a depletion in free ubiquitin pools (Swaminathan et al., 1999). This in turn impairs proteasome-mediated protein degradation and may result in a loss of cell viability (Papa and Hochstrasser, 1993; Swaminathan et al., 1999). More recently, it has been shown that Doa4 deubiquitinates uracil permease, a plasma membrane protein, prior to its endosomal degradation. Other membrane proteins are also missorted in Doa4 mutant cells (Reggiori and Pelham, 2001). This suggests an important role for Doa4 in the regulation of the endocytic pathway.

Once the polyubiquitin chain is released from the protein remnant, it is further broken down to monomeric form. The disassembly of this so called unanchored polyubiquitin chain is carried out by Ubp14 in yeast and by isopeptidase T, the mammalian homolog (Hadari et al., 1992). The chains are cleaved one ubiquitin monomer at a time starting from the

proximal end with the free carboxy terminus. A null yeast for a Ubp14 strain accumulates polyubiquitin chains and displays a phenotype of depletion of monomeric ubiquitin (Amerik et al., 1997). Therefore, depletion of Ubp14 has an overall effect on cellular ubiquitination and can affect the half-life of substrates that are degraded via the UPP.

Unlike isopeptidases T and doa4, the vast majority of DUBs do not affect overall cellular ubiquitination and are thought to have more target specificity. It is assumed that they modulate the protein half-life of certain specific substrates by editing their ubiquitination status and hence protecting them from proteasome degradation. To date, only a few substrates that are modified by USP proteins have been elucidated. An example is the deubiquitinating enzyme USP7 (otherwise known as HAUSP) which acts on the tumor suppressor p53. p53 is a short lived protein that is maintained at low levels by the ubiquitin protein ligase Mdm2 whose function is to target p53 for proteasome-dependent destruction (Brooks and Gu, 2004).

Another example of a deubiquitinating enzyme whose substrate has been identified is Faf, *Drosophila* fat facets, a large UBP/USP essential for early eye development in *Drosophila* (Fischer-Vize et al., 1992). Mutants of Faf have an abnormal number of photoreceptors in each eye facets. This phenotype can be suppressed by a mutation in a proteasome subunit gene suggesting that Faf is involved in the deubiquitination of certain substrates to protect them from proteolysis. One known substrate of Faf is Lqf, Liquid Facets, which is a member of the epsin family of proteins and is involved in the endocytic pathway (Chen et al., 2002b). There is growing evidence to suggest that Lqf may not be the only substrate of Faf. Considering its role in the regulation of synaptic development at the *Drosophila* neuromuscular junction (DiAntonio et al., 2001) it is conceivable that Faf acts on proteins involved in this process. The mouse homolog of Faf, USP9 (otherwise known as

Fam), has been shown to co-localize and interact with β-catenin (Murray et al., 2004; Taya et al., 1999). Depletion of Fam in mouse embryos leads to a decrease in β-catenin activity suggesting that Fam may stabilize this protein through its deubiquitinating activity (Pantaleon et al., 2001).

Tre-2, a mammalian proto-oncogene, is a deubiquitinating enzyme for which the substrate still remains unknown. However, a truncated isoform of Tre-2 lacking the His domain, the domain partially responsible for its deubiquitinating activity, has been found to be transforming (Nakamura et al., 1992). This suggests that at least one of the substrates of Tre-2 is a tumor suppressor (Nakamura et al., 1992; Papa and Hochstrasser, 1993).

Usp4, a gene related to the tre oncogene, is a mouse deubiquitinating enzyme that was discovered during an analysis of genes linked to the Mpv20 retroviral insertion site (Gupta et al., 1993). It was originally named *Unp* (ubiquitously expressed nuclear protein) but has since been renamed *Usp4* to be in accordance with the new nomenclature for deubiquitinating enzymes (Baker et al., 1999). The mouse *Usp4* gene contains 22 exons spanning 47.2 kb and is located on chromosome 9 which is homologous to human 3p21 (Gupta et al., 1993). A human *Usp4* gene (previously named Unph), which is 91 % identical to the mouse gene, has also been identified and maps to 3p21.3, a chromosomal locus implicated in lung cancer (Naylor et al., 1987; Yamakawa et al., 1993). Human USP4 mRNA levels are elevated in primary tissues of small cell tumors and adenocarcinomas (Gray et al., 1995). In addition, overexpression of murine *Usp4* from the active pgk promoter can transform NIH-3T3 cells in a nude mouse assay (Gupta et al., 1994).

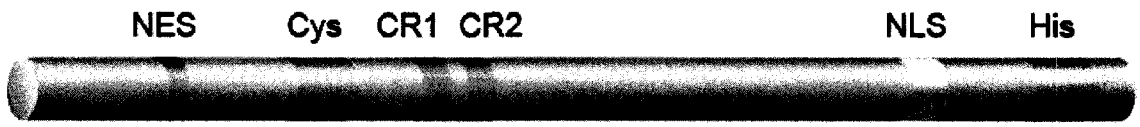
Like other DUBs, USP4 contains conserved Cys and His domains that constitute the deubiquitinating activity of the enzyme (Gilchrist and Baker, 2000). The deubiquitinating activity of USP4 has since been demonstrated in various cleavage assays (Frederick et al.,

1998; Gilchrist and Baker, 2000; Gilchrist et al., 1997). USP4 also contains a nuclear localization signal (NLS) and has recently been found to possess a nuclear export signal (NES) (Soboleva et al., 2005). USP4 has originally been described as a nuclear protein, however, subsequent studies have placed USP4 in the cytoplasm. A recent study has shown that fluorescent tagged exogenous USP4 can be found in the nucleus as well as the cytoplasm (Soboleva et al., 2005). The same conclusion was reached while analyzing the localization of the endogenous USP4 protein suggesting that it may be a nucleocytoplasmic protein (Soboleva et al., 2005). The equilibrium of USP4 in the nucleus versus the cytoplasm varies between cell lines (Soboleva et al., 2005). USP4 also contains CR1 and CR2 domains that are highly homologous to viral protein motifs (such as E1A) which are responsible for binding the tumor suppressor pRb. In fact, USP4 has been shown to interact with pRb, p107 and p130 (Blanchette et al., 2001; DeSalle et al., 2001). This suggests that USP4 may be involved in stabilizing a protein in the pRb pathway and may have a role in cell cycle regulation. USP4 also contains a domain rich in acidic residues the role of which is unknown. A schematic representation of functional domains identified in USP4 is shown in Figure 3.

Recent findings have suggested a role for USP4 in the regulation of cell surface receptor expression (Milojevic et al., 2006). The endoplasmic reticulum (ER) exerts a quality control system that only allows properly folded membrane proteins to be exported. A large fraction of proteins, including membrane proteins, are misfolded during synthesis. Misfolded membrane proteins are ubiquitinated at the surface of the ER and retro-translocated to the cytoplasm by a process known as ERAD (endoplasmic reticulum associated degradation) where they are degraded.

Figure 3. Functional domains identified in USP4

The Cys and His domains of USP4 join to constitute the catalytic domain of the enzyme. The CR1 and CR2 domains are identical to viral protein motifs involved in the binding of tumor suppressor, pRb. The nuclear localization (NLS) and the nuclear export (NES) signals serve in the shuttling of the protein. The acidic domain is of unknown function.



This quality control system ensures that misfolded receptors do not reach the plasma membrane (Pankevych et al., 2003; Petaja-Repo et al., 2001). USP4 has been shown to interact with the C-terminus of the A2a adenosine receptor, a Gs-coupled receptor (Milojevic et al., 2006). USP4 rescues the receptor from degradation through its deubiquitinated activity resulting in an enhanced cell surface expression of the receptor (Milojevic et al., 2006). Ro52, a classic autoantigen involved in Sjögen's syndrome, has also been proposed as a substrate of USP4. Ro52 is a zinc-finger protein that can act as an E3 ubiquitin ligase. Ro52 can ubiquitinate itself as well as USP4 with the help of the E2 ubiquitin-conjugating enzyme UbcH5B (Wada and Kamitani, 2006). A yeast-two hybrid screening of a human cDNA library using Ro52 as bait has lead to the discovery that USP4 binds Ro52 in yeast cells. Recent evidence has shown that USP4 deubiquitinates Ro52 in human cells (HEK293T) (Wada et al., 2006). In addition, USP4 and Ro52 co-localize at the cytoplasmic rod-like structures where it stabilized Ro52 by maintaining its unubiquitinated state. The USP4/Ro52 complex possesses opposing activities and is thought to be involved in modulating the ubiquitination state of certain yet unidentified substrates.

Roles of the UPP in transcription

Although transcription and proteolysis are on opposing ends of a protein's life, the transcriptional machinery and the UPP work together in regulating gene expression (Conaway et al., 2002). Many transcription factors, especially those involved in cell growth, are unstable and are degraded by the UPP (Conaway et al., 2002; Nakagawa and Yokosawa, 2000). For example, many E3 ligases have been shown to have Pol II transcription factors as their targets which results in ubiquitin-dependent degradation of these factors. In addition, Pol II transcription factors as well as nuclear factor kappa B1 (NF-κB1) are synthesized as

inactive precursor proteins (Heusch et al., 1999; Lewis and Manley, 1986). The proteasome processes these precursors into their active forms in an ubiquitin-dependent manner (Hayashi and Faustman, 2000; Karin and Ben-Neriah, 2000; Orian et al., 2000).

Regulation of RNA stability and translation by the UPP

The mRNA levels in the cytoplasm reflect transcription rates, mRNA processing and export, as well as mRNA steady-state stability (Laroia et al., 2002; Wilusz et al., 2001). AU-rich elements (AREs) are located in the 3' noncoding region of certain mRNAs and have been shown to exert a destabilizing effect by a mechanism which has not been elucidated (Espel, 2005; Mitchell and Tollervey, 2000; Wilusz et al., 2001). Some proteins can bind these ARE sequences resulting in either the stabilization or destabilization of the ARE containing-mRNA (Espel, 2005; Raineri et al., 2004). For example, HuR is a nucleocytoplasmic protein that can bind and stabilize certain mRNAs (Chen et al., 2002a; Myer et al., 1997; Tran et al., 2003). On the other hand, AUF1 and tristetrapolin are ARE-binding proteins that have been associated with an increased rate of mRNA degradation (Paschoud et al., 2006; Sarkar et al., 2003). A more recent report has implicated USP4 in the stabilization of ARE containing mRNAs (Laroia et al., 2002). In these studies, overexpression of USP4 resulted in increased stability of granulocyte-macrophage colony-stimulating factor (GM-CSF). This suggests that USP4 may be involved in mRNA stability.

A role for the UPP has also been proposed in translation based on findings which have indicated that many ribosomal proteins as well as eEF1A, a translation elongation factor, are substrates of ubiquitin (Peng et al., 2003). In addition, it has been shown that ribosomes engaged in translation are ubiquitinated and that proper assembly of ribosomes requires the modification of the L28 ribosomal subunit by a K63 linked polyubiquitin chain

(Spence et al., 2000). K63R mutant strains, which have the lysine at position 63 mutated to a arginine, have been shown *in vitro* to possess an altered sensitivity to translational inhibitors and a reduced rate in translation (Spence et al., 1995).

Aims of the research

Although a few interacting partners of USP4 have been discovered in the past few years and a few possible substrates have been proposed, the physiological role of USP4 still remains unknown. For example, it is currently unclear why elevated levels of expression of USP4 have been detected in lung tumors and how this finding can not be explained by the recently proposed roles of USP4. pRb has previously been shown to bind E1A (adenovirus early-region 1A) which contains two conserved motifs namely CR1 and CR2. The interaction between E1A and pocket proteins occurs primarily through the CR2 domain although CR1 has also been shown to be involved in this interaction (Helt and Galloway, 2003). Subsequently, USP4 which also contains CR1 and CR2 motifs has been found to bind pocket proteins including pRb, p107 and p130 (Blanchette et al., 2001; DeSalle et al., 2001). Interestingly, E1A binding to pocket proteins has been shown to down-regulate transcription by the PARP (poly (ADP-ribose) polymerase) gene (Pacini et al., 1999). Similarly, the USP4 and pRb interaction might be important in transcriptional regulation. In addition, E1A also binds p300 and CREB-binding protein (CBP) proteins through its amino terminus and a portion of the CR1 motif (Barbeau et al., 1994). Therefore, we speculate that USP4 might be involved in the regulation of gene expression levels through interactions with CR binding proteins such as pRb and p300/CBP.

The objective of the thesis was to determine whether manipulation of USP4 levels affects RNA levels, and to elucidate if this is due to RNA stability effects or effects on

transcription. The data presented in this thesis argue that although fluctuations in USP4 levels may affect global transcription, perhaps through binding with transcriptional activator CBP, they do not support a role for USP4 in ARE-mRNA stability.

MATERIALS AND METHODS

Cell culture and transfections

NIH-3T3 cells, Cos-7 and U87MG cells were cultured in 5 % CO_2 at 37 °C in α-MEM containing 10 % of a mixture of donor bovine serum and fetal calf serum. For transient transfection experiments in NIH-3T3 cells, the cells were plated in 6 well dishes the day before transfection at a density of 50 000 cells per well. The cells were transfected using GeneJuice (Novagen, LaJolla, CA) and 3 μg of plasmid DNA according to the supplier's protocol. For transfections in 10 cm dishes, cells were plated at a density of 75 000 cells per dish and 9 μg of plasmid DNA were used. Transfections were incubated for 48 hours before analysis. Cos-7 cells and U87MG cells were plated at a density of 200 000 cells per well in 6 well dishes and transfections were incubated for 24 hours before analysis.

Generation of expression constructs

A UbC promoter was inserted into the multiple cloning site of pGL3-basic R2.1 and pGL3-basic R2.2 expression vectors (Sigma-Aldrich Co., St-Louis, Missouri, USA) using BglII (NEB Biolabs) and HindIII (NEB Biolabs) sites to generate UbC-Luc and UbC-Luc-ARE. The GM-CSF ARE sequence was inserted into pGL3-basic containing a UbC promoter. Briefly, the following GM-CSF sense and non-sense primers were annealed together prior to insertion into the vector: GM-CSF ARE sense primer 5'-CTAGATTTATTTATTTATTTATTTACGCGT-3' and GM-CSF ARE non-sense primer 5' CTAGACGCGTAAATAAATAAATAAATA AAT-3'. The annealing step was performed following phophorylation of each primer using T4 polynucleotide kinase (NEB Biolabs). The annealing step was performed at 95 °C for 2 min. followed by a 5 min. incubation at

room temperature. The UbC pGL3-basic vector was linearized using Xba1 digestion and dephosphorylated by shrimp alkaline phosphatase (SAP) (NEB Biolabs) prior to ligation with the ARE insert. Ligation was performed overnight at 16 °C using T4 DNA ligase (NEB Biolabs).

Reporter assays

NIH-3T3, Cos-7 or U87MG cells were plated in 6 well dishes and transiently transfected using GeneJuice as recommended by the supplier (Novagen, LaJolla, CA). Each well was transfected with a constant amount of plasmid DNA containing 4xCRE-Luc (Strategene, LaJolla, CA), *Renilla* expression construct (Promega, Madison, WI) and either with empty control vector (pcDNA3.1) or the appropriate expression vectors. Expression vectors consisted of pGL3-basic R2.1 (Sigma-Aldrich Co., St-Louis, Missouri, USA) containing the UbC promoter (UbC-Luc), pGL3-basic R2.2 (Sigma-Aldrich Co., St-Louis, Missouri, USA) containing the UbC promoter (UbC-Luc-ARE), pGL3 basic with a UbC promoter as well as GM-CSF ARE sequence (UbC-Luc GM-CSF ARE). In addition, cells were transfected with myc wt USP4, Flag wt USP4, myc Cys USP4 mutant, myc CR1 USP4 mutant, myc CR2 USP4 mutant, myc CR1/CR2 USP4 mutant, myc NLS (nuclear localization signal) USP4 mutant, myc acidic domain USP4 mutant, shControl, shUSP4D, UCHL1, Cys UCHL1 mutant or Flag-HuR. Cells were harvested in passive lysis buffer 24 (Cos-7 and U87MG) or 48 hours (NIH-3T3) post-transfection and 20 µl of cell extract was assayed for luciferase activity using a Dual-Luciferase™ reporter assay system as per the supplier's protocol (Promega, Madison, WI). Data analysis was performed using Prism (Graph pad software Inc., San Diego, CA).

ELISA assays

Cos-7 cells were plated in 6 well dishes and transiently transfected with a constant amount of pcDNA3.1 control, myc wt USP4, Flag HuR or co-transfected with Flag HuR and myc wt USP4 using GeneJuice as per the manufacturer's protocol (Novagen, LaJolla, CA). Cells were harvested in lysis buffer 24 hours post-transfection and 100 µl of cell extract was assayed for GM-CSF expression levels using a human GM-CSF immunoassay as per the manufacturer's protocol (Quantikine, R&D Systems Inc., Minneapolis, USA). The optical density was read using a microplate reader set to 450 nm.

Flow cytometry

For flow cytometry experiments, adherent NIH-3T3 cells were plated in 10 cm dishes at a density of 75 000 cells per dish and transiently transfected with expression plasmids encoding the shControl, shUSP4D, or wtUSP4. Forty-eight hours post-transfection, cells were harvested and fixed o/n with 80% (v/v) ethanol in PBS. Cell nuclei were stained with propidium iodide. Briefly, cells were resuspended in 1 ml of staining buffer (1mM EDTA, 0.2 % Triton X-100 in PBS) containing 50 µg/ml of propidium iodide and 50 µg/ml of RNaseA and incubated for 1 hour at room temperature in the dark. DNA content was analyzed by flow cytometry using a BD LSR flow cytometer (Becton Dickinson, San Jose, CA). Data was acquired using Cell Quest software (Becton Dickinson, San Jose, CA) and were analyzed using Mod Fit LT software (Verity Software House, Inc., Sopsham, ME).

USP4 depletion experiments

1. shRNA lentivirus method

NIH-3T3 cells were plated in 6 well dishes at a density of 50 000 cells per well and transiently transfected using GeneJuice as recommended by the manufacturer. Cells were transfected with either 1 μg of MISSION shControl lentiviral plasmid (SHC002) or 1 μg of one of the MISSION shUSP4 lentiviral plasmid clones (NM_011678): TRCN0000030739 (clone A), TRCN0000030740 (clone B), TRCN0000030741 (clone C), TRCN0000030743 (clone D) (Sigma-Aldrich Co., St-Louis, Missouri, USA). Alternatively, cells were co-transfected with myc wt USP4 and shControl or shUSP4 clones. Cells were collected 48 hours post-transfection for RNA or protein analysis.

2. siRNA method

NIH-3T3 cells were plated in 6 well dishes at a density of 50 000 cells per well. Cells were transfected with 50 pmol or 100 pmol of *SMART*pool USP4 siRNA (Dharmacon Inc., Chicogo, Illinois, USA) using oligofectamine as per the supplier's protocol (Invitrogen Canada Inc., Burlington, Ontario, Canada). Cells were collected 48 hours post-transfection for RNA or protein analysis.

RNA extraction

Total RNA was extracted from NIH-3T3 or U87MG cells using the Qiagen RNeasy Mini Kit (P/N 74104) as per the supplier's protocol (Qiagen Inc., Canada Mississauga, Ontario, Canada). The quantity and quality of the RNA was assessed using a spectrophotometer (Eppendorf BioPhotometer).

DNA Affymetrix Microarray analysis

Total RNA was extracted from NIH-3T3 as previously described. 120 µl of RNA from each sample was concentrated by addition of 12 µl of 3 M sodium acetate pH 5.2 and 400 µl of 100 % ethanol. The samples were precipitated overnight at – 20 °C. The RNA samples were centrifuged at 14 000 rpm for 20 minutes at 4 °C. The supernatants were discarded, pellets were washed with 500 µl of 70 % ethanol and centrifuged at 14 000 rpm for 20 minutes at 4 °C. The supernatants were discarded and the pellets were air dried. The RNA in each sample was resuspended in 11 µl of RNase free water. The RNA integrity was analyzed using the Agilent Bioanalyzer (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). A total of 20 µg of total RNA at a concentration of 2 µg /µl was sent to the Ottawa Genome Centre (The OHRI Gene Expression Facility, Ottawa, Ontario, Canada) for microarray analysis. Gene expression of NIH-3T3, transfected shControl and transfected shUSP4D were assessed using Mouse Genome 430 2.0 Array (MG-430_2) (Affymetrix, Santa Clara, CA, USA). Microarray data analysis was performed using R project for statistical computing and Stratagene ArrayAssist 3.0 (La Jolla, CA, USA).

Quantitative RT-PCR analysis

A total of 1 μg of RNA of cell lysates was used to prepare the first strand cDNA using M-MLV Reverse Transcriptase and Oligo(dT)$_{12-18}$ primers (Invitrogen Canada Inc., Burlington, Ontario, Canada) as per the manufacturer's protocol. Gene expression levels were assessed with the LightCycler® 1.5 instrument using SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany) as the detection format.

The following primer sets were used for the amplification of the target genes by RT-PCR:

USP4: forward primer 5'-TGCAATGGCTCTAGGAG-3'
reverse primer 5'-TCCTCATCTGGTTTGAG-3'

TGF-beta1: forward primer 5'-TGAGTGGCTGTCTTTTGACG-3'
reverse primer 5'-TCTCTGTGGAGCTGAAGCAA-3'

TGFbeta2: forward primer 5'-AGCTGGCCAGTACCTTTGAA-3'
reverse primer 5'-ACAGAACCATCCATCCCAGA-3'

TGF-beta receptor 2: forward primer 5'-GACTTGACCTGTTGCCTGTG-3'
reverse primer 5'-CTCGGTCTCTCAGCACACTG-3'

Smad3: forward primer 5'-ACTAGATGCGTGAGCGCTTT-3'
reverse primer 5'- CGGCAGAATGCTAAACATCA-3'

Smad4: forward primer 5'-CAGGGTTTCTTCCCCAAGAC-3'
reverse primer 5'-GCAGAACAGTGAAGCAGCAG-3'

Smad7: forward primer 5'-AGAAGGCTGAACCAGAACCA-3'
reverse primer 5'- TTCCACATCTGGGAGAAACC-3'

Runx1: forward primer 5'- GCCAAGGATTTGCTCTGAAG-3'
reverse primer 5'- TTGGATCTTTGGGGTACAGC-3'

Elf1: forward primer 5'- TCTCAGGTAGCCGTCAAACA-3'
reverse primer 5'- ACTTGGGTTCGCCTCCTTAT-3'

GAPDH: forward primer 5'-TTGATGTCATCATACTTGGCAGGT-3'
reverse primer 5'-CAGTCAAGGCTGAGAATGGGA-3'

The following LightCycler® cycling conditions were used for the USP4 primers: denaturation program (95 °C for 30 s); amplification and quantification programs were repeated 35 times (95 °C for 2 s, 60 °C for 10 s, 72 °C for 40 s with a single fluorescent measurement); melting curve program (65 °C to 99 °C continuous fluorescent measurement); and a cooling step to 40 °C. The following LightCycler® cycling conditions were used for the TGF-beta1, TGF-beta2, TGF-beta receptor 2, Smad3, Smad4, Smad7, Runx1 and Elf1 primers: denaturation program (95 °C for 30 s); amplification and quantification programs were repeated 35 times (95 °C for 2 s, 58 °C for 10 s, 72 °C for 24 s with a single fluorescent measurement); melting curve program (65 °C to 99 °C continuous fluorescent measurement); and a cooling step to 40 °C. The following LightCycler® cycling conditions were used for the USP4 primers: denaturation program (95 °C for 30 s); amplification and quantification programs were repeated 35 times (95 °C for 2 s, 58 °C for 10 s, 72 °C for 40 s with a single fluorescent measurement); melting curve program (65 °C to 99 °C continuous fluorescent measurement); and a cooling step to 40 °C. PCR reactions were a total of 20 µl and contained 4 pmols of forward and reverse primer, 0.4 µl of Taq Polymerase (Invitrogen Canada Inc., Burlington, Ontario, Canada), 1.6 µl of 10 mM dNTPs, 0.4 µl SYBR Green I at a dilution of 1/1000 (Cambrex, Crambrex Bioscience Rockland Inc., Rockland, ME, USA), 0.4 µl of 25 mg/ml BSA (Sigma-Aldrich Co., St-Louis, Missouri, USA), 4 mM $MgCl_2$ and 2 µl of cDNA dilution or H_2O for the blank reaction. The LightCycler® software package Version 5.3.2 was used to determine the relative expression levels of the target genes (Roche Molecular Biochemicals, Mannheim, Germany). Quantification of gene expressions was extrapolated from a standard curve constructed from a serial dilution of cDNA. Fold changes were calculated relative to untransfected NIH-3T3 values.

Protein extraction

Cells were lysed in a protein lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Nonidet P-40 (Sigma, St. Louis, Missouri, USA), 0.5 mM EDTA and 20 % glycerol) containing the following protease and phosphates inhibitors: 1mM phenylmethylsulfonyl fluoride, 5 µg/mL leupeptin (Sigma-Aldrich Co., St-Louis, Missouri, USA), 2 µg/mL aprotinin (Sigma-Aldrich Co., St-Louis, Missouri, USA), 200 µM sodium fluoride (NaF) and 200 µM sodium pyrophosphate (NaPPi). The extracts were sonicated three times for 10 seconds each and placed on ice. The extracts were centrifuged at 14 000 rpm at 4°C for 30 minutes and the supernatants were recovered. The protein concentration of each sample was determined using the Bradford protein assay (Bio-Rad Laboratories Canada Inc., Mississauga, Ontario, Canada)

Western blot analysis

A total of 30 µg of protein lysate was resolved on an 8 % SDS-polyacrylamide gel and electroblotted onto a hydrobond C nitrocellulose membrane (Amersham Pharmacia Biotechnologies, Baie D'Urfée, Québec, Canada). Membranes were stained with Ponceau S (Sigma-Aldrich Co., St-Louis, Missouri, USA) in order to ensure complete transfer of proteins. Non specific binding was reduced by incubating the membrane in 5 % skim milk diluted in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 % Tween-20) overnight at 4 °C. The respective primary antibody, diluted in 5 % skim milk in TBST, was added to the membrane and incubated on the shaker for 1 hour or overnight. The membrane was washed 3 times with TBST for 10 min. Subsequently, the corresponding secondary antibody, also diluted in 5 % skim milk in TBST, was incubated on the membrane for 45 min. The membrane was again washed 3 times in TBST for 10 min. The proteins were visualized by

the horseradish peroxidase method using the ECL kit purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, Mayland, USA).

Immunoprecipitations

NIH-3T3 cells were plated in 10 cm dishes at a density of 750 000 cells. Twenty-four hours after seeding, cells were transfected with a myc-tagged wt USP4 expression construct. Cells were harvested in protein lysis buffer and sonicated 3 times for 5 seconds. Cell extracts were centrifuged at 14 000 rpm for 20 minutes at 4 °C to eliminate cell debris. Protein lysates were precleared with G-sepharose gamma bind beads (Amersham, Pharmacia Biotechnologies, Baie D'Urfée, Québec, Canada) for 1 hour at 4 °C. Lysates were subsequently incubated with an anti-CBP antibody and G-sepharose gamma bind beads overnight at 4 °C. Immunoprecipitates and total cell lysates were analyzed by western blot analysis with the appropriate antibodies.

Antibodies for Western blot analysis

The mouse polyclonal antibody used in the detection of myc-tagged USP4 proteins was a gift from Dr. John Bell (Ottawa Research Institute, Ottawa). The rabbit polyclonal antibody raised against CBP was purchased from Upstate (Upstate Biotechnologies, Chicago, IL, USA). The rabbit polyclonal Smad2 and Smad3 antibodies were gifts from Dr. Barbara Vanderhyden (Ottawa Research Institute, Ottawa). EGFP was detected using a rabbit polyclonal antibody purchased from Santa Cruz (Santa Cruz Biotechnologies Inc., Santa Cruz, CA). The antibody used in the detection of HuR was a gift from Dr. Bruce McKay (Ottawa Research Institute,Ottawa). The mouse monoclonal actin antibody was purchased from Sigma (Sigma-Aldrich Co., St-Louis, Missouri, USA).

RESULTS

Effect of USP4 on stabilization of ARE-mRNAs

DUBs have previously been implicated in the control of rapid degradation of ARE-mRNAs (Laroia et al., 2002). Exogenous expression of USP4 has been previously demonstrated to prevent degradation of ARE containing mRNAs (Laroia et al., 2002). In an attempt to further elucidate the mechanism/domains by which USP4 exerts this stabilizing effect, a luciferase assay was employed. NIH-3T3 cells transiently co-transfected with wt or various USP4 mutants in conjunction with either a luciferase construct (UbC-Luc) in which an ARE sequence had been introduced or a luciferase without the ARE sequence (Figure 4). As expected the UbC-Luc construct containing the ARE sequence was destabilized when compared to the UbC-Luc construct which did not contain the ARE sequence (Figure 5). In contrast to what has previously been shown, overexpression of wt or USP4 mutants did not appear to significantly affect the expression levels of the Ubc-Luc containing the ARE sequence in NIH-3T3 cells. UCHL-1, another deubiquitinating enzyme which served as a control, also did not demonstrate any effect. In order to rule out the possibility that the lack of an effect was due to expression levels in NIH-3T3 cells, twice the amount of wt USP4 was transfected (Figure 6A). Increasing the amount of transfected USP4 did not seem to affect the luciferase readout; no increase in the expression of the luciferase reporter was observed. The transfection efficiency in NIH-3T3 cells (approx. 30 %) was assessed by western blot (Figure 6C, lanes 2-5) with an antibody directed against the myc epitope.

Considering that Laroia et al. demonstrated an increase in the stability of a particular ARE-mRNA (GM-CSF) in Cos-7 cells in the presence of exogenous wt USP4, it was conceivable that the discrepancy in our data can be explained by the difference in the cell

Figure 4. Schematic representation of the UbC-Luciferase expression constructs

A) Schematic representation of the UbC-Luciferase (R2.1) expression plasmid containing an hPEST degradation sequence. B) Representation of the UbC-Luciferase-ARE (R2.2) expression construct containing two degradation sequences (hCL1 and hPEST) and an ARE sequence (ARE).

A

B

Figure 5. Effect of wt and mutant USP4 on the stabilization of ARE containing mRNAs

NIH-3T3 cells were co-transfected with UbC-Luc and UbC-Luc-ARE expression constructs with control vector pcDNA3.1, myc wt USP4, myc USP4 mutants or UCHL-1 and analyzed for luciferase activity. As expected, the activity of the UbC-Luc-ARE luciferase is lower when compared to the activity of the the UbC-Luc alone. No significant change in ARE luciferase activity was observed in cells overexpressing wt or USP4 mutants when compared to cells expressing pcDNA3.1 vector. Expressing UCHL-1, which served as a control, also did not result in any change in luciferase activity when compared to cells expressing pcDNA3.1 plasmid alone. Lysates were assayed in triplicates. wt = wild-type; NLS = nuclear localization signal; AD = acidic domain.

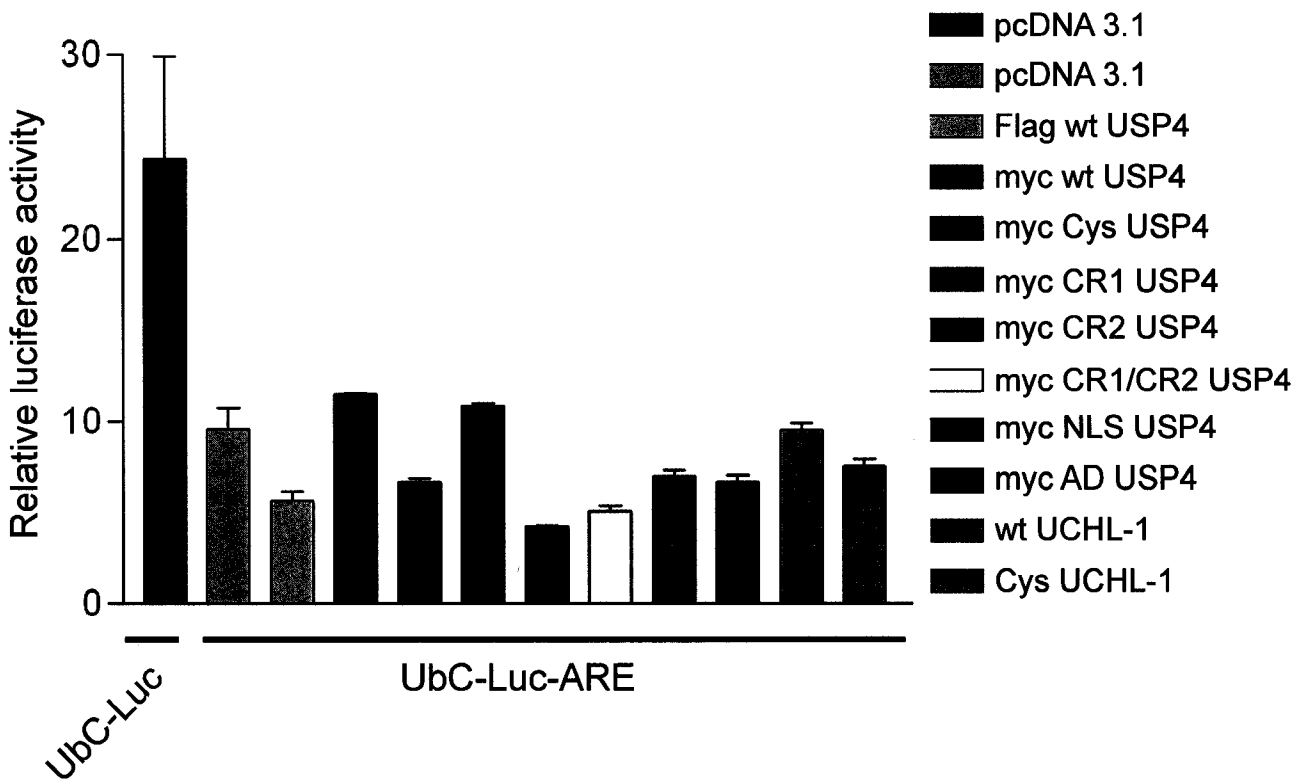
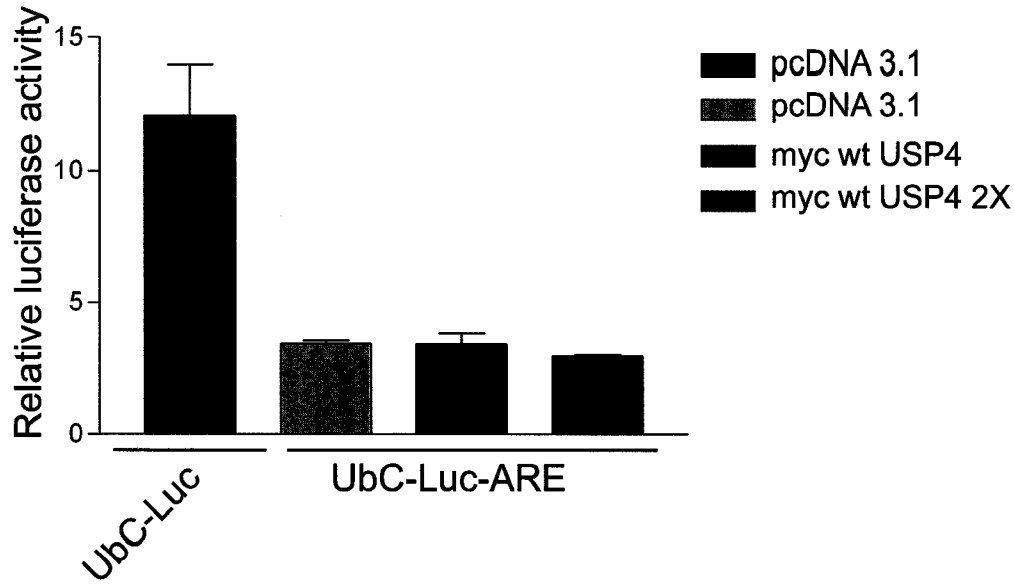


Figure 6. Effect of wt USP4 on the stabilization of ARE-mRNAs in NIH-3T3 and Cos7

A) NIH-3T3 cells were assayed for expression of a luciferase reporter using a dual luciferase assay system. As expected, transfection with the UbC-Luc ARE showed decreased luciferase expression compared to UbC-Luc alone. No significant change in luciferase activity was observed in cells overexpressing different amounts of wt USP4 when compared to cells expressing pcDNA3.1 vector. Lysates were assayed in triplicates. B) Cos-7 cells were assayed for expression of a luciferase reporter by dual luciferase assay. The transfection with the UbC-Luc ARE showed a decrease in luciferase expression when compared to the UbC-Luc without the ARE sequence. No significant change in luciferase activity resulted from overexpression of wt USP4 or Cys USP4 constructs compared to cells expressing pcDNA3.1 vector. Lysates were assayed in triplicates. C) Western blot analysis with an anti-myc antibody demonstrated good transfection efficiency of myc wt USP4 in NIH-3T3 cells (lanes 2-5) and very high transfection efficiency in Cos-7 cells (lanes 6-7).

A



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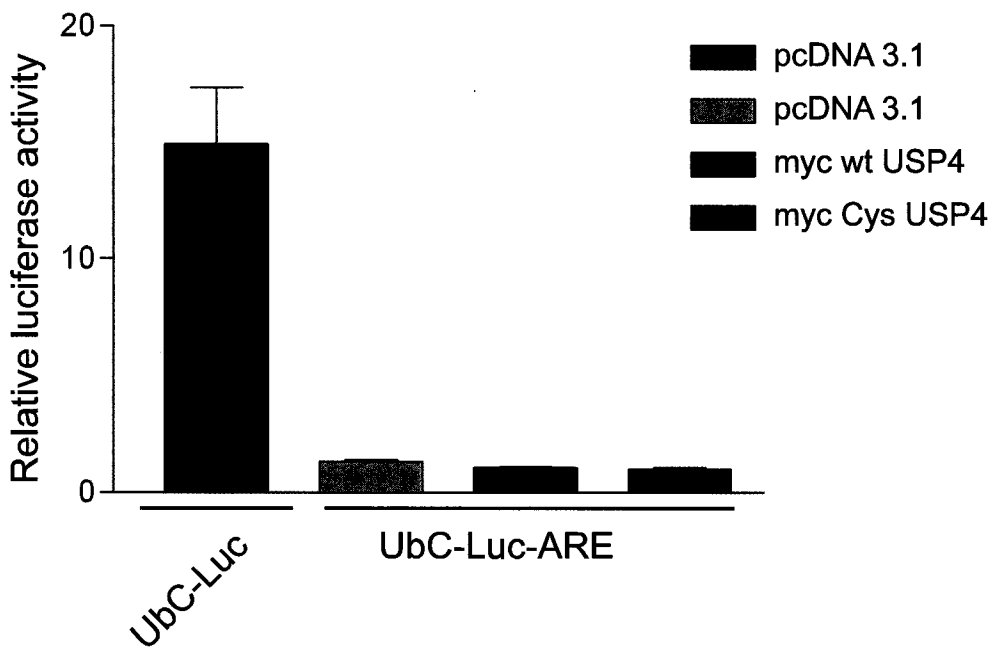

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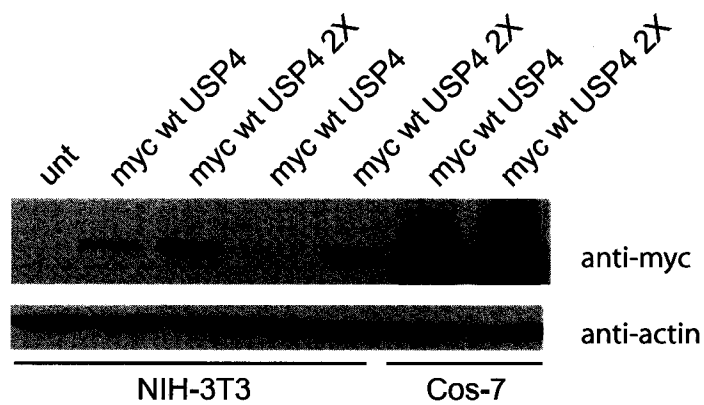


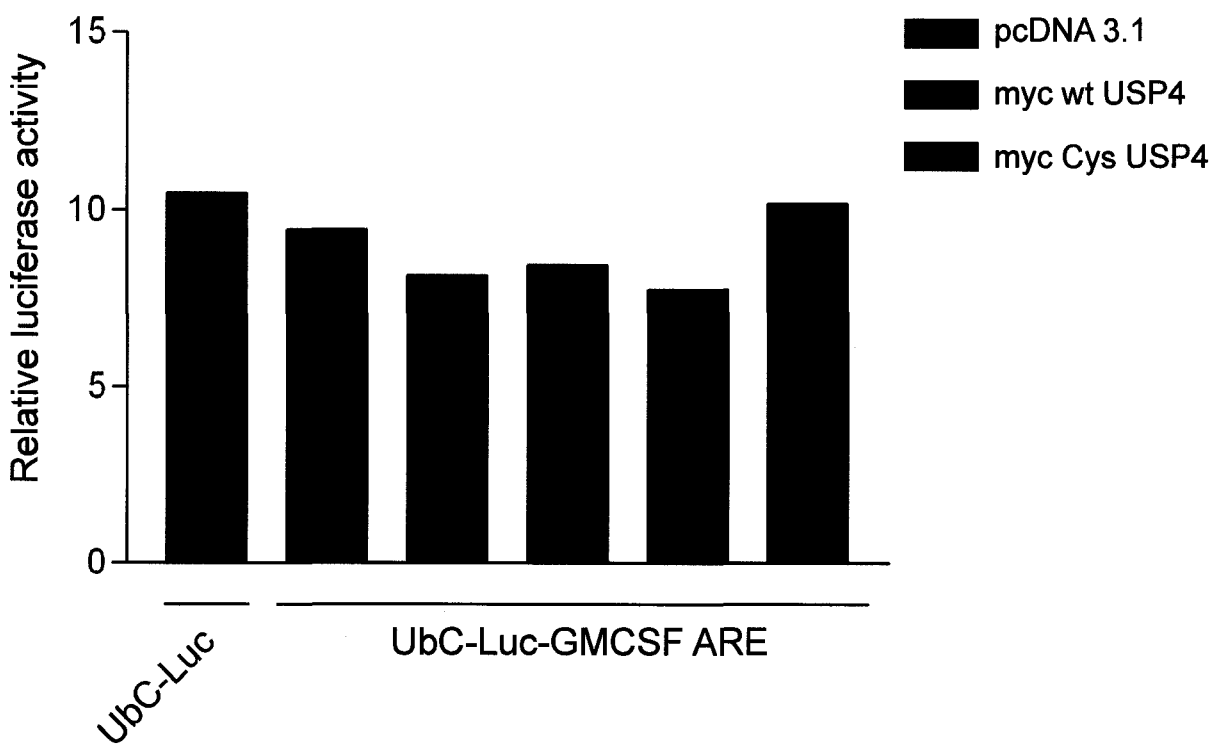
line employed. Therefore, the experiment was repeated in Cos-7 cells. The transfection efficiency of Cos-7 (over 80 %) proved to be higher than that of 3T3 cells when transfected with an equivalent amount of DNA (Figure 6C, compare lanes 2-5 with 6-7) and the original luciferase experiment yielded only background readings (data not shown). In order to bypass this, the experiment was repeated in Cos-7 cells with less DNA. In accordance with what was observed in NIH-3T3 cells, overexpression of wt USP4 in Cos-7 cells did not increase the expression level of the UbC-Luc-ARE construct (Figure 6B). Having ruled out the cell line specificity, it was reasoned that USP4 was not acting as a general modifier of ARE containing mRNAs (and hence would not stabilize the commercial vector which contains a generic ARE) but rather acting to stabilize the GM-CSF message. To test this supposition, the GM-CSF ARE sequence was inserted downstream of the luciferase reporter of the UbC-Luciferase expression construct. The presence of the ARE sequence was confirmed by DNA digest (see material and methods). Co-transfection of this modified UbC-Luciferase containing the GM-CSF ARE with either wt or Cys USP4 did not result in a change in luciferase expression when compared to the UbC luciferase control plasmid (Figure 7).

Effect of USP4 on endogenous GM-CSF mRNA stability

To confirm the data presented above, an ELISA assay was employed to assess the ability of USP4 to stabilize endogenous GM-CSF mRNA in U87MG cells (Figure 8A). U87MG cells were selected due to their well documented ability to basally synthesize GM-CSF (Tweardy et al., 1990). U87MG cells were either transfected with pcDNA3.1 vector, a wt USP4 plasmid or a plasmid encoding HuR (positive control) or co-transfected with wt USP4 and HuR plasmids. Transfection efficiency was above 80 %. Lysates were subsequently analyzed by ELISA. As shown in figure 8A, no significant change in the levels

Figure 7. Effect of wt USP4 on the stabilization of GM-CSF ARE-mRNA in Cos7 cells

Cos-7 cells were assayed for expression of a luciferase reporter using a dual luciferase assay. Contrary to what was expected, the UbC-Luc containing the GM-CSF ARE sequence did not demonstrate a decrease in luciferase activity when compared to the UbC construct without an ARE sequence. No significant change in luciferase activity was observed following overexpression of wt USP4 or Cys USP4 mutant constructs compared to cells expressing pcDNA3.1 vector.



of secreted GM-CSF protein was observed in cells exogenously expressing wt USP4 or HuR. Co-transfection of wt USP4 and HuR expression constructs resulted in a significant change in the protein levels of GM-CSF when compared to cells expressing pcDNA3.1 alone. The protein levels of USP4 (Figure 8B) and HuR (Figure 8C) were assessed by western blot analysis of extracts from transfected cells.

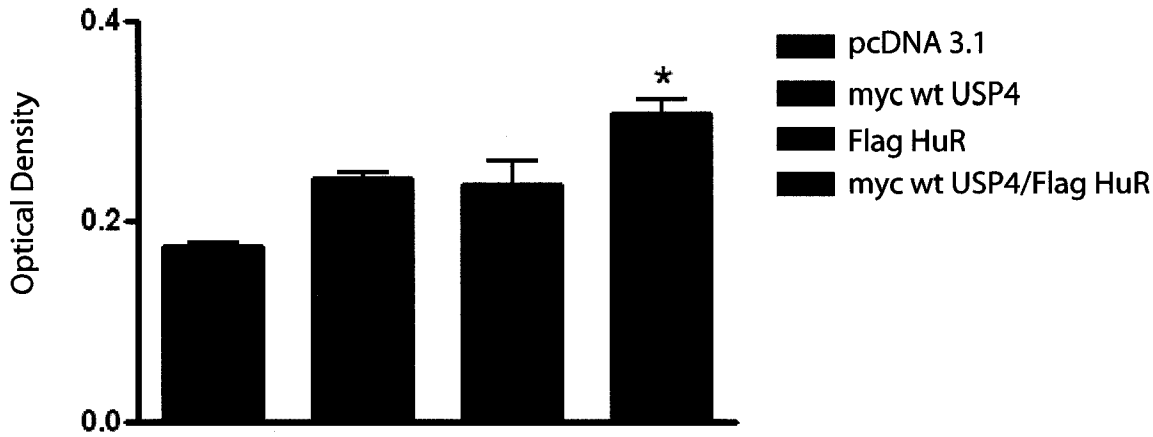
USP4 knockdown using a short-hairpin lentivirus

Previous attempts to generate USP4 stable overexpressors in cultured mammalian cells resulted in overt toxicity and cell death (Paola Blanchette, thesis 2002). Hence the role of USP4 in stable overexpression studies was not a feasible approach. A knockdown approach was adopted instead using four commercially available shUSP4 lentivirus clones (hereafter referred to as shUSP4A, B, C, D). A shRNA lentivirus plasmid containing an irrelevant hairpin was used as a control. In order to verify the knockdown efficiency of USP4 by lentivirus transfection, NIH-3T3 cells were transiently co-transfected with the various shUSP4 expression constructs and myc wt USP4 and cell extracts were analyzed by western blot analysis using the antibody directed towards the myc epitope. The analysis revealed that the protein levels of exogenously expressed USP4 were significantly decreased in cells expressing shUSP4D and to a lesser extent in cells expressing shUSP4C (Figure 9). No significant difference in USP4 protein levels was observed in cells expressing the control lentivirus or other shUSP4 clones (Figure 9). Furthermore, western blot analysis of cell extracts from cells co-expressing shUSP4D and GFP with a GFP specific antibody revealed a mild change in the protein level of GFP when compared to cells co-transfected with the shControl and GFP plasmids (Figure 10). The optimal amount of shUSP4D required for efficient knockdown of exogenously expressed USP4 was assessed by transfecting

Figure 8. Effect of ectopic expression of wt USP4 on endogenous GM-CSF

A) Endogenous GM-CSF expression levels were assayed in U87MG cells using a human GM-CSF immunoassay. Cell extracts overexpressing wt USP4 did not demonstrate a significant change in GM-CSF levels when compared to cells lysates expressing pcDNA3.1 vector alone. Cells overexpressing Flag HuR, used as a positive control also did not demonstrate an increase in GM-CSF levels. GM-CSF levels demonstrated a significant increase in cell lysates co-transfected with myc wt USP4 and Flag HuR constructs. Lysates were assayed in triplicates. The optical density was read at 450 nm. B) Western blot analysis with an anti-myc antibody demonstrating good transfection efficiency of the myc wt USP4 construct in Cos-7 cells. C) Western blot analysis using anti-HuR showing good transfection efficiency of Flag HuR in Cos-7 cells. The arrow represents endogenous HuR and the asterisk represents exogenous Flag HuR.

A



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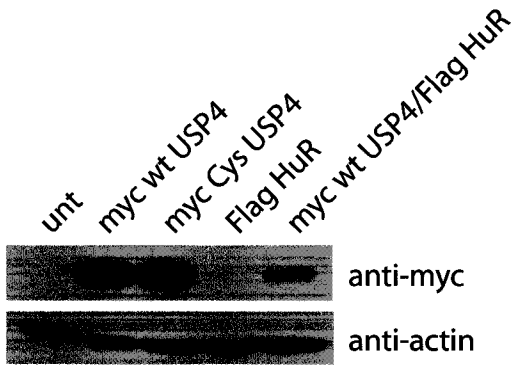

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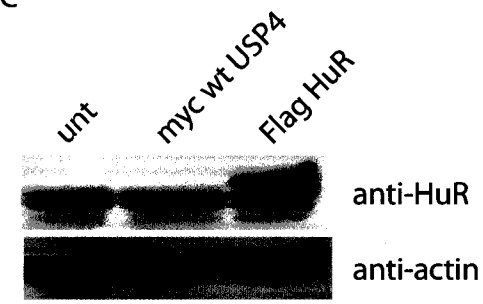


Figure 9. USP4 depletion using shUSP4 lentivirus target clones

Western blot analysis of NIH-3T3 cell extracts co-transfected with a total of 3 μg of DNA including 2 μg of myc wt USP4D and 1 μg of one of the shUSP4 clones (A, B, C or D) using an anti-myc antibody. Protein expression levels of myc-tagged wt USP4 was observed to be significantly decreased following overexpression of shUSP4D and to a lesser extent with expression of shUSP4C when compared to overexpression of other shUSP4 clones, shControl or pcDNA3.1 vector.

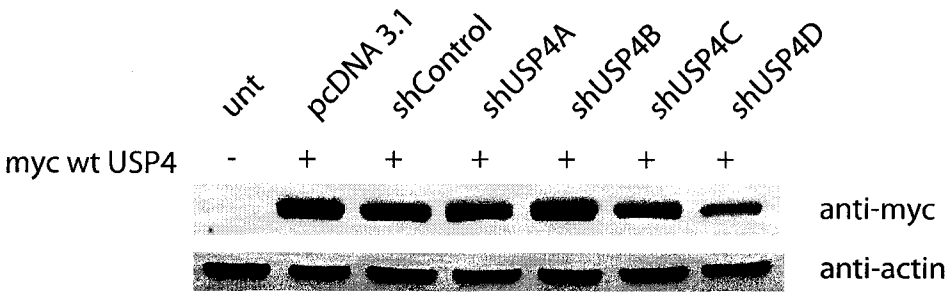


Figure 10. shUSP4 is specific to USP4

Western blot analysis of NIH-3T3 cell extracts co-transfected with a total of 3 μg of DNA including 1.5 μg of GFP, the amount of shUSP4D indicated and the remainder being pcDNA3.1 using an anti-GFP antibody. A slight decrease in GFP protein expression levels was noted following transfection of the shUSP4D construct when compared to the shControl construct or pcDNA3.1 vector.

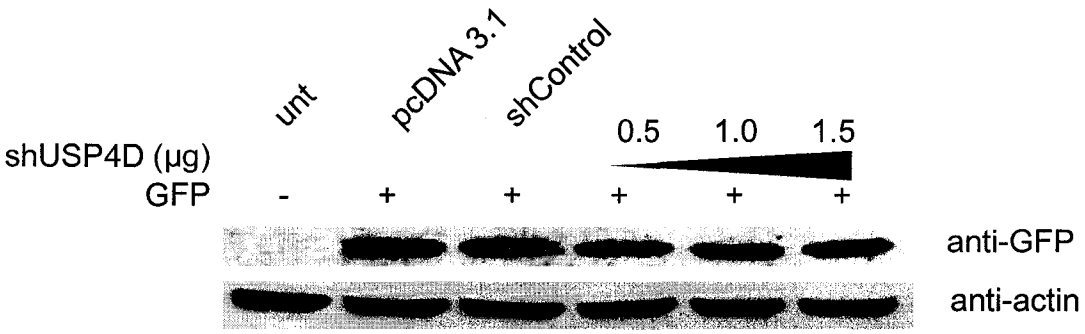
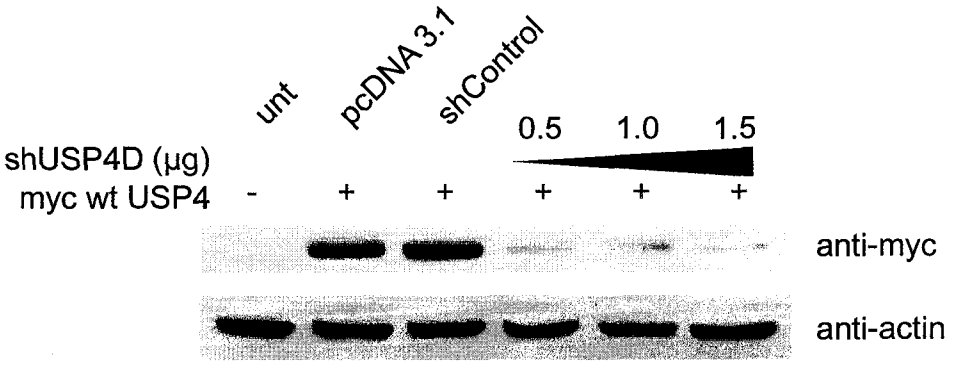


Figure 11. Optimization of USP4 knockdown using shUSP4D lentivirus

Western blot analysis of NIH-3T3 cells co-transfected with a total of 3 μg of DNA including 1.5 μg of myc wt USP4, the amount of shUSP4D indicated and the remainder being pcDNA3.1 using an anti-myc antibody. A significant decrease in myc-tagged USP4 protein levels was observed with all transfected amounts of shUSP4 when compared to proteins levels of cell extracts transfected with shControl or pcDNA3.1 vector.



increasing amounts of the shUSP4D expression construct in NIH-3T3 cells. By western blot analysis with the antibody raised against the myc epitope it was found that cells transfected with the minimal amount of shUSP4D plasmid showed a similar level of USP4 depletion when compared to cells transfected with higher amounts of shUSP4 plasmid DNA. (Figure 11). To ensure maximal knockdown, all subsequent USP4 depletion experiments were performed using 1μg of shUSP4D plasmid DNA. The depletion of endogenous USP4 mRNA levels in cells expressing shUSP4D was assessed by Real-time PCR. The analysis revealed that the mRNA levels of USP4 did not significantly differ when compared to cells expressing shControl or untransfected NIH-3T3 cells (Figure 12A). The presence of the RT-PCR products was confirmed by agarose gel (Figure 12 B).

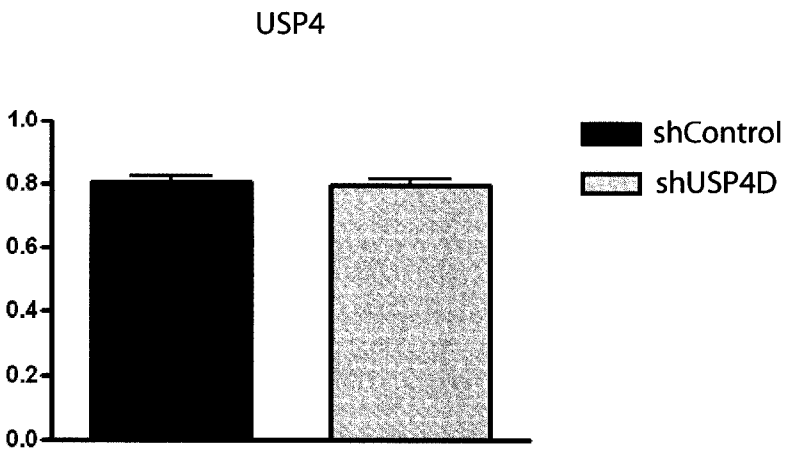
Analysis of gene expression in USP4 knockdown cells

The identification of genes affected by depletion of USP4 would be useful in elucidating the physiological role of USP4 in the regulation of cellular processes. In order to achieve this, we profiled mRNA of untransfected NIH-3T3 and cells transfected with either the shControl or shUSP4 by independently hybridizing each sample to an Affymetrix microarray. The analysis using R project for statistical computing and ArrayAssist revealed a large repertoire of mRNAs that were differentially expressed in cells transiently transfected with the shUSP4 plasmid when compared to untransfected NIH-3T3 and cells transfected with the shControl plasmid. Of interest were genes involved in TGF-beta signaling, a pathway whose dysregulation has been implicated in the onset/progression of lung tumorgenesis. These genes included TGF-beta2, the TGF-beta receptor 2, downstream effectors such as Smad2, Smad3, Smad4, Smad7, Smurf1, Runx1, Elf1 and Elf4 (Table 1). Given the association of the TGF beta family to lung cancer and the presence of

Figure 12. RNA analysis of USP4 depletion using shUSP4D

A) Quantitative RT-PCR analysis of endogenous mRNA USP4 levels following transfection with shControl (green) or shUSP4D (blue) in NIH-3T3 cells. Values represent triplicates and are normalized to untransfected NIH-3T3 cells. No depletion of endogenous USP4 was noted using the shUSP4D compared to the shControl. B) Agarose gel depicting the product of the RT-PCR reaction of A) for an shControl sample, an shUSP4D sample and an H_2O blank showing presence of the RT-PCR products.

A



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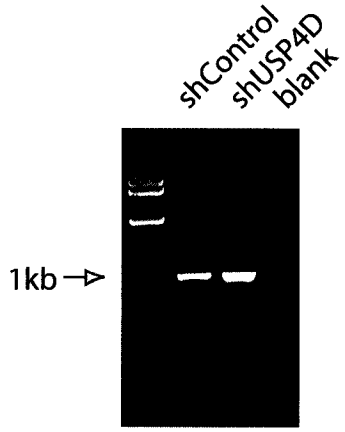


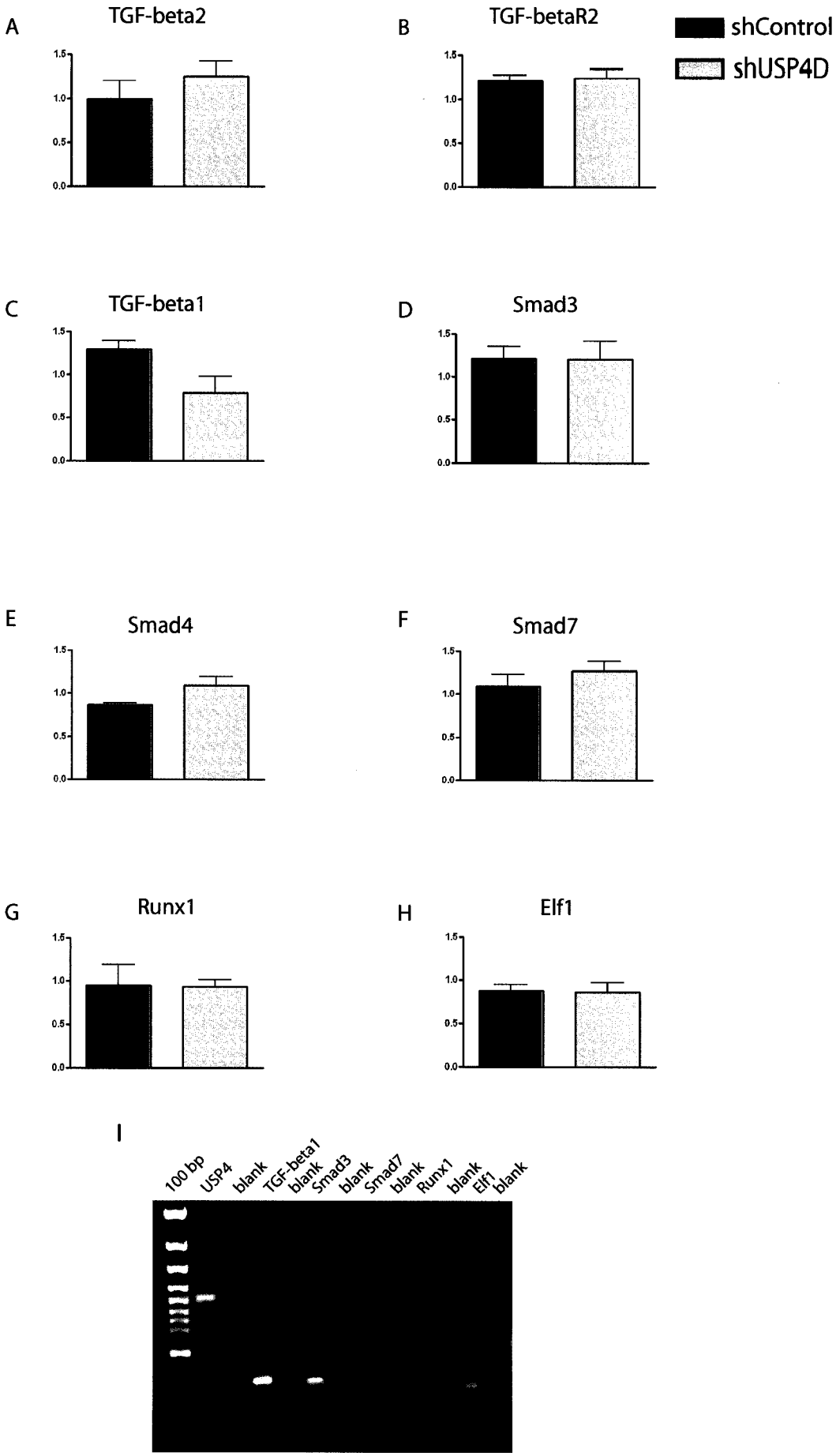
Table 1. Summary of altered genes following shUSP4D knockdown in NIH-3T3

A number of genes involved in the transforming growth factor-beta signaling pathway revealed dysregulation following depletion of USP4 by transfection of shUSP4D. Numbers represent logarithmic fold changes relative to untransfected NIH-3T3 cells. Genes marked with an asterisk were investigated by Real-time PCR.

shUSP4D	Untransfected		
- 1.99	0.00	*	Transforming growth factor-beta2 (TGF-beta2)
- 4.76	0.00	*	Transforming growth factor-beta receptor2 (TGF-betaR2)
-1.09	0.00		MAD homolog 2 (Drosophila) (Smad2)
- 1.04	0.00	*	MAD homolog 3 (Drosophila) (Smad3)
- 2.98	0.00	*	MAD homolog 4 (Drosophila) (Smad4)
- 2.23	0.00	*	MAD homolog 4 (Drosophila) (Smad7)
-1.42	0.00		SMAD specific E3 ubiquitin protein ligase 1 (Smurf1)
+ 3.91	0.00	*	E74-like factor 1 (Elf1)
-2.19	0.00		E74-like factor 4 (Elf4)
- 1.35	0.00	*	Runt-related transcription factor 1(Runx1)

Figure 13. RNA analysis of genes in the TGF-beta signaling in USP4 depleted cells

Quantitative RT-PCR analysis of genes involved in the TGF-beta pathway following USP4 depletion using shUSP4D. Values represent triplicates and are normalized to untransfected NIH-3T3. Patterns of expression did not reveal any significant change in the mRNA levels of selected genes when comparing untransfected NIH-3T3 and shControl expressing cells to cells expressing shUSP4D. A) transforming growth factor-beta2 (TGF-beta2) B) transforming growth factor-beta receptor 2 (TGF-betaR2) C) transforming growth factor-beta 1 (TGF-beta1) D) MAD homolog 3 (Drosophila) (Smad3) E) MAD homolog 4 (Drosophila) (Smad4) F) MAD homolog 7 (Drosophila) (Smad7) G) Runt-related transcription factor 1(Runx1) and H) E74-like factor 1 (Elf1) I) Agarose gel depicting the product of some of the above RT-PCR reactions for a shUSP4D transfected sample and an H_2O blank showing presence of the RT-products.



elevated levels of USP4 in lung tumors, genes in the TGF family were selected to validate the Affymetrix microarray data using the real-time PCR LightCycler® from Roche. Investigated genes are marked by an asterisk in Table 1. As shown in Figure 13, the Real-time PCR analysis did not reveal a significant change in the mRNA levels of any of the selected genes when comparing untransfected NIH-3T3 and shControl expressing cells to cells expressing shUSP4D. The presence of the RT-PCR products was confirmed by agarose gel (Figure 13I). Due to the availability of the Smad2 and Smad3 antibodies, the protein levels of Smad2 and 3 were analyzed by western to further confirm the PCR results. Cell extracts from shUSP4D transfected cells with the Smad2 antibody did not reveal a significant difference in the protein level of Smad2 when compared to extracts derived from untransfected NIH-3T3 cells (Figure 14A). However, analysis of cell extracts from shUSP4D expressing cells revealed an increase in the protein levels of Smad3 when compared to extracts from untransfected NIH-3T3 cells (Figure 14B). When the extracts of shControl expressing cells were analyzed by western analysis with the Smad3 antibody along side with extracts from shUSP4D cells, a similar increase in Smad3 protein levels was noted suggesting that the effect previously observed was due to transfection rather than the depletion of USP4 (Figure 14C). Therefore, a second approach to knockdown USP4 was undertaken using *SMART*pool siRNA targeting USP4. The USP4 siRNA was co-transfected into NIH-3T3 cells and cell extracts were analyzed by western blot with the Smad3 antibody. The analysis revealed that when compared to extracts from cells expressing the shControl or the shUSP4D, cells expressing the USP4 siRNA did not show an increase in Smad3 protein levels (Figure 14D). To confirm the efficient depletion of USP4 in siRNA transfected cells, myc-tagged wt USP4 was transfected in USP4 siRNA expressing cells and cell extracts were analyzed by western blot with the myc antibody (Figure 15A).

Figure 14. Western analysis of Smad2 and Smad3 following depletion of USP4

A) Western blot analysis of untransfected NIH-3T3 lysates or extracts overexpressing shUSP4D with an antibody raised against Smad2. No difference in Smad2 protein expression levels were observed in cell extracts expressing shUSP4D when compared to untransfected cell lysates. B) Western blot analysis of untransfected NIH-3T3 lysates or extracts overexpressing shUSP4D with an antibody raised against Smad3. An increase in Smad3 protein levels was observed in cell extracts overexpressing USP4D compared to untransfected cell lysates. C) Western blot analysis of untransfected, shControl or shUSP4D overexpressing NIH-3T3 cells with an antibody raised against Smad3. An increase in Smad3 protein levels was observed in shUSP4D overexpressing lysates as well as in shControl overexpressing extracts when compared to untransfected lysates. D) Western blot analysis of untransfected NIH-3T3 cell lysates or cell extracts expressing different amounts of USP4 siRNA, shUSP4D or shControl with an antibody raised against Smad3. An increase in Smad3 protein levels was observed in cell lysates transfected using shControl or shUSP4D plasmids compared to untransfected cell lysates. No increase in Smad3 protein levels were noted in cells transfected with the siRNA when compared to untransfected lysates.

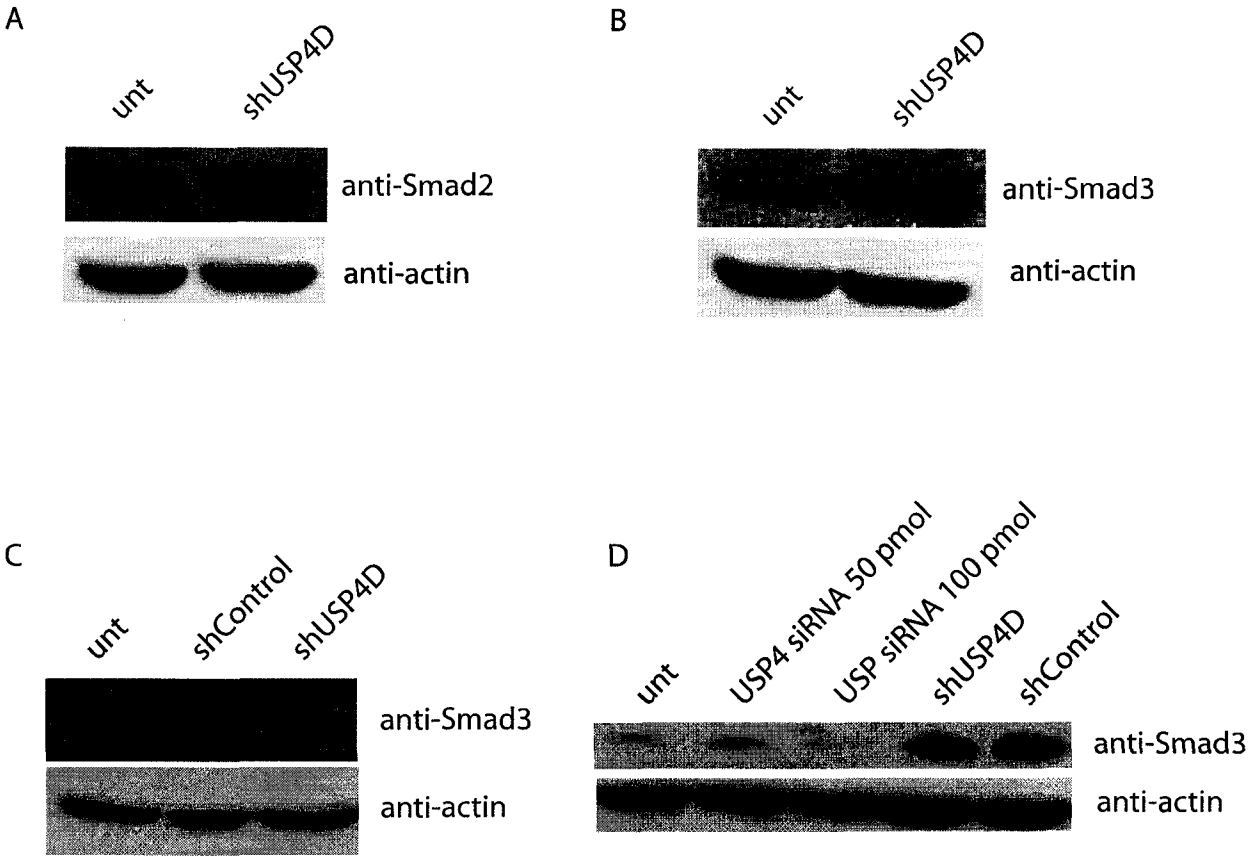
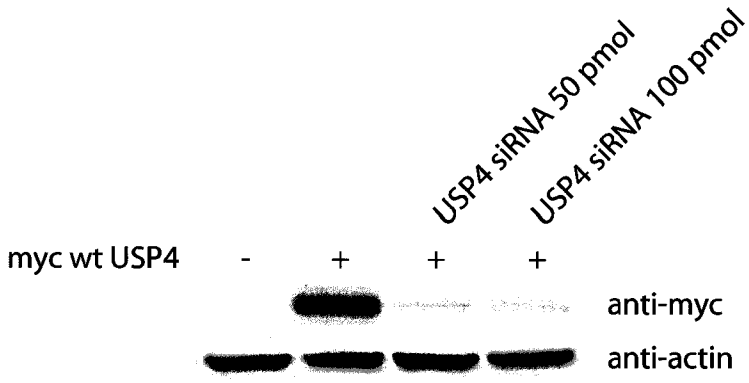


Figure 15. Depletion of USP4 using siRNA

A) Western blot analysis of untransfected NIH-3T3 cell lysates or cell lysates overexpressing myc wt USP4 or myc wt USP4 in conjunction with different amounts of USP4 siRNA using an anti-myc antibody. A significant decrease in myc-tagged wt USP4 protein levels was observed when co-transfecting the siRNA compared to cells overexpressing myc wt USP4 alone. B) Quantitative RT-PCR analysis of endogenous mRNA USP4 levels following transfection of USP4 siRNA, myc wt USP4 or co-transfection of myc wt USP4 and USP siRNA. Values represent triplicates and are normalized to untransfected NIH-3T3. No depletion of endogenous USP4 mRNA levels was observed following transfection of the siRNA when compared to untransfected cell extracts. A significant increase in USP4 mRNA levels was detected in cell lysates overexpressing myc wt USP4 in comparison with untransfected NIH-3T3. A significant decrease in USP4 mRNA expression levels was observed in cell lysates co-transfected with the myc wt USP4 and the USP4 siRNA when compared to USP4 mRNA levels of myc wt USP4 transfected lysates.

A



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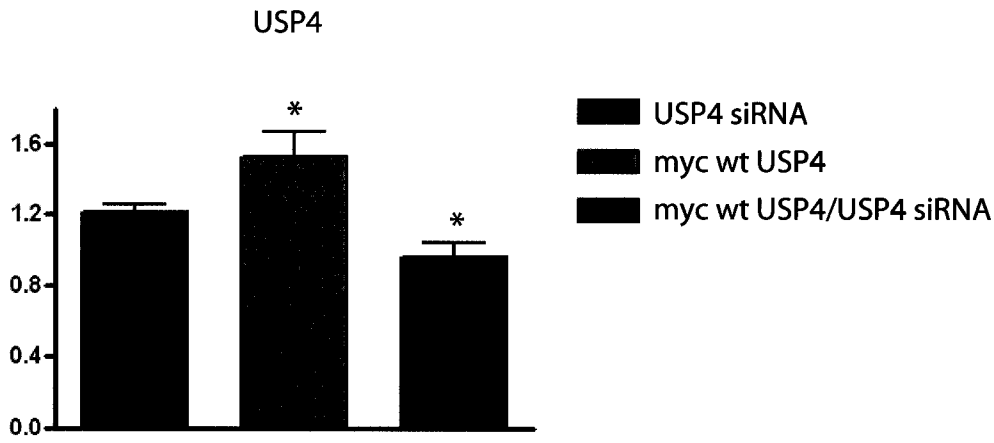


The analysis revealed that in cells expressing the USP4 siRNA, the protein levels of exogenous USP4 were significantly reduced (Figure 15A). The efficient depletion of both endogenous and exogenous USP4 mRNA levels using the siRNA was assessed by Real-time PCR (Figure 15B). No decrease in endogenous USP4 mRNA levels was detected. However, the exogenous levels of USP4 mRNA increased when expressing myc wt USP4 compared to NIH-3T3 cells. In addition, USP4 mRNA levels were significantly reduced compared to cells expressing myc wt USP4 when myc wt USP4 was co-transfected with USP4 siRNA (Figure 15B).

Analysis of USP4 knockdown or overexpression on cell morphology and cell cycle

USP4 contains two motifs (namely CR1 and CR2), which are common to viral protein motifs responsible for binding the retinoblastoma gene product pRb. Given the known interaction of USP4 with pocket proteins pRb, p107 and p130 (Blanchette et al., 2001; DeSalle et al., 2001) and the well characterized role of pRb in the regulation of the G1 to S phase transition of the cell cycle (Dannenberg and te Riele, 2006; Grana et al., 1998) the effects of shUSP4D transfection on cell cycle progression of NIH-3T3 cells were assessed. Untransfected NIH-3T3 cells, shControl or shUSP4 transfected cells were subjected to flow cytometric analysis which revealed that transfection of shUSP4D did not affect the G1/S phase transition of the cell cycle (Figure 16). In agreement with the flow cytometry data, examination of cells transiently transfected with shUSP4D or cells overexpressing either wt or Cys USP4 by light microscopy revealed no significant differences in the general appearance of cells. In addition, no increase in mitotic or arrested cells was noted (Figure 17).

Figure 16. Effect of USP4 depletion on cell cycle by flow cytometry

Analysis of cell cycle parameters in NIH-3T3 cells transfected using shControl or shUSP4D by flow cytometry using propidium iodide staining. No significant difference in G1/S transition and cell cycle profiles were observed between untransfected NIH-3T3 cells, and cells transfected with shControl or shUSP4D. Representative cell cycle profiles are shown for A) untransfected NIH-3T3 B) shControl C) shUSP4D

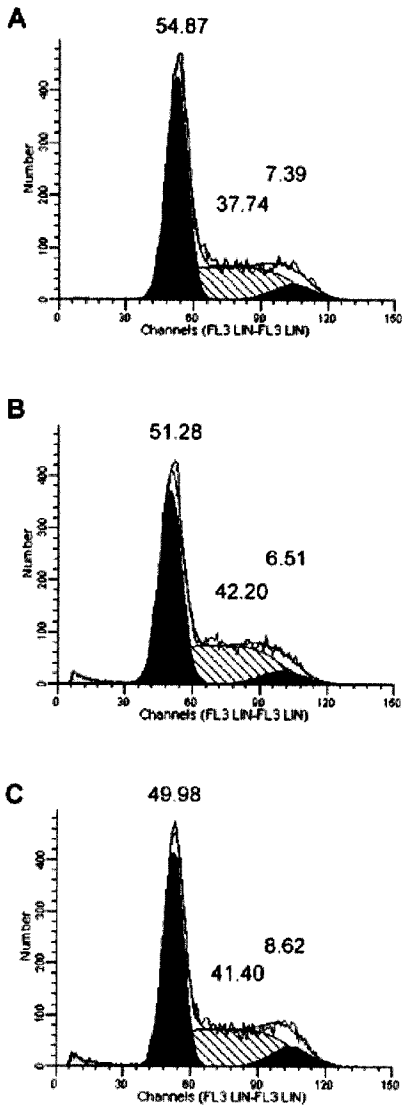
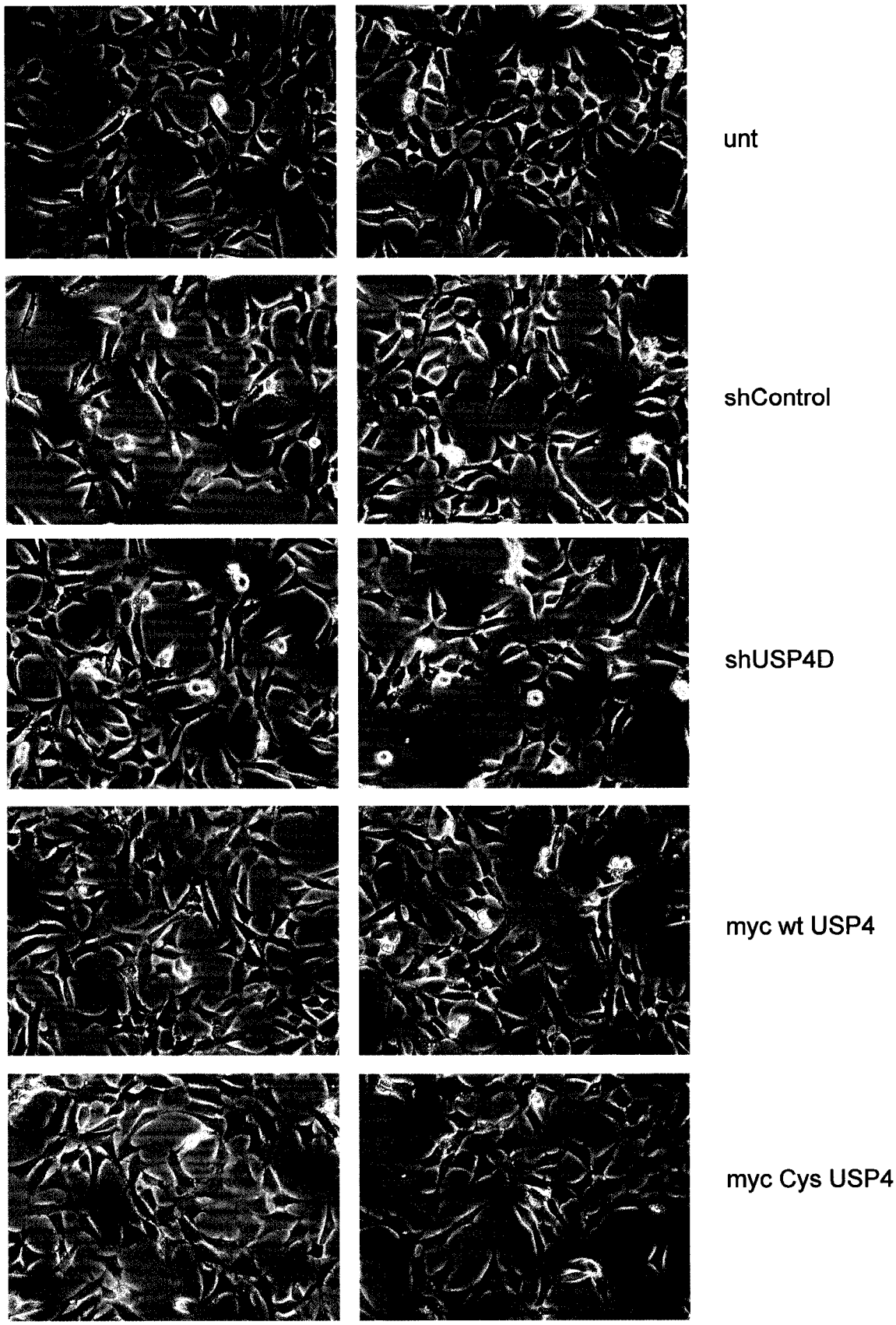


Figure 17. Cell morphology in cells depleted or overexpressing USP4

Visualization of NIH-3T3 cells by phase contrast microscopy. No morphology changes were observed between untransfected cells or cells overexpressing shControl, shUSP4, myc wt USP4 or myc Cys USP4. Two representative panels of each transfections are depicted A) untransfected NIH-3T3 B) shControl C) shUSP4 D) myc wt USP4 E) Cys USP4 mutant.



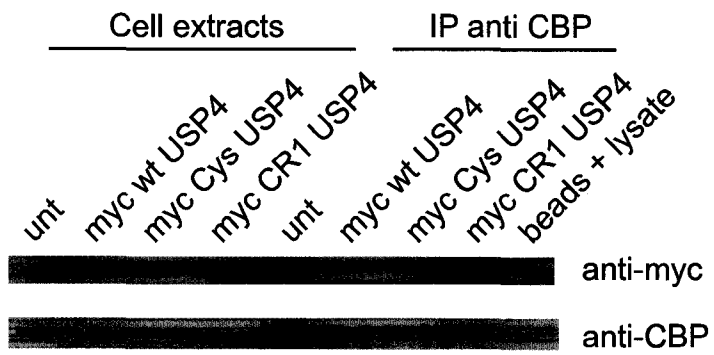
USP4 affects transcription

Given the exceptionally large number of genes that were modified by expression of the shUSP4, the possibility of the involvement of USP4 in modulating global transcription was assessed by luciferase assay. The expression of a luciferase reporter driven by a TATA box was assessed in cells expressing wt USP4 or depleted of USP4 using the shUSP4D. The analysis revealed that whereas transfection of shUSP4D decreased basal transcription of the reporter protein, ectopic expression of wt USP4 had the opposite effect. Overexpression of the catalytically inactive version of USP4 (Cys mutant) had no effect on transcription (Figure 18). This data proposes a role for USP4 in gene transcription regulation. Since CREB-binding protein (CBP) is a transcriptional co-activator and has been shown to bind the amino terminus and the CR1 motif of E1A (Barbeau et al., 1994), we explored the possibility that USP4 and CBP interact. The interaction of USP4 with CBP was investigated by co-immunoprecipitation assays in cell extracts from NIH-3T3 cells transfected with myc tagged-versions of USP4. The analysis of the immunoprecipitates by western blot analysis with the myc antibody revealed that CBP interacts with USP4 and that this interaction is not dependent on the Cys or CR1 domains. The membrane was re-probed with an antibody against CBP to confirm its presence in the lysates and immunoprecipitates.

Figure 18. USP4 modifies transcription

A) Western blot analysis with the anti-myc antibody of total cells extracts or immunoprecipitates from NIH-3T3 cells transfected with myc wt USP4, myc Cys USP4 or myc CR1 USP4. CBP was observed to be present in immunoprecipitates of myc wt USP4, myc Cys USP4 and myc CR1 USP4 but absent in untransfected immunoprecipitates and in the beads plus lysate negative control. B) NIH-3T3 cells were assayed for expression of a luciferase reporter containing a TATA box using a dual luciferase assay. Depletion of USP4 using shUSP4D showed a significant decrease in luciferase activity while lysates overexpressing myc wt USP4 demonstrated an increase in luciferase activity when compared to cells transfected with the pcDNA3.1 vector alone. Cells overexpressing the myc Cys USP4 construct did not show a significant change compared to cells transfected with the pcDNA3.1 vectore alone.

A



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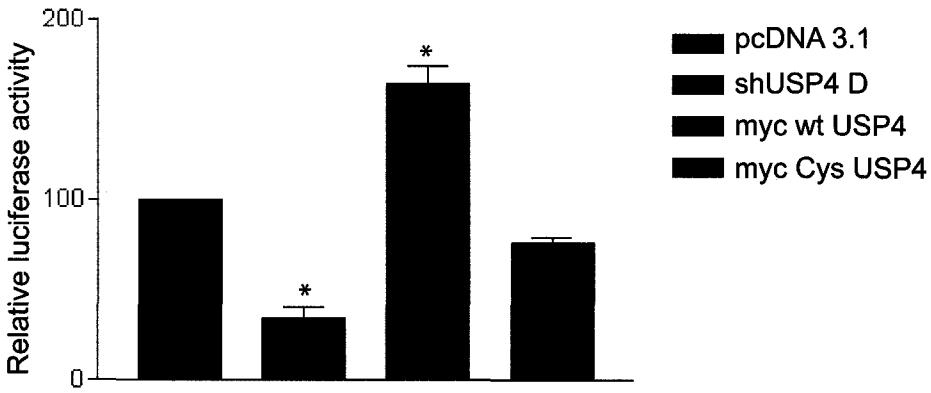

DISCUSSION

USP4 and ARE-mRNA stability

A large number of proteins that belong to different biological processes and are important in various diseases are encoded by ARE-mRNAs (Bakheet et al., 2006; Hollams et al., 2002). Some tumors stabilize oncogene mRNAs which leads to protein overproduction and a predisposition for unrestrained growth. For example, the mRNA of the c-myc proto-oncogene undergoes rapid turnover due to the presence of an ARE sequence in its 3' UTR (Brewer, 1991; Brewer, 1998; Jones and Cole, 1987). Translocation or loss of the c-myc 3' UTR renders the c-myc mRNA much more stable and has been observed in some myelomas and human T-cell leukemias (Aghib et al., 1990; Hollis et al., 1986). Various cytokines and growth factors including GM-CSF, IL-1, IL-3 and IL-6 also show an increased mRNA stability in different tumors or cell lines (Ross et al., 1991; Schuler and Cole, 1988). ARE-binding proteins have been implicated in different cancers (Gouble et al., 2002; Guo and Hartley, 2006). HuR, an ARE-binding protein, antagonizes mRNA decay (Myer et al., 1997; Peng et al., 1998). HuR is predominantly nuclear but can translocate to the cytoplasm which suggests that it binds to an ARE-mRNA in the nucleus and that the complex shuttles to the cytoplasm where HuR stabilizes the transcript and facilitates translation (Fan and Steitz, 1998). Increased HuR expression and an increase in its cytoplasmic presence has been observed in many cancers including induced mouse mammary tumors as well as in human breast carcinomas (Heinonen et al., 2005) and colon carcinomas (Denkert et al., 2006; Lopez de Silanes et al., 2003). Interestingly, both HuR and AUF1, a destabilizing ARE-binding protein, have increased cytoplasmic expression levels in induced neoplasia and hyperplasia of mouse lung tissue. In addition, the study suggests that the alterations in HuR

and AUF1 expression levels might act on highly regulated mRNAs and that this impacts on the neoplastic pathology (Blaxall et al., 2000). The explanation behind the HuR or AUF1 overexpression or cytoplasmic distribution still remains unclear.

USP4 has previously been implicated in the control of rapid degradation of ARE-mRNAs (Laroia et al., 2002). The study demonstrated that USP4 overexpression in mammalian Cos-7 cells prevents degradation of ARE-mRNAs. The mechanism by which USP4 accomplishes these effects remains unknown. One possibility by which USP4 could exert ARE-mRNAs stability control could be through the deubiquitination and subsequent stabilization of ARE-binding proteins. HuR is likely to be a substrate of the UPP since proteasomal inhibition increases its levels (Bonelli et al., 2004). Since HuR is a stabilizing ARE-binding protein of GM-CSF (Fan and Steitz, 1998; Peng et al., 1998; Raineri et al., 2004), it is possible that it may be stabilized through deubiquitination by USP4. Therefore, increased levels of USP4, seen in human lung tumors (Gray et al., 1995), could lead to increased expression levels of HuR which in turn would result in increased mRNA stability of HuR-targeted ARE-mRNAs such as GM-CSF. The increased mRNA stability would allow increased translation of GM-CSF protein. It has been shown that during tumor development, GM-CSF stimulates cell proliferation and affects the microenvironment of the tumor as well as modulates the immune response (Diament et al., 2003). GM-CSF has also been attributed a role in tumor progression of lung cancer (Uemura et al., 2006). Therefore, abnormal GM-CSF mRNA stability exerted by USP4 may cause increased cell proliferation and tumorigenesis.

The role of USP4 in ARE-mRNA stability was investigated in this study in order to elucidate if the catalytic activity of USP4, binding domains or localization signals are required and to explore if this effect is specific to GM-CSF or can be extended to other ARE

sequences. Overexpression of USP4 or USP4 mutants did not result in increased luciferase activity of a construct containing a generic ARE sequence in NIH-3T3 cells (Figure 5 and Figure 6A). It is possible that USP4 acts on a protein that is not expressed to the same level in NIH-3T3 cells compared to Cos-7 cells. Interestingly, HuR expression levels are different across cell lines and have been found to be low in NIH-3T3 cells (Manjithaya and Dighe, 2004). Therefore, the effects of USP4 on the generic ARE sequence were assessed in Cos-7 cells. Once again, USP4 overexpression did not demonstrate an increased luciferase activity (Figure 6B) suggesting that this is not due to a cell type specific effect. Since the ARE sequence encoded in the luciferase construct is generic, these results suggest that the stabilizing effects of USP4 on ARE-mRNAs are not global and can not be attributed to every ARE-mRNAs. Rather, USP4 might be stabilizing certain specific ARE-mRNAs, one of which could be GM-CSF. In fact, if USP4 exerts its effect by interacting or deubiquitinating an ARE-binding protein, its effects are most likely going to be specific to the AREs bound by that protein. When interpreting these results, it is important to keep in mind that the commercially available UbC-Luc-ARE construct contains hCL1 and PEST protein degradation sequences (Figure 4). Since the mRNA stability effect was assessed by luciferase reporter assay which measures protein levels, the lack of stability effect could be attributed to the fact that the protein degradation sequences are degrading the proteins before an effect could be observed, therefore overriding the mRNA stabilization effect of USP4. An attempt to overcome rapid protein degradation was made by using a proteasome inhibitor in order to block protein degradation (data not shown) however, this approach yielded unconvincing results. Thus, the results obtained with the commercial UbC-Luc-ARE construct were inconclusive and a different approach was undertaken.

ARE-binding proteins bind selectively to different ARE sequences (Chen et al., 2002a). Since USP4 may act on an ARE-binding protein involved in the mRNA stability of GM-CSF, the effects of overexpression of USP4 were investigated on a luciferase construct (without protein degradation sequences) containing the GM-CSF ARE sequence. The observation that the UbC-LuC construct containing the ARE does not show a decrease in luciferase activity like expected by an ARE-containing mRNA sequence (Figure 7) could be explained by the strong UbC promoter driving expression of the construct. The UbC promoter could be saturating the system to the point that rapid mRNA decay of the construct is masked. Therefore, endogenous GM-CSF levels were assessed following overexpression of UPS4. Overexpression of USP4 or HuR did not prevent rapid decay of GM-CSF whereas co-transfection of USP4 and HuR resulted in increased GM-CSF protein levels (Figure 8). It is important to note when interpreting these results that HuR, used as a positive control, did not result in increased levels of GM-CSF. Depending on endogenous levels of USP4 and HuR, it is possible that in order to observe an increase in the stability effect under the conditions used, both USP4 and HuR must be elevated. The most parsimonious explanation for these findings is that whereas endogenous HuR may be stable in its normal physiological location and/or complex, the exogenous HuR is recognized, ubiquitinated, and rapidly degraded by the proteasome, but can be protected by the deubiquitinating activity of USP4. Because the endogenous HuR is not ubiquitinated under our cell culture conditions, the addition of more USP4 protein does not lead to an increase in HuR levels and subsequent stability and increase of GM-CSF. It is also important to note that more than one protein can bind the ARE sequence of GM-CSF. AUF1 is also an ARE-binding protein that binds the GM-CSF ARE sequence but leads to the destabilization of the construct (Buzby et al., 1999). This suggests that HuR and AUF1 compete in order to determine the stability of GM-CSF

mRNA. Without knowing whether this competition occurs in our experimental system, whether HuR, AUF1, or both are substrates of USP4, and whether USP4 alters the stoichiometry or physical dynamics of the competition it is difficult to predict how HuR/AUF1 competition might affect GM-CSF mRNA stability in our assay.

The data in this study fails to find any support for the stability effects of USP4 on GM-CSF mRNA proposed in (Laroia et al., 2002). Further studies are required in order to convincingly evaluate if USP4 is involved in mRNA stability. For instance, investigation of endogenous GM-CSF, and other ARE-containing transcripts, at the RNA levels using Real-time PCR following overexpression or depletion of USP4. It would also be useful to determine if HuR or other ARE-binding proteins are substrates of USP4.

Generation of USP4 knockdown

Considering the fact that no commercially antibody to USP4 is available, depletion of endogenous USP4 levels following shUSP4 expression could not be analyzed by western. Alternatively, myc wt USP4 was overexpressed and subsequently depleted using the shUSP4 in order to assess knockdown of USP4. The shUSP4D proved to be successful in knocking down exogenous myc wt USP4 (Figures 9 and 11) but to decrease GFP protein levels only very slightly (Figure 10). Therefore, it was assumed that endogenous USP4 would be targeted and depleted. However, no depletion of endogenous USP4 at the mRNA level (Figure 12) was observed when transfecting shUSP4D. A few reasons could account for the observation that endogenous USP4 is not depleted. First, it is possible that endogenous levels of USP4 are already at the limits of detection and therefore too low to detect a significant knockdown. Second, although the system was designed to maximize specificity there is the possibility that it is unable to discriminate between USP4 and the highly related

USP15 (Baker et al, 1999). Third, since USP4 has splicing variants (Gray et al., unpublished), some of them could be escaping depletion by the shUSP4D. In addition, a cryptic promoter may be present in the USP4 sequence and result in a truncated transcript that is not targeted by the knockdown. Despite the suggestive evidence from the tagged exogenous USP4 knockdown there is no definitive evidence at this point demonstrating that endogenous USP4 is being depleted. Due to time limitations this issue was unfortunately left unresolved, but a sensitive non-PCR based strategy such as RNAse protection could be used to determine if endogenous USP4 levels were actually affected.

USP4 depletion and gene dysregulation

Transforming growth factor-beta (TGF-beta) signaling is a suppressor of cell growth and loss of this pathway can result in tumorigenesis (Bian et al., 2003; Kaklamani and Pasche, 2004). A number of studies have focused on inactivation of the TGF-beta signaling cascade in lung cancer (Baek et al., 2006; Domagala-Kulawik et al., 2006; Kang et al., 2006; Park et al., 2002).

TGF-beta signaling (reviewed in (Massague and Gomis, 2006; Massague and Wotton, 2000)) is activated by a family of cytokines divided into two subfamilies: the TGF-beta/Activin/Nodal subfamily and the BMP (bone morphogenetic protein)/GDF (growth and differentiation factor)/MIS (Mullerian inhibiting substance). The signaling cascade is initiated when a TGF-beta ligand binds and brings together type I and type II receptor serine/threonine kinases on the cell surface. Subsequently, receptor II phosphorylates the kinase domain of receptor I which results in phosphorylation of downstream Smad transcription factor proteins and propagation of the signal. Smad proteins are divided in three functional classes: 1) receptor-regulated Smad (R-Smad) which includes Smad1, 2, 3, 5

and 8; 2) co-mediator Smad (Co-Smad) which has Smad4 as its sole member; 3) inhibitory Smad (I-Smad) which includes Smad6 and 7. R-Smads are directly phosphorylated and activated by the receptor I kinase activity. These Smads can homotrimerize or form a complex with Co-Smad4. Once the complexes are activated, they move into the nucleus where, in cooperation with other nuclear co-factors, they regulate transcription of target genes. Smad6 and Smad7 negatively regulate signaling by competing with R-Smad for the receptors or Smad4 interactions and by selecting the receptors for degradation by the proteasome. Tight regulation of TGF-beta signaling is crucial in maintaining homeostasis and preventing carcinogenesis. The UPP is one mechanism through which cells regulate the expression levels and activity of TGF-beta signaling effectors (Izzi and Attisano, 2004; Izzi and Attisano, 2006). Smurfs are responsible for the regulation of the cascade through ubiquitination and proteasomal degradation of TGF-receptor and Smads (Arora and Warrior, 2001; Izzi and Attisano, 2004). A recent report has demonstrated that the deubiquitinating enzyme UCH37 (ubiquitin C-terminal hydrolase) interacts with Smad2, Smad3 and Smad7. In addition, UCH37 has been shown to deubiquitinate and stabilize the TGF-beta1 receptor (Wicks et al., 2005). These findings support the role of ubiquitin and DUBs in the control of TGF-beta signaling. In light of the fact that USP4 mRNA levels are elevated in human primary tissue of small cell tumors and adenocarcinomas (Gray et al., 1995) and that a number of genes involved in the TGF-beta pathway were observed to be dysregulated following depletion of USP4, TFG-beta signaling was investigated. USP4 could be implicated in the TGF-beta cascade at a number of possible levels. USP4 could interact with TGF-beta itself or one of the TGF-beta receptors potentially stabilizing them. Therefore, depletion of USP4 would lead to inhibition of the TGF-beta pathway. Alternatively, USP4 could act on downstream effectors of the TGF-beta signaling cascade. The data presented

here demonstrates no change in mRNA expression levels of investigated genes of the TGF-beta pathway (Table 1) transfection of shUSP4D (Figure 13). The observation that Smad3 protein levels (Figure 14B) increased following shUSP4D transfection but also increased with the shControl transfection suggests that the observed upregulation of Smad3 protein levels were due to the short-hairpin transfection itself through unspecific interactions of the hairpin or viral protein effects. This was confirmed by the fact that Smad3 demonstrated no increase in protein level in cells expressing USP4 siRNA (Figure 14D) compared to untransfected NIH-3T3. This suggests that the gene deregulation observed by microarray analysis was a result of the short-hairpin transfection and explains why the investigated genes did not demonstrate any mRNA changes when validated by Real-time PCR. Therefore, the data presented here does not support the proposed model of USP4 in TGF-beta signaling.

Cell cycle and cell morphology following changes in USP4 expression levels

USP4 contains domains, CR1 and CR2, which are identical to viral protein motifs responsible for binding the retinoblastoma gene product pRb. In fact, USP4 has been shown to interact with pocket proteins pRb, p107 and p130 (Blanchette et al., 2001; DeSalle et al., 2001). The pRb tumor suppressor is involved in the regulation of the G1 to S phase transition of the cell cycle (Dannenberg and te Riele, 2006; Grana et al., 1998). It has been proposed that pRb is unlikely to be a substrate of USP4. Rather, pRb is thought to bring USP4 in close association with other proteins found in complex with it (Blanchette et al., 2001). Some pRb-binding proteins are degraded through the UPP including *c-myc* (Gregory and Hann, 2000), E2F-1 (Hofmann et al., 1996) and E2F-4 (Hateboer et al., 1996). USP4 could potentially provide a mode of regulation for these proteins via deubiquitination. Given

the fact that pRb is involved in cell cycle regulation, changes in USP4 expression could affect levels of a protein involved in cell cycle and lead to a dysregulation in cell cycle progression. The data presented in this study does not support a role for USP4 in the G1/S phase transition of cell cycle (Figure 16). One explanation for this could be related to the fact that the shRNA lentivirus transfection itself is affecting the cells and that the endogenous USP4 mRNA levels were not shown to be depleted. Therefore, the fact that USP4 is not involved in cell cycle can not be ruled out. Over a hundred pRb-binding proteins have been identified to date suggesting that pRb possess many different functions (reviewed in (Morris and Dyson, 2001). Therefore, USP4 could be regulating a pRb-binding protein which is implicated in cell cycle or in a different pathway involving pRb such as transcription. These experiments need to be repeated using the USP4 siRNA in order to overcome the lentivirus effect. As well, a combination of siRNAs targeting different regions of USP4 could be used in an attempt to deplete endogenous USP4. Visualization of cells following shUSP4D transfection or USP4 overexpression revealed no significant differences in the general appearance of cells (Figure 17). Once again it is important to note that the shUSP4D transfection is not totally reliable. However, overexpression of myc wt USP4 also revealed no change in cell appearance suggesting that USP4 is not acting on a gene affecting cell morphology.

Role for USP4 in transcription

The assumed depletion of USP4 and analysis of gene expression by microarray has demonstrated dysregulation of a great number of genes. This suggests that USP4 exerts a global effect which could be attributed by involvement in transcription or translation. This study demonstrates that the deubiquitinating enzyme USP4 and the transcriptional co-

activator CBP interact in a mouse fibroblast cell line. Interestingly, CBP is a target of the ubiquitin-proteasome pathway (Lonard et al., 2000) which has recently been shown to be accomplished via UBC3 (Yan et al., 2003). It is possible that ubiquitination and deubiquitination of transcriptional co-activators such as CBP provide a transcriptional mode of regulation by ensuring appropriate protein levels of these co-activators. Since CBP is such an important player in transcriptional regulation, the fact that USP4 is in the same complex as CBP supports the observation that depletion of USP4 leads to the mRNA deregulation of a large number of genes. The role of USP4 in transcriptional regulation is supported by the fact that shUSP4D transfection decreased and USP4 overexpression increased basal transcription activity. It is conceivable that USP4 stabilizes CBP, or another protein in the same transcription complex, through deubiquitination. Consequently, depletion of USP4 would lead to a decrease of transcription activity through increased ubiquitin-dependent degradation of CBP which is what is observed in regards to basal transcription activity. On the other hand, overexpression of USP4 would stabilize CBP and result in increased basal transcription like observed. Further studies need to be conducted to assess if USP4 binds other proteins in the CBP complex. It would be interesting to know if USP4 directly deubiquitinates CBP or if CBP acts as a bridge between USP4 and some other unidentified component of the transcriptional complex that it stabilizes. CBP and its homolog p300 are known to interact with many proteins involved in transcription, general transcription and nuclear receptors (see Table 2) (reviewed in Shikama et al., 1997) which suggests many possible substrates for USP4. In order to identify such a substrate, a pull-down using anti-CBP (from cells overexpressing or depleted in USP4) followed by mass spectrometry could be contemplated.

Table 2. p300/CBP target proteins

List of multiple p300/CBP binding partners including transcription factors, viral oncoproteins and nuclear receptors. The p300/CBP regions responsible for the binding activity with the target proteins are depicted.

Target	Binding domain		Comment
	p300	CBP	
CREB	1-663	590-669	Transcription factor
c-Fos	-	1621-1877	Transcription factor
c-jun, v-jun	1596 1752-2370	461-662	Transcription factor
JunB	744-1571 1572-2370	-	Transcription factor
YY1	1572-2370	-	Transcription factor
E1A	1767-1816	1805-1854	Viral oncoprotein
SV large T	1709-1913	1746-1951	Viral oncoprotein
c-myb	-	461-661	Transcription factor
Nuclear receptors (RXR, RAR, TR, ER, PR, GR)	1-117	1-101	Transcription factor
$p160^{SRC-1}$	2046-2157	2058-2163	Coactivator
Sap-1a	-	451-721	Transcription factor
Stat2	CH-1	-	Transcription factor
MyoD	1514-1922	-	Transcription factor
E2F-1	-	1621-1877	Transcription factor
TBP	1-743 1737-2414	-	General transcription factor
TFIIB	1737-2414	1680-1812	General transcription factor
P/CAF	1763-1966	1801-1880	Acetyltransferase
$pp90^{Rsk}$	1737-1836	1805-1891	Kinase
Tax	566-663	451-682	Viral transactivator
SREBP-2	1-663	451-682	Transcription factor

adapted from Shikama et al., 1997

Conclusions

In the past few years, studies have been identifying binding partners of UPS4 and have shown Ro52 to be a substrate of USP4. To date, none of these findings have explained the increased lung carcinoma pathology associated with elevated USP4 mRNA levels. This study demonstrates that the mammalian deubiquitinating enzyme USP4 is in complex with the CBP transcriptional co-activator. This interaction could suggest a role for USP4 in transcriptional regulation. In addition, changes in USP4 expression levels were shown to impact on basal transcriptional activity. Further studies need to be conducted in order to explore this interaction further and elucidate its physiological relevance.

Aghib, D. F., Bishop, J. M., Ottolenghi, S., Guerrasio, A., Serra, A., and Saglio, G. (1990). A 3' truncation of MYC caused by chromosomal translocation in a human T-cell leukemia increases mRNA stability. Oncogene *5*, 707-711.

Aguilar, R. C., and Wendland, B. (2003). Ubiquitin: not just for proteasomes anymore. Curr Opin Cell Biol *15*, 184-190.

Amerik, A., Swaminathan, S., Krantz, B. A., Wilkinson, K. D., and Hochstrasser, M. (1997). In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. Embo J *16*, 4826-4838.

Amerik, A. Y., and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta *1695*, 189-207.

Amerik, A. Y., Li, S. J., and Hochstrasser, M. (2000). Analysis of the deubiquitinating enzymes of the yeast Saccharomyces cerevisiae. Biol Chem *381*, 981-992.

Arora, K., and Warrior, R. (2001). A new Smurf in the village. Dev Cell *1*, 441-442.

Baek, H. J., Kim, S. S., da Silva, F. M., Volpe, E. A., Evans, S., Mishra, B., Mishra, L., and Marshall, M. B. (2006). Inactivation of TGF-beta signaling in lung cancer results in increased CDK4 activity that can be rescued by ELF. Biochem Biophys Res Commun *346*, 1150-1157.

Baker, R. T., Tobias, J. W., and Varshavsky, A. (1992). Ubiquitin-specific proteases of Saccharomyces cerevisiae. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J Biol Chem *267*, 23364-23375.

Baker, R. T., Wang, X. W., Woollatt, E., White, J. A., and Sutherland, G. R. (1999). Identification, functional characterization, and chromosomal localization of USP15, a novel human ubiquitin-specific protease related to the UNP oncoprotein, and a systematic nomenclature for human ubiquitin-specific proteases. Genomics *59*, 264-274.

Bakheet, T., Williams, B. R., and Khabar, K. S. (2006). ARED 3.0: the large and diverse AU-rich transcriptome. Nucleic Acids Res *34*, D111-114.

Barbeau, D., Charbonneau, R., Whalen, S. G., Bayley, S. T., and Branton, P. E. (1994). Functional interactions within adenovirus E1A protein complexes. Oncogene *9*, 359-373.

Bian, Y., Kaklamani, V., Reich, J., and Pasche, B. (2003). TGF-beta signaling alterations in cancer. Cancer Treat Res *115*, 73-94.

Blanchette, P., Gilchrist, C. A., Baker, R. T., and Gray, D. A. (2001). Association of UNP, a ubiquitin-specific protease, with the pocket proteins pRb, p107 and p130. Oncogene *20*, 5533-5537.

Blaxall, B. C., Dwyer-Nield, L. D., Bauer, A. K., Bohlmeyer, T. J., Malkinson, A. M., and Port, J. D. (2000). Differential expression and localization of the mRNA binding proteins, AU-rich element mRNA binding protein (AUF1) and Hu antigen R (HuR), in neoplastic lung tissue. Mol Carcinog *28*, 76-83.

Bochtler, M., Ditzel, L., Groll, M., Hartmann, C., and Huber, R. (1999). The proteasome. Annu Rev Biophys Biomol Struct *28*, 295-317.

Bonelli, M. A., Alfieri, R. R., Desenzani, S., Petronini, P. G., and Borghetti, A. F. (2004). Proteasome inhibition increases HuR level, restores heat-inducible HSP72 expression and thermotolerance in WI-38 senescent human fibroblasts. Exp Gerontol *39*, 423-432.

Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P. M., Finley, D., and

Schmidt, M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat Cell Biol *1*, 221-226.

Brewer, G. (1991). An A + U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. Mol Cell Biol *11*, 2460-2466.

Brewer, G. (1998). Characterization of c-myc 3' to 5' mRNA decay activities in an in vitro system. J Biol Chem *273*, 34770-34774.

Brooks, C. L., and Gu, W. (2004). Dynamics in the p53-Mdm2 ubiquitination pathway. Cell Cycle *3*, 895-899.

Buzby, J. S., Brewer, G., and Nugent, D. J. (1999). Developmental regulation of RNA transcript destabilization by A + U-rich elements is AUF1-dependent. J Biol Chem *274*, 33973-33978.

Chen, C. Y., Xu, N., and Shyu, A. B. (2002a). Highly selective actions of HuR in antagonizing AU-rich element-mediated mRNA destabilization. Mol Cell Biol *22*, 7268-7278.

Chen, X., Zhang, B., and Fischer, J. A. (2002b). A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets. Genes Dev *16*, 289-294.

Chung, C. H., and Baek, S. H. (1999). Deubiquitinating enzymes: their diversity and emerging roles. Biochem Biophys Res Commun *266*, 633-640.

Ciechanover, A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. Embo J *17*, 7151-7160.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol *6*, 79-87.

Conaway, R. C., Brower, C. S., and Conaway, J. W. (2002). Emerging roles of ubiquitin in transcription regulation. Science *296*, 1254-1258.

Cressman, D. E., and Taub, R. (1994). Physiologic turnover of nuclear factor kappa B by nuclear proteolysis. J Biol Chem *269*, 26594-26597.

D'Andrea, A., and Pellman, D. (1998). Deubiquitinating enzymes: a new class of biological regulators. Crit Rev Biochem Mol Biol *33*, 337-352.

Dannenberg, J. H., and te Riele, H. P. (2006). The retinoblastoma gene family in cell cycle regulation and suppression of tumorigenesis. Results Probl Cell Differ *42*, 183-225.

Denkert, C., Koch, I., von Keyserlingk, N., Noske, A., Niesporek, S., Dietel, M., and Weichert, W. (2006). Expression of the ELAV-like protein HuR in human colon cancer: association with tumor stage and cyclooxygenase-2. Mod Pathol *19*, 1261-1269.

DeSalle, L. M., Latres, E., Lin, D., Graner, E., Montagnoli, A., Baker, R. T., Pagano, M., and Loda, M. (2001). The de-ubiquitinating enzyme Unp interacts with the retinoblastoma protein. Oncogene *20*, 5538-5542.

Diament, M. J., Stillitani, M. I., Puricelli, L., Bal de Kier-Joffe, E., and Klein, S. (2003). GM-CSF secreted by murine adenocarcinoma cells modulates tumor progression and immune activity. Oncol Rep *10*, 1647-1652.

DiAntonio, A., Haghighi, A. P., Portman, S. L., Lee, J. D., Amaranto, A. M., and Goodman, C. S. (2001). Ubiquitination-dependent mechanisms regulate synaptic growth and function. Nature *412*, 449-452.

Dice, J. F., Chiang, H. L., Spencer, E. P., and Backer, J. M. (1986). Regulation of catabolism of microinjected ribonuclease A. Identification of residues 7-11 as the essential pentapeptide. J Biol Chem *261*, 6853-6859.

Dice, J. F., Terlecky, S. R., Chiang, H. L., Olson, T. S., Isenman, L. D., Short-Russell, S. R.,

Freundlieb, S., and Terlecky, L. J. (1990). A selective pathway for degradation of cytosolic proteins by lysosomes. Semin Cell Biol *1*, 449-455.

Domagala-Kulawik, J., Hoser, G., Safianowska, A., Grubek-Jaworska, H., and Chazan, R. (2006). Elevated TGF-beta1 concentration in bronchoalveolar lavage fluid from patients with primary lung cancer. Arch Immunol Ther Exp (Warsz).

Espel, E. (2005). The role of the AU-rich elements of mRNAs in controlling translation. Semin Cell Dev Biol *16*, 59-67.

Fan, X. C., and Steitz, J. A. (1998). Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. Embo J *17*, 3448-3460.

Finley, D., and Chau, V. (1991). Ubiquitination. Annu Rev Cell Biol *7*, 25-69.

Fischer-Vize, J. A., Rubin, G. M., and Lehmann, R. (1992). The fat facets gene is required for Drosophila eye and embryo development. Development *116*, 985-1000.

Fischer, J. A. (2003). Deubiquitinating enzymes: their roles in development, differentiation, and disease. Int Rev Cytol *229*, 43-72.

Frederick, A., Rolfe, M., and Chiu, M. I. (1998). The human UNP locus at 3p21.31 encodes two tissue-selective, cytoplasmic isoforms with deubiquitinating activity that have reduced expression in small cell lung carcinoma cell lines. Oncogene *16*, 153-165.

Gilchrist, C. A., and Baker, R. T. (2000). Characterization of the ubiquitin-specific protease activity of the mouse/human Unp/Unph oncoprotein. Biochim Biophys Acta *1481*, 297-309.

Gilchrist, C. A., Gray, D. A., and Baker, R. T. (1997). A ubiquitin-specific protease that efficiently cleaves the ubiquitin-proline bond. J Biol Chem *272*, 32280-32285.

Glickman, M. H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev *82*, 373-428.

Gouble, A., Grazide, S., Meggetto, F., Mercier, P., Delsol, G., and Morello, D. (2002). A new player in oncogenesis: AUF1/hnRNPD overexpression leads to tumorigenesis in transgenic mice. Cancer Res *62*, 1489-1495.

Grana, X., Garriga, J., and Mayol, X. (1998). Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. Oncogene *17*, 3365-3383.

Gray, D. A., Inazawa, J., Gupta, K., Wong, A., Ueda, R., and Takahashi, T. (1995). Elevated expression of Unph, a proto-oncogene at 3p21.3, in human lung tumors. Oncogene *10*, 2179-2183.

Gray, D. A., Tsirigotis, M., and Woulfe, J. (2003). Ubiquitin, proteasomes, and the aging brain. Sci Aging Knowledge Environ *2003*, RE6.

Gregory, M. A., and Hann, S. R. (2000). c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol *20*, 2423-2435.

Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997). Structure of 20S proteasome from yeast at 2.4 A resolution. Nature *386*, 463-471.

Guo, X., and Hartley, R. S. (2006). HuR Contributes to Cyclin E1 Deregulation in MCF-7 Breast Cancer Cells. Cancer Res *66*, 7948-7956.

Gupta, K., Chevrette, M., and Gray, D. A. (1994). The Unp proto-oncogene encodes a nuclear protein. Oncogene *9*, 1729-1731.

Gupta, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Gray, D. A. (1993). Unp, a mouse gene related to the tre oncogene. Oncogene *8*, 2307-2310.

Guterman, A., and Glickman, M. H. (2004). Deubiquitinating enzymes are IN/(trinsic to proteasome function). Curr Protein Pept Sci *5*, 201-211.

Hadari, T., Warms, J. V., Rose, I. A., and Hershko, A. (1992). A ubiquitin C-terminal isopeptidase that acts on polyubiquitin chains. Role in protein degradation. J Biol Chem *267*, 719-727.

Haglund, K., Di Fiore, P. P., and Dikic, I. (2003). Distinct monoubiquitin signals in receptor endocytosis. Trends Biochem Sci *28*, 598-603.

Hateboer, G., Kerkhoven, R. M., Shvarts, A., Bernards, R., and Beijersbergen, R. L. (1996). Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. Genes Dev *10*, 2960-2970.

Hayashi, T., and Faustman, D. (2000). Defective function of the proteasome in autoimmunity: involvement of impaired NF-kappaB activation. Diabetes Technol Ther *2*, 415-428.

Heinonen, M., Bono, P., Narko, K., Chang, S. H., Lundin, J., Joensuu, H., Furneaux, H., Hla, T., Haglund, C., and Ristimaki, A. (2005). Cytoplasmic HuR expression is a prognostic factor in invasive ductal breast carcinoma. Cancer Res *65*, 2157-2161.

Helt, A. M., and Galloway, D. A. (2003). Mechanisms by which DNA tumor virus oncoproteins target the Rb family of pocket proteins. Carcinogenesis *24*, 159-169.

Hershko, A. (1997). Roles of ubiquitin-mediated proteolysis in cell cycle control. Curr Opin Cell Biol *9*, 788-799.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu Rev Biochem *67*, 425-479.

Heusch, M., Lin, L., Geleziunas, R., and Greene, W. C. (1999). The generation of nfkb2 p52: mechanism and efficiency. Oncogene *18*, 6201-6208.

Hilt, W., and Wolf, D. H. (1995). Proteasomes of the yeast S. cerevisiae: genes, structure and functions. Mol Biol Rep *21*, 3-10.

Hofmann, F., Martelli, F., Livingston, D. M., and Wang, Z. (1996). The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. Genes Dev *10*, 2949-2959.

Hollams, E. M., Giles, K. M., Thomson, A. M., and Leedman, P. J. (2002). MRNA stability and the control of gene expression: implications for human disease. Neurochem Res *27*, 957-980.

Hollis, G. F., Gazdar, A. F., and Kirsch, I. R. (1986). DNA rearrangement and expression of the c-myc gene in a human myeloma. Curr Top Microbiol Immunol *132*, 225-226.
Hu, M., Li, P., Song, L., Jeffrey, P. D., Chenova, T. A., Wilkinson, K. D., Cohen, R. E., and

Shi, Y. (2005). Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. Embo J *24*, 3747-3756.

Izzi, L., and Attisano, L. (2004). Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. Oncogene *23*, 2071-2078.

Izzi, L., and Attisano, L. (2006). Ubiquitin-dependent regulation of TGFbeta signaling in cancer. Neoplasia *8*, 677-688.

Jones, T. R., and Cole, M. D. (1987). Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. Mol Cell Biol *7*, 4513-4521.

Kaklamani, V. G., and Pasche, B. (2004). Role of TGF-beta in cancer and the potential for therapy and prevention. Expert Rev Anticancer Ther *4*, 649-661.

Kang, H. G., Chae, M. H., Park, J. M., Kim, E. J., Park, J. H., Kam, S., Cha, S. I., Kim, C. H., Park, R. W., Park, S. H., *et al.* (2006). Polymorphisms in TGF-beta1 gene and the risk of lung cancer. Lung Cancer *52*, 1-7.

Kao, C. F., Hillyer, C., Tsukuda, T., Henry, K., Berger, S., and Osley, M. A. (2004). Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B. Genes Dev *18*, 184-195.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol *18*, 621-663.

Kim, J. H., Park, K. C., Chung, S. S., Bang, O., and Chung, C. H. (2003). Deubiquitinating enzymes as cellular regulators. J Biochem (Tokyo) *134*, 9-18.

Lam, Y. A., Xu, W., DeMartino, G. N., and Cohen, R. E. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature *385*, 737-740.

Laroia, G., Sarkar, B., and Schneider, R. J. (2002). Ubiquitin-dependent mechanism regulates rapid turnover of AU-rich cytokine mRNAs. Proc Natl Acad Sci U S A *99*, 1842-1846.

Layfield, R., Franklin, K., Landon, M., Walker, G., Wang, P., Ramage, R., Brown, A., Love, S., Urquhart, K., Muir, T., *et al.* (1999). Chemically synthesized ubiquitin extension proteins detect distinct catalytic capacities of deubiquitinating enzymes. Anal Biochem *274*, 40-49.

Lewis, E. D., and Manley, J. L. (1986). Polyadenylylation of an mRNA precursor occurs independently of transcription by RNA polymerase II in vivo. Proc Natl Acad Sci U S A *83*, 8555-8559.

Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell *5*, 939-948.

Lopez de Silanes, I., 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*, 7146-7154.

Manjithaya, R. R., and Dighe, R. R. (2004). The 3' untranslated region of bovine follicle-stimulating hormone beta messenger RNA downregulates reporter expression: involvement of AU-rich elements and transfactors. Biol Reprod *71*, 1158-1166.
Massague, J., and Gomis, R. R. (2006). The logic of TGFbeta signaling. FEBS Lett *580*, 2811-2820.

Massague, J., and Wotton, D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. Embo J *19*, 1745-1754.

Milojevic, T., Reiterer, V., Stefan, E., Korkhov, V. M., Dorostkar, M. M., Ducza, E., Ogris, E., Boehm, S., Freissmuth, M., and Nanoff, C. (2006). The ubiquitin-specific protease Usp4 regulates the cell surface level of the A2A receptor. Mol Pharmacol *69*, 1083-1094.
Mitchell, P., and Tollervey, D. (2000). mRNA stability in eukaryotes. Curr Opin Genet Dev *10*, 193-198.

Morris, E. J., and Dyson, N. J. (2001). Retinoblastoma protein partners. Adv Cancer Res *82*, 1-54.

Mosesson, Y., and Yarden, Y. (2006). Monoubiquitylation: a recurrent theme in membrane protein transport. Isr Med Assoc J *8*, 233-237.

Murray, R. Z., Jolly, L. A., and Wood, S. A. (2004). The FAM deubiquitylating enzyme localizes to multiple points of protein trafficking in epithelia, where it associates with E-cadherin and beta-catenin. Mol Biol Cell *15*, 1591-1599.

Myer, V. E., Fan, X. C., and Steitz, J. A. (1997). Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay. Embo J *16*, 2130-2139.

Nakagawa, K., and Yokosawa, H. (2000). Degradation of transcription factor IRF-1 by the ubiquitin-proteasome pathway. The C-terminal region governs the protein stability. Eur J Biochem *267*, 1680-1686.

Nakamura, T., Hillova, J., Mariage-Samson, R., Onno, M., Huebner, K., Cannizzaro, L. A., Boghosian-Sell, L., Croce, C. M., and Hill, M. (1992). A novel transcriptional unit of the tre oncogene widely expressed in human cancer cells. Oncogene *7*, 733-741.

Naylor, S. L., Johnson, B. E., Minna, J. D., and Sakaguchi, A. Y. (1987). Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. Nature *329*, 451-454.

Orian, A., Gonen, H., Bercovich, B., Fajerman, I., Eytan, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A. L., and Ciechanover, A. (2000). SCF(beta)(-TrCP) ubiquitin ligase-mediated processing of NF-kappaB p105 requires phosphorylation of its C-terminus by IkappaB kinase. Embo J *19*, 2580-2591.

Ozkaynak, E., Finley, D., and Varshavsky, A. (1984). The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. Nature *312*, 663-666.

Pacini, A., Quattrone, A., Denegri, M., Fiorillo, C., Nediani, C., Ramon y Cajal, S., and Nassi, P. (1999). Transcriptional down-regulation of poly(ADP-ribose) polymerase gene expression by E1A binding to pRb proteins protects murine keratinocytes from radiation-induced apoptosis. J Biol Chem *274*, 35107-35112.

Pankevych, H., Korkhov, V., Freissmuth, M., and Nanoff, C. (2003). Truncation of the A1 adenosine receptor reveals distinct roles of the membrane-proximal carboxyl terminus in receptor folding and G protein coupling. J Biol Chem *278*, 30283-30293.

Pantaleon, M., Kanai-Azuma, M., Mattick, J. S., Kaibuchi, K., Kaye, P. L., and Wood, S. A. (2001). FAM deubiquitylating enzyme is essential for preimplantation mouse embryo development. Mech Dev *109*, 151-160.

Papa, F. R., Amerik, A. Y., and Hochstrasser, M. (1999). Interaction of the Doa4 deubiquitinating enzyme with the yeast 26S proteasome. Mol Biol Cell *10*, 741-756.

Papa, F. R., and Hochstrasser, M. (1993). The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. Nature *366*, 313-319.

Park, C., Kim, W. S., Choi, Y., Kim, H., and Park, K. (2002). Effects of transforming growth factor beta (TGF-beta) receptor on lung carcinogenesis. Lung Cancer *38*, 143-147.

Paschoud, S., Dogar, A. M., Kuntz, C., Grisoni-Neupert, B., Richman, L., and Kuhn, L. C. (2006). Destabilization of interleukin-6 mRNA requires a putative RNA stem-loop structure, an AU-rich element and the RNA-binding protein AUF1. Mol Cell Biol.

Pavri, R., Zhu, B., Li, G., Trojer, P., Mandal, S., Shilatifard, A., and Reinberg, D. (2006). Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. Cell *125*, 703-717.

Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003). A proteomics approach to understanding protein ubiquitination. Nat Biotechnol *21*, 921-926.

Peng, S. S., Chen, C. Y., Xu, N., and Shyu, A. B. (1998). RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. Embo J *17*, 3461-3470.

Petaja-Repo, U. E., Hogue, M., Laperriere, A., Bhalla, S., Walker, P., and Bouvier, M. (2001). Newly synthesized human delta opioid receptors retained in the endoplasmic

reticulum are retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. J Biol Chem *276*, 4416-4423.

Pickart, C. M., and Rose, I. A. (1985). Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides. J Biol Chem *260*, 7903-7910.

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.

Redman, K. L., and Rechsteiner, M. (1988). Extended reading frame of a ubiquitin gene encodes a stable, conserved, basic protein. J Biol Chem *263*, 4926-4931.

Redman, K. L., and Rechsteiner, M. (1989). Identification of the long ubiquitin extension as ribosomal protein S27a. Nature *338*, 438-440.

Reggiori, F., and Pelham, H. R. (2001). Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. Embo J *20*, 5176-5186.

Ross, H. J., Sato, N., Ueyama, Y., and Koeffler, H. P. (1991). Cytokine messenger RNA stability is enhanced in tumor cells. Blood *77*, 1787-1795.

Sarkar, B., Xi, Q., He, C., and Schneider, R. J. (2003). Selective degradation of AU-rich mRNAs promoted by the p37 AUF1 protein isoform. Mol Cell Biol *23*, 6685-6693.

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

Shikama, N., Lyon, J. and La Thangue, N.B. (1997). The p300/CBP family: integrating signals with transcription factors and chromatin. Trends Cell Biol *7*, 230-236

Soboleva, T. A., and Baker, R. T. (2004). Deubiquitinating enzymes: their functions and substrate specificity. Curr Protein Pept Sci *5*, 191-200.

Soboleva, T. A., Jans, D. A., Johnson-Saliba, M., and Baker, R. T. (2005). Nuclear-cytoplasmic shuttling of the oncogenic mouse UNP/USP4 deubiquitylating enzyme. J Biol Chem *280*, 745-752.

Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D. (2000). Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. Cell *102*, 67-76.

Spence, J., Sadis, S., Haas, A. L., and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Mol Cell Biol *15*, 1265-1273.

Stone, M., Hartmann-Petersen, R., Seeger, M., Bech-Otschir, D., Wallace, M., and Gordon, C. (2004). Uch2/Uch37 is the major deubiquitinating enzyme associated with the 26S proteasome in fission yeast. J Mol Biol *344*, 697-706.

Sun, L., and Chen, Z. J. (2004). The novel functions of ubiquitination in signaling. Curr Opin Cell Biol *16*, 119-126.

Swaminathan, S., Amerik, A. Y., and Hochstrasser, M. (1999). The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. Mol Biol Cell *10*, 2583-2594.

Taya, S., Yamamoto, T., Kanai-Azuma, M., Wood, S. A., and Kaibuchi, K. (1999). The deubiquitinating enzyme Fam interacts with and stabilizes beta-catenin. Genes Cells *4*, 757-767.

Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. Embo J *19*, 94-102.

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*, 7177-7188.

Turner, G. C., and Varshavsky, A. (2000). Detecting and measuring cotranslational protein degradation in vivo. Science *289*, 2117-2120.

Tweardy, D. J., Mott, P. L., and Glazer, E. W. (1990). Monokine modulation of human astroglial cell production of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. I. Effects of IL-1 alpha and IL-beta. J Immunol *144*, 2233-2241.

Uemura, Y., Kobayashi, M., Nakata, H., Kubota, T., Bandobashi, K., Saito, T., and Taguchi, H. (2006). Effects of GM-CSF and M-CSF on tumor progression of lung cancer: roles of MEK1/ERK and AKT/PKB pathways. Int J Mol Med *18*, 365-373.
van der Horst, A., de Vries-Smits, A. M., Brenkman, A. B., van Triest, M. H., van den

Broek, N., Colland, F., Maurice, M. M., and Burgering, B. M. (2006). FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. Nat Cell Biol.

von Mikecz, A. (2006). The nuclear ubiquitin-proteasome system. J Cell Sci *119*, 1977-1984.

Wada, K., and Kamitani, T. (2006). UnpEL/Usp4 is ubiquitinated by Ro52 and deubiquitinated by itself. Biochem Biophys Res Commun *342*, 253-258.

Wada, K., Tanji, K., and Kamitani, T. (2006). Oncogenic protein UnpEL/Usp4 deubiquitinates Ro52 by its isopeptidase activity. Biochem Biophys Res Commun *339*, 731-736.

Wicks, S. J., Haros, K., Maillard, M., Song, L., Cohen, R. E., Dijke, P. T., and Chantry, A. (2005). The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling. Oncogene *24*, 8080-8084.

Wilkinson, K. D. (1995). Roles of ubiquitinylation in proteolysis and cellular regulation. Annu Rev Nutr *15*, 161-189.

Wilkinson, K. D. (1997). Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. Faseb J *11*, 1245-1256.

Wilusz, C. J., Wormington, M., and Peltz, S. W. (2001). The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Biol *2*, 237-246.

Wing, S. S. (2003). Deubiquitinating enzymes--the importance of driving in reverse along the ubiquitin-proteasome pathway. Int J Biochem Cell Biol *35*, 590-605.

Yamakawa, K., Takahashi, T., Horio, Y., Murata, Y., Takahashi, E., Hibi, K., Yokoyama, S., Ueda, R., Takahashi, T., and Nakamura, Y. (1993). Frequent homozygous deletions in lung cancer cell lines detected by a DNA marker located at 3p21.3-p22. Oncogene *8*, 327-330.

Yan, F., Gao, X., Lonard, D. M., and Nawaz, Z. (2003). Specific ubiquitin-conjugating enzymes promote degradation of specific nuclear receptor coactivators. Mol Endocrinol *17*, 1315-1331.