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Analysis of Interactions with the Mammalian Deubiquitinating Enzyme, USP4

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ABSTRACT

We have previously reported the cloning of the murine *USP4* (previously *Unp*) and human *USP4* (previously *Unph*) genes. These genes encode ubiquitin specific proteases capable of cleaving ubiquitin tags from synthetic substrates *in vitro*. However, the natural substrates of this deubiquitinating enzyme are not known. It has been shown that USP4-overexpressing NIH3T3 cells consistently produce tumors in nude mice experiments and USP4 has been implicated in specific types of human lung tumors such as small cell and adenocarcinomas.

Unlike some other deubiquitinating enzymes, USP4 does not have an overall affect on cellular ubiquitination. Therefore, it is thought that USP4 has only one or a few substrates and that it may be functioning by stabilizing a protein by editing its ubiquitin degradation signal. As a first step in attempting to identify USP4's substrate(s), we have been looking for proteins that may interact with USP4. We have done this using an *in vitro* pull down system and mass spectrometry analysis. The translational elongation factor, EF1A, was identified by mass spectrometry and this interaction was confirmed *in vivo* using immunoprecipitations and immunofluorescence.

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LIST OF ABBREVIATIONS

aa-tRNA	aminoacyl-transfer ribonucleic acid
Amp	Ampicillin
ApoB	Apolipoprotein B
ARE	AU rich element
ATP	Adenine triphosphate
Cdk	Cyclin dependent kinase
CFTR	Cystic fibrosis transmembrane conductance regulator
Cki	Cyclin dependent kinase inhibitor
CMV	Cytomegalovirus
CR1/2	Conserved region 1/2
Cys	Cysteine
DLD	Dihydrolipoamide dehydrogenase
DNA	Deoxyribonucleic acid
DriP	Defective ribosomal products
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
EDTA	Ethyl diaminetetraacetic acid
EF-1A, 2	Elongation factor-1A, 2
Faf	Fat facets
Lqf	Liquid facets
GDP	Guanine diphosphate
GFP	Green fluorescent protein
Gly	Glycine
GST	Glutathione S-transerfase
GTP	Guanine triphosphate
H2A, B	Histone 2A, B
HECT	Homologous to the E6-AP COOH terminus
HIF	Hypoxia inducible factor
His	Histidine
HPV	Human papillomavirus
HRP	Horseradish peroxidase
IPTG	Isopropylthio-β-D-galactosidase
IκB	Inhibitor κB
Kda	Kilodalton
Lys	Lysine
LB	Lauria broth
MEM	Minimal essential medium
mRNA	messenger ribonucleic acid
NAC	Nascent-polypeptide associated complex
NLS	Nuclear localization signal
NFκB	Nuclear factor κB
ODC	Ornithine decarboxylase
ODO1,2	2-oxoglutarate dehydrogenase e1 component 1,2

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RAF	Ribosome associated factor
RB	Retinoblastoma
RGS4	Regulator of G protein signaling 4
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SRP	Signal recognition particle
TBST	Tris buffered saline with Tween
Ub	Ubiquitin
UCH	Ubiquitin C-terminal hydrolase
UBA	Ubiquitin activating
UBC	Ubiquitin conjugating
UBP	Ubiquitin specific protease
USP	Ubiquitin specific protease
UTR	Untranslated region
VHL	von Hippel-Lindau
YFP	Yellow fluorescent protein
Zn	Zinc

1. INTRODUCTION

1.1 The Ubiquitin-Proteasome Pathway

The discovery and ongoing study of the ubiquitin-proteasome proteolytic pathway in eukaryotes has made it clear that degradation of cellular proteins is a complex, tightly regulated process. It is apparent that cells require a highly regulated pathway for the degradation of intracellular proteins. First of all, different proteins have half-life times that range from a few minutes (e.g. the tumor suppressor p53) to several days (the muscle proteins actin and myosin), to up to a few years (crystallin). Also, the proteolytic machinery and its substrates reside in the same cellular compartment (reviewed in 1). These observations reveal a need for a specific proteolytic trigger, which is provided by the ubiquitin pathway.

This pathway is important for a variety of biological processes. Among these are elimination of abnormal proteins, cell cycle progression, signal transduction, transcriptional activation, antigen presentation, and DNA repair. There are many known substrates of the ubiquitination pathway including protein kinases, cyclins, transcription factors, and inhibitors of cyclin-dependent protein kinases. Degradation of a protein via the ubiquitin proteasome pathway involves two successive steps. The first is the conjugation of multiple ubiquitin moieties to the substrate and, subsequently, the degradation of the tagged protein by the downstream 26S proteasome complex, with the release of free and reusable ubiquitin (reviewed in 2).

Ubiquitin, a highly evolutionarily conserved 76-residue polypeptide, is usually conjugated via a bond between its C terminus G76 and the ε-amino group of a substrate lysine residue. This reaction occurs via a three-step mechanism as follows (see Figure 1

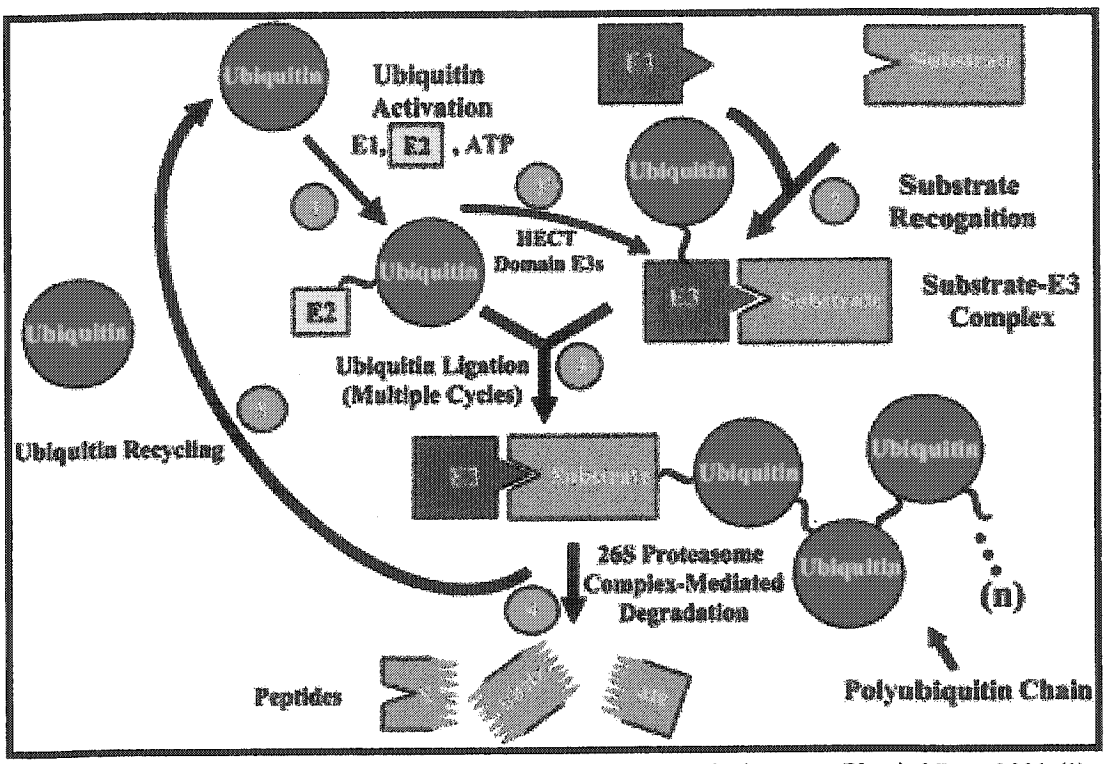
for schematic). 1) An activating enzyme, or E1, forms a high-energy thiol ester with the carboxyl group of G76 in an ATP dependent reaction. This activates the C terminus of ubiquitin for nucleophilic attack. 2) A conjugating enzyme (UBC), or E2, transfers the activated ubiquitin moiety from E1 via an additional thiol-ester bond. 3) A ubiquitin-protein ligase, or E3, transfers the activated ubiquitin from the E2 to the substrate (or ubiquitin) lysine residue. By successively adding activated ubiquitin moieties to internal lysine residues, a polyubiquitin chain is formed. This chain is recognized by the 26S proteasome complex (reviewed in 1).

In yeast, there is a single ubiquitin activating enzyme called UBA1. It contains a nuclear localization signal (4, 5), it is phosphorylated (6) and inactivation of the enzyme is lethal (5). On the other hand, there have been eleven conjugating enzymes identified in the yeast genome (Ubc1-8, 10, 11, 13) and many more in higher organisms. Ubiquitin conjugating enzymes share an active-site ubiquitin-binding Cys residue and a UBC domain required for binding of distinct E3s (7). Typically, each E2 interacts with a variety of ligases, thus helping target numerous substrates for ubiquitination. For example, Ubc2/Rad6 acts along with Ubr1/E3α to target N-end rule substrates (later described) (9). However, Ubc2/Rad6 has also been implicated in the ubiquitination of histone H2B and Bre1 was recently identified as the E3 for Ubc2/Rad6's role in transcription (10). Therefore, conjugating enzymes can be involved in many different processes by interacting with different ligases.

The ubiquitin ligase is a protein or protein complex that binds both the E2 and the substrate and is responsible for the specific recognition of the wide range of substrates. There are a number of different classes of E3 enzymes. For the HECT (homologous to

Figure 1. The ubiquitin proteolytic pathway

1: Activation of ubiquitin by the ubiquitin-activating enzyme E1, a ubiquitin-carrier protein, E2, and ATP, producing a high-energy E2-ubiquitin thiol ester.
2: Binding of a protein substrate to a specific ubiquitin-protein ligase, E3.
3: When RING finger E3s are involved, E2 transfers the first activated ubiquitin moiety directly to the E3-bound substrate, and in following cycles, to ubiquitin.
3': When HECT domain E3s are involved, the activated ubiquitin moiety is transferred from E2 to a high-energy thiol on E3 before its conjugation to the E3-bound substrate or ubiquitin.
4: Degradation of the ubiquitin-tagged substrate by the 26S proteasome with the release of short peptides.
5: Ubiquitin is recycled via the activity of deubiquitinating enzymes.



Michael H. Glickman and Aaron Ciechanover, Physiol Rev, 2001 (1)

the E6-AP COOH terminus) domain proteins, the ubiquitin is transferred from the E2 enzyme to an active site Cys residue on the E3 (12). The NH2-terminal domain, which is variable among HECT domain proteins, is thought to be involved in specific substrate recognition. E6-AP was the first protein described in this family. It targets p53 for degradation in the presence of the HPV oncoprotein E6 (11). NEDD4 is also a HECT domain E3 but targets the kidney epithelial Na+ channel (13, 14).

Another ubiquitin protein ligase family is the RING finger motif-containing E3s. RING fingers contain a pattern of conserved Cys and His residues that form a cross-brace structure that most likely bind two Zn cations (15, 16). c-Cbl is a RING finger ligase that recruits the E2 UbcH7 and is involved in targeting activated receptor kinases (17). The RING domain binds the RING through contacts between a groove in the RING and two loops in the E2 fold of UbcH7 (18). The SCF complexes are E3 catalytic complexes that contain RING finger proteins. There are variables to these complexes, however, a typical complex would usually contain and E2, Hrt1/Rbx1/Roc1, Skp1, and members of the Cdc53/Cullin-1 family. A substrate binding F-box protein is also present in the complex and it is the most variable component (19). Thus the F-box component gives the complex its specificity.

It is apparent that these E3 complexes can be quite complex. Modifying these complexes is one mechanism that gives the ubiquitin pathway its specificity. In some cases, the E3 enzyme must be "switched on" in order for it to recognize its substrate. Another way to obtain this specificity is by creating changes in the substrate so that it is recognizable (or unrecognizable) to the ubiquitination machinery.

One example of how the ubiquitin pathway is regulated to acquire specific substrate recognition is through the N-end rule pathway. In this pathway, substrates bind directly to the ligase via their NH_2 terminal residue (20). E3α/Ubr1, the E3 involved in this pathway, has two N-end rule recognition sites. Site I recognizes substrates with basic NH_2 termini and site II recognizes substrates with hydrophobic NH_2 termini (21, 22). RGS4 is a protein involved in specific G protein activation and is a target for Ubr1-mediated ubiquitination. It is first arginylated posttranslationally at the NH_2 terminus, which renders it sensitive to Ubr1 recognition (23).

Another posttranslational modification that can affect substrate recognition by the ubiquitination machinery is phosphorylation. The NFκB pathway is one of many that demonstrates this type of regulation. This protein is inhibited by IκBα, which binds to it and sequesters it in an inactive form in the cytosol. Upon stimulation, IκBα is phosphorylated and degraded, which releases NFκB to be translocated to the nucleus (24).

The ubiquitin system is known to selectively degrade abnormal, mutated or misfolded proteins. It is responsible of ridding the cell of denatured or misfolded proteins that arise from mutations or posttranslational environmental stress (25, 8). It also must degrade cotranslationally nascent peptide chains that do not attain native structure due to errors in translation. 30% of nascent chains are degraded; however, the signals and E3s involved are not evident (26, 27).

In some cases, substrates are recognized via a specific sequence. Ubiquitination of mitotic cyclins is mediated by an NH_2-terminal motif known as the "destruction box" (R-A/T-A-L-G-X-I/V-G/T-N). The purpose of the destruction box is not clear, as it is not

phosphorylated or ubiquitinated. It has also been reported that many short-lived proteins contain a sequence enriched in PEST residues (Pro, Glu, Ser, Thr). This sequence has also been implicated in the destabilization of proteins. Phosphorylation of its Ser/Thr residues may be involved in this destabilization (28). These modes of specific substrate recognition only touch on the many ways the ubiquitin pathway is regulated. It is apparent that these intricate signals are crucial for appropriate protein turnover and cellular quality control.

Once the substrate is recognized and ubiquitinated, it is ready for destruction. The proteasome is a large protease that degrades polyubiquitinated proteins into small peptides. It is composed of two major subcomplexes, a core catalytic 20S particle and a regulatory 19S particle. The core particle is made up of four heptameric rings, two identical outer alpha rings and two identical inner beta rings. The two inner beta rings contain the proteolytic active sites facing inward into a proteolytic chamber (29, 30). The 19S regulatory particle is made up of two subcomplexes, the lid and the base (31). The base consists of six homologous ATPase subunits and three non-ATPase subunits (Rpn1, 2, and 10). The lid is a complex made up of eight non-ATPase subunits (Rpn3, 5, 6, 7, 8, 9, 11, 12) that can dissociate from the proteasome, resulting in a truncated base-core particle complex (32).

The 19S complex recognizes ubiquitinated proteins and other substrates of the proteasome. The subunit, Rpn10/S5a/Mcb1, has been shown to have affinity for ubiquitin chains (33, 34, 35). However, this protein is not essential in yeast so there must be other ubiquitin binding proteins within the complex (36). Recently, it was shown that a 19S ATPase subunit, S6' (also known as Rpt5), contacts the bound polyubiquitin signal

and this interaction is modified by ATP hydrolysis (129). It is thought that another function of the 19S particle is to unfold the polypeptide chain so it can be inserted into the proteolytic chamber. The 19S complex, probably through its ATPase subunits, creates a gate in the alpha ring through which the polypeptides are inserted and exposed to the proteolytic machinery (37).

For the proteasome to recognize a ubiquitin chain, the chain must be linked by G76-K48 isopeptide bonds. In these links, the first ubiquitin moiety is anchored via its COOH terminal glycine residue (G76) to an NH_2 group of a lysine residue in the target substrate. Subsequently, a ubiquitin chain is formed in which moieties are linked to one another via a Gly-76-Lys-48 bond. Chains containing at least 4 ubiquitin moieties are necessary for efficient binding to the proteasome (38). It is apparent that a surface of the four-subunit structure of the chain is recognized by the proteasome and there is evidence indicating that hydrophobic interactions are involved in this recognition (39, 40).

Cases of ubiquitination via Lys-63 chains are also known. Instead of degradation, these links appear to play a role in processes such as endocytosis of cell surface receptors, postreplicative DNA repair, stress response and ribosomal function. In the case of the ribosome, it was found that L28, a component of the large ribosomal unit, is strongly ubiquitinated in the S-phase of the yeast cell cycle. This ubiquitination was inhibited by a Lys63 to Arg substitution in ubiquitin, indicating that L28 is modified by Lys63 links (43). There are also many cases where proteins have been shown to be monoubiquitinated, which may serve as a cellular localization signal, rather than a degradation signal. Recently, it was shown that monoubiquitination is sufficient to serve

as an internalization signal for several cell surface receptors. Such is true for the yeast alpha- (41) and a- (42) pheromone factor receptors.

Interestingly, simple versions of the proteasome are present in certain bacteria, while ubiquitin and ubiquitin pathway components are not (44, 45). In bacteria, the proteasome is involved in the degradation of damaged and misfolded proteins during the heat shock response (44, 46). This would suggest that the proteasome may be able to recognize substrates other than ubiquitin chains. Although in eukaryotes, the bulk of proteins are removed by the proteasome in a ubiquitin dependent manner, there have been proteins identified to be degraded in a ubiquitin-independent manner. The enzyme ornithine decarboxylase (ODC), upon binding to the protein antizyme, is recognized and degraded by the proteasome without ubiquitination (47). It is possible that antizyme's stability is affected by the proteasome and this, in turn, may influence ODC's stability. Also, p21, a CDK inhibitor, is normally ubiquitinated and degraded by the proteasome. However, mutants of p21 have been generated which are not ubiquitinated but still efficiently degraded by the proteasome (48).

There are also ubiquitinated proteins that are efficiently recognized by the proteasome but are not efficiently degraded. The transcription factor Met-4 is polyubiquitinated by SCF^{Met-30} but not subsequently degraded (49). Yet another function of the proteasome is to process certain proteins into a truncated form, rather than completely hydrolyzing them. The best studied case of this is the processing of the of the p105 precursor to p50, a component of the transcription factor, NFκB (50). p105 is ubiquitinated, and consequently, the COOH terminal half is degraded by the proteasome. The NH_2 terminal 50 kDa protein is then released (51).

There are other small proteins that can posttranslationally modify proteins. These have been termed ubiquitin-like proteins although some bear little resemblance to ubiquitin. They do, however, form an isopeptide bond with an internal lysine of a substrate. In fact, in all cases, the modes of conjugation and sites of attachment to the substrate are similar to that of ubiquitin. Such small proteins that show homology with ubiquitin include the SUMO/Sentrin/Smt3p family of proteins (~20% homology) and NEDD8/Rub1 (~60% homology). They are activated by an activating enzyme, E1, and then transferred to an E2 enzyme via a thiol-ester bond (reviewed in 1). Usually, the E2 directly conjugates the ubiquitin-like protein to a lysine, however, in the case of SUMO, a specific E3 has been identified (108).

Many functions of ubiquitin-like protein conjugation have been reported. However, directly targeting the substrate for degradation by the proteasome does not seem to be one of these functions. One of the functions of SUMO conjugation is to alter the cellular localization of the substrate. Conjugation of SUMO to p53, RanGAP1, SP100, and HIPK2 increases their levels in the nucleus compared to the cytoplasm (52). RanGAP1, a GTPase activating protein, is localized at the cytoplasmic periphery of the nuclear pore complex. The sumoylation of RanGAP1 allows it to associate with RanBP2, which serves to target RanGAP1 to the nuclear pore complex (53). In some cases the conjugation of SUMO may affect protein stability by modifying its ubiquitination. Mdm2, an E3 ubiquitin ligase for p53, is sumolated at Lys-446, which is located within the RING finger domain. This reduces Mdm2 self-ubiquitination, thus reduces its degradation. This, in turn, elicits increased degradation of p53 (54).

It is clear that the ubiquitin pathway is involved in many processes in the cell. Therefore, it is not surprising that abnormalities in this pathway, directly, or indirectly result in the onset of many diseases. These can be caused by mutations in a ubiquitin system enzyme or substrate that lead to either increased stability or accelerated degradation of a protein. The following touches on a few mechanisms by which the ubiquitin pathway is involved in tumorigenesis.

1.2 Ubiquitin in Cancer

Deregulation of cell cycle regulators play a large role in oncogenesis. The regulator genes that are most often altered in tumors are those that control the G1/S transition. This phase of the cell cycle is regulated by the retinoblastoma tumor suppressor (RB) and by overcoming the inhibitory effect of RB, tumor cells obtain a growth advantage (reviewed in 55, 56). The eukaryotic cell cycle is regulated by a family of serine/threonine protein kinases, called cyclin-dependent-kinases (cdks). Their activity requires association with regulatory proteins called cyclins, however, they also associate with cdk inhibitors (ckis). Ckis mediate cell cycle arrest in response to many antiproliferative signals including serum deprivation, DNA damage, cytokines and contact inhibition. There are two families of ckis termed the Cip/Kip family, which includes p21, p27, and p57, and the Ink family, which includes p15, p16, p18, and p19 (reviewed in 58). The ubiquitin pathway regulates the degradation of cyclins and ckis along with upstream regulators of cdks (e.g., the phosphatase Cdc25) (57). Proper temporal destruction of these proteins by the ubiquitin pathway is a critical factor in controlling the cell cycle.

The cki, p27, binds and negatively regulates the activity of CDK2/cyclinE and CDK2/cyclinA complexes, thus preventing cell cycle progression from G1 and entrance in to S phase (reviewed in 58). Indeed, low levels of the p27, which result from increased ubiquitin-mediated degradation, have been demonstrated in colorectal, prostate, and breast cancers (reviewed in 59). The tumor suppressor p53 is targeted for ubiquitin-mediated degradation by the human papillomavirus (HPV) oncoprotein E6 (11). It's been observed that p53 levels are extremely low in uterine cervical carcinoma tumors caused by high-risk strains of HPV (E6-16 or 18) (60). However, low risk strains that encode different E6 proteins, do not target p53 for degradation and do not transform cells 61).

These two examples show how tumor suppressors can be destabilized to cause malignancies. However, there are also many ubiquitin-regulated growth-promoting factors that could potentially transform cells. A few such proteins are N-myc, c-myc, c-fos, and c-jun. Interestingly, c-Jun, but not its viral counterpart, v-Jun is ubiquitinated and rapidly degraded. The amino acid sequence that spans residues 31-57 of c-Jun, which confers protein instability, is lacking in v-Jun. This region of the protein does not contain lysine residues (so cannot be ubiquitinated), however, it may serve as a binding site for ubiquitination machinery (62). Although mutated Jun has been identified in tumors, it is not known whether it is involved directly in tumorigenesis.

1.3 Deubiquitinating Enzymes

The conjugation of ubiquitin is a reversible process. The enzymes responsible for the reverse reaction are called deubiquitinating enzymes (DUBs). They are proteases that

specifically hydrolyze ester, thiol ester, and amide bonds of the G76 carboxyl group of ubiquitin. There are at least 19 in yeast and many more in higher eukaryotes. These enzymes have been shown to be involved in numerous biologically important processes such as growth and oncogenesis, differentiation and development, memory, chromosome structure and neurodegenerative disease (reviewed in 63). This suggests that deubiquitination is an essential regulatory process in the ubiquitin pathway that cannot be overlooked.

DUBs have many important roles, including processing of the primary ubiquitin gene product. Ubiquitin and several ubiquitin-like proteins are synthesized as fusion proteins either as multi-ubiquitin fusions or ribosomal fusions. DUBs are responsible for cleavage of these proteins at the C-terminal glycine. This proprotein processing yields ubiquitin and the ribosomal proteins L40 and S27a. DUBs are also responsible for the regeneration of the C-terminus of ubiquitin after degradation of the attached protein or after reactions of ubiquitin thiol esters with cellular amines and thiols. Another role for DUBs is the editing of polyubiquitin or ubiquitin from modified proteins. Some enzymes have been shown to affect a protein's stability by modifying its ubiquitin chain. Yet another role is to disassemble the polyubiquitin chain after use. This replenishes the ubiquitin pool and prevents accumulation of these chains that could act as competitive inhibitors of ubiquitinated proteins to the proteasome (reviewed in 63).

There are two classes of DUBs, the ubiquitin C-terminal hydrolases (UCHs) and the ubiquitin-specific processing proteases (UBPs). Both were first identified based on their ability to hydrolyze esters or fusion proteins containing ubiquitin (64, 65, 66). The UCH family of DUBs was the first to be identified and there have been at least 12 family

members identified from different organisms. UCHs have a 230 amino acid core catalytic domain with four very highly conserved sequence blocks identifying these enzymes. They are more active on ubiquitin extended by small peptides and are thought to be involved in processing ubiquitin-fusion peptides and removing small peptides from the polyubiquitin degradation signal (67). A number of mutations in UCH family members have been shown to be associated with disease. For example, mutations in the neuronal specific, UCH-L1 (also known as PGP9.5), may be associated with human Parkinson's disease (68) and gracile exonal dystrophy in mice (69).

The second family of DUBs, the UBP family, is thought to be responsible for removing ubiquitin from larger proteins, disassembling polyubiquitin chains and ubiquitin fusion processing. There are 16 UBPs in yeast and a total of at least 80 UBPs identified from many different organisms such as *C. elegans*, *Aplysia*, *Drosophila*, mouse and human. These enzymes contain a catalytic domain of about 450 amino acids flanked by the catalytic Cys and His domains, which center around a key catalytic cysteine residue and two catalytic histidine residues, respectively. UBPs vary from 50 to 250 kDa with a variety of N-terminal extensions, an occasional C-terminal extension and insertions in the catalytic domain, all of which have the potential to control localization and substrate specificity. UBP enzymes have significant diversity, and therefore may have a broad range of substrate specificity (reviewed in 63).

The specific UBPs responsible for the processing of the ubiquitin proproteins and ubiquitin fusion proteins in cells are unknown. However, for other functions of UBPs, some of the enzymes have been identified and characterized. The first of this family to be characterized was Ubp4p (also known as Doa4p) in yeast. Cells that express mutants

of this protein accumulate polyubiquitin chains with peptide remnants still attached, show impaired proteolysis, have lower free ubiquitin levels (70), and have defects in DNA replication (71). It is likely that this enzyme is responsible for removing the polyubiquitin chain from the protein remnant following degradation. This role overlaps with the proposed substrate specificity for UCHs. This may be explained by the fact that UCHs and Ubp4 are differentially expressed or localized. For instance, there are clear tissue specific patterns in many UCHs, such as UCH-L1, which is expressed in the brain and testis, and UCH-L3, which is expressed in brain, lung and red cells (72).

After the peptide remnant removal, the free polyubiquitin chain must be disassembled. This appears to be catalyzed by Ubp14 in yeast and the homologous isopeptidase T in mammalian cells (73, 74). Isopeptidase T has been shown to trim polyubiquitin chains by releasing one subunit at a time from the proximal end of the chain. The reaction only proceeds if the proximal end has a free carboxy terminus. Ubiquitin chains with peptides or substrates still attached are not good substrates for Isopeptidase T (75).

Interestingly, there is also an isopeptidase activity associated with the PA700 regulatory subcomplex of the 26S proteasome (76). It appears that this activity shortens the polyubiquitin chain one ubiquitin at a time from the distal end. It is postulated that this could be a proofreading mechanism. Because the ubiquitin chain is being hydrolyzed, only the substrates with an adequate ubiquitin chain length will be degraded. Proteins that have short chains may have this chain removed before proteolysis can occur.

The DUBs described above play housekeeping roles in the ubiquitin pathway.

For the most part, they do not discriminate among polyubiquitin chains as long as the substrate has associated with the proteasome. However, it has been postulated that some DUBs remove ubiquitin chains from specific protein targets, and thus may act as substrate specific regulators of ubiquitination. For at least one of these DUBs, namely, the Drosophila fat facets (Faf), the substrate has been identified. Faf has been identified as a DUB required for patterning the compound eye. There is genetic evidence that Faf antagonizes ubiquitination and proteasomal degradation and that Liquid facets (Lqf), an endocytic protein, is the critical substrate of Faf in the eye. Initial experiments showed that 1) faf and lqf loss-of-function mutations have similar mutant eye phenotypes, 2) the faf^+ and lqf^+ genes are required in the same group of cells in the eye, and 3) one extra copy of the lqf^+ gene eliminates the need for the faf^+ gene in the eye (77, 78). Further experiments showed 1) a physical interaction between Faf and Lqf, 2) there is less Lqf protein in the developing eye in the absence of functional Faf protein, and 3) Lqf is ubiquitinated in developing eyes and deubiquitinated by Faf (79).

A human UBP, HAUSP (herpes virus-associated Ub-specific protease) has also been shown to cleave ubiquitin from a specific substrate (80). In this case, the substrate is p53, a short-lived tumor suppressor. p53 is maintained at low levels in normal cells by Mdm2-mediated ubiquitination and subsequent proteolysis. It was shown that HAUSP stabilizes p53 and induces p53-dependent cell growth repression and apoptosis, whereas a catalytically inactive HAUSP destabilizes p53. It was also shown that HAUSP binds and deubiquitinates p53 *in vivo* and *in vitro*. This implies that, through deubiquitinating a tumor suppressor, HAUSP itself may function as a tumor suppressor.

It has been shown that histones (mainly H2A and H2B) are ubiquitinated and that deubiquitination of histones is important for chromatin condensation (reviewed in 81). Crude preparations of enzymes were previously demonstrated to remove ubiquitin from the histone, H2A. Recently, a human recombinant Ubp-M was cloned and shown to deubiquitinate H2A *in vitro* (82). Although transfected Ubp-M localizes to the cytoplasm, non-active mutant forms associate closely with mitotic chromosomes. The significance of this is not yet known, however it remains that Upb-Y may deubiquitinate proteins that are involved in the condensation of mitotic chromosomes. Another DUB that may affect DNA is Ubp3. It associates with the gene silencer, SIR4 and seems to inhibit gene silencing. It is proposed that Ubp3 regulates silencing by controlling either the activity or assembly of the SIR4 protein complex (83). It is possible that Ubp3 is accomplishing this by promoting the stabilization of an inhibitor of silencing.

The human ubiquitin isopeptidase, UBPY, was recently cloned and characterized (84). When this DUB was increased or decreased in cells, substantial changes in the total pattern of ubiquitination were observed. That is, when UBPY levels were decreased or when a catalytic mutant form of UBPY expressed, overall cellular ubiquitination was substantially increased. When UBPY was overexpressed, a decrease in ubiquitination was found. Also, upon decreasing UBPY expression by antisense plasmid microinjection, cells were prevented from entering S-phase in response to serum-starvation. This suggests that UBPY plays a role in regulating the overall function of the ubiquitin pathway, and that it may take part in controlling mammalian cell proliferation.

The mammalian proto-oncogene tre-2 has been shown to encode a deubiquitinating enzyme that disassembles multiubiquitin chains from peptides still bound to the

proteasome. The tre-2 oncoprotein exhibits a transforming activity in NIH3T3 fibroblasts (70, 85). However, no malignancies have been associated with mutations in tre-2. About ten years ago, *USP4*, a mouse gene related to the tre oncogene, was cloned in our lab and remains one of our areas of interest. This gene was discovered during a survey of genes near the Mpv 20 retroviral insertion site and was originally designated Unp for *u*biquitous *n*uclear *p*rotein. The protein contains motifs that are consistent with its presence in the nucleus. The first of these is a putative nuclear localization signal very similar to that of p53. It also contains a bipartite motif common to proteins that associate with RB. This motif has the consensus sequence LHE (spacer) LXCXE, where X is any amino acid. USP4 contains a bipartite motif identical to those in the adenoviral E1A protein and the HPV16 E7 proteins (86). *USP4* is expressed in various mouse tissues such as heart, kidney, brain, lung and testes. Also, the expression of *USP4* from a highly active promoter (the pgk promoter) resulted in tumorigenic transformation of NIH3T3 cells injected into nude mice (87). It therefore fulfils at least one criterion of a proto-oncogene.

The mouse *USP4* gene is located on chromosome 9 (86) in a region that is homologous to the human 3p21, a region frequently rearranged in human tumors, particularly of the lung (130, 131, 132). The human *USP4* was identified and it is, indeed, located on chromosome 3p21.3 (88). It was also found that human *USP4* mRNA levels are consistently elevated in small cell tumors and adenocarcinomas of the lung (88). Upon protein alignment, it was found that the human USP4 amino acid sequence is 90% identical to the mouse USP4 sequence. Both these proteins contain amino acid similarities to enzymes that cleave ubiquitin tags from substrates, specifically, the His

Figure 2. Amino acid sequence of USP4

The putative domains in the USP4 protein are highlighted as follows:
Cys domain (303-315), CR1 domain (405-407), CR2 domain (459-463), Acidic domain (657-691), Nuclear Localization Signal (765-772), His domain (864-881)

```
    10         20         30         40         50         60
     |          |          |          |          |          |
MAEGRGSRER PDVETQKTEL GALMGTTLQR GAQWYLIDSR WFKQWKKYVG FDSWDMYTNVG

    70         80         90        100        110        120
     |          |          |          |          |          |
EHNLFPGPID NSGLFSDPES QTLKEHLIDE LDYVLVPAEA WNKLLNWYGC VEGQQPIVRK

   130        140        150        160        170        180
     |          |          |          |          |          |
VVDDGLFVKH CKVEVYLLEL KLCENSDPTN VLSCHFSKAD TIATIEKEMR KLFNIPAERE

   190        200        210        220        230        240
     |          |          |          |          |          |
TRLWNKYMSN TYEQLSKLDN TIQDAGLYQG QVLVIEPQNE DGTWPRQSLQ SKSSTAPSRN

   250        260        270        280        290        300
     |          |          |          |          |          |
FTTSSKPSAS PYCSVSASLI ANGDSTNSSG MHSSGVSRGG SGFSASYNCQ EAPSPHIQPG

   310        320        330        340        350        360
     |          |          |          |          |          |
LCGLGNLGNT CFMNSALQCL SNTAPLTEYF LKDEYEAEIN RDNPLGMKGE IAEAYAELIK

   370        380        390        400        410        420
     |          |          |          |          |          |
QMWSGRDTHV APRMFKTQVG RFAPQFSGYQ QQDSQELLAF ILDGLHEDLN RVKKKPYLEP

   430        440        450        460        470        480
     |          |          |          |          |          |
KDANGRPDAV VAKEAWENHR LRNDSVIVDT FHGLFKSTLV CPECAKVSVT FDPFCYLTLP

   490        500        510        520        530        540
     |          |          |          |          |          |
LPLKKDRIME VFLVPADPQC RPIQYRVTVP LMGAISDLCE ALSKLSGIAA ENMVVTDVYN

   550        560        570        580        590        600
     |          |          |          |          |          |
HRFHKIFQMD EGLSHITPRD DIFVYEVCNT SMDGSECITL PVYFREKKSR PSSASSGAVL

   610        620        630        640        650        660
     |          |          |          |          |          |
YGQPLLVSVP KHKLTLESLY QAVCDRISRY IKQPLPDEFL SSPLEPGACN GSRSSYEGDE

   670        680        690        700        710        720
     |          |          |          |          |          |
EEEMDHQEEG KEQLSEVEGS GEDDQGDDHS ESAQKVKGQP RHKRLFTFSL VNSCGTADIN

   730        740        750        760        770        780
     |          |          |          |          |          |
SLATDGKLLK LNSRSTLAID WDSETRSLYF DEQESEACEK HLSMSQPQKK KKAAVALREC

   790        800        810        820        830        840
     |          |          |          |          |          |
IELFTTMETL GEHDPWYCPT CKKHQQATKK FDLWSLPKIL VVHLKRFSYN RYWRDKLDTV

   850        860        870        880        890        900
     |          |          |          |          |          |
VEFPVRALNM SEFVCDRSAR PYVYDLIAVS NHYGAMGVGH YTAYAKNRLN GKWYYFDDSS

   910        920        930        940        950        960
     |          |          |          |          |          |
VSLASEDQIV TKAAYVLFYQ RRDDECSSTS SLGSFPGSDG GVKLSSSHQG MGDEEAYNMDTN
```

and Cys domains (88). Figure 2 shows the location of the putative domains found in the USP4 amino acid sequence: the His and Cys domains, the CR1 and CR2 domains (which make up the bipartite domain) and an acidic domain. Also, as expected, USP4 was shown to have ubiquitin protease activity in cleavage assays and when the Cys domain was mutated, the activity was lost (unpublished data).

Although there have been ongoing studies with USP4, it remains somewhat of a mysterious protein. USP4 does not seem to have an overall effect on cellular ubiquitination (3) as previously shown for Ubp-Y. Therefore, it is likely that USP4 has only one or a specific group of substrates, and that USP4 may be functioning by stabilizing these by editing their ubiquitin degradation signal. We think it is important that its substrate(s) be found, as this may lead to information on its mechanism of transformation. We have found that the CR1 and CR2 domains of USP4 do, indeed, mediate an interaction between it and the pocket proteins RB (3, 89), p107 and p130 (89). However, the relevance of this interaction has not yet been resolved. In this study, further studies on USP4's interactions have been carried out using mass spectrometry. We have identified the translational elongation factor, EF1A, as a USP4-associated protein *in vitro* and *in vivo*.

1.4 Elongation Factor 1A

In mammals, the elongation factor, EF-1 consists of 4 different subunits, EF-1α,β,γ,δ. EF-1α (or EF1A) is a GTP-binding protein. It plays a principle role in translation by catalyzing GTP-dependent binding of aminoacyl-tRNA (aa-tRNA) to the A-site of the ribosome (91). EF-1β catalyzes GDP/GTP exchange on EF-1α •GDP to

regenerate EF-1α•GTP (92) and EF-1γ has recently been shown to have glutathione S-transferase activity (93). EF-2 catalyzes the translocation of peptidyl-tRNA form the A-site to the P-site on the ribosome (94). EF-1A is encoded by a small gene family consisting of two genes in humans. Its bacterial homologue, EF-Tu also has two genes in its family, whereas there are more than 10 family members in maize. Elongation factors are well conserved among different organisms and have been widely used in phylogenetic analyses.

EF-Tu and EF1A are composed of three domains, I, II, and III. Domain I is comprised of three motifs, which together are responsible for binding GTP. Motif I (G-1) contains the consensus sequence, GXXXXGKS, which is involved in binding the phosphate and is also called the P box. Motif II (or G-3), also called the S (switch) box, contains the consensus sequence DXXG and is involved in GTP hydrolysis. Motif III (G-4), or the G (guanine) box, is involved in binding the guanine ring and contains the consensus sequence NKXD. An α-helix called helix B that follows motif II in the protein sequence undergoes a conformational change upon exchange between GTP and GDP, called the GTPase switch. Upon binding GTP, domain I rotates toward domains II and III to form a cavity for the binding of aa-tRNA (reviewed in 95, 96).

EF1A has been shown to have many functions in addition to its role in translation. It is involved in various processes such as apoptosis (100-102), and F-actin (104) and microtubule (105) bundling and severing. It has also been implicated in diseases such as adult atopic dermatitis (136) and other autoimmune diseases (137, 138), cancer (97, 99, 125), and diabetes (139). Large increases in its mRNA levels have been observed in rapidly proliferation cells, such as cultured cells and cancer cells. Specifically, EF1A

mRNA has been shown to be overexpressed in human pancreas, colon, breast, lung, ovarian, and gastric tumors (97).

The two isoforms, EF1A1 and EF1A2 share greater than 90% DNA sequence and amino-acid identity. EF1A2 has recently been found to be amplified in 25% of primary ovarian tumors and is highly expressed in approximately 30% of ovarian tumors and cell lines. The oncogenic properties of EF1A2 were also explored. NIH3T3 cells stabily expressing EF1A2 under control of the CMV promoter were created. These cells exhibited many properties of a transformed cell line. They grew as colonies in soft agar, a property that was not seen with parental NIH3T3 cells or cells transfected with the empty vector. They also had an accelerated growth rate relative to the parental cells. Equal numbers of both populations were plated and four days later, there were approximately four times as many EF1A2 cells as parental cells. Also, EF1A2 was transfected into Rat1 fibroblasts and assayed for foci formation. As a positive control, the constitutively active Ras^{Val12} allele was used, as a general oncogenic property of Ras is foci formation. The EF1A2 overexpressing cells did form foci and had similar morphology as the RAS^{Val12}-induced foci. Finally, EF1A2-overexpressing cells formed tumors in nude mice, whereas no tumor growth was observed in parental or empty vector-expressing cells (98).

The human prostate tumor-inducing gene I (PTI-1) has been shown to be similar in sequence to EF1A truncated in the N-terminal residues. The expression of a PTI-1 cDNA clone in nude mice led to tumor formation and the antisense expression led to reversal of this phenotype (99). Taken together, these observations have led to the belief

that the amplification/overexpression of EF1A isoforms may be contributing to the formation of certain kinds of tumors.

Conversely, EF1A has also been implicated in apoptosis. Immunofluorescence studies with *Trypanosoma cruzi* showed that EF1A is in the cytoplasm of the parasites in exponential growth phase. However, during the stationary phase or when apoptosis was induced with geneticin, EF1A was found in the nucleus (100). It has also been suggested that EF1A is upregulated by p53, a transcription factor that is known to transactivate many apoptotic genes, and is a major tumor suppressor. In a study using a temperature sensitive form of p53, EF1A was expressed at a level more than two fold higher at the permissive temperature. Also, there is a p53-responsive element conserved in human, rat, and frog EF1A promoters (101). In NIH3T3 cells, overexpression of EF1A resulted in increased susceptibility to serum-deprivation-induced apoptosis and the opposite was found with the antisense RNA (102).

Other studies with EF1A have shown a strong correlation between the cellular distribution of EF1A and F-actin. Furthermore, actin-bundling activity has been associated with EF1A. It has been suggested that F-actin is required for protein synthesis (103). Studies with wounded potato tubers have shown that F-actin is organized around the wound and translational machinery is gathered at the filament for efficient protein synthesis (104). Microtubule severing activity has also been associated with EF1A. EF1A expressed in bacteria has been shown to have microtubule severing activity *in vitro* and when this protein was microinjected into fibroblasts, it induced fragmentation of the cytoplasmic microtubule arrays (105).

Given this information, it seems as though EF1A can have both bundling and severing effects on the cytoskeleton. Cytoskeletal structures are important for supporting the motility required for cell growth and metastasis. On the other hand, it is speculated that microtubule severing is one of the causes of apoptosis. This could be a hint as to why EF1A may be involved in both cell proliferation/oncogenesis and apoptosis. The mechanism of switching between the two activities is still unknown. There are many possibilities of how EF1A is contributing to oncogenesis. As just discussed, it could be related to cytoskeleton reorganization. It also could be that EF1A is translationally stimulating growth-related factors, or that it has roles in the nucleus that we are unaware of.

The observation that EF1A and USP4 interact in cells brings up some interesting points. It is shown in this study that the subcellular localization of USP4 may not be as clear as was once thought. It is suggested here that USP4 actually shuttles between the cytoplasm and nucleus and that USP4's and EF1A's shuttling properties may be linked in some way. We also show that EF1A does not seem to be ubiquitinated. However, EF1A may act as a physical link to USP4's substrate(s). One possibility shown here is that EF1A is bringing USP4 to active translational sites (polysomes), which may imply a role for USP4 in translation. Based on the data presented, several possibilities will be discussed concerning the reason and significance of the interaction between USP4 and EF1A.

2. MATERIALS & METHODS

2.1 Cell Culture and Transfections

H1299 cells (a human lung carcinoma cell line) were maintained in α-MEM containing 7.5% donor bovine serum and 2.5% fetal bovine serum. Transfection of H1299 cells was carried out with either Fugene6 (Roche) or GeneJuice (Novagen) using the same protocol for both. For transfection in 60 mm dishes, $7x10^5$ cells were plated in 60mm dishes the day before transfection. The cells were transfected with 15 μL of either Fugene or GeneJuice and 7.5 μg of DNA according the company protocol. For 35 mm dishes, $3x10^5$ cells were plated and 6 μL of transfection reagent and 3 μg of DNA were used. Transfections were incubated for 24 hours before analysis.

2.2 Western Blots

Cells were lysed in a lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X, and 5% glycerol) supplemented with Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals). Lysis buffer was added directly to cell culture dishes and incubated on ice for 20 minutes. Cells were collected by scraping and the lysate was centrifuged at 4°C for 10 minutes at 14,000 rpm to pellet cellular debris. 3X SDS sample buffer (New England Biolabs) supplemented with DTT was added to lysates, the samples were boiled, and proteins were separated by SDS-PAGE (7.5%, 8.5%, or 10% acrylamide, see figure legends). The proteins were transferred to a nitrocellulose membrane (Hybond C+, Amersham) at 400 mA for 1 hour and blocked overnight at 4°C.

Membranes were incubated with a primary antibody diluted in 5% skim milk in TBST (10 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20) for 1 hour at room temperature. Membranes were washed with TBST 3 times for 7 minutes each. The membrane was then incubated with an HRP-conjugated secondary antibody for 45 minutes at room temperature and washed 3 times again. The membrane was incubated with a chemiluminescent substrate (Kirkegaard and Perry Laboratories) for one minute and exposed to X-ray film. Antibody dilutions are summarized in Table 1.

2.3 Immunoprecipitations

Approximately 1200 µL of lysate at a protein concentration of 4 mg/mL (cells were lysed as in western blots) were precleared with 50 µL of a 50% slurry of protein G sepharose beads (Gammabind G sepharose, Amersham Pharmacia) for 2 hours. The resulting lysate was then incubated overnight with the appropriate antibody. 50 µL of sepharose beads were added to the lysate-antibody mixture and incubated for another 2 hours. Beads were spun down and washed 4 times with lysis buffer. Proteins were eluted by adding 3X SDS sample buffer with DTT and boiling the samples. The samples were separated by SDS-PAGE and analyzed. Antibody dilutions are summarized in Table 1.

2.4 Immunofluorescence

Cells were plated on coverslips and transfected. 24 hours later, they were rinsed with PBS and fixed with 4% paraformaldehyde for 20 minutes. Coverslips were washed 5

times with PBS and incubated with 0.5% Triton X-100 for 25 minutes. They were then incubated with the primary antibody for 1 hour and washed 4 times for 5 minutes each. The coverslips were then incubated (in the dark) with the secondary antibody for 30 minutes and washed as above. Coverslips were mounted on mounting media (Kirkegaard and Perry Laboratories). Triton X-100 and antibodies were diluted in PBS + 5% normal blocking serum (Santa Cruz) and all steps were carried out at room temperature. Antibody dilutions are summarized in Table 1.

2.5 Cellular Fractionation

Cells were trypsinized, washed, and resuspended in hypotonic lysis buffer (10mM HEPES, 10 mM KCl, 3 mM MgCl2, 0.05% NP-40, 1mM EDTA) supplemented with Complete Protease Inhibitor Cocktail (Roche). The cells were gently pipeted up and down and incubated on ice for 30 minutes. Nuclei were pelleted by centrifugation at 2500 rpm for 5 minutes at 4°C. The cytoplasmic fraction was kept and the nuclear fraction was washed with hypotonic buffer and pelleted as above. The nuclei were then lysed in hypotonic buffer supplemented with 1% SDS and sonicated. Debris was cleared from both the nuclear and cytoplasmic fractions by centrifugation for 5 minutes at 14,000 rpm.

2.6 Antibodies

The companies from which the antibodies were obtained and the working dilutions of the antibodies are summarized in Table 1.

Table 1. Antibody Companies and Dilutions

Antibody	Company	Western dilution	IP[1] dilution	IF[2] dilution
FLAG (M2)	Sigma	1/500	1/100	1/100
His	Qiagen	1/2000	-	1/200
GFP	Santa Cruz	1/1000	-	-
EF1A	US Biological	1/500	1/100	1/100
USP4	Zymed	1/300	1/100	1/50
E2F-4	Santa Cruz	1/1000	-	-
HRP mouse	Cedarlane	1/5000	N/A	N/A
HRP rabbit	Cedarlane	1/5000	N/A	N/A
Cy3 mouse	Jackson Immuno Research	N/A	N/A	1/1000
Cy3 rabbit	Jackson Immuno Research	N/A	N/A	1/1000
AF594 mouse	Molecular Probes Inc.	N/A	N/A	1/500

1 - Immunoprecipitation
2 - Immunofluorescence
N/A – not applicable
Dash – did not use the antibody for this technique

2.7 Silverstains

Proteins were subject to SDS-PAGE and incubated overnight in fixative (50% (v/v) methanol, 5% (v/v) acetic acid). The gel was then washed 3 times for 15 minutes each. It was stained for 20 minutes in 2 g/L silver nitrate and rinsed with water briefly. The gel was then incubated in reducing agent (275 mM sodium carbonate, 0.02% formaldehyde) until bands appeared.

2.8 Preparation of GSTUSP4 Fusion Beads

The GSTUSP4Myc expressing pGEX vector was transformed into TG1 bacteria. In 6 tubes, 500 μL of an overnight culture was added to 1.5 mL of LB-Amp and grown to exponential phase for 45 minutes at 37°C. The cultures were induced by shaking for 3 hours at room temperature in the presence of 1.5 mM IPTG. Bacteria was pelleted and

lysed in lysis buffer supplemented with Complete Protease Inhibitor Cocktail (Roche). Lysis was completed by a freeze/thaw cycle followed by sonication. The lysates were centrifuged for 5 minutes at 14,000 rpm to clear cellular debris. 200 μL of a 50% slurry of glutathione-agarose beads (Sigma) was added to the lysate and mixed by inversion for 2 hours at 4°C. The beads were then washed 5 times and resuspended in 100 μL of lysis buffer to make a 50% slurry of fusion beads.

2.9 GST Pulldown

Approximately 1200 μL of lysate at a protein concentration of 4 mg/mL (cells were lysed as in western blots) was mixed with beads by inversion for 2 hours at 4°C. 80 μL of GSTUSP4Myc beads and 30 μL of GST beads were used for the pulldowns. This is because the GST protein was expressed at a higher level and the purification consistently produced more GST protein than GSTUSP4Myc protein. Following the incubation, beads were washed 5 times with lysis buffer. Beads alone and pulldowns were subject to SDS-PAGE and silver stained.

2.10 Purification of His-tagged Proteins

HisUSP4 and HisUSP15 bacterial expression plasmids (donated by Dr. Rohan Baker) were transformed into TG1 bacteria. Induction and lysis were carried out according to the above protocol for GSTUSP4Myc purification. However, instead of the previously described lysis buffer, this lysis buffer used consisted of 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole according to the QIAexpressionist Handbook (Qiagen). 100 μL of

Ni-NTA Agarose beads (Qiagen) were added to the lysate and mixed by inversion for 2 hours at 4°C. Beads were washed twice with lysis buffer (10 mM imidazole) and washed once with lysis buffer containing an increased concentration imidazole (15 mM). This step is to rid the beads of non-specific binding without ridding the beads of the 6xHis-tagged protein. The beads were then resuspended in lysis buffer containing 250 mM imidazole and boiled to elute the proteins. Eluted proteins were separated by SDS-PAGE.

2.11 Mass Spectrometry

Following silver staining, protein bands of interest were excised from the gel. These bands were kindly analyzed by Dr. Steven Gygi's lab at Harvard University.

2.12 Preparation of ^{35}S-labeled Ubiquitin-GST Fusion Protein

Bacteria were transformed with a bacterial vector expressing the UbGST fusion protein (pRB307, donated by Dr. Rohan Baker). 500 µL of an overnight culture was added to 50 mL LB-Amp and grown to until the OD600 reading was 0.9. Bacteria was pelleted at 6000 rpm and washed in M9 broth (6 g/L Na_2PO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 0.2% glucose, 1 mM $MgSO_4$, 0.00005% thiamine, 0.1 mM $CaCl_2$) and resuspended in M9 broth supplemented with 40 µg/mL Amp, 1 mM IPTG and 0.0625% Methionine Assay Medium (Difco) which contains most amino acids, but lacks methionine. 800 µCi of ^{35}S methionine and cysteine (Translabel, ICN) was added to the culture and incubated for 3 hours. 1 mM of methionine was then added, this was incubated for 10 minutes and the bacteria were pelleted. The pellets were washed with

M9 broth and pelleted again. Lysis was carried out by sequentially adding in 25% sucrose/50 mM Tris pH 8, lysozyme, and Triton X-100. The lysate was centrifuged and the supernatant was diluted in 50 mM Tris pH 7.2. At this step, 20 μL of the "crude extract" was kept for SDS-PAGE analysis. 500 μL of glutathione agarose beads (Sigma) was added and mixed for 2 hours at 4°C. The beads were centrifuged and "flowthrough" was also kept for analysis. The beads were washed 5 times and the UbGST protein was eluted by resuspension in 50 mM Tris/5 mM glutathione pH 9.6. The elution was performed 3 times and the 3 fractions, crude extract and flowthrough, were analyzed by SDS-PAGE and autoradiography. Fractions 1 and 2 were pooled, dialysed against 50 mM Tris pH 7.2/1 mM β-mercaptoethanol, aliquoted and stored at -80°C.

2.13 *In vitro* Deubiquitinating Assay

The protein preparation in question (i.e. immunoprecipitation, GSTUSP4Myc fusion, or lysate) was incubated with 15 μL of labeled substrate for 1 hour at 37°C. The reaction was then boiled with SDS sample buffer with DTT (NEB) and separated by SDS-PAGE (10%). The gel was then dried and exposed to X-ray film.

2.14 Polysome Isolation

Cells were transfected, treated with 0.1 mg/mL cyclohexamide for 3 minutes, and washed twice with PBS + 0.1 mg/mL cyclohexamide. Lysis buffer (15 mM Tris pH 7.4, 15 mM MgCl2, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/mL cyclohexamide) was added directly to cell dishes, cells were collected by scraping, and incubated on ice for 20 minutes with occasional vortexing. Nuclei were cleared by centrifugation at 3000 rpm

for 5 minutes. Nuclei were resuspended in lysis buffer supplemented with 1% SDS and sonicated. Both cytoplasmic and nuclear fractions were cleared of debris by centrifugation for 5 minutes at 14,000 rpm.

2.14.1 Pelleting Method

Cytoplasmic lysates were carefully added to the top of a 2.5 mL sucrose cushion (lysis buffer + 35% sucrose) and centrifuged (in a TLA 100.3 rotor) for 30 minutes at 86,000 rpm. The supernatant was carefully decanted and the polysome pellet was resuspended in lysis buffer and analyzed by SDS-PAGE and western blotting.

2.14.2 Fractionation Method

10% sucrose and 50% sucrose solutions were made (in lysis buffer) and combined with a gradient maker to produce a 10 mL 10-50% sucrose gradient. Cytoplasmic lysates were carefully added to the gradient and centrifuged (in a SW41 rotor) for 90 minutes at 39,000 rpm. Gradients were reversed and fractions were collected every 30 seconds (yielding approximately 1 mL per fraction) while continually taking spectrophotometer readings at an absorbance of 254. Fractions were TCA precipitated by adding 500 µL of TCA, mixing, incubating at -20°C for 5 minutes and then at 4°C for 15 minutes. Mixtures were centrifuged for 15 minutes at 14,000 rpm and supernatant was carefully decanted. Precipitated protein was washed with 500 µL of acetone and centrifuged as above. Acetone was decanted and protein was resuspended in 8M urea + 1% NP-40 and subject to SDS-PAGE and western blotting.

3. RESULTS

3.1 Transfected USP4 localization

Although USP4's subcellular localization has previously been studied, it continues to be an interesting aspect of USP4, as there seems to be contradicting data. It was originally thought that it resided solely in the nucleus. However, the transfected USP4 studies presented here would greatly argue against this idea. In these experiments, His-tagged and GFP-tagged versions of USP4 were transfected into H1299 cells. Cellular fractionation was carried out and microscopy was used to confirm that the nuclei were intact, following the hypotonic lysis. Western blots (Figure 3A, B) and corresponding immunofluorescence of transfected USP4 are also displayed (Figure 4A, B). Both the western blots and the immunofluorescence indicate that transfected USP4 resides in the nucleus and the cytoplasm. However, immunofluorescence gives us further information that the distribution of USP4 varies from cell to cell. In Figure 4A, cells indicated by 1 and 3 have USP4 primarily in the cytoplasm whereas in cell 2 it is mainly in the nucleus. In Figure 4B, USP4 is dispersed quite evenly throughout cell 1, whereas in cell 2, it is concentrated in the nucleus. Taken together, these experiments suggest that USP4 shuttle's between the nucleus and the cytoplasm. Indeed, this aspect of USP4 has been observed by another group (Dr. Rohan Baker, personal communications). Endogenous USP4 experiments are not shown here due to reasons discussed below.

3.2 Endogenous USP4 antibody recognizes ODO1 in immunoprecipitation

Identifying proteins that interact with USP4 *in vivo* is an important stepping-stone in determining its cellular substrate(s). In an attempt to achieve this, H1299 lysates were

Figure 3. Subcellular localization of transfected USP4

H1299 cells were transfected with a USP4His expression plasmid (A) or a USP4GFP expression plasmid (B). They were then lysed with lysis buffer and the nuclei were fractionated from the cytoplasm. Nuclear fraction was inspected to ensure nuclear integrity. The lysates were then separated by SDS-PAGE (7.5%) followed by immunoblotting with an anti-His antibody (A) or an anti-GFP antibody (B).

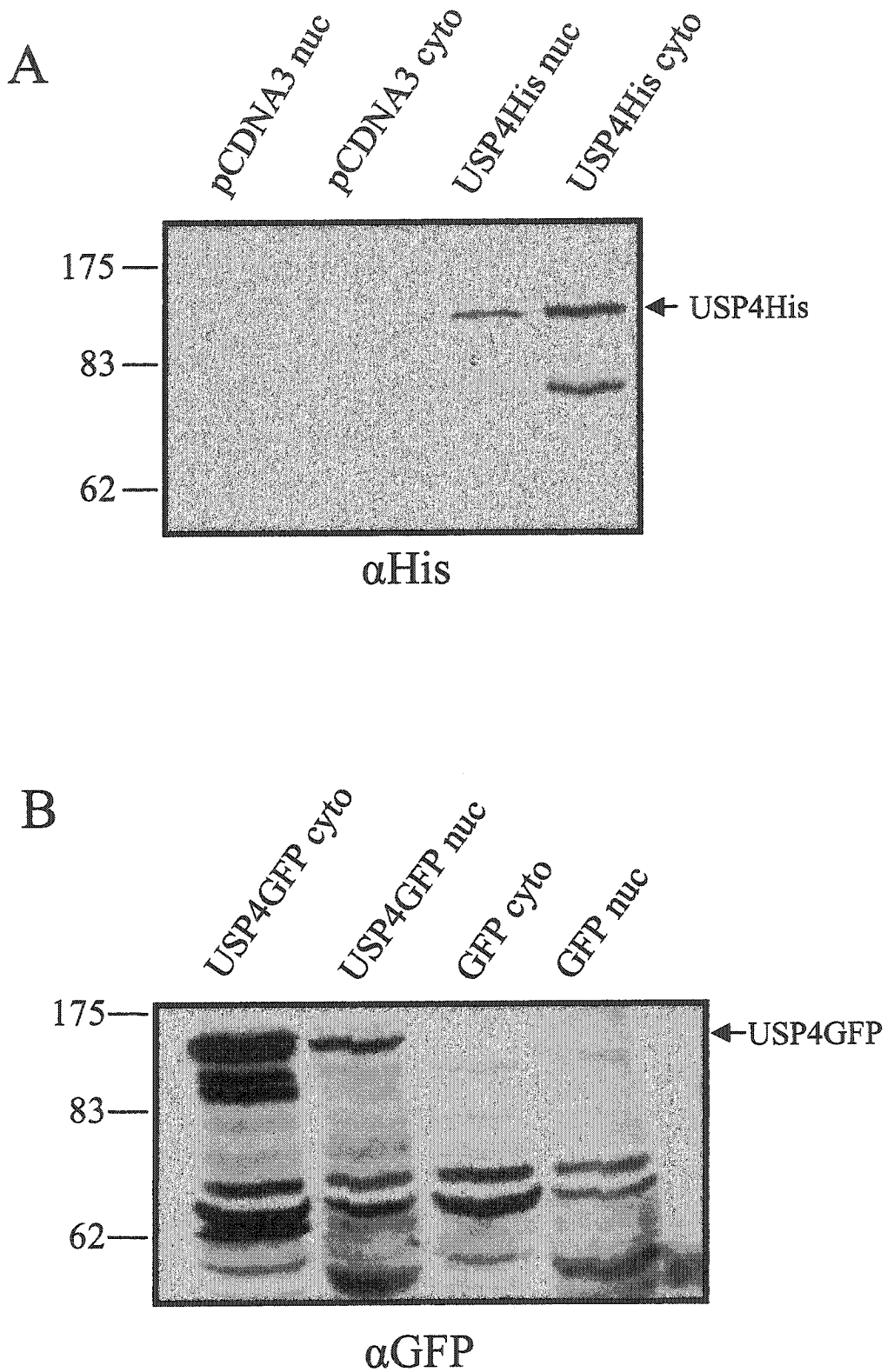
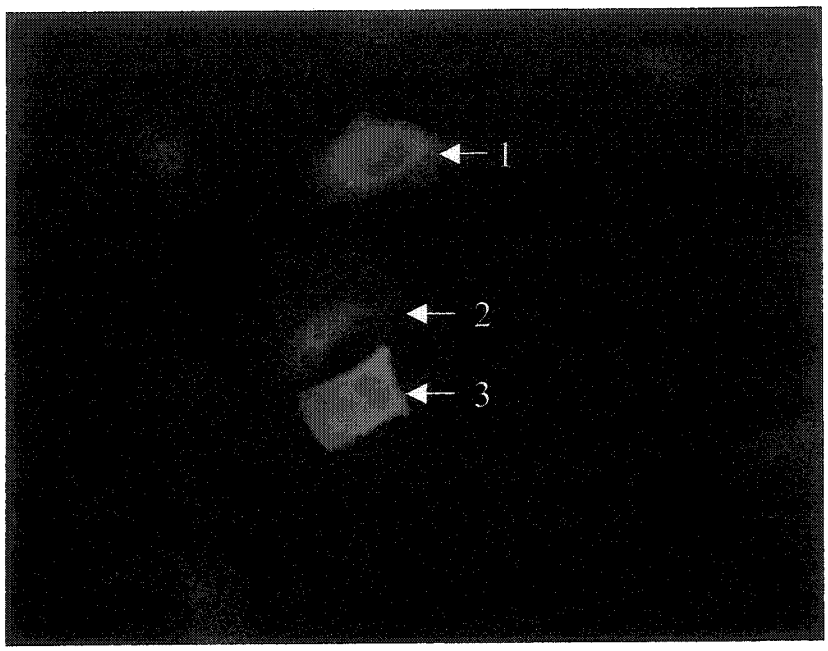

A
pCDNA3 nuc
pCDNA3 cyto
USP4His nuc
USP4His cyto
175
83
62
USP4His
αHis
B
USP4GFP cyto
USP4GFP nuc
GFP cyto
GFP nuc
175
83
62
USP4GFP
αGFP

Figure 4. Subcellular localization of transfected USP4

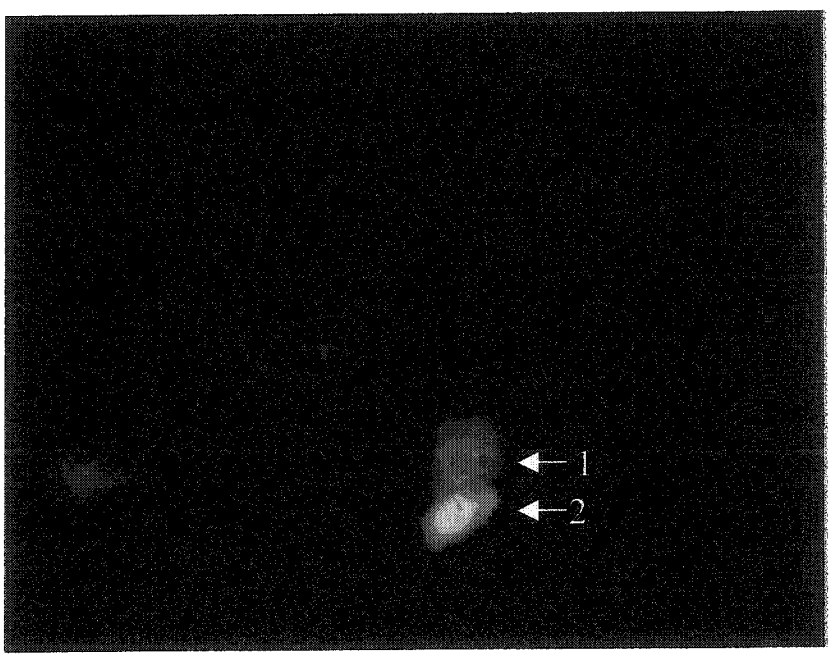
(A) H1299 cells were transfected with a USP4His expression plasmid. Cells were then fixed and subjected to immunofluorescence with a primary anti-His antibody and a secondary Cy3-conjugated anti-mouse antibody.
(B) H1299 cells were transfected with a USP4GFP expression plasmid and fixed. The images show the distribution of transfected USP4. Cells are numbered for the sake of the Results section.

A



αHis

B



immunoprecipitated with an αUSP4 antibody (Zymed) and this was visualized by silver stain (Figure 5). The bands indicated by arrows were excised from the gel and analyzed by mass spectrometry (Dr. Gygi, Harvard University). The band expected to be USP4 (~110 kDa) was identified as the mitochondrial protein, 2-oxoglutarate dehydrogenase e1 component (ODO1), also known as alpha-ketoglutarate dehydrogenase (Table 2). Because of the large amount of this protein, we believe that the anti-USP4 antibody was preferentially immunoprecipitating ODO1. However, it cannot be said whether it also immunoprecipitated USP4. As seen in Table 2, the other proteins that were identified using mass spectrometry analysis are the mitochondrial proteins dihydrolipoamide dehydrogenase (DLD) and the dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (ODO2).

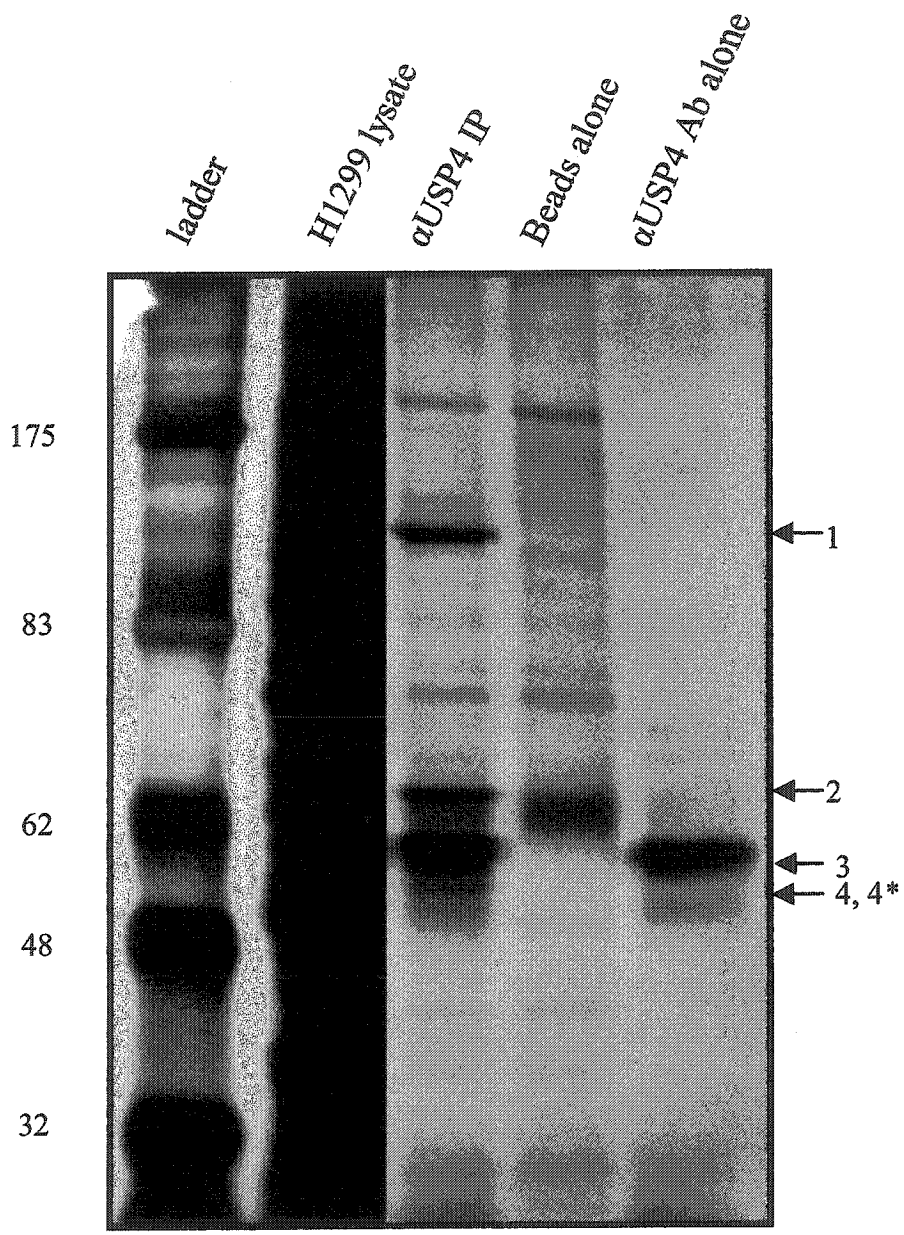
Table 2. Mass Spectrometry Results #1
Identification of protein bands shown in Figure 5

Band #	**Protein Name**	**Protein Size (kDa)**	**# Peptides Identified**
1	2-oxoglutarate dehydrogenase e1 component (ODO1)	~113	37
2	Keratin contamination	N/A	N/A
3	Dihydrolipoamide dehydrogenase (DLD)	~54	19
4	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (ODO2)	~49	12
4*	Elongation factor 1A (EF1A)	~50	4

*discussed later (see Table 3)

Figure 5. Silver Stain of endogenous USP4 and interacting proteins

USP4 was immunoprecipitated from H1299 lysates using an anti-USP4 antibody. The immunoprecipitate was subject to SDS-PAGE (7.5%), along with the antibody alone and an immunoprecipitation with only beads (no antibody). The gel was then silver stained. The arrows indicate the bands that appear in the anti-USP4 immunoprecipitation only. These bands were excised from the gel and sent for mass spectrometry (Dr. Gygi, Harvard University).


ladder
H1299 lysate
αUSP4 IP
Beads alone
αUSP4 Ab alone
175
83
62
48
32
1
2
3
4, 4*

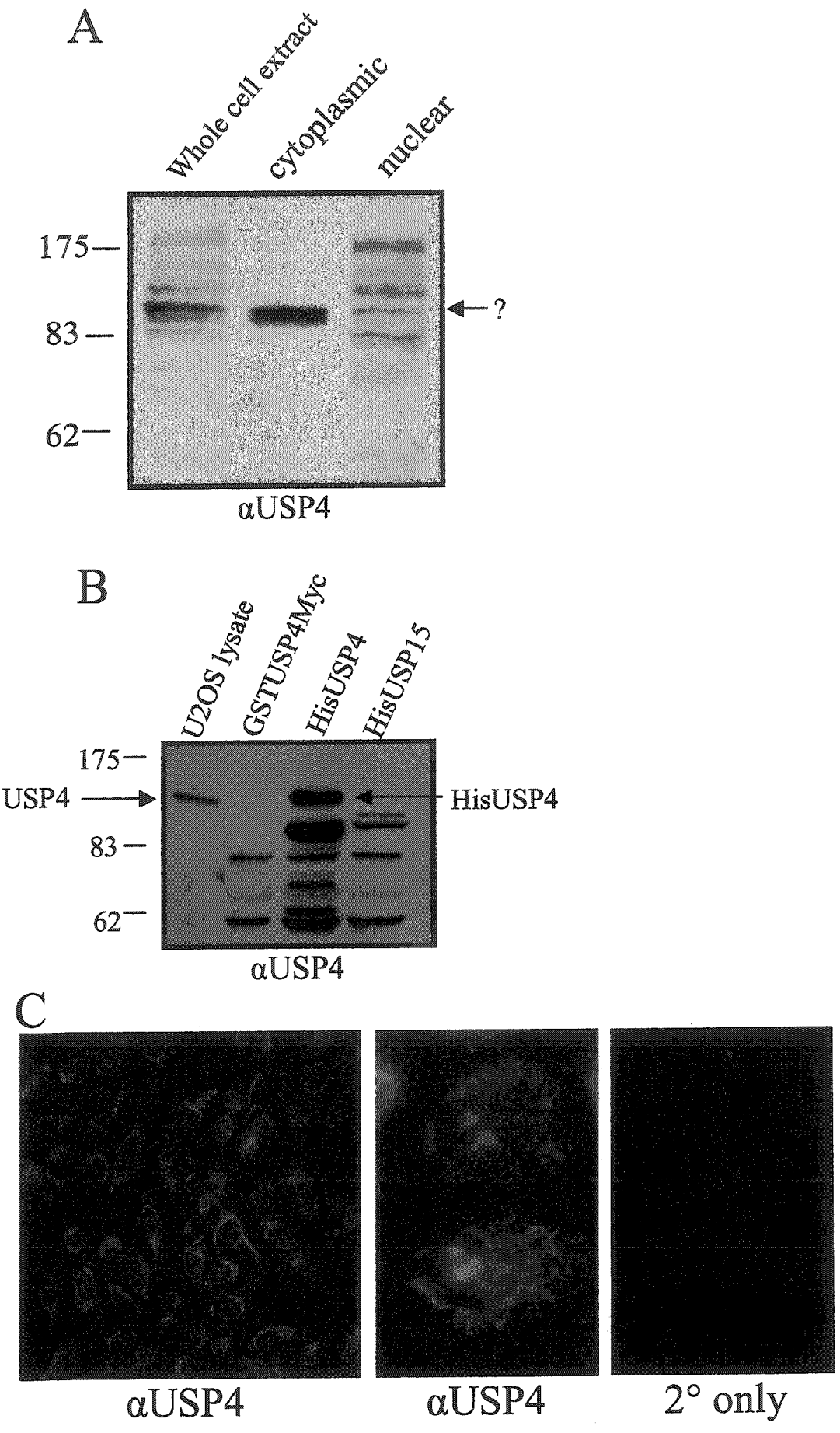
These three proteins have been shown to reside together in the 2-oxoglutarate dehydrogenase complex, which consists of multiple copies of each. The complex is localized in the mitochondria within the inner membrane/matrix compartment and it catalyzes an oxidative decarboxylation sequence converting 2-oxoglutarate to succinyl-CoA and CO_2 (106, 107). We have unintentionally confirmed that these three proteins interact *in vivo*.

3.3 anti-USP4 antibody analysis

To further analyze the anti-USP4 antibody, western blots and immunofluorescence were carried out (Figure 6). First, H1299 cells were fractionated and immunoblotted with the anti-USP4 antibody (Figure 6A). The majority of the detected protein was shown to be localized in the cytoplasm and a doublet pattern was seen here. It is not known what this doublet represents, however, it is possible that one band represents USP4 and the other, ODO1. To definitively determine if the antibody does recognize USP4 in a western blot, bacterially-expressed USP4 was nickel purified and immunoblotted (Figure 6B). The antibody is supposed to recognize the carboxyl-terminus of USP4. Therefore, as a negative control, GSTUSP4Myc, which has a carboxyl-terminus truncation, was purified. A His-tagged version of USP15, a deubiquitinating enzyme with a high degree of homology to USP4 (and with a similar molecular weight), was also purified and included as a negative control. As seen in Figure 6C, anti-USP4 did recognize HisUSP4 without recognizing GSTUSP4Myc or HisUSP15. Taken together, it was concluded that the anti-USP4 antibody does recognize USP4 in western blots, however, perhaps not exclusively.

Figure 6. anti-USP4 antibody analysis

(A) H1299 lysates were fractionated, subjected to SDS-PAGE (7.5%) and immunoblotted with the anti-USP4 antibody.
(B) anti-USP4 recognizes the carboxy-terminal of USP4 in western blots.
Bacterially expressed USP4 and USP15 were purified using a nickel column. Bacterially expressed GSTUSP4Myc, which has a carboxy-terminal truncation, was purified with glutathione agarose beads. All were subjected to SDS-PAGE (7.5%) and immunoblotted with anti-USP4.
(C) H1299 cells were fixed and immunofluorescence was performed using the anti-USP4 antibody and a Cy3-conjugated anti-rabbit secondary antibody. The middle panel shows a higher magnification of the left panel. The right panel shows cells that were only exposed to the Cy3 anti-rabbit antibody, thus showing the low level of secondary antibody background signal.


A
Whole cell extract
cytoplasmic
nuclear
175
83
62
?
αUSP4
B
U2OS lysate
GSTUSP4Myc
HisUSP4
HisUSP15
175
USP4
83
62
HisUSP4
αUSP4
C
αUSP4
αUSP4
2° only

The immunofluorescence staining of H1299 cells with the anti-USP4 antibody showed a pattern unlike transfected USP4. The staining looked granular and was mostly in the cytoplasm (Figure 6C). Upon higher magnification, a pattern typical of mitochondrial staining was observed. It is likely that the antibody is recognizing the ODO1 molecule in the immunofluorescence assay.

For the reasons stated above, USP4 has been studied by transfecting an epitope tagged form of USP4 into cells so that the use of the anti-USP4 antibody can be avoided.

3.4 USP4 interacts with EF1A

As an alternative to the immunoprecipitation, an *in vitro* system was used to determine any binding partners that USP4 may have. To do this, USP4GST was purified from bacteria using GSH agarose beads and a pull down assay was performed with the purified protein and H1299 cell lysates (Figure 7). It is expected that when the GSTUSP4Myc purified protein is subject to SDS-PAGE, a single band would be revealed. However, for reasons unknown, the purification consistently produces a multi-band pattern as seen in the "GSTUSP4Myc beads" lane in Figure 7A. Unfortunately, this makes the visualization of interacting proteins very difficult. However, when the proteins are separated for a considerable amount of time on a large gel, proteins present in the pulldown, but lacking in the beads only column, could be visualized. The silver stained pulldown is shown in Figure 7A and the band indicated was analyzed by mass spectrometry (Dr. Gygi, Harvard University). The results identified a protein called elongation factor 1 alpha, or EF1A (Table 2 and 3). This result was confirmed by performing the same pulldown and immunoblotting for the EF1A protein (Figure 7B).

Figure 7. EF1A interacts with GSTUSP4Myc *in vitro*.

GSTUSP4Myc and GST alone bound to glutathione beads were incubated with equal amounts of H1299 lysate. Pull downs and beads alone were run on an SDS-PAGE gel (8.5%).
(A) The gel was silver stained to view USP4 interacting proteins. The arrowhead shows the band present in the GSTUSP4Myc pull down lane only. This band was excised and sent for mass spectrometry (Dr. Gygi, Harvard University). Mass spectrometry identified the protein to be EF1A, as shown in Table 2 and 3.
(B) A parallel gel was immunoblotted with an anti-EF1A antibody to confirm the mass spectrometry data.

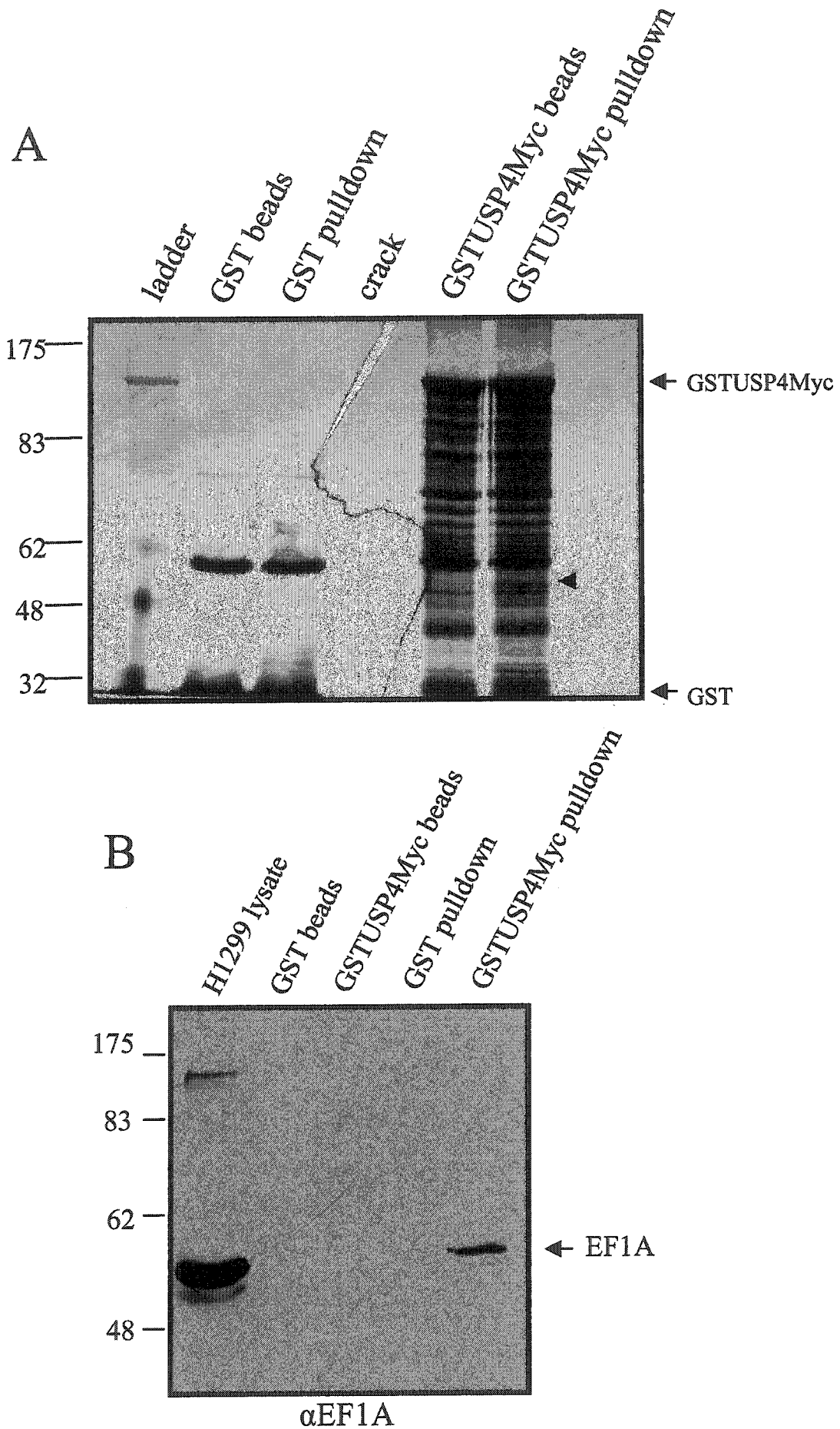

A
ladder
GST beads
GST pulldown
crack
GSTUSP4Myc beads
GSTUSP4Myc pulldown
175
83
62
48
32
GSTUSP4Myc
GST
B
H1299 lysate
GST beads
GSTUSP4Myc beads
GST pulldown
GSTUSP4Myc pulldown
175
83
62
48
EF1A
αEF1A

Table 3. Mass Spectrometry Results #2
Identification of EF1A in two independent experiments

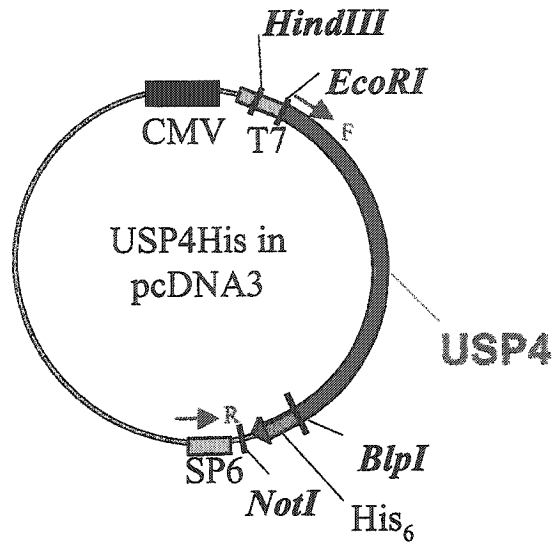
Band	Protein Name	Protein Size (kDa)	# Peptides Identified
Figure 5, Band 4*	EF1A	~50	4
Figure 7A	EF1A	~50	3

To confirm these results *in vivo*, immunoprecipitations were carried out with transfected USP4. Initially, the USP4His expressing plasmid was used, but the experiments were unsuccessful in immunoprecipitating USP4His with the anti-His antibody. For that reason, a FLAG-tagged version of USP4 was constructed using the USP4His plasmid as a template (Figure 8). Consequently, a double-tagged (FLAG and 6xHis) form of USP4 was constructed. The protein product is recognized by the anti-FLAG antibody in both western blots and immunofluorescence (Figure 9A, and B, respectively). Successful immunoprecipitations were also carried out with the FLAGUSP4His protein with an anti-FLAG antibody, as seen on a Ponceau S stain (data not shown).

To ensure that the FLAGUSP4His protein product was active, a deubiquitinating activity assay was carried out. First, a ^{35}S labeled Ubiquitin-GST fusion protein was purified from bacteria and separated on a gel (Figure 10A). This was then used as the substrate to measure deubiquitinating activity. In this assay, the higher the degree of UbGST cleavage, the greater the deubiquitinating activity in the sample. FLAGUSP4His was immunoprecipitated and incubated with UbGST. It can be seen that the FLAGUSP4His protein is able to cleave the substrate to yield GST and ubiquitin. When the cells were

Figure 8. Strategy for FLAGUSP4His construction

Shown is a schematic of the approach taken in the construction of FLAGUSP4His. The USP4His plasmid was used as a template to amplify USP4His with a forward primer containing a HindIII site and a FLAG sequence. The reverse primer was complementary to part of the SP6 sequence 3' to USP4. The resulting FLAGUSP4His linear product and the original template were digested with HindIII and NotI and ligated.



F – forward primer

USP4 complementary

5'AAAAAGCTTACCATGGACTACAAAGATGACGATGACAAGATGGCGGAAGGCCGG3'

D Y K D D D D K

HindIII

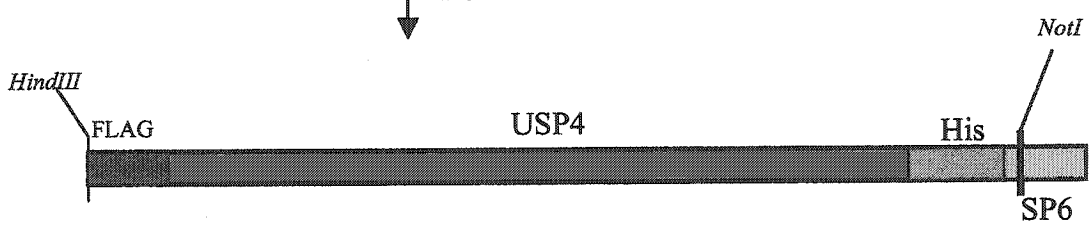
FLAG sequence

R – reverse primer

5'ATTTAGGTGACACTATAGAAT3'

SP6 complementary

PCR



Digest PCR product and USP4His vector with *HindIII*, *NotI* and ligate

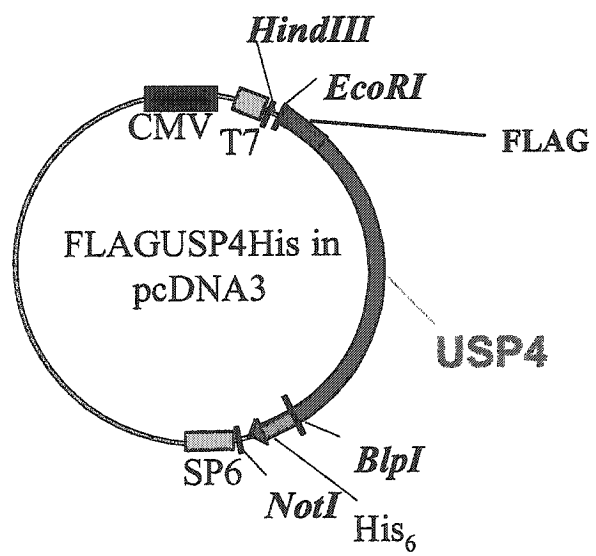
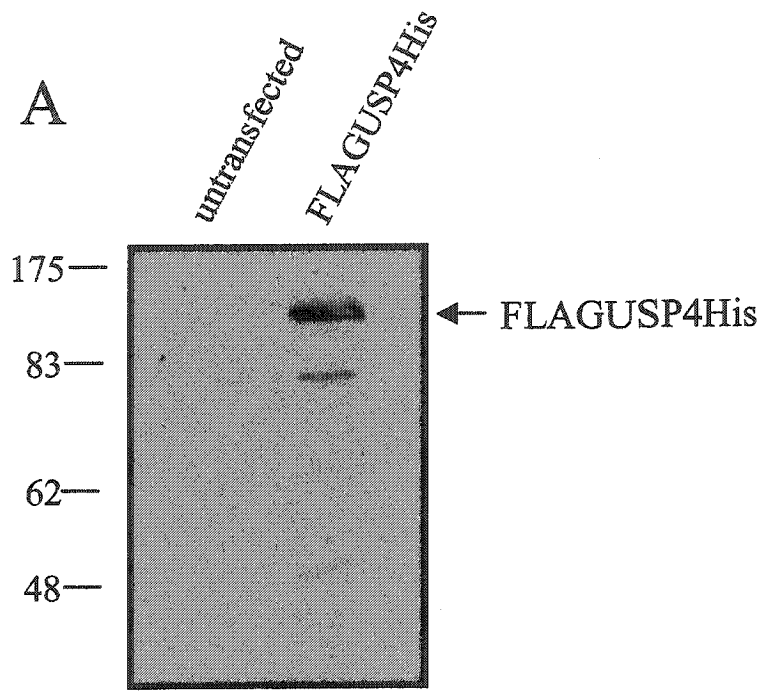


Figure 9. The anti-FLAG antibody recognizes the FLAGUSP4His protein.

The FLAGUSP4His expression plasmid was transfected into H1299 cells.
(A) Cells were lysed with lysis buffer and separated by SDS-PAGE (7.5%), followed by immunoblotting with an anti-FLAG antibody.
(B) Cells were fixed and immunofluorescence was performed with the same antibody. The image shows the distribution of transfected USP4.

A

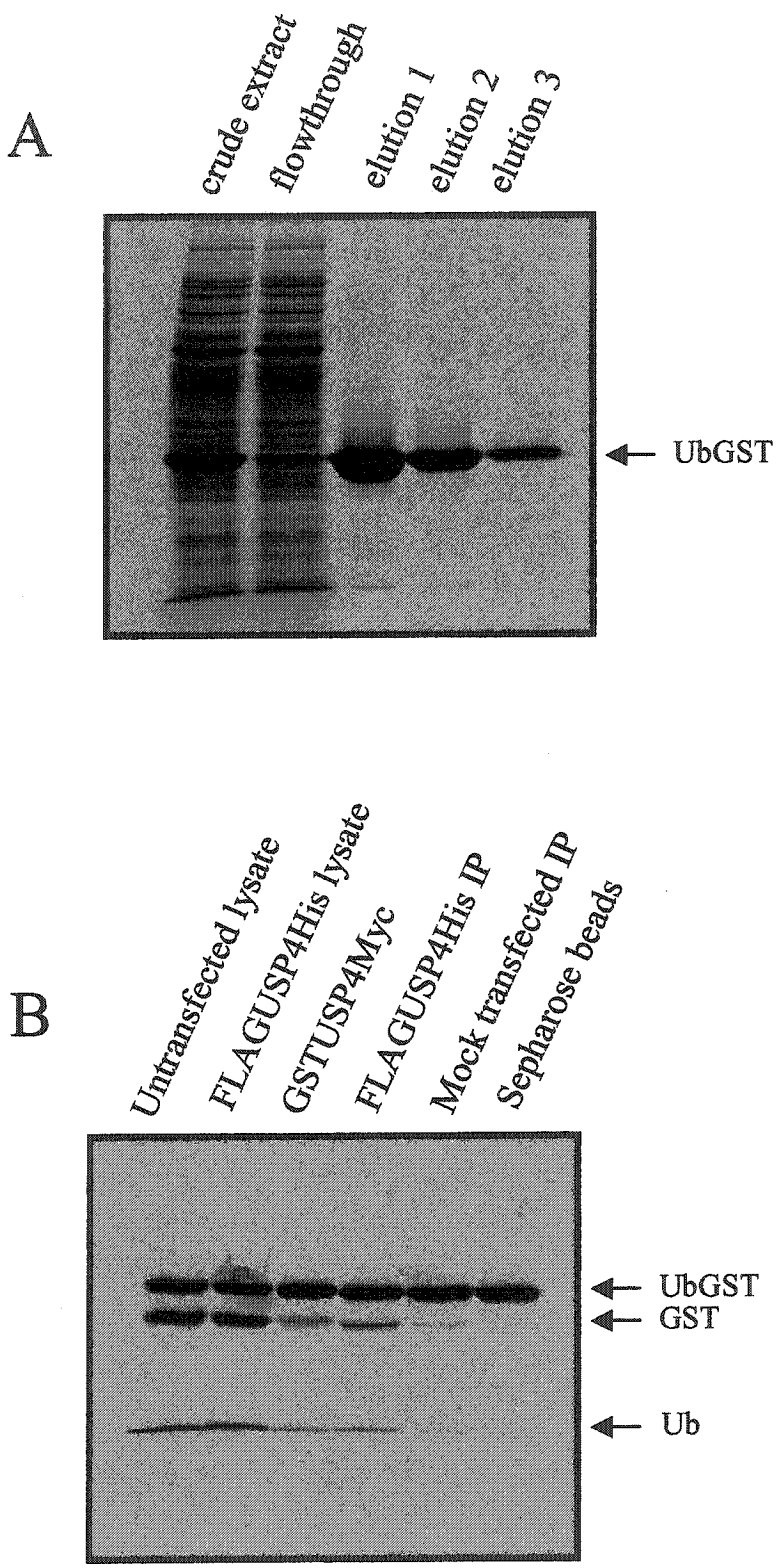

untransfected
FLAGUSP4His
175
83
62
48
FLAGUSP4His
αFLAG

B


αFLAG

Figure 10. FLAGUSP4His has deubiquitinating activity *in vitro*.

(A) Preparation of ^{35}S labeled UbGST fusion protein.
Shown is an autoradiograph of products obtained from sequential steps in the purification of UbGST. Products were separated on a 10% SDS-PAGE gel. The gel was fixed and exposed to an X-ray film.
(B) The FLAGUSP4His expression plasmid was transfected into H1299 cells and immunoprecipitation was carried out with the anti-FLAG antibody. The same was done with mock transfected cells. The immunoprecipitates, along with corresponding lysates, GSTUSP4Myc and sepharose beads alone, were incubated with the labeled substrate. They were then separated on a 10% SDS-PAGE gel. The gel was fixed and exposed to an X-ray film.


A
crude extract
flowthrough
elution 1
elution 2
elution 3
UbGST
B
Untransfected lysate
FLAGUSP4His lysate
GSTUSP4Myc
FLAGUSP4His IP
Mock transfected IP
Sepharose beads
UbGST
GST
Ub

untransfected, there was only a very small level of cleavage seen as compared to the transfected immunoprecipitate. H1299 lysates and purified GSTUSP4Myc were used as positive controls.

To begin to study whether the interaction between USP4 and EF1A is biologically significant, immunoprecipitations were done to determine if this interaction takes place *in vivo*. FLAGUSP4His was immunoprecipitated from H1299 lysates and a western blot was done with the anti-EF1A antibody (Figure 11A). The opposite experiment was also done (Figure 11B). In both experiments, FLAGUCHL1 was separately transfected as a negative control. UCHL1 is also a deubiquitinating enzyme, and being FLAG-tagged, it makes for an appropriate control. In both cases, EF1A and FLAGUSP4His specifically co-immunoprecipitate.

Immunofluorescence was also used to show this interaction by visualizing transfected USP4GFP and endogenous EF1A in H1299 cells. Figures 12A, B, and C show USP4GFP (green, top panel), EF1A (red, middle panel), and the two images merged (bottom panel). In each case, it appears that the two signals are similarly localized. Not only this, in select cases, it appears that when USP4GFP is primarily localized in the nucleus (B and C), EF1A is also in the nucleus. This is surprising since EF1A is normally localized in the cytoplasm. Figure 12D shows the same experiment with GFP instead of USP4GFP. As expected, it does not appear that GFP is colocalizing with EF1A, nor does it have the same localization effect as on EF1A. Taken together, these experiments suggest that USP4 and EF1A interact *in vivo*, and that USP4 may be affecting EF1A's localization.

Figure 11. Transfected FLAGUSP4His binds EF1A *in vivo*.

H1299 cells were transfected with either the FLAGUSP4His expression plasmid, the FLAGUCHL1 expression plasmid, or mock transfected. They were then lysed with lysis buffer.
(A) Lysates were immunoprecipitated with an anti-Flag antibody and separated on an 8.5% SDS-PAGE gel along with the same IPs lacking the antibody, an IP lacking lysate and lysates alone. The membrane was blotted with an anti-EF1A antibody.
(B) The opposite was done using the anti-EF1A antibody and immunoblotting with an anti-FLAG antibody.

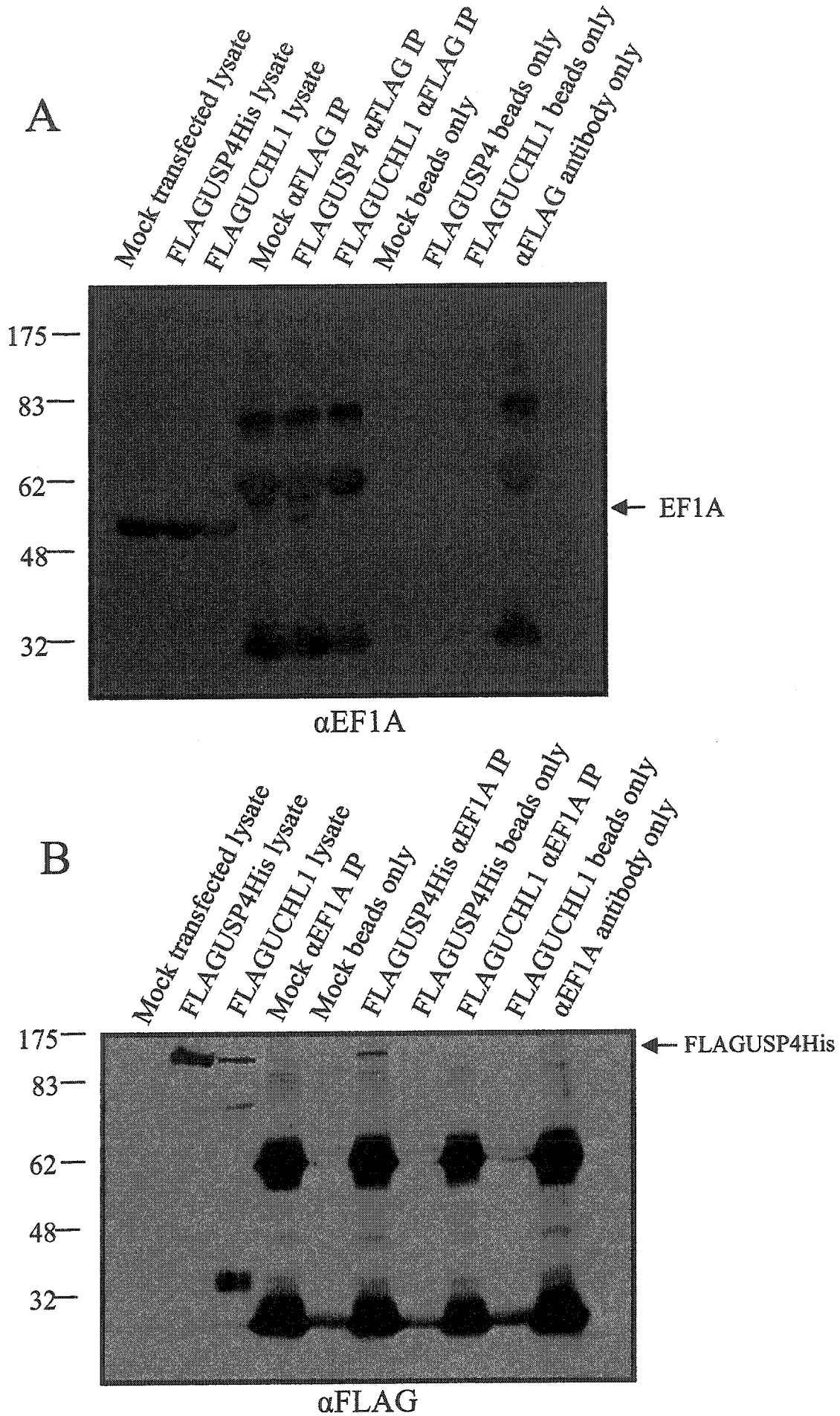
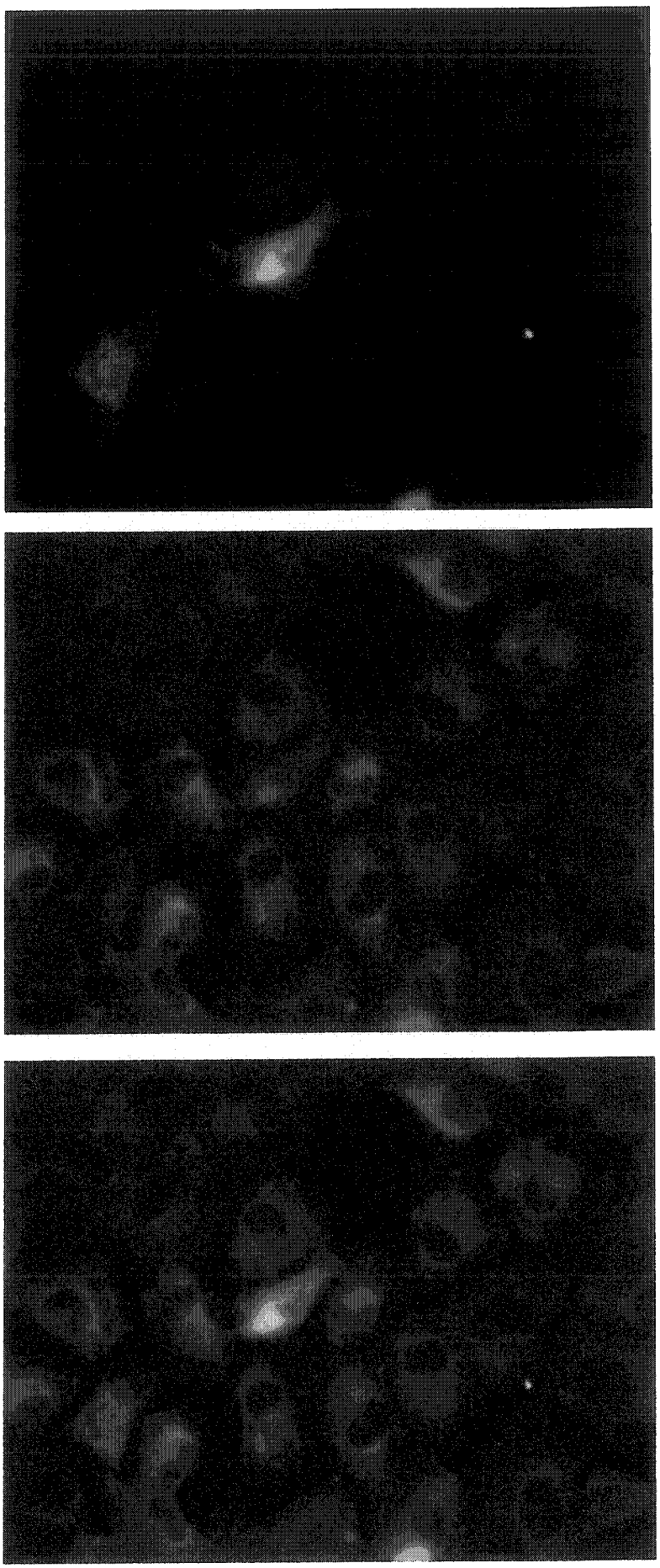

A
Mock transfected lysate
FLAGUSP4His lysate
FLAGUCHL1 lysate
Mock αFLAG IP
FLAGUSP4 αFLAG IP
FLAGUCHL1 αFLAG IP
Mock beads only
FLAGUSP4 beads only
FLAGUCHL1 beads only
αFLAG antibody only
175
83
62
48
32
EF1A
αEF1A
B
Mock transfected lysate
FLAGUSP4His lysate
FLAGUCHL1 lysate
Mock αEF1A IP
Mock beads only
FLAGUSP4His αEF1A IP
FLAGUSP4His beads only
FLAGUCHL1 αEF1A IP
FLAGUCHL1 beads only
αEF1A antibody only
175
83
62
48
32
FLAGUSP4His
αFLAG

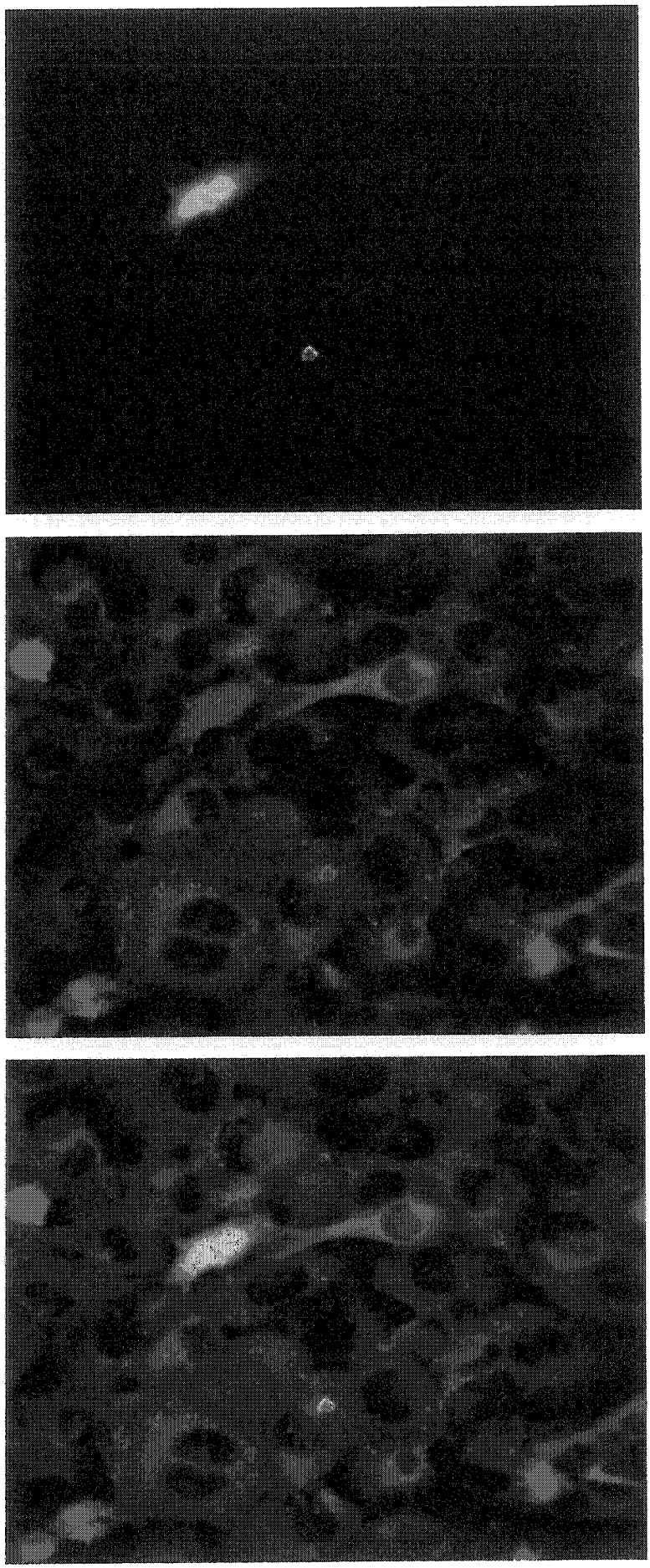
Figure 12. Visualization of USP4GFP and EF1A in H1299 cells

A, B, and C show three different fields of view of H1299 cells transfected with a USP4GFP expression plasmid. D shows cells transfected with a GFP expression plasmid. Immunofluorescence was carried out with an anti-EF1A primary antibody and an Alexafluor-594 conjugated anti-mouse secondary antibody. Cells were viewed under FITC (top) and AF594 (middle) channels. The bottom panels show the merged images.

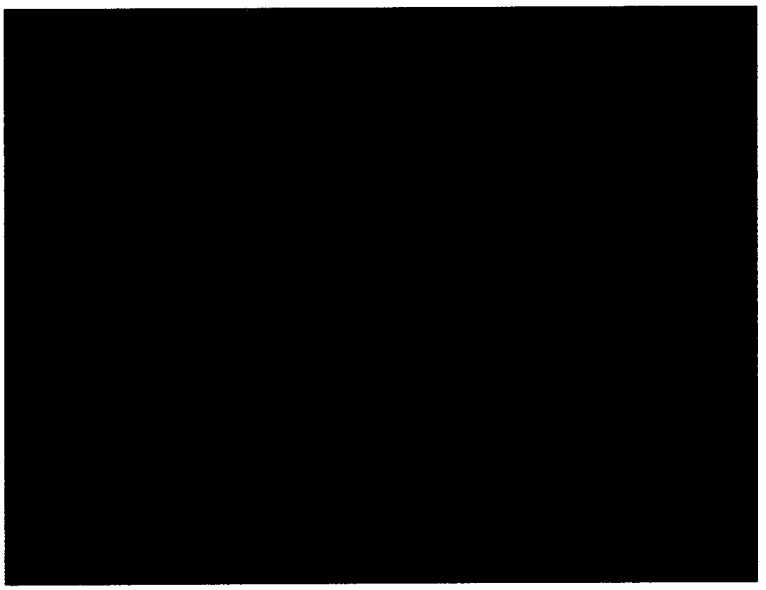
A

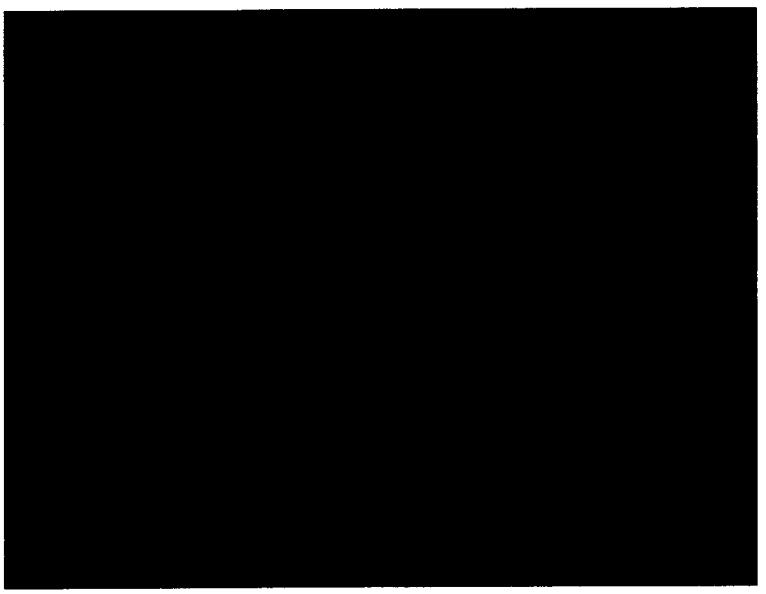


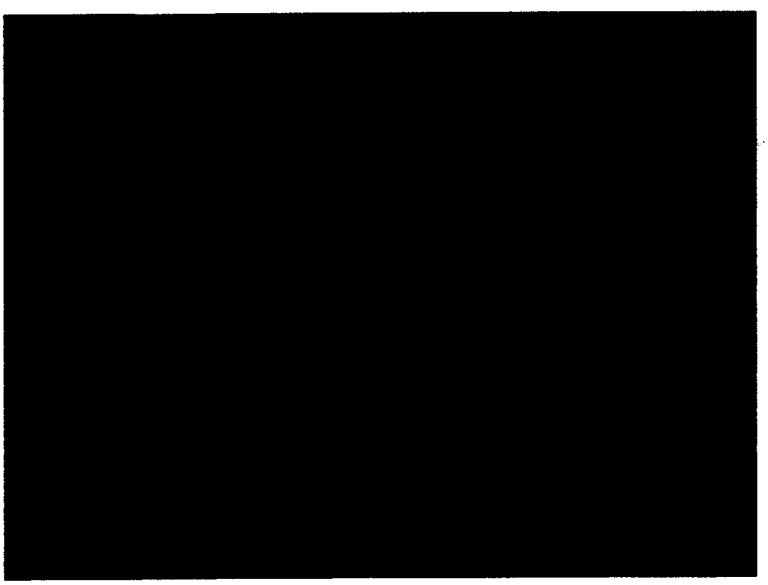
B



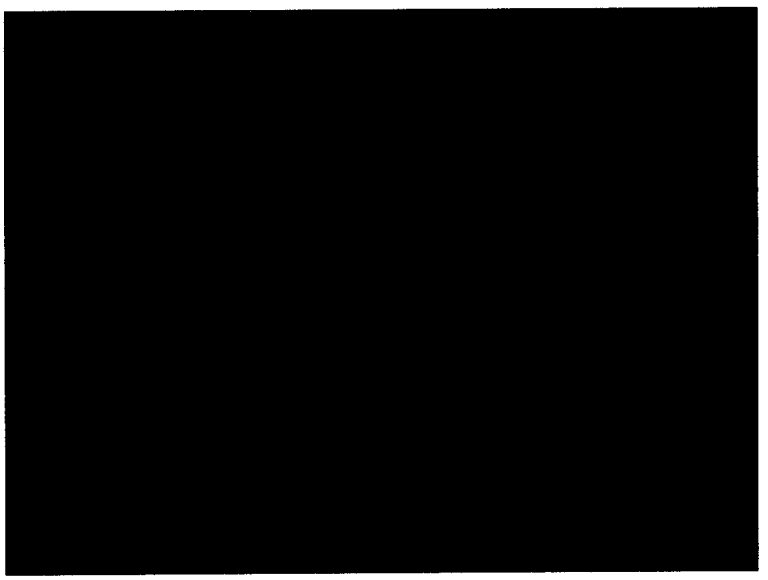
C

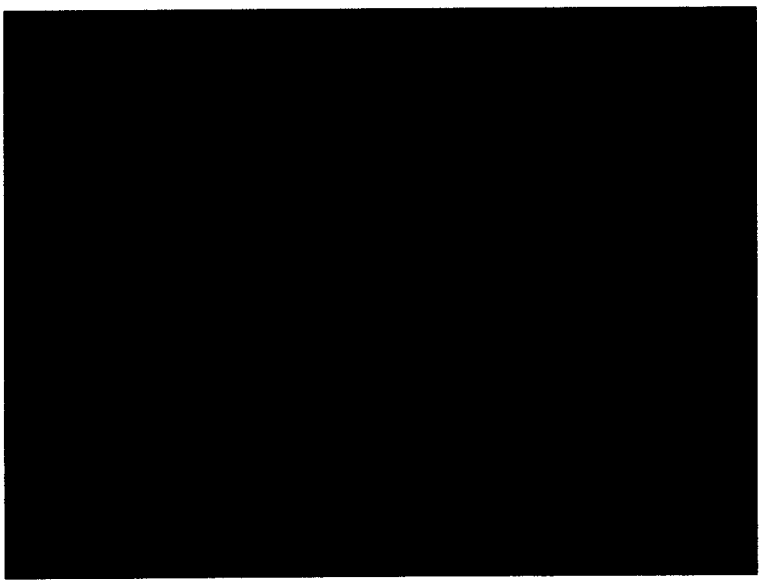


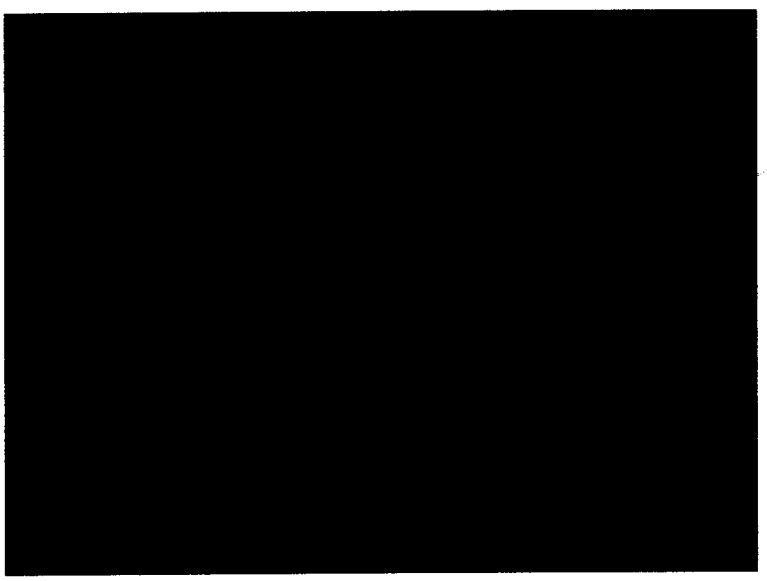




D







3.5 EF1A is not ubiquitinated

One explanation for why USP4 and EF1A interact is that USP4 deubiquitinates EF1A. Therefore, the next experiments were to determine whether EF1A is ubiquitinated. H1299 cells were transfected with a plasmid expressing HisUbGFP. When HisUbGFP is expressed, GFP is cleaved and HisUb is conjugated to substrates (133). Therefore, in this system, ubiquitin-conjugated proteins are detected with an anti-His antibody. Often when a protein is ubiquitinated, the ubiquitin-conjugates cannot be detected due to their degradation by the proteasome. Therefore, proteasome inhibitor was used to stabilize the ubiquitinated proteins in the cell. In these experiments, EF1A was immunoprecipitated and analyzed by western blot. As seen in Figure 13A, even in the presence of proteasome inhibitor, there were no ubiquitin conjugates detected by the anti-His antibody, compared to the controls.

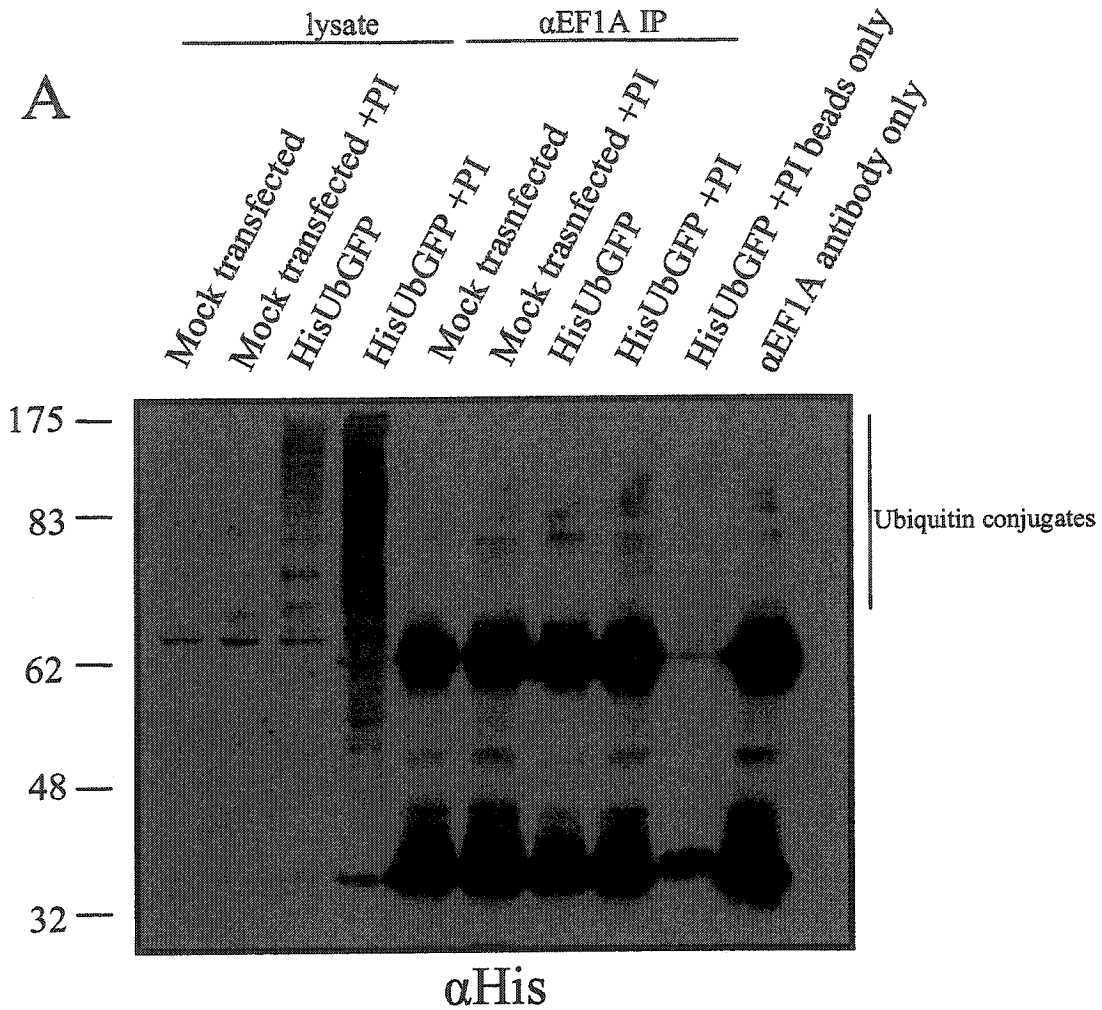
In Figure 13B, H1299 cells were treated with proteasome inhibitor and transfected with either pcDNA3 (empty vector) or FLAGUSP4His. Neither proteasome inhibition nor the overexpression of USP4 changed the levels of EF1A. This indicates again that EF1A is not ubiquitinated. This also shows that USP4 does not affect the stability of EF1A.

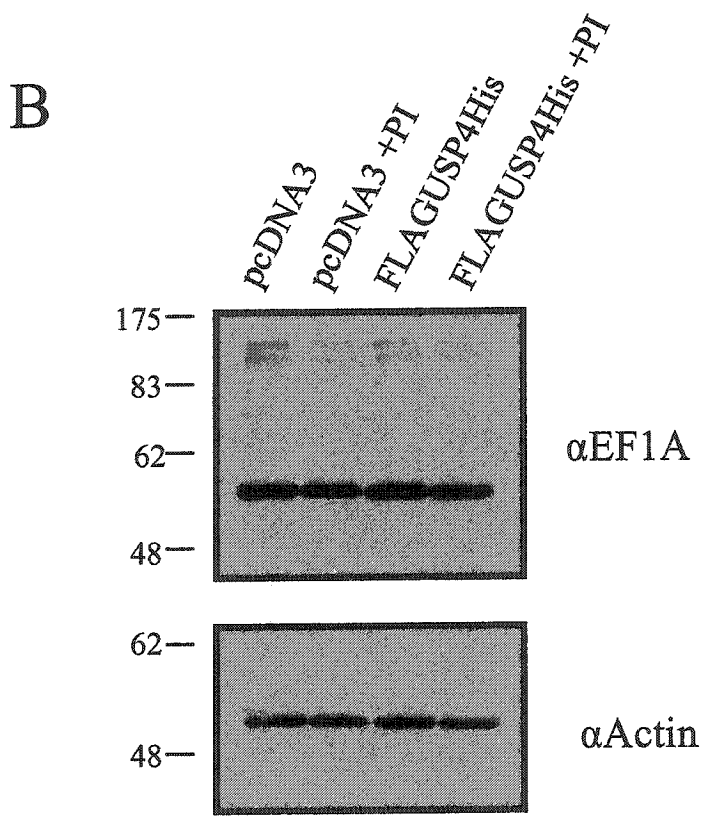
3.6 FLAGUSP4His is enriched in polysomes

EF1A is found in polysomes and its involvement in translation is one of its primary functions. Therefore, we wanted to determine whether USP4 is in a complex with EF1A in polysomes and perhaps also involved in translation. Initial polysome pelleting with a sucrose cushion was carried out as a crude method to determine if

Figure 13. EF1A is not ubiquitinated under conditions specified.

(A) EF1A-ubiquitin conjugates were not detected.
H1299 cells were either mock transfected or transfected with a HisUbGFP expression plasmid. Cells were either untreated or treated with proteasome inhibitor (PI) for 3 hours and were lysed. EF1A was immunoprecipitated with an anti-EF1A antibody and separated on an 8.5% SDS-PAGE gel along with corresponding lysates and immunoprecipitation controls. This was followed by immunoblotting with an anti-His antibody.
(B) EF1A levels do not change with proteasome treatment or USP4 overexpression.
H1299 cells were either transfected with pcDNA3 (empty vector) or the FLAGUSP4His expression plasmid and either untreated or treated with PI. Equal amounts of lysate were separated on an 8.5% SDS-PAGE gel followed by immunoblotting with an anti-EF1A antibody. An anti-Actin blot is shown as a control for equal loading.


A
lysate
αEF1A IP
Mock transfected
Mock transfected +PI
HisUbGFP
HisUbGFP +PI
Mock trasnfected
Mock trasnfected +PI
HisUbGFP
HisUbGFP +PI
HisUbGFP +PI beads only
αEF1A antibody only
175
83
62
48
32
Ubiquitin conjugates
αHis


B
pcDNA3
pcDNA3 +PI
FLAGUSP4His
FLAGUSP4His +PI
175
83
62
48
αEF1A
62
48
αActin

transfected FLAGUSP4His is pelleted with the polysomes. H1299 cells were transfected with either the FLAGUSP4His expression plasmid or the FLAGUCHL1 expression plasmid. The resulting polysome pellets were analyzed by western blot with an anti-FLAG antibody (Figure 14A). The blot shows that while there was little UCHL1, there was a large portion of USP4 found in the polysome pellet. Although the overall levels of UCHL1is lower (probably due to lower transfection efficiency), a pattern is observed. It is apparent that USP4 is enriched in the polysome fraction compared to the UCHL1 polysome fraction.

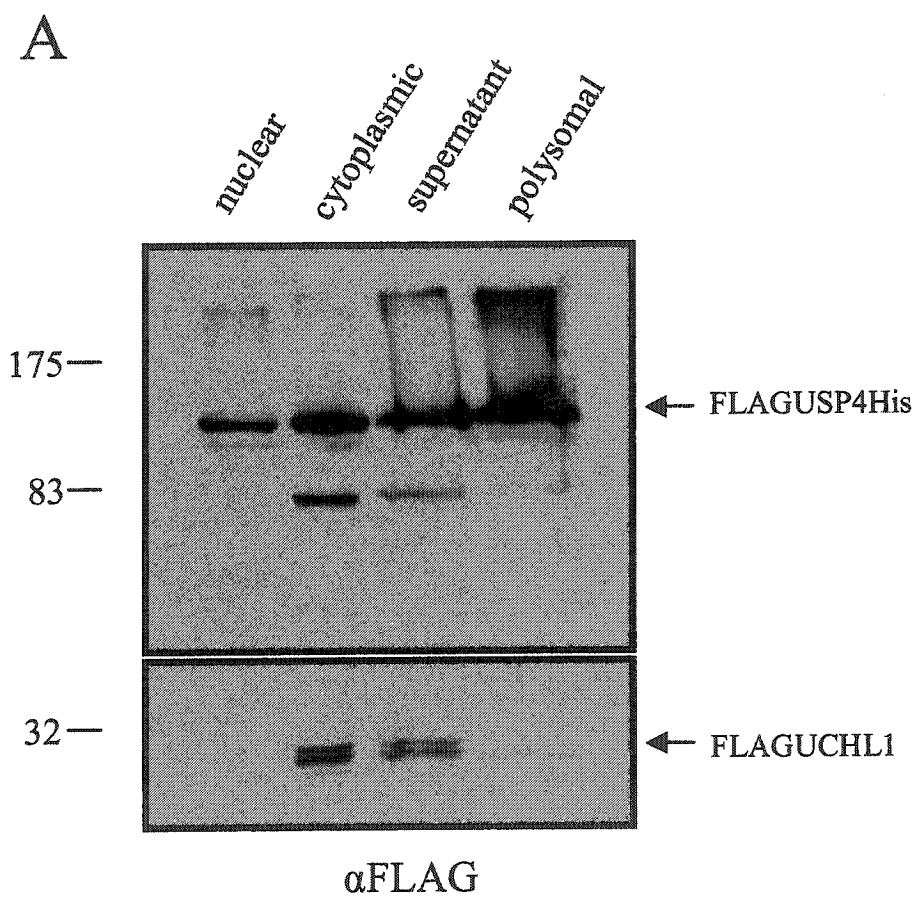
A more common and sophisticated way to isolate polysomes is by using a sucrose gradient to fraction the lysates. When the gradient is spun at the appropriate speed and time, the polysomes will move through the gradient due to their large molecular weight. The other cytoplasmic components not associated with polysomes will presumably stay on top of the gradient. The gradients are then reversed and the RNA readings are taken, which provides a polysome profile. In this case, H1299 cells were again transfected with the FLAGUSP4His expression vector and the lysates were fractionated and reversed while taking spectrophotemeter readings. The fractions were then TCA precipitated and analyzed by western blot. The polysome profile and corresponding western blots are shown in Figure 14B. Anti-FLAG, anti-EF1A, and anti-E2F-4 blots were carried out. E2F-4 was included as a negative control because it is localized throughout the cell and is not known to be associated with polysomes. The profile shows that fractions 5 through 10 represent the polysomal fractions. FLAGUSP4His and EF1A both seem to be enriched in these fractions compared to the E2F-4 control. A long exposure of the

western blot shows that there is E2F-4 present in some of the polysomal fractions, however the level is lower than the levels of USP4 and EF1A.

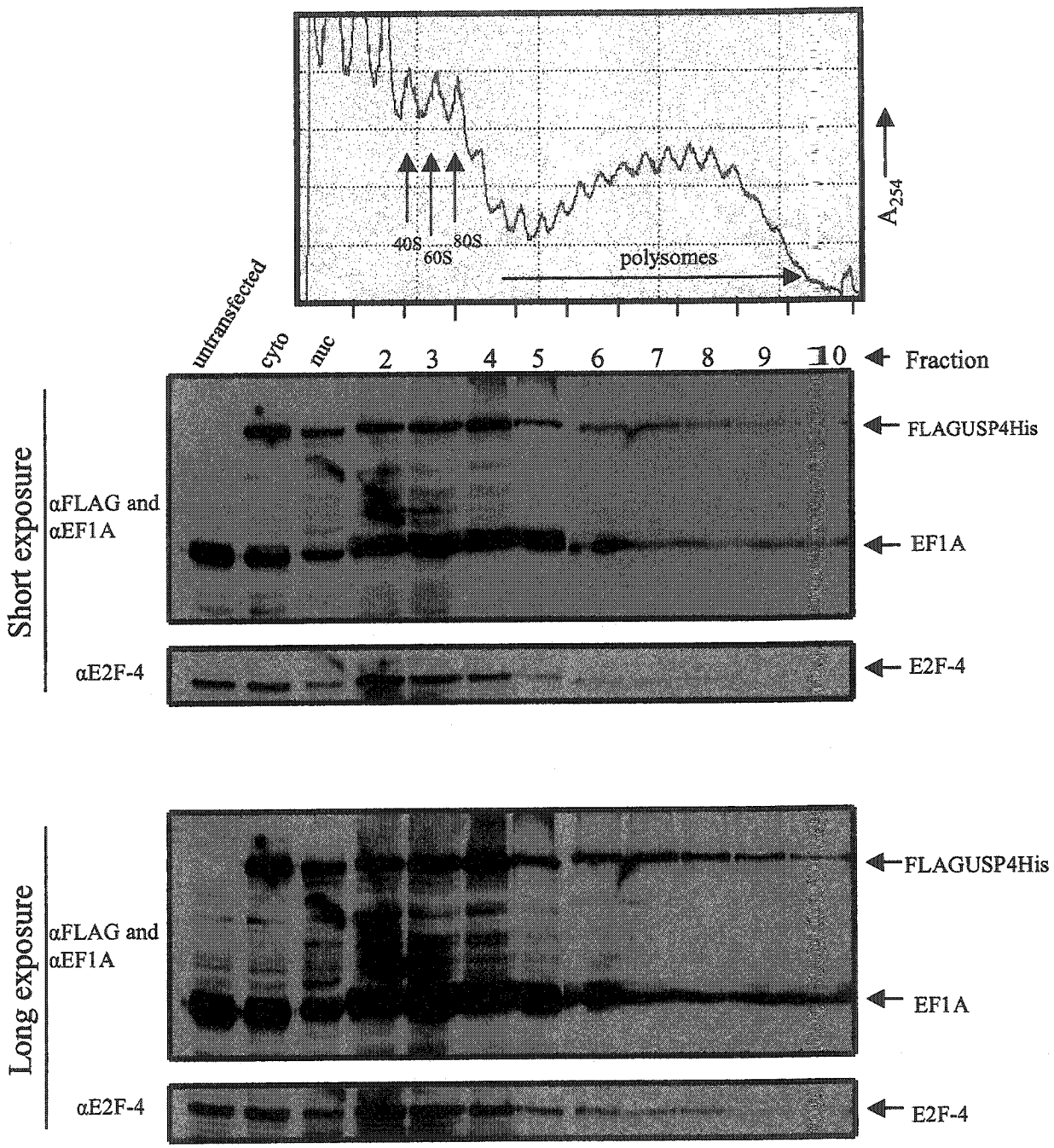
These experiments suggest that USP4 may be associated with polysomes; however, further investigation is required.

Figure 14. FLAGUSP4His is enriched in polysomes.

(A) Polysome isolation by pelleting polysomes using a sucrose cushion
H1299 cells were transfected with a plasmid expressing either FLAGUSP4His or FLAGUCHL1. After incubation for 24 hours, cells were treated with cyclohexamide for three minutes and lysed. Cytoplasmic extracts were loaded on a 35% sucrose cushion and centrifuged. Polysomal pellets were resuspended and separated on an 8.5% SDS-PAGE gel. Along with this, the supernatant from the polysomal pelleting, the nuclear fraction and the cytoplasm fraction were separated. The membrane was immunoblotted using an anti-FLAG antibody.
(B) Polysome isolation by fractionation using a sucrose gradient
H1299 cells were transfected with the FLAGUSP4His expression plasmid, incubated for 24 hours and treated with cyclohexamide for 3 minutes. Cytoplasmic extracts were loaded on a 10%-50% sucrose gradient and centrifuged. The gradient was reversed and fractions were taken every 30 seconds while continually taking spectrophotometer readings at an absorbance of 254. Fractions were TCA precipitated and separated on an 8.5% SDS-PAGE gel along with cytoplasmic and nuclear fractions. Membranes were immunoblotted with anti-FLAG, anti-EF1A and anti-E2F-4 antibodies. E2F-4 is shown as an arbitrary protein not known to be associated with polysomes.


A
nuclear
cytoplasmic
supernatant
polysomal
175
83
32
FLAGUSP4His
FLAGUCHL1
αFLAG

B


40S
60S
80S
polysomes
A_{254}
untransfected
cyto
nuc
2
3
4
5
6
7
8
9
10
Fraction
Short exposure
αFLAG and αEF1A
FLAGUSP4His
EF1A
αE2F-4
E2F-4
Long exposure
αFLAG and αEF1A
FLAGUSP4His
EF1A
αE2F-4
E2F-4

4. DISCUSSION

We have demonstrated here that the deubiquitinating enzyme, USP4 and the elongation factor, EF1A interact in a human cancer cell line. Interestingly, EF1A has previously been linked to the proteasome pathway. One group showed that a specific factor they named FH, is required for the proteolysis of certain N-α-acetylated proteins such as the histone, H2A, actin and α-crystallin (126). They identified this protein as EF1A. In this study, a radioactive labeled multiubiquitinated, H2A was incubated with purified EF1A proteins from yeast and rabbit reticulocytes, and 10 units of 26S protease complex. They suggested that EF1A stimulates the degradation of ubiquitinated H2A. However, the control that contained proteasome, but lacked purified EF1A was not included. This reaction may have had the same degree of substrate degradation. This study also showed that the bacterial EF-Tu can substitute for EF1A in this proteolytic system. When they added ubiquitin aldehyde (which inhibits certain UCHs) to these systems, the "EF1A-dependent degradation" of ubiquitinated H2A was inhibited. However, they did not account for the possibility that the ubiquitin aldehyde may have been affecting a proteasome-associated activity. This study was not convincing in its argument that EF1A has ubiquitin-isopeptidase activity. It was suggested that bacterial EF-Tu has isopeptidase activity, however, components of the ubiquitin pathway have not been detected in bacteria. Although further studies are needed to convincingly demonstrate this aspect of EF1A, it is still possible that EF1A and the ubiquitin-proteasome pathway are somehow linked.

The experiments presented in this study have suggested that EF1A is interacting with a deubiquitinating enzyme rather than behaving as one. Due to the observation that

USP4 is enriched in polysomes, it is conceivable that USP4, when interacting with EF1A, is involved in active translation. Recent studies (described below) have shown protein ubiquitination at sites of active translation. The possible roles of USP4 in these cases will be discussed.

First of all, it is known that the ubiquitin pathway is responsible for degrading misfolded or toxic proteins as a result of mutations or errors in transcription or translation. The proteins that do not attain native structure have been designated DRiPs (defective ribosomal products). It has been suggested that 30% of newly synthesized proteins are DRiPs and at least some of these are ubiquitinated proteins (26).

There has been recent data implicating the ubiquitin-proteasome pathway in the degradation of misfolded integral membrane proteins, including CFTR (109). CFTR folding is an inefficient process (110) and 50-75% of newly synthesized CFTR molecules are rapidly degraded by a pathway involving ubiquitin and the proteasome (109). This degradation occurs without a detectable lag following synthesis (110). Also, the rate of CFTR synthesis is increased 2-3 fold in the presence of proteasome inhibitor. This led to the suggestion that the recognition of CFTR misfolding by ubiquitin conjugation machinery may be closely linked to translation. Indeed, a recent study (111) indicated that nascent CFTR can become ubiquitinated prior to the release from the translation apparatus. This was also observed with the protein Apolipoprotein B (ApoB), the sole protein component in low- density lipoproteins. This protein has been previously shown to be degraded by the ubiquitin pathway. However, recently, it was also shown that nascent partial-length ApoB peptides are ubiquitinated cotranslationally (112). These data suggest that the ubiquitin machinery can recognize nascent chains during translation.

Interestingly, EF1A has been shown to interact with newly synthesized polypeptides. This protein is one of several known to be a ribosome-associated factor (RAF). The nascent-polypeptide associated complex (NAC) is a heterodimeric complex that can reversibly bind to eukaryotic ribosomes. It is located in close proximity to nascent polypeptide chains as they emerge from the ribosomes, and it is thought to be the first complex that the nascent chain encounters (reviewed in 113). Another RAF is the signal recognition particle (SRP), which is necessary for cotranslational translocation into the lumen of the endoplasmic reticulum (reviewed in 114). Chaperones can likely bind nascent chains as they emerge from the ribosome, but their more characterized function is posttranslational protein-association. Recent data demonstrate that EF1A binds ribosome nascent chain complexes and unfolded proteins that are no longer associated with the ribosome, but not to correctly folded proteins (115).

Another study showed that EF1A interacts with polyglutamine aggregates (119). The presence of mutant proteins containing an abnormal length of polyglutamine tracts leads to formation of intracellular aggregates, probably caused by irregular protein-protein interactions. These "inclusions" are a common pathological feature in the group of neurodegenerative disorders known as polyglutamine diseases (e.g. Huntington disease). Aggregates have been shown to be highly ubiquitinated in the brains of transgenic mice (134) and patients of Huntington Disease (135). EF1A may be recognizing the aggregates as misfolded proteins and binding accordingly.

We have confirmed in the lab that EF1A binds a YFP-tagged huntingtin protein containing an extended poly-glutamine repeat (103 repeats) but not huntingtin containing only 25 glutamine repeats (see Figure A in Appendix). This would be a useful system to

determine whether USP4 is also in a complex with misfolded proteins. However, due to time constraints, this aspect of USP4 was not examined.

It is possible that USP4 is in a complex with EF1A, nascent polypeptides and other unidentified proteins such as ubiquitination machinery. Because EF1A also binds nascent misfolded chains no longer associated with the ribosome, this complex may be present with and without ribosomes. In some way, ubiquitinating proteins and deubiquitinating proteins (such as USP4) could be providing a balance so that only the appropriate nascent chains are ubiquitinated to a point of being targeted to the proteasome. Therefore, nascent chain ubiquitination/deubiquitination could potentially be a mechanism of cotranslational regulation. If this model is true, the over/under-expression of proteins such as USP4 could lead to deregulation of certain translational products. USP4's overexpression could potentially cause the stabilization of misfolded/toxic proteins (see Figure 15A for model).

There are many translationally regulated genes whose gene products are important for cell growth. A few such genes are the TGF-β family, c-myc, ribosomal proteins, elongation factors, cyclin D1 and p53. Also, there are many mechanisms of translational control, such as, 1) additional initiation codons within the 5' UTR, 2) long and highly structured 5'-untranslated regions, 3) internal ribosomal entry sites (IRESes) and, 4) targeted mRNA degradation (reviewed in 125). The ubiquitination/deubiquitination of nascent chains could be an additional mechanism.

Interestingly, the ubiquitin ligase, von Hippel-Lindau (VHL), has also been found to associate with polysomes and EF1A (116). VHL is a tumor suppressor gene that, when mutated, causes VHL disease. Patients of this syndrome can develop renal cell

carcinoma's, retinal angiomas, pheochromocytomas, hemangioblastomas of the cerebellum and spine, endolymphatic sac tumors, and pancreatic adenomas (reviewed in 117). VHL associates with a complex that functions as an E3 ubiquitin ligase and is believed to play a key role in the ubiquitination and subsequent degradation of specific cellular proteins. The best-studied substrates of VHL-mediated ubiquitination are the hypoxia-inducible factor (HIF) alpha subunits (reviewed in 118). cDNA arrays with cells that lacked or expressed VHL were recently carried out using polysome-bound or unbound mRNA. This revealed that VHL can inhibit the translation of certain mRNAs, such as tumor necrosis factor-α (TNF-α), cyclins and c-myc (116). The observations that VHL is a ubiquitin ligase, interacts with EF1A, and is present in polysomes, suggests a possible connection between it and USP4 (see Figure 15A for model).

Other polysomal proteins that have been shown to be ubiquitinated are ribosomes (43). One study showed that ubiquitin is conjugated to L28, a component of the large ribosomal subunit, to form the most abundant ubiquitinated protein is *S. cerevisiae*. L28 ubiquitination is inhibited by a Lys63 to Arg (K63R) substitution, indicating that L28 is modified by Lys63-linked ubiquitin. It was shown that heavily ubiquitinated ribosomes are active in translation *in vitro*. Also, K63R mutants displayed defects in translation (shown by polysome profiles), including a hypersensitivity to translational inhibitors and polysome instability (induced by reduced $MgCl_2$). If USP4 is involved in this process and deubiquitinates ribosomes, it could conceivably have a polysome-destabilizing affect. Also, this would be the first example of the hydrolysis of K63-linked ubiquitin (see Figure 15B for model).

USP4 has recently been linked to mRNA in a different circumstance. In a recent paper, it was shown that both proteasome inhibition and overexpression of USP4 led to the stability of AU rich element (ARE) mRNA (120). Normally, an ARE in the 3' noncoding region promotes the rapid degradation of mammalian cytokine and proto-oncogene mRNA such as TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). Although USP4 was shown to stabilize this mRNA, UBPY was shown to have the opposite effect. The author's rationale is that the two DUBs have opposite effects on total cellular ubiquitination. That is, USP4 has a general effect of impairing addition of ubiquitin to proteins and UBPY has the effect of promoting ubiquitin addition. However, USP4 has not been shown to have this effect in cells, and UBPY has actually been shown to decrease total cellular ubiquitination. Although the explanation is up for discussion, USP4's effect on AU rich mRNAs remains. It may be involved in stabilizing a specific protein that affects AU rich mRNA.

Interestingly, the carboxy-terminus of the mammalian GTP-binding protein GSPT/eRF3, is homologous to, and covers almost the full length of EF1A. This protein has been shown to bind mRNA binding proteins including polyadenylate-binding protein (PABP), which is a major component of eukaryotic mRNA turnover. PABP binds the 3' poly(A) tail to prevent exoribonuclease activity to mRNA (121). It would be interesting to know if USP4 also binds eRF3, as this could be a mechanism by which USP4 could affect mRNA stability.

USP4's subcellular localization is a controversial issue. This study suggests that USP4 resides in both the nucleus and the cytoplasm and that USP4 is dispersed differently in different cells. Immunofluorescence of transfected USP4 showed that

USP4 is either 1) mainly nuclear, 2) mainly cytoplasmic, or 3) dispersed quite evenly throughout the cell. This would suggest that USP4 is a shuttling protein and the fact that USP4 contains an NLS would support this notion. This idea is also supported by the observation that USP4 interacts with the nuclear protein, retinoblastoma (RB). Interestingly, some cells that show nuclear localization of USP4, also show nuclear localization of EF1A. Although EF1A is thought of as a cytoplasmic protein, the following cases have linked this protein to the nucleus.

A recent paper looked at EF1A in relation to the importin β transport receptor family (122). This family accounts for most, but not all, nuclear transport pathways. This study demonstrated that EF1A is the predominant nuclear export substrate of RanBP21/exportin4 (Exp5) and that Exp5 binds EF1A indirectly via aa-tRNA. They also observed by immunofluorescence, that EF1A is strictly excluded from the nuclei. However, if EF1A is being exported, it would have had to be in the nuclei at one point. This nuclear localization is probably not observed due to its transient nature.

EF1A has also been shown to interact with a zinc finger protein, ZPR1 (123). ZPR1 is located in the cytoplasm of quiescent mammalian cells, and upon treatment with mitogens such as epidermal growth factor (EGF), ZPR1 redistributes to the nucleus. ZPR1 binds to a group of tyrosine kinase receptors including the EGF receptor (EGFR), and the binding of ZPR1 to the receptor is blocked after mitogen stimulation. It is possible that ZPR1 is sequestered in the cytoplasm by these tyrosine kinase receptors. EGF stimulation of mammalian cells triggers the formation of ZPR1/EF1A complexes and translocation of these complexes into the nucleus.

Another study has clearly shown the presence of EF1A in nuclei. Vigilin is a ubiquitous protein that contains multiple RNA-binding motifs called heterogeneous nuclear ribonucleoprotein K homologous (KH) domains. It was demonstrated that vigilin is part of a novel large tRNA-binding ribonucleoprotein complex (tRNP), found in both the cytoplasm and the nuclei of human cells. Moreover, it was found that EF1A is enriched in the purified nuclear, as well as cytoplasmic, vigilin-containing tRNPs (124). Vigilin is highly expressed in cells that are known to produce high quantities of protein and anti-vigilin antibodies have been shown to inhibit translation *in vitro*. Therefore, vigilin may have a role in translation.

USP4 and EF1A seem to be interacting in the context of the nucleus and the cytoplasm. It appears as though USP4 is interacting with EF1A in the cytoplasm and causing the translocation of EF1A into the nucleus. Given the observation that USP4 interacts with RB, it is possible that once this complex moves into the nucleus, it associates with RB. Interestingly, RB has been shown to be involved in the suppression of polymerase III (pol III) (127), which is responsible for transcription of small stable RNAs including tRNA, and 5S rRNA. This relationship was first considered because loss of RB function and elevated pol III activity are both common features of transformed and tumor cells. Using many different systems (e.g. transfections, *in vitro* assays, cell lines that only express a truncated form of RB, and RB-knockout mice fibroblasts), it was shown that RB directly represses pol III transcription (127). It appears to be regulating tRNA and rRNA synthesis by specifically inactivating the transcription initiation factor, TFIIIB (128). If USP4 is interacting with RB in the nucleus, it could be interfering with RB's effect on transcriptional machinery, thus lifting RB's suppressing activity. This

would cause an upregulation of tRNA, which would then be available to bind EF1A. It would be of interest to us to determine if USP4 and RB interact and whether overexpression of USP4 has an effect on pol III activity/tRNA synthesis.

Further experiments must be done to clarify if any of these models hold true and there are many questions still to be answered. It would be of interest to determine whether USP4 has an overall effect on cellular translation. It is also possible that USP4 has an effect on the translation of only specific mRNAs, such as cytokines, cell cycle regulatory genes, and proto-oncogenes. Such is the case for the ubiquitin ligase, VHL. Since VHL is in a complex with EF1A, it would be worthwhile to determine if USP4 is also in this complex. As mentioned, it is also important that experiments be done to determine whether USP4 is in a complex with misfolded or nascent proteins.

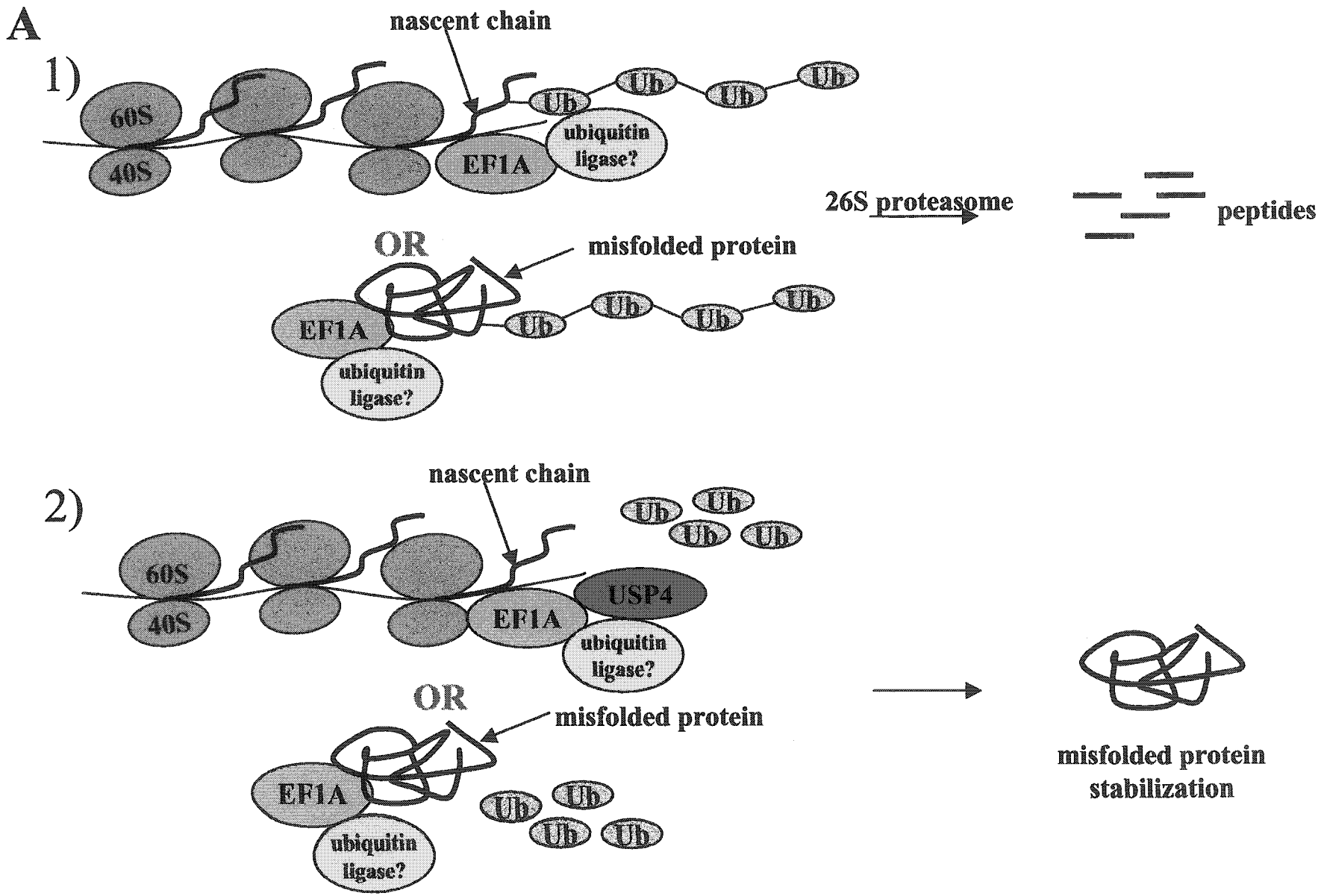
Overexpression of proteins can sometimes force them into artificial interactions or functions. In this case, the overexpression of USP4 is of interest to us because of our original observation this overexpression causes tumorigenesis. It would be interesting to investigate whether the translocation of EF1A into the nucleus is due to the overexpression of USP4, or if endogenous USP4 has the same effect. It is possible that the presence of "too much" USP4 is causing the translocation of this complex and that this is one of USP4's oncogenic effects. Unfortunately, it was not possible to study endogenous USP4 due to the described antibody predicament.

We have shown here, a novel interaction with the mammalian deubiquitinating enzyme, USP4. This interaction with EF1A, along with its previously described interaction with RB, have not yet revealed a substrate for USP4. However, we hope that

these interactions will help to elicit a biological function for USP4. We think this function may be important for cell growth regulation.

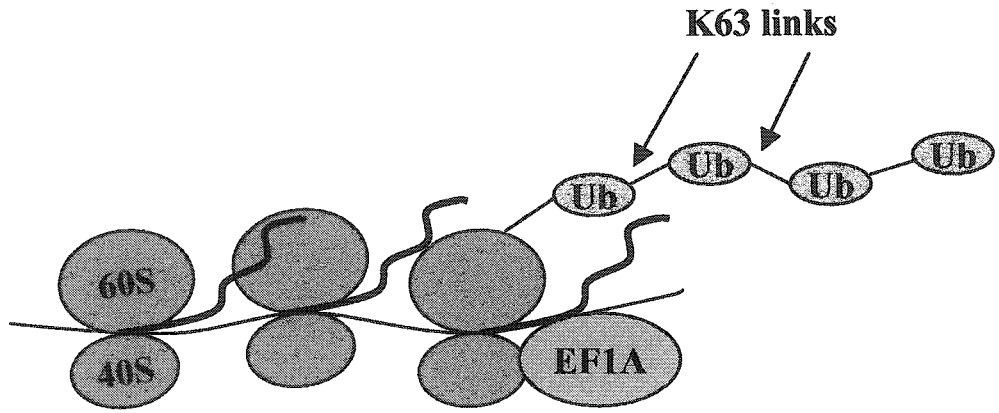
Figure 15. Two possible models of USP4's function in translation

A. 1) EF1A binds nascent chains or misfolded proteins. Ubiquitination machinery (e.g. VHL) may accompany EF1A and ubiquitinate nascent/misfolded proteins. In this case, the protein will be targeted for proteasome degradation.
   2) USP4 binds to EF1A. This will result in the deubiquitination of nascent chains or misfolded proteins and the protein will be stabilized.
B. 1) The large ribosomal subunit is ubiquitinated by K63-linked ubiquitin moieties.
   2) USP4 binds EF1A and deubiquitinates ribosomes, leading to polysome destabilization.

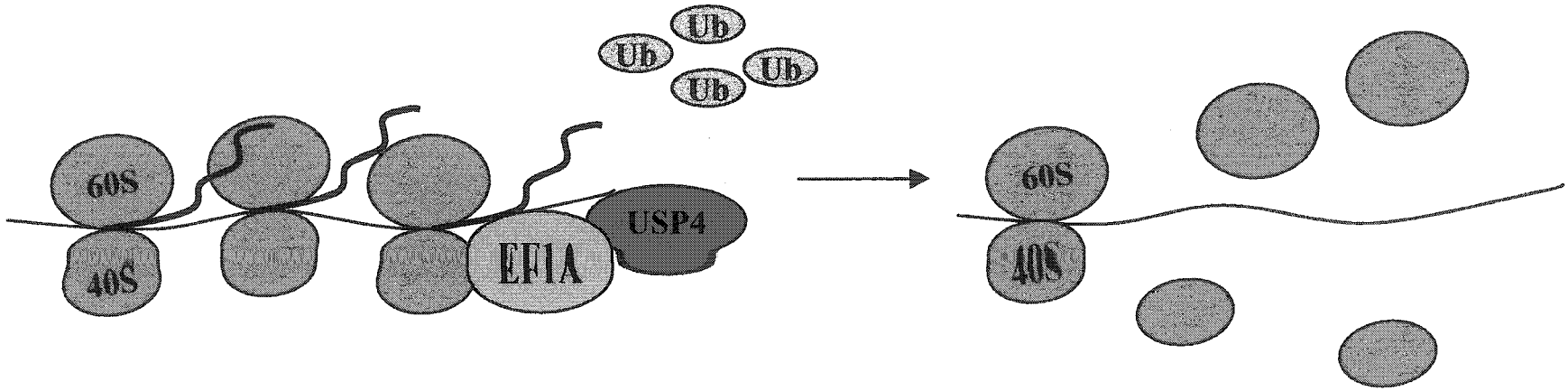

A
1)
nascent chain
60S
40S
EF1A
ubiquitin ligase?
Ub
Ub
Ub
Ub
26S proteasome
peptides
OR
misfolded protein
EF1A
ubiquitin ligase?
Ub
Ub
Ub
Ub
2)
nascent chain
60S
40S
EF1A
USP4
ubiquitin ligase?
Ub
Ub
Ub
Ub
OR
misfolded protein
EF1A
ubiquitin ligase?
Ub
Ub
Ub
Ub
misfolded protein stabilization

B

1)



2)

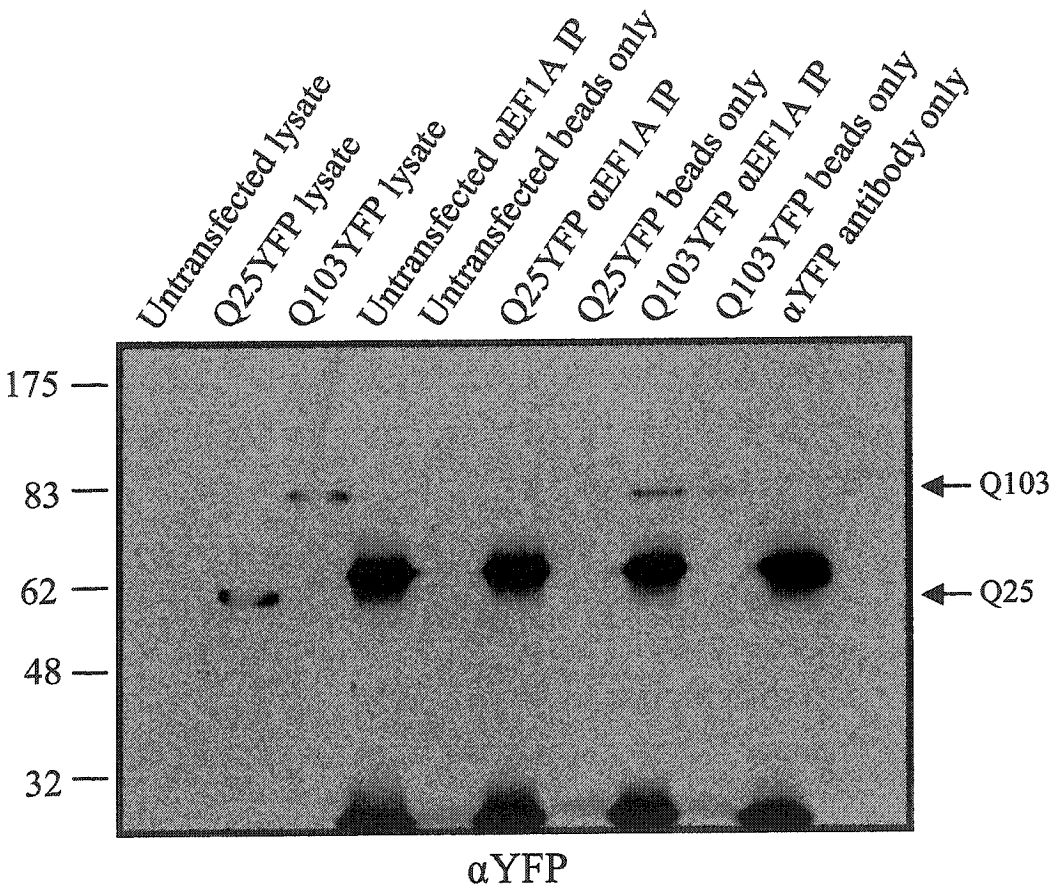


Destabilization of polysomes

5. APPENDIX

Figure A. EF1A binds HuntingtinQ103, but not HuntingtinQ25 *in vivo*.

H1299 cells were either untransfected, transfected with the HuntingtinQ103YFP plasmid, or the HuntingtinQ25YFP plasmid. They were then lysed with lysis buffer. Lysates were immunoprecipitated with an anti-EF1A antibody and separated on an 8.5% SDS-PAGE gel along with the same IPs lacking the antibody, an IP lacking lysate and lysates alone. The membrane was blotted with an anti-YFP antibody.


Untransfected lysate
Q25YFP lysate
Q103YFP lysate
Untransfected αEF1A IP
Untransfected beads only
Q25YFP αEF1A IP
Q25YFP beads only
Q103YFP αEF1A IP
Q103YFP beads only
αYFP antibody only
175
83
62
48
32
Q103
Q25
αYFP

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