[Frontiers in Bioscience 10, 2701-2722, September 1, 2005]

ISG15: A UBIQUITIN-LIKE ENIGMA

Chinh T. Dao and Dong-Er Zhang

Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

TABLE OF CONTENTS

1. ABSTRACT

ISG15 is a 17 kDa protein encoded by an interferon stimulated gene. Described in 1979, it was the first ubiquitin-like modifier to be identified, and its discovery followed the first reports of ubiquitin by only four years. While many important functions for ubiquitin have been reported, the functions for ISG15 and its conjugation are still largely unknown. Evidence suggests that ISG15 and its modification system play important roles in the innate immune response, regulation of interferon signaling, pregnancy, and several cancers. Modification of proteins by ISG15 occurs in a manner similar to that of ubiquitin and other ubiquitin-like modifiers. The enzymes which help perform the activation and conjugation of ISG15 have recently been identified. The conjugation enzyme identified for ISG15 was revealed to be an enzyme that was also involved in ubiquitin conjugation. Identification of an ISG15 specific protease has also been reported. Knockout of this protease in mice decreases the lifespan of these mice and makes them hypersensitive to treatment with interferon or lipopolysaccharide. The study of ISG15 and its modification system may yield a set of potentially useful therapeutic targets and thus, there is an increasing awareness and interest in this protein modifier. This review will highlight the history of its discovery, describe more recent observations about the enzymes involved in ISG15 modification, and summarize new findings which have important implications for the ISG15 system in signal transduction and immunology. This review will also point out important questions that remain to be answered and identify the major roadblocks which currently obstruct the understanding of ISG15 biologic functions.

2. INTRODUCTION

2.1. IFNs

Interferons (IFNs) are a group of related cytokines that were originally discovered to have a role in the defense against viral infections (1-3). IFNs have been classified into two major groups based on their cellular origins and based on the types of receptors they bind. Type I IFNs include -α, -β, -ε, -κ, -δ, -ω, and -τ, whereas IFNγ is the only occupant of the type II family (4). IFN -α, -β, -ε, -κ, -ω, and -γ are all expressed in humans, while IFN-δ, and -τ, are not. IFNγ is mainly produced by activated T cells and NK cells while virtually all nucleated cells are capable of producing type I IFNs (2). The two classes of IFNs share some functional similarities but they lack any obvious structural homology. The two IFN families bind differing receptors, there is a partial overlap in their signal transduction pathways (5). In humans, IFN-β is the predominant subtype, being produced by a variety of non-hematopoietic cells in response to viral infections (6). IFN-α and IFN-ω are more readily produced by hematopoietic cells, but these cells will also secrete variable levels of IFN-β (7). Interestingly, while the IFN-α subfamily in most species is quite large, the IFN-β subfamily in most species consists of a single gene. IFN-β is the most divergent subtype of the type I family and it shares only approximately 25% to 30% protein homology with the members of the IFN-α subfamily. At the DNA level, IFN-β shows approximately 45% homology with the coding sequences of IFN-α genes (8). Structurally, IFN-α appears more similar to IFN-τ and IFN-τ seems most similar to IFN-ω (9). IFN-τ appears to play a role in some animal species to promote implantation of the embryo. IFN-κ is expressed in human keratinocytes and it exhibits low specific anti-viral activity (10). Human IFN-ε is not well-characterized. The promoter regions of human and murine IFN-ε possess reproductive endocrine regulatory elements, and appears perhaps to play a role in reproductive function in placental mammals (4).

Double-stranded RNA (dsRNA) complexes that are formed during the life cycle of many viruses can activate IFN expression (11;12). Experimentally, injection of dsRNA or polyinosinic acid-polycytodylic acid (polyIC), a synthetic form of dsRNA, can induce IFN production by binding to cellular Toll-like receptor 3 (13;14). Lipopolysaccharide, a cell wall component of Gram-negative bacteria, can also stimulate IFN secretion, via binding of Toll-like receptor 4 and the other components of the LPS receptor complex (15).

IFNs are used to treat a variety of human ailments including some viral infections, autoimmune diseases, and many cancers (16-18). IFN-α2b has proven to be an effective agent in treating symptomatic acute hepatitis C (19) and is also used to treat chronic hepatitis B virus infection (20). IFN-β is used to treat the autoimmune disorder multiple sclerosis (MS) (21). IFN treatment is thought to modify the focal disruption of the blood-brain barrier which is characteristic of MS, preventing the transmigration of T cells into the brain parenchyma (22). IFN-α has proven to be useful in the treatment of hematological malignancies including leukemias, lymphomas, and myelomas (23). IFN-α has also been utilized as a therapeutic agent in the treatment of solid tumors such as melanoma, Kaposi's sarcoma, renal cancer, and breast cancer (24). The anti-cancer effects of IFN are thought to stem from its ability to curtail cellular proliferation, induce cellular differentiation, alter oncogene expression, and augment immune responses.

2.2. Ubiquitin-like modifiers

IFN treatment induces a variety of cellular changes and alters the expression of over 1000 genes (3;25;26). As its name suggests, ISG15 is a protein whose expression is regulated by IFN; and in fact, it is one of the most abundant gene products expressed following IFN stimulation (27;28). ISG15 also belongs to the family of ubiquitin-like modifiers whose growing list includes neuronal precursor cell-expressed developmentally downregulated protein 8 (NEDD8)/related to ubiquitin 1 (RUB1), small ubiquitin-related modifier (SUMO), Homologous to ubiquitin 1 (HUB1), Autophagy (APG) 12, and APG8 (29). Modification by ubiquitin-like modifiers plays important roles in many processes including cell cycle progression, nuclear transport, and autophagy (30). The role in which ISG15, itself, plays in the myriad of IFN effects is not yet clear.

3. DISCOVERY AND HISTORY OF ISG15

ISG15 has been reported in the literature for over two decades. In the first experiments describing ISG15, a ~15 kDa protein with an isoelectric point of 7.1 was found in the *in vitro* translation products generated from IFN-stimulated murine tumor cell RNA (27). This protein was not noted again until 1984 when it was reported that both human and bovine cell lines produced this protein in response to IFN treatment (31). This response was dependent on RNA synthesis, and it was observed that IFN-α and IFN-β were more efficient than IFN-γ at inducing ISG15 expression.

In 1986 the cDNA for human ISG15 was first cloned from Daudi cells (32). The original sequence included an extra nucleotide which would have resulted in a premature stop codon. The first reported sequence would have generated a translation product with molecular weight ~15 kDa, and ironically, this estimate was in agreement with the observed experimental molecular weight (27). In 1987, when ISG15 was re-cloned it was discovered that the full length sequence actually contained 165 amino acids giving it a calculated molecular weight of 17,890 daltons (33). In 1988, it was discovered that the full length 165 amino acid human ISG15 protein was a precursor to a shorter, more mature form (34). Eight C-terminal amino acids are removed from human ISG15 giving it a C-terminus ending with the amino acid sequence'…Leu Arg Leu Arg Gly Gly' (LRLRGG) (34). The mature form of ISG15 also lacks an N-terminal methionine and has a calculated molecular weight of 17,145 Daltons. Ubiquitin-like modifiers are often translated as longer precursors with C-terminal extensions which prevent their direct conjugation (30).

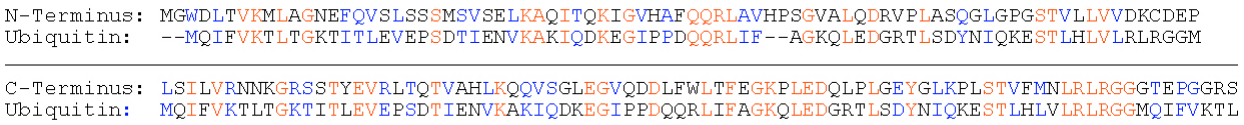

Figure 1. Comparison of human ISG15 (accession number P05161) and ubiquitin (accession number AAH14880) sequences. ISG15 is comprised of two ubiquitin-like domains. N-terminal and C-terminal sequence was aligned with the ubiquitin protein sequence via the ClustalW method (185). The N-terminal and C-terminal ubiquitin like domains share approximately ~30% homology with ubiquitin. Residues highlighted in red represent identical amino acids shared by ubiquitin and ISG15. Residues highlighted in blue show amino acids which are conserved.

Figure 2. Comparison of human (accession number P05161), murine (accession number NP_056598.1), bovine (accession number NP_776791.1), and goldfish (accession number AAP68826.1) ISG15 protein sequences. Majority sequence derived from these four sequences is also listed. Sequences were aligned via the ClustalW method (185). Amino acids identical in all four sequences appear within the majority sequence in red. Residues shared by three of the four species are highlighted in yellow, and residues shared by two are printed in blue letters.

In 1987, a study was done to compare the cellular ubiquitin (Ub) levels in A549 cells, a human carcinoma cell line, and in L929 cells, a murine fibroblast cell line, following infection of these cells with encephalomyocarditis virus (35). No changes in ubiquitin pools were found, but upregulated levels of a ubiquitin cross-reactive protein (UCRP) were observed. It was discovered that this protein was identical to the protein originally identified in the first reports of ISG15 and, it was in this report that the protein was originally dubbed UCRP due to its cross-reactivity with anti-ubiquitin antibodies. It was also in 1987 that the name ISG15 was coined (33).

4. ISG15 SEQUENCE HOMOLOGY

ISG15 contains two ubiquitin-like domains connected in tandem. Sequence analysis has revealed the N-terminal domain shares approximately 33% and the C-terminal domain possesses approximately 32% homology with ubiquitin (figure 1). A three-dimensional structure for ISG15 has not yet been reported. An NMR structure for SUMO-1 is available (36) and an X-ray crystal structure for NEDD8 has been solved (37). NEDD8 has approximately 57% identity (38) and SUMO proteins share only about 18% sequence identity with ubiquitin (30). The three-dimensional structures of these two ubiquitin-like modifiers are very similar to ubiquitin. Based on the sequence similarities between ISG15 and ubiquitin, the two domains of ISG15 are predicted to have similar 3-D structures. Moreover, it is unclear how the two ubiquitin-like domains of ISG15 pack together to form a singular structure. High quality crystals of ISG15 that can be used to solve its structure have not been reported. It is possible that the intervening sequence linking the two ubiquitin-like domains confers ISG15 with a greater 'flexibility' that may inhibit its crystallization.

ISG15 has only been found in vertebrates. It was originally discovered in humans, but its expression has also been reported for mouse, rat, cow, and sheep. ISG15 has only a 47% conservation among the five mammalian sequences reported. Comparatively, its cross-species conservation is low (figure 2) when one considers that ubiquitin has a virtual 100% conservation cross-species and other ubiquitin-like molecules are more conserved. ISG15 has also been cloned from fish (39), including goldfish, zebrafish, catfish, and pufferfish. It is interesting to note that the ISG15 protein sequences reported for fish, cow, and sheep lack amino acids following the highly conserved C-terminal 'LRLRGG' sequence (40).

5. EXTRACELLULAR ISG15

Like ubiquitin, ISG15 modifies proteins post-translationally, forming covalent conjugates with its targets. ISG15 can also be found extracellularly in an unconjugated form. The first reports of extracellular ISG15 were made in 1991. Human lymphocytes and monocytes were reported to release free ISG15 following treatment with IFN-β (41). More than 50% of the total ISG15 could be detected in the culture medium twenty-four hours following IFN stimulation. The human monocytic cell line THP-1 was also found to release ISG15 following induction by IFN.

ISG15 has also been reported to be released by several other cell lines. Raji cells (a human Burkitt's lymphoma B cell line) and Jurkat cells (a human acute T cell leukemia line) both release extracellular ISG15 (42). Human cornea fibroblastic keratocytes, A549 (a human lung epithelial carcinoma cell line), and OVCAR-3 (a human ovary epithelial adenocarcinoma cell line) also release ISG15 (42). Our lab has detected free ISG15 released by the murine macrophage cell line RAW264.7 following treatment with LPS (unpublished results).

ISG15 can also be detected in human serum. IFN treatment induces a dose-dependent increase in the levels of ISG15 in human serum. A 7.3-fold increase in serum ISG15 concentration (up to 3,000 pg/ml) was observed in healthy volunteers receiving an IFN regimen consisting of four injections of 8.0×10^6 international units (IU) over the course of nine days (43).

ISG15 has been reported to be released by cultured endometrium on days 15 through 26 of pregnancy (44). By western blot, marginal levels of extracellular bovine ISG15 were detected at day 15. Maximal extracellular ISG15 was detected at day 18 and ISG15 was present until day 26 of pregnancy.

ISG15 lacks a signal peptide sequence which is common to other secreted proteins (45). Furthermore, ISG15 shows no homology to other cytokines. It is not clear if ISG15 is secreted from cells or if it is released by some other mechanism. It is possible that ISG15 may be released from dead or apoptotic cells. It is also possible that there is a novel mechanism for its extracellular release. Fibroblast growth factor (FGF)1, FGF2, interleukin (IL)-1α, and IL-1β are only a few examples of proteins that are released via a non-classical pathway (46;47). The export of FGF1 and IL-1α relies on the Cu^{+2}-dependent formation of protein complexes containing the S100A13 protein (48;49). Under normal circumstances, cells that produce FGF1 and IL-1α do not release these pro-angiogenic polypeptides. However, following cellular stresses such as heat shock (50), hypoxia (51), cultivation of cells under low serum (52), or treatment with low-density lipoproteins (53), FGF1 release from NIH3T3 cells can be induced. Release of IL-1α from human promonocytic leukemia cells and from activated peripheral mononuclear cells can be induced by heat shock (49;54). It is thought that both FGF1 and IL-1α translocate across cellular membranes as 'molten globules' (46). The depletion of intracellular Cu^{+2} with the specific chelator tetrathiomolybdate (TTM) can attenuate the stress-induced release (48;49). It is possible that ISG15 could utilize a similar mechanism for its extracellular release. Unlike FGF1 and IL-1α, release of IL-1β is sensitive to the inhibitor of exocytosis, methylamine (55). It would be interesting to determine if this set of chemical inhibitors has any effect on the extracellular presence of ISG15.

6. REGULATION OF ISG15 EXPRESSION

ISG15 has been detected as early as two hours post-IFN stimulation and reaches maximal expression near 18 hours post-stimulation (56). IFN signaling begins at the cell surface with the binding of IFN to its receptor. The receptor for type I IFN is a heterodimer of IFNAR1 and IFNAR2 subunits, which assemble upon binding of IFN (4). Janus kinase 1 (Jak1) and protein-tyrosine kinase 2 (TYK2) become activated by transphosphorylating themselves and the tyrosine residues of the IFN receptor. The phosphorylated receptor binds signal transducers and activators of transcription (STAT) 1 and 2 via their Src homology domains. Stat1 and Stat2, in turn, are phosphorylated by Jak1 causing the STAT molecules to heterodimerize. Phosphorylated Stat1 and Stat2 form a complex with IFN regulated factor (IRF) 9. This complex (ISGF3) translocates to the nucleus and binds to the IFN stimulated response element (ISRE)/IFN response factor binding site (IRFE) in the regulatory regions of IFN-stimulated genes, activating their expression (57;58). The promoter of ISG15 contains two ISRE/IRFE binding sites.

The expression of ISG15 also can be regulated by IRFs. ISG15 was one of the first reported targets of IRF3 (59). IRF3 is a 55 kDa protein that is expressed constitutively in all tissues. Viral infection and IFN treatment appear not to stimulate IRF3 expression; however, viral infection seemingly plays a role in phosphorylation and activation of IRF3. Phosphorylated IRF3 translocates into the nucleus and activates the expression of IFN-stimulated genes also by binding to the ISRE/IRFE elements (60).

The ISG15 promoter also contains a PU.1 binding site, which overlaps with the ISRE/IRFE sequence (61). PU.1 is a member of the Ets family of transcription factors. PU.1 expression is B cell and myeloid cell-specific and plays an important role in hematopoiesis and blood cell functions (62-64). PU.1 has been reported to synergistically activate the ISG15 promoter with IRF4 or IRF8 (61), which supports the significantly higher level of ISG15 expression in myeloid cells relative to other cell types. Interestingly, IRF8 knockout mice show increased ISG15 expression in their macrophages but not their B cells (65).

ISG15 expression has been reported to be upregulated following cellular stress signals. ISG15 expression is upregulated by bacterial and viral infections. These infections activate both IRF3 and ISGF3 transcription factors (66;67). Increased ISG15 expression has been observed in the human colorectal cancer cell line HCT116 following gamma irradiation (68), in acute multiple sclerosis lesions (69), in colorectal and breast cancer cells treated with the anti-cancer drug camptothecin (CPT, topoisomerase I inhibitor) (70), in human fibroblasts from patients with ataxia telangiectasia (71), in constitutively activated JNK1β expressing mouse fibroblasts (72), and in leukemic T cells overexpressing the chemokine I-309 (73). The role of ISG15 in these cellular stresses is not known. It is likely that these cellular stresses induce IFN secretion which would result in increased ISG15 expression. Interestingly, additional work related to CPT induced ISG15 expression has been reported recently, indicating an IFN independent induction of ISG15 by CPT (74). According to this report, a much lower level of

ISG15 expression was induced by CPT than by IFN treatment and a very specific 50 kDa ISG15 conjugate was formed upon CPT stimulation (74).

7. ISG15 CONJUGATION, DECONJUGATION, AND MODIFICATION ENZYMES

Protein ubiquitylation involves the coordinated activities of three modification enzymes. They are the activating or 'E1', the conjugating or 'E2', and the ligating or 'E3' enzymes (75). E1 molecules possess conserved ATP-binding domains and active-site cysteine residues that are essential components for the activation of substrates. The E1 enzyme activates ubiquitin in an ATP-dependent manner, adenylating the C-terminus of ubiquitin and then forming a reactive thioester bond between the ubiquitin C-terminus and the catalytic E1 cysteine residue. The activated ubiquitin is then transferred to the active-site cysteine residue of an E2 enzyme. Again, the activated ubiquitin forms a thioester bond with the E2 molecule. With the aid of an E3 ligase, the activated ubiquitin is transferred to a lysine residue of a substrate. In most cases, the modification by the E2 enzyme is mediated via an E3 molecule which acts as a sort of adaptor protein, interacting with the E2 enzyme and the substrate. The roles of E3 molecules can vary, but they all serve to promote the transfer of ubiquitin. The end result is an isopeptide bond formed between the C-terminal carboxylate of ubiquitin and the ε-amino group of a substrate lysine.

Only a single E1 enzyme has been identified for mammalian protein ubiquitylation. Similarly, each of the ubiquitin-like modifiers is activated by a single and a separate E1 molecule. In contrast, there is a multitude of ubiquitin E3 molecules. Database searches indicate that there are on the order of hundreds of E3 molecules (76). E3 molecules recognize and bind specific amino acid sequences or degrons (75;77-79). The large numbers of ligating enzymes imbue the conjugation system with the capacity for fine substrate specificity. Ubiquitin E2 molecules are intermediate in number and more than twenty E2 enzymes have been reported. Using a conjugation system controlled by enzymes at three successive levels allows for careful regulation of ubiquitylation. Additionally, ubiquitin modification can be regulated by a set of deconjugating enzymes.

Ubiquitin deconjugating enzymes (Dubs) play critical roles in the recycling of ubiquitin. These enzymes help ensure that a pool of ubiquitin is made again available for conjugation. Dubs can also play important roles in the processing of newly synthesized ubiquitin, making these molecules available for conjugation as well. Deconjugating enzymes fall into four classes: the carboxyl-terminal hydrolases (UCHs), the ubiquitin-processing proteases (UBPs/USPs), ubiquitin-specific JAMM motif containing metalloproteases, and proteases containing an OTU domain (80-84).

Much of the recent progress in the study of ISG15 has been in the identification of enzymes involved in ISG15 conjugation and deconjugation. The modification of protein substrates with ISG15 utilizes a set of enzymes analogous to the ubiquitin modification system (figure 3). Ubiquitin activating enzyme E1-like (UBE1L) is a 112 kDa protein that has E1 activity specific for ISG15 (85). Human UBCH8 and the murine homologue UBCM8 have been identified as E2 molecules for ISG15 (86;87). UBP43 is an ISG15 specific protease (88). Cellular ISG15 E3 ligases and a putative ISG15 processing enzyme have not been reported.

7.1. ISG15 activating enzyme UBE1L

UBE1L was originally cloned from a human pre-B cell library in 1993 (89). UBE1L is located 140 kb from the D3F15S2 locus in chromosomal region 3p21. Deletion of this 3p21 region was found to be associated with small cell lung carcinoma (SCLCs), non-SCLCs, and other solid tumors (90). A gene within this chromosomal region was thought to be a putative tumor suppressor gene (90-92). Further analysis showed that expression of UBE1L itself was present in normal lung, but interestingly, largely absent from SCLC and non-SCLC (93). By quantitative PCR, it was observed that UBE1L expression in lung cancer cell lines was consistently below 3% of normal lung levels. It is interesting to note that an IFN insensitive leukemia cell line K562 also lacks functional UBE1L (94).

UBE1L possesses a striking homology with the ubiquitin E1, sharing 45% amino acid sequence identity and possessing a conserved active site cysteine (figure 4) (89). In 2001, UBE1L was demonstrated to be an E1 enzyme for ISG15 (85). Using the yeast two-hybrid assay, the influenza B virus protein NS1B was found to interact with ISG15 (85). NS1B was shown to bind to ISG15 and block ISG15 conjugation. A GST-ISG15 fusion protein was then made and used to identify UBE1L as an ISG15 interacting protein. UBE1L was also shown to be capable of forming an adduct with radiolabeled ISG15, to catalyze the activation of ISG15, and to function as an E1 enzyme for ISG15 (85). Ubiquitin E1 cannot replace UBE1L in protein ISGylation (86;87).

Immunostaining of MG37, an EBV-transformed B-cell line, and immunostaining of transfected COS-7 cells revealed that UBE1L was a cytosolic protein (95). However, staining for UBE1L in transfected GLC-45 cells, a SCLC cell line, revealed both a nuclear and cytoplasmic localization. In these experiments, it was noted that not all MG37 cells were positive for endogenous UBE1L expression. Moreover, the staining of transfected GLC-45 yielded a pattern that was described as diffuse. It was hypothesized that the localization and distribution of UBE1L may be cell cycle dependent, as has been reported for the ubiquitin activating enzyme E1 (96).

The expression of UBE1L is upregulated by IFN treatment (97). The upstream regulatory sequence of the UBE1L gene has a conserved ISRE/IRFE binding site which may be responsible for its upregulation following IFN treatment (40). Knowing now that UBE1L is an IFN regulated gene and that it functions as an E1 for ISG15 conjugation, it will be interesting and informative to re-examine UBE1L cellular localization following IFN

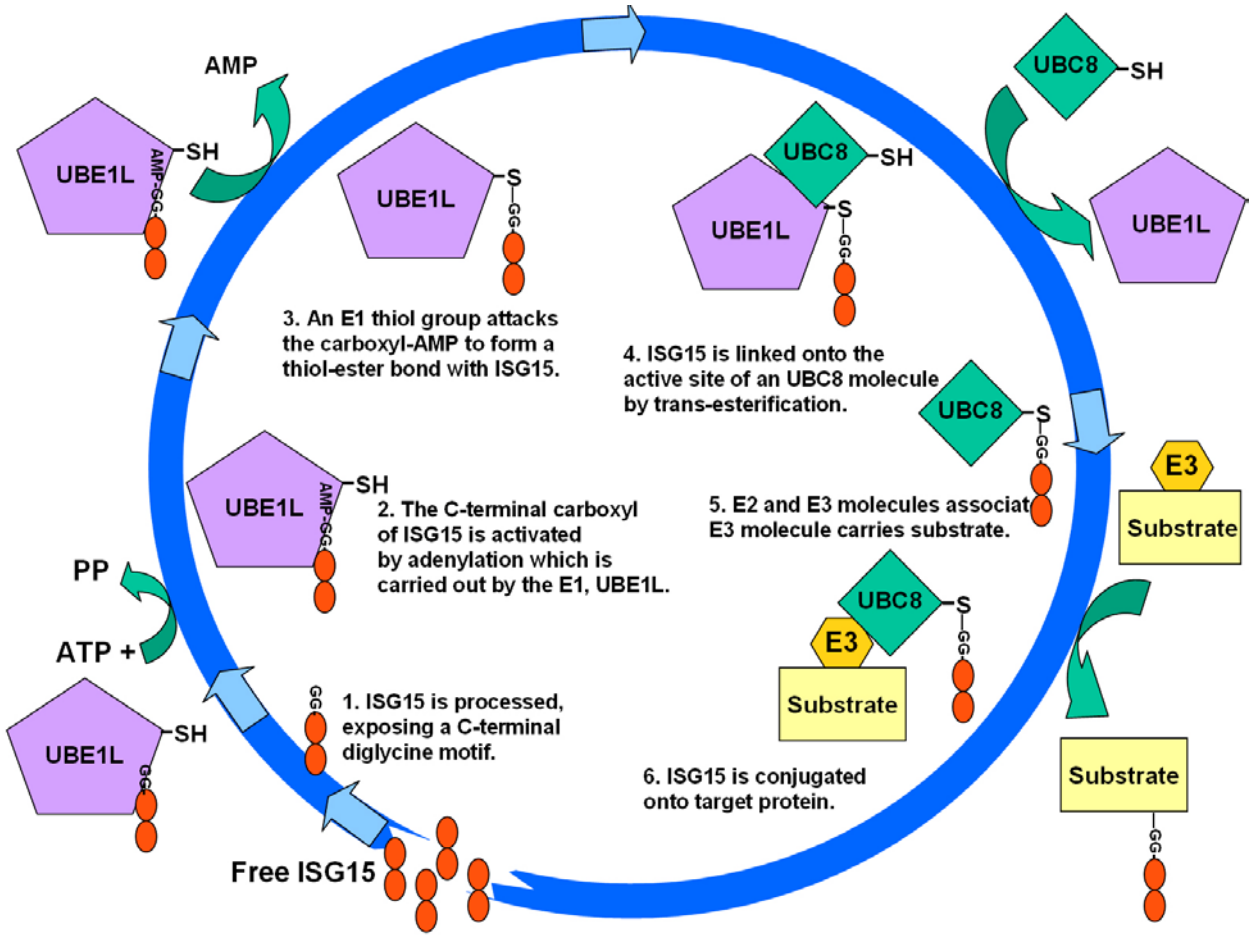

Figure 3. Schematic of the ISG15 conjugation system. ISGylation is proposed to occur in a manner analogous to the ubiquitin modification system. 1. Prior to conjugation, ISG15 is processed, possibly by the protease UBP43 or by a yet to be identified processing enzyme. 2. The E1 or activating enzyme for ISG15 is UBE1L. UBE1L catalyzes the adenylation of ISG15. 3. UBE1L also forms a thioester bond with ISG15. 4. Activated ISG15 is passed onto the E2 enzyme, UBC8, by a trans-esterification reaction. UBC8 can serve as an E2 enzyme for ISG15 and ubiquitin. 5, 6. ISGylation is proposed to occur in a similar manner with the aid of an E3 molecule. RING E3 molecules act as adaptor proteins, bringing substrate into close proximity with E2 enzymes. HECT E3 molecules have the capacity to directly transfer ubiquitin onto substrates. Our lab has identified an E3 enzyme for ISG15 and a substrate which is modified by this E3.

stimulation. Possibly the staining pattern observed in GLC-45 cells would appear less diffuse and more definitive following IFN treatment.

7.2. ISG15 conjugating enzyme UBC8

Two groups have recently and independently reported the identification of UBC8 as an E2 conjugating enzyme for ISG15 using differing approaches (86;87).

In the presence of the activation enzyme and in the presence of ATP, ubiquitin can form a thioester bond with an E2 conjugation enzyme. With the hypothesis that ISG15 would behave in the same manner and form a thioester bond with an E2, Zhao *et al.* performed GST-ISG15 pull down experiments to identify proteins that would covalently link to ISG15 which could be found in protein extracts from IFN treated cells. Through this approach, they identified human UBC8 (UBCH8) as a candidate and went on to further demonstrate that UBCH8 could function as an ISG15 E2 enzyme (86). In addition, they showed *in vitro* that UBCH8 could promote both the ubiquitylation and ISGylation of human WBP2 in the presence of the yeast ubiquitin E3 enzyme Rsp5p.

Kim *et al.* began with the hypothesis that an E2 enzyme for ISG15 was potentially an E2 molecule already identified for the ubiquitin system and that the expression of an ISG15 enzyme would be IFN inducible. This hypothesis was based on the high degree of homology shared between the ubiquitin activating enzyme UBE1 and the ISG15 activating enzyme UBE1L and between the ubiquitin deconjugating enzymes and the ISG15 deconjugating enzyme UBP43, especially within the critical domains of these proteins. Moreover, both UBE1L and UBP43 were known to be IFN-inducible genes (119,172). Therefore, it would be natural to hypothesize

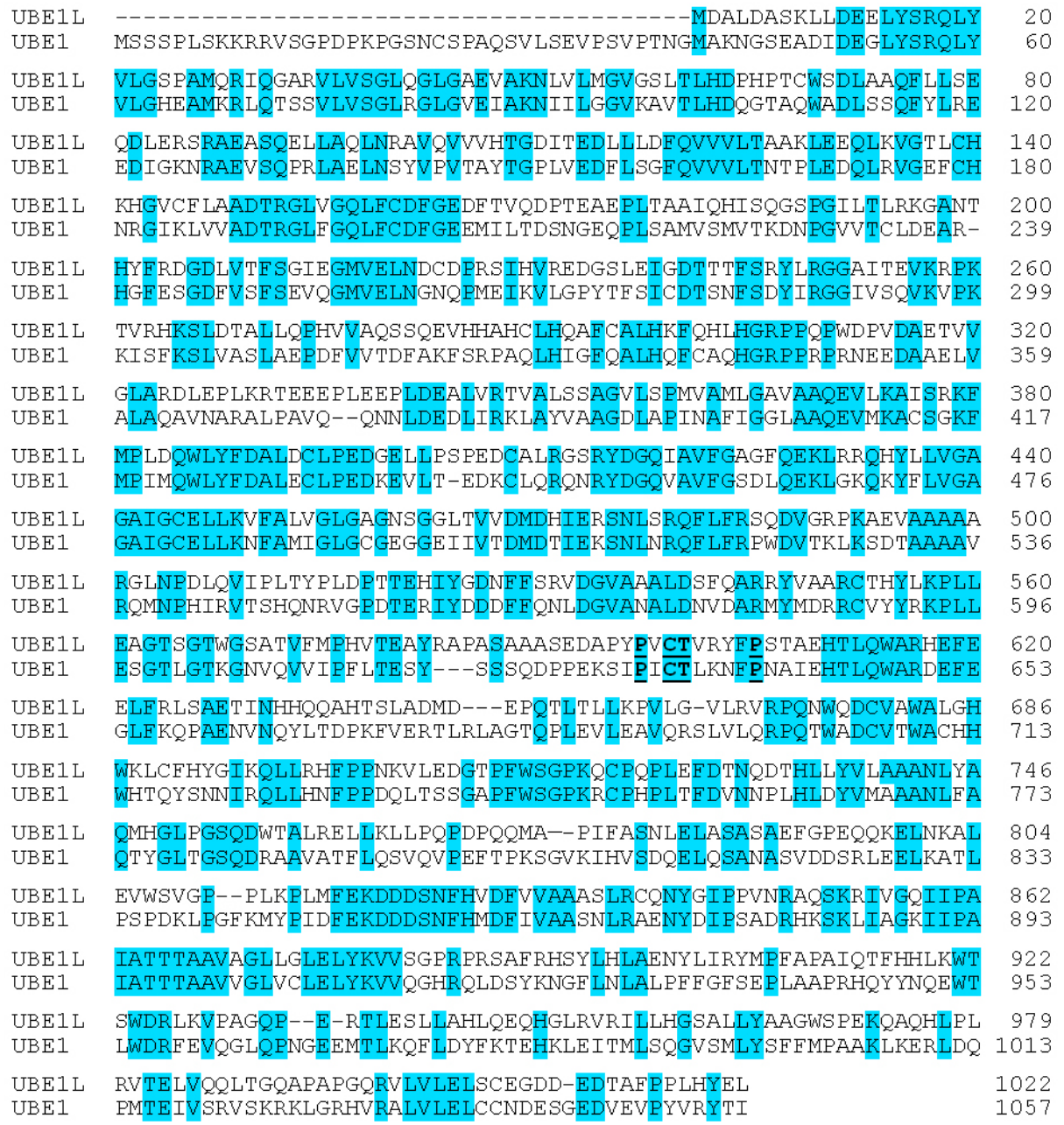

Figure 4. Sequence homologies of the human ISG15 E1 molecule UBE1L (accession number NP_003326.2) and the human ubiquitin E1 molecule UBE1 (accession number AAH13041.1). Sequences were aligned based on the ClustalW method (185). Identical amino acids are highlighted in blue. The conserved active residues are underlined and in bold.

that an E2 enzyme would share the same property. UBC8 and the E2-like molecules 1-8U, 9-27 (also known as Leu13), fit these criteria. 1-8U and 9-27 are among two of the seven earliest IFN inducible genes (98;99). Interestingly, 1-8U and 9-27 have been linked to the antiproliferative effects of IFN (100;101). Protein sequence analysis identified conserved ubiquitin E2 motifs in these proteins and it was proposed that they might function as ISG15 E2s (98;99). Fractionation and microscopy studies indicated that 1-8U is a membrane associated protein and that it is also possibly located in the exosome membrane (100). 9-27 has been reported as a cell surface protein that forms a complex with CD19, CD21, and CD81 in B lymphocytes (102;103).

Using a proteomic approach, UBCH8 and ISG15 were identified as primary proteins induced following stimulation of T cells with IFN-α (104). Enhanced UBCH8

mRNA expression was observed in purified T cells and in macrophages following treatment with IFN-α or IFN-γ and by infection with Sendai virus. UBCH8 mRNA expression was also upregulated in A549 (lung carcinoma), HepG2 (hepatoma), and NK-92 (natural killer) cells. Experiments using the protein synthesis inhibitor cyclohexamide suggested that UBCH8 was among the primary genes upregulated following IFN-α stimulation (104). UBCH8, along with UBCH7, was originally described as a protein that interacted with Papilloma virus E6 associated protein (E6-AP) in a yeast two-hybrid screen (105). UBCH8 possesses 45.7% homology to UBCH7. UBCH7 functions with viral E6 protein and E6-AP in the ubiquitylation of p53 (106-108). The murine homolog, UBCM8, interacts with both the N-terminal and the C-terminal halves of ISG15 (87).

To test whether 1-8U, 9-27, and UBC8 are ISG15 E2 conjugating enzymes, Kim *et al.* developed a transfection based system to assay for ISG15 E2 activity. Expression vectors for ISG15, UBE1L, and a potential E2 were transiently transfected into 293T cells and cell lysates from these transfectants were used in western blot analysis to determine the relative levels of protein ISGylation (87). Only transfection with UBC8 (both human UBCH8 and murine UbcM8), but not 1-8U or 9-27, enhanced ISG15 conjugation in this assay. Furthermore, an active site cysteine mutant of UBC8 lost the ability to increase protein ISGylation. Substitution of UBC8 with the ubiquitin E2 UBCM4 (murine homolog of UBCH7) or the SUMO E2 UBC9 did not result in enhanced ISG15 conjugation. Utilizing RNA interference and both 1D- and 2D-electrophoresis, Kim *et al.* further demonstrated that decreasing UBC8 expression leads to a general reduction of all ISGylated protein detectable in HeLa cells (87). These experiments demonstrated that UBC8 is an ISG15 E2 conjugating enzyme and that it is the major ISG15 E2 in HeLa cells. More importantly, the transfection based assay also provides a system to easily identify and to verify potential ISG15 target proteins. Three known ISG15 target proteins, but not the protein AML1, were shown to be ISGylated using this assay (87).

While several E2 molecules for ubiquitin have been identified, no more than one E2 molecule has been reported for any of the other ubiquitin-like modifiers. Despite this observation, it is still possible that ISG15 conjugation may be served by additional E2 molecules which have yet be identified. Other E2s which have been identified as serving the ubiquitin system may also possess dual activity as ISG15 conjugation enzymes. E2 molecules which may be capable of regulating ISG15 conjugation would be expected to be IFN induced genes. In addition to UBCH8, the mRNA expression of UBCH5 and UBCH6 in both human T cells and macrophages has been shown to increase dramatically several hours post-IFN stimulation (104). While UBCH5 was shown not to form an adduct with ISG15 (86), the observation that its expression is enhanced by IFN-α still makes it an attractive candidate for further examination. Likewise, it would be interesting to determine if UBCH6 can serve as an E2 for ISG15 conjugation. Having now a transfection system used to identify ISG15 conjugating enzymes, it would be interesting to determine if other ubiquitin E2 molecules possess ISG15 conjugating activity.

UBCH8 is able to associate with both ubiquitin E1 and UBE1L perhaps because these activating enzymes share much homology. Of all the E1 enzymes reported for the ubiquitin-like modifiers, UBE1L most closely resembles ubiquitin E1 in sequence. The crystal structure for ubiquitin E1 has not been solved. The sequences for APPBP2-UBA3, the E1 molecule for Nedd8, has been solved, and mutational analysis has been done to map its functions in adenylation, thioester bond formation, and E2 binding (109). The structures of the ubiquitin E1 and the E1 enzymes for other ubiquitin-like modifiers are thought to resemble APPBP2-UBA3, and by analogy the molecular features and critical residues important to their E1 functions are also thought to be very similar. The hypothesis is that the interaction is similar to that of the NEDD8 E1 and E2. Given this hypothesis, the structural basis of the interaction between UBE1L and UBCH8 has not yet been fully demonstrated. Targeted structure-function studies designed to map this interaction could be used to address this hypothesis. UBCH7 is most similar in primary sequence to UBCH8. UBCH7 does not form a thioester with ISG15 in an *in vitro* analysis (86), nor does UBCH7 enhance ISGylation in 293T cells (87). Careful comparison of UBCH8 and UBCH7 sequences may reveal possible insights into the structural basis for ISG15 and E2 association.

The conjugation systems of other ubiquitin-like modifiers generally utilize separate and distinct sets of enzymes. It has been shown that recombinant human NEDD8 can be activated by the ubiquitin E1 *in vitro* (37). It was noted that the activation in this artificial system is very inefficient. The observation that UBC8 is an E2 molecule for ubiquitin and ISG15 is the major exception to this paradigm. The ability of UBC8 to serve as E2 molecules for both ubiquitin and ISG15 underscores the overlap of these two systems. The affinities of UBC8 for ubiquitin and for ISG15 are not known, nor is the efficiency of ubiquitin conjugation relative to ISG15 conjugation known. Certainly the answers to these two questions are of much interest. Since UBC8 and ISG15 expression are activated by IFN, it is reasonable to hypothesize that ISG15 is probably the major substrate of UBC8.

UBCH8 is located on human chromosome 11q12. Deletion of the chromosomal region on which UBE1L is located is associated with human cancer. If the lack of UBE1L ISG15 conjugation activity is somehow related to tumorigenic potential and if UBCH8 is the only E2 which serves ISGylation, the expectation might be that deletion or mutation of UBCH8 might also be associated with cancer development. It is not known if this gene is associated with cancer as a careful analysis of a possible relationship has not been reported.

7.3. Putative ISG15 E3 ligases

E3 enzymes play primary roles in dictating substrate specificity. Mechanistically, ubiquitin E3

enzymes have been divided into two major groups, molecules containing homologous to E6AP carboxy terminus (HECT) domains (110), and non-HECT E3 ligases, all of which contain a really interesting new gene (RING) finger domain or structurally-related domains such as the U-box domain (38;111;112). HECT E3 molecules accept ubiquitin from an E2 molecule forming a thioester adduct between the HECT E3 and ubiquitin. Ubiquitin is transferred from the active-site cysteine of the HECT E3 ligase to a substrate via a covalent attachment to an ε-amino group of a lysine side chain (113). RING E3 molecules serve as docking proteins, bringing together E2 molecules and substrate (114). RING domains contain two zinc ions coordinated by cysteines and histidines in a cross-brace structure (115). Unlike HECT E3s, RING E3 molecules are non-catalytic.

The similarities between the ubiquitin and ISG15 conjugation systems are uncanny, and the observation that UBC8 is an E2 enzyme for ubiquitin and ISG15 highlights the similarities. The observation that UBC8 can serve as an E2 molecule for ISG15 may suggest that E3 molecules which associate with UBC8 may also be E3s for ISG15. Several HECT and RING E3 molecules have been reported to associate with UBC8 (105;116-119). Double ring finger protein (Dorfin), E6AP, human homologue of *Drosophila* ariadne (HHARI), UBCH7-associated protein 1 (H7-AP1), and Parkin are E3 molecules which have been reported to bind to UBC8.

UBCH7 and its mouse ortholog UbcM4 are highly homologous (55%) to UBC8. It is possible that the reported E3 enzymes which act in conjunction with UBCH7/M4 to ubiquitylate proteins could also function as ISG15 E3 enzymes. As E3 enzymes are finely tuned to recognize specific substrates, the possibility that known protein targets of UBC8 and UBCH7/M4 ubiquitylation may also be targets of ISG15 modification, is an intriguing one. Our lab has identified both an ISG15 E3 and an associated ISG15 substrate based on this hypothesis (Zou *et al.* paper in preparation). ISG15 modification of this substrate can clearly be seen following type I IFN stimulation. The identification of additional ISG15 ligases and substrates modified by these enzymes is eagerly anticipated.

7.4. ISG15 deconjugating enzyme UBP43

UBP43, also known as USP18, possesses cysteine and histidine boxes that are highly conserved and characteristic of enzymes of the UBP family (120;121). Members of the UBP family of proteases are involved in the removal of ubiquitin from conjugated proteins (84;122). Analysis using ^{125}I-labelled ubiquitin-like modifer fusion peptides, including ubiquitin-gsPESTc, SUMO-gsPESTc, Nedd8-gsPESTc, and ISG15-gsPESTc, demonstrated that UBP43 preferentially removes ISG15 from its conjugates (88). Additional studies using UBP43 expressed cells confirmed that UBP43 is an ISG15 specific protease (88). UBP43 was first reported by our laboratory during the analysis of abnormally expressed genes in AML1-ETO knock-in mice (120;121). AML1-ETO is a fusion protein generated from the 8;21 chromosomal translocation and is associated with abnormal blood cell differentiation and proliferation. Heterozygous AML1-ETO knock-in mice die around the E12.5 stage of embryo development (123). UBP43 is highly expressed in hematopoietic related organs, including both the yolk sac and fetal liver, in AML1-ETO knock-in embryos relative to the wild type controls. The cloning of UBP43 was also independently reported by three other groups. Zhang *et al.* discovered the cDNA encoding a 43 kDa protein in pig alveolar macrophages infected with porcine reproductive and respiratory syndrome virus. The expression of this gene was also found to be highly upregulated *in vivo* by infection of pigs with the same virus (124). Li *et al.* cloned UBP43 as a potential RNase-L substrate in a differential display of N1E-115 cell RNA (125). They determined that UBP43 was an IFN stimulated gene. UBP43 was also identified by Kang *et al.* as an IFN-induced gene in an analysis of differentially expressed genes of type I IFN treated human melanoma cells. They demonstrated that UBP43 was induced by IFN treatment in over 20 human cell lines and that its induction occurred via the JAK-STAT signaling pathway (126). UBP43 promoter possesses highly conserved ISRE/IRFE sequences (127).

Although UBP43 is the only currently known ISG15 deconjugating enzyme, it is possible that additional ISG15 proteases exist. Recombinant ISG15 modified with a C-terminal vinyl sulfone moiety was generated and used to trap and identify associating enzymes (128). Using this approach, three ISG15 adducts of varying molecular weights were identified. These adducts had estimated molecular weights of ~70, ~110, and ~210 kDa. The 210 kDa adduct was identified by tandem mass spectrometry analysis as isopeptidase T (IsoT)/USP5. IsoT/USP5 is a 93 kDa ubiquitin-specific cysteine protease from the UBP family involved in the disassembly of polyubiquitin chains (129). It has been proposed that IsoT has two (possibly four) binding sites for ubiquitin (130;131). It was speculated that the multiple binding sites may allow IsoT to accommodate ISG15 which resembles two ubiquitin molecules connected in tandem and that IsoT may have dual specificity for ubiquitin and ISG15 (128). However, no activity toward ISG15 conjugates was reported.

A UBP43 knockout mouse was generated and, UBP43 deficient cells from these mice showed higher basal levels and higher IFN inducible levels of ISG15 conjugation. Furthermore, no obvious change in protein ubiquitin conjugation was detected (132). These data further confirmed that UBP43 is a bona fide ISG15 protease. Homozygous UBP43 knockout mice having a C57B and 129 mixed strain background are born alive but suffer from a decreased lifespan and from neurological abnormalities, including convulsions, tremor, loss of balance, rolling, and repeated tight circling. More detailed histological analyses reveal that the UBP43 deficient mice have ependymal cell death in their brains and that this cell death disrupts the normal boundary between neuronal tissue and the brain cavity, leading to aqueduct stenosis and subsequent hydrocephalus due to blockage of normal cerebral spinal fluid flow (132). Hydrocephalus could also be observed in the brains of some 14.5 day post coitum UBP43 deficient embryos (our unpublished data). UBP43

null mice with increased C57 strain background showed less neurological related problems, however, UBP43 null mice backcrossed more than 10 generations into the C57 background showed abnormal embryonic development and were embryonic lethal (our unpublished data).

Recently, UBP43 was reported to associate with the Skp2 oncoprotein and UBP43 was also found to be a novel substrate for Skp2-mediated ubiquitylation (133). The association between the two proteins was discovered via a yeast two-hybrid screen. Skp2 belongs to the family of F-box proteins which comprise the Skp1, cullin, F box proteins (SCF) E3 ubiquitin ligase complexes. Binding of UBP43 to SCF^{Skp2} resulted in its multi-ubiquitylation and proteasomal degradation. Furthermore, in Skp2 deficient MEF cells, levels of UBP43 are upregulated and levels of ISG15 conjugates are reduced. These findings demonstrated the control of UBP43 protein levels by SCF^{Skp2}.

7.5. ISG15 processing enzymes

The candidate processing enzyme which generates the mature form of ISG15 ending in a di-glycine motif has not been cloned but has been described (134). The processing enzyme was purified to homogeneity from cell extracts of A549 cells using a combination of ion exchange and hydrophobic chromatography. This enzyme, whose activity is unaffected by IFN treatment, but interestingly is stimulated 12-fold by micromolar concentrations of ubiquitin, was determined to be approximately 100 kDa in size. Moreover, its processing activity is irreversibly inhibited by thiol-specific alkylating agents and exhibits pH dependence, suggesting that the enzyme may be a thiol protease. Partial sequencing indicated that the enzyme could be the human ortholog to yeast Ubp1 (134).

UBP43 may also be capable of processing the immature form of ISG15. Since UBP43 knockout cells still process ISG15, it is at least not the major processing enzyme for ISG15.

8. TARGETS OF ISG15 MODIFICATION

Evidence for ISG15 protein conjugates was first reported in 1992. Multiple heterogeneous high molecular weight conjugates of ISG15 were detected. Of the seven cell lines tested, all experienced an increase in free ISG15 following IFN stimulation. Enhanced protein ISGylation was only detected in four of the seven tested cell lines, including A549, MG-63, U937, and Molt-4 (56). ISG15 conjugates were not detected in three other cell lines, including the BCR/ABL+ leukemic cell line K562 and two Burkitt's lymphoma cell lines, Daudi and Namalwa cells. Interestingly, transfection of functional UBE1L into K562 cells restores ISGylation within these cells (94).

Upon type I IFN stimulation, protein ISGylation can be induced in HeLa, COS-7, RAW264.7, NIH3T3, 2fTGH, L929, A431 and KT-1 cells, but not in Vero and 293T cells (127;135) (our unpublished data). The reason these cells express free ISG15 but lack conjugates is not known. Potentially these cells may lack functional ISG15 E1 or E2 enzymes. It is also possible that these cells express factors that may inhibit ISG15 processing, or E1 and E2 activities. Stat1-deficient U3A cells lack ISG15 conjugation following IFN treatment, but the Stat1-positive human cell line 2fTGH (136), which is the parental cell line of U3A, shows the formation of ISG15 conjugates following IFN treatment (our unpublished data), emphasizing the importance of IFN signaling cascade in this process. ISGylation is detected in many primary tissues of mice including liver, lung, heart, kidney, thymus, spleen, bone marrow, and brain following injection with LPS or polyI-C. ISGylation can also be induced in murine embryonic fibroblast (MEF) cells by IFN treatment (94;132;137;138).

The functions for ISG15 modification are currently not clear. Possibly, one of the keys to understanding ISGylation is the identification of substrates and specific ISG15 modification sites within these proteins. To date, only five ISG15-conjugated proteins have been reported in the literature. Serpin 2a was the first ISG15 target to be identified. In 2002, it was demonstrated by mass spectrometric analysis that serine protease inhibitor 2a (Serpin 2a) was ISG15 modified (139). The sequence of murine serpin 2a most closely resembles human anti-chymotrypsin, sharing ~60% identity (140). Serpin 2a is highly activated in macrophages upon bacterial infection (139). In FDCP-Mix cells, Serpin 2a becomes dramatically down-regulated following differentiation (141). Serpin 2a is an intracellular protein which localizes to both the cytoplasm and nucleus and has a prominent nuclear localization in FDCP-Mix cells and in COS-7 cells transfected with Serpin 2a (140). Interestingly, ISG15-modification of Serpin 2a did not appear to alter its localization (139).

Jak1, Stat1, phospholipase C (PLC)-γ1, extracellular regulated kinase (ERK) 1/2 were also identified as ISG15 targets (138). Jak1, PLC γ1, and ERK1/2 were identified by a combination of immunoprecipitation and high throughput western blotting. ISG15 modification of Stat1 was tested following the identification of the other three signaling molecules. Upon IFN stimulation, the endogenous ISG15 modified forms of these proteins were identified (138).

Jak1 and Stat1 are important molecules involved in the transduction of signals following type I IFN stimulation (3). They also play important roles in signal transduction cascades of other cytokines (142). Stat1 and Jak1 deficient mice have been generated (143-145). Stat1 and Jak1 deficient cells lack IFN related functions and are sensitive to viral infections.

PLCγ1 is an important enzyme in many cellular processes, including proliferation, differentiation, embryonic development, oxidative stress responses, many hormone dependent responses, and activation of specific immune responses (146). At least 11 phospholipase C isozymes have been identified in mammalian cells. Members of the PLC family hydrolyze phosphatidylinositol

(4, 5) biphosphate into inositol (1, 4, 5) triphosphate (IP3) and diacylglycerol (DAG) (147-149). IP3 and DAG serve as important second messengers by mobilizing calcium flux and activating protein kinase C, respectively (149).

ERK1 and ERK2 are protein kinases that are involved in the regulation of signal transduction following stimulation by growth factors and by other environmental signals. ERK1 and ERK2 pathways can be activated by several different stimuli, including growth factors, viral infection, cytokines, ligands for G-protein coupled receptors, transforming agents, and some carcinogens. Both ERK molecules are components of a signal transduction cascade which can be activated by ras (150).

It is possible that ISG15 modification may play an important role in modulating cellular signals because these four targets identified play important roles in signal transduction cascades; however, because the high throughput approach used to identify ISG15 targets was specialized to analyze signal transduction molecules, there is much hesitation about reaching this conclusion. Identification of additional targets may give a clearer idea about possible functions for ISGylation.

With the development of an *in vitro* conjugation system (86) and a transfection-based conjugation system (87), it is likely that more ISG15 modified proteins will be reported. However, it is important to emphasize that those proteins identified using these systems should be verified *in vivo* to ensure that ISG15 modification of these proteins is biologically relevant.

The molecular sites of ISG15 modification are not known. Identification of the sites of modification is an important step in elucidating the function of protein ISGylation. Comparison of protein sequences between the limited number of ISG15 modification targets have failed to reveal a consensus motif. Possibly the identification of more targets will help to reveal a common site. One of the roadblocks in determining additional targets of modification and in deducing the sites of ISG15 modification is the relative amount of ISG15-modified target protein versus total target protein. It is estimated that only one to five percent of the currently known target proteins become ISG15-modified following IFN stimulation. It is possible that analysis of ISG15 targets will show proteins that are more abundantly modified by ISG15. SUMO-modified targets range in the extent to which their proteins are modified. Modification of some SUMO targets is barely detectable while modification of RanGAP1 under some circumstances can approach ~90% of total protein (151). Further work in developing the *in vitro* conjugation system with inclusion of ISG15 E3 ligases may be an invaluable set of tools in the generation of large quantities of ISG15-modified protein.

9. POSSIBLE ROLES OF ISG15 AND PROTEIN MODIFICATION BY ISG15

A definitive role for ISG15 conjugates has not yet been demonstrated. ISG15 conjugation almost certainly has significant roles in vertebrate biology. The evolution and preservation of ISG15 itself along with its conjugation and deconjugation enzymes strongly suggest the importance of this modification system. Several possible roles for protein ISGylation have been reported in recent literature.

9.1. Possible role as a cytokine

Extracellular ISG15 has been reported to have cytokine-like activity. Recombinant ISG15 produced in *E. coli* stimulated IFNγ production by $CD3^+$ cells but not by $CD14^+$ or $CD56^+$ cells harvested from human peripheral blood (42;152). Recombinant ISG15 stimulates the proliferation of natural killer (NK) cells and enhances NK cell cytotoxicity. $CD3^+$ T cells, but not B cells, were required for the ISG15-induced proliferation and cytotoxicity of NK cells. Exogenous ISG15 did not activate the production of IL-2 or IL-12 by T cells or NK cells. IL-2 and IL-12 are known to induce NK cell proliferation (45). Interestingly, ISG15 cytokine-like activity was observed with only the smaller processed form and not with the unprocessed pro-ISG15 molecule (43). The cytokine activity of extracellular ISG15 also shows species specificity, as human ISG15 is unable to stimulate murine cells (43).

ISG15 can also be released by human peripheral blood mononuclear cells (PBMCs) (152). ISG15 released by PBMCs had no effect on growth or 2'5'-A synthetase activity in Daudi, U937, or HL60 cells. However, when incubated with fresh PBMCs, free ISG15 augmented LPS-induced monocyte cytotoxicity against WEHI-164 targets. This enhanced cytotoxicity could be inhibited with a rabbit anti-ISG15 polyclonal. Cytotoxicity induced by ISG15 could be virtually eliminated using neutralizing anti-TNF-α antibody. This implied that ISG15-mediated enhancement of cytotoxicity acted via secretion of TNF-α.

More recently, extracellular ISG15 was identified to be a novel chemotactic factor for neutrophils (153). Neutrophils are the most abundant circulating leukocytes in humans and are often the first effectors to the site of infection. A novel neutrophil chemotactic factor was isolated from *Plasmodium yoelii*-infected red blood cells, and this factor turned out to be ISG15 (153).

Ubiquitin, which is also found in human serum, has been reported to have cytokine-like activity. Released ubiquitin has been reported to have anti-inflammatory properties, inhibiting TNFα production by LPS-activated peripheral blood mononuclear cells (154). Released ubiquitin is reported to be involved in regulating immune responses to sepsis and trauma. Like extracellular ubiquitin, released ISG15 lacks a reported receptor. Description of a cellular receptor and an associated signal transduction cascade would go great lengths towards making ISG15 a *bona fide* cytokine. It is, however, tempting to speculate on the nature of a putative ISG15 receptor. Having been reported as a chemotactic factor of neutrophils and as being able to activate NK cells, these cells may be good candidates for the identification of an ISG15 receptor. Extracellular ISG15 and its receptor may

be used to signal states of cellular stress. While IFNs are generally thought to act in a paracrine or autocrine fashion, extracellular ISG15, which can be found at high levels in serum, may serve as a mechanism whereby a danger signal can be transmitted longer distances.

9.2. Possible role in regulation of IFN signaling

Binding of type I IFN to its receptor initiates the JAK-STAT signaling pathway. In mice lacking the ISG15 specific protease UBP43, several events associated with IFN signaling appear dysregulated (94). Cells from ubp43$^{-/-}$ mice are hypersensitive to type I IFN stimulation. Bone marrow cells from ubp43$^{-/-}$ mice undergo apoptosis following IFN treatment. ISG15 conjugates can be detected in UBP43 deficient cells even in the absence of exogenous IFN stimulation and following IFN treatment, the levels of ISG15 conjugation are dramatically increased in comparison to wild-type cells. In cells lacking UBP43, there is an increased and prolonged phosphorylation of Stat1. There are also increases in DNA binding of ISGF3 and expression of IFN-stimulated genes (94). The enhanced and prolonged signaling events observed in knockout cells result from the lack of UBP43. Possibly, these events are related to the increased presence of ISG15 conjugates, but this remains to be demonstrated. Since the two key players in type I IFN signaling, Jak1 and Stat1, can be modified upon IFN stimulation, it is natural to link their ISGylation with the IFN hypersensitivity observed in UBP43 deficient cells. However, there is no current direct experimental data support this notion. Furthermore, the increased phosphorylated Stat1 observed in UBP43 deficient cells following IFN treatment was not observed to be ISG15 modified. It is possible that ISGylation of Jak1 and Stat1 are not related to the increased IFN signaling in UBP43 deficient cells.

Analyses of the UBP43 knockout mice and of UBP43 deficient cells have produced information about the possible roles for protein ISG15 conjugation. However, it should be noted that UBP43 may have functions independent of protein ISGylation. ISG15 and UBE1L knockout mice would be very useful in elucidating the functions for ISG15 and of ISG15 modification. Since free ISG15 has been reported to have cytokine function in stimulating IFN-γ production, UBE1L knockout mice would more clearly demonstrate the role of protein ISGylation.

9.3. Possible role in innate immunity and anti-viral and -bacterial infections

IFNs possess important immunologic roles. Treatment of type I IFN increases the expression of major histocompatibility complex (MHC) molecules, activates the cytotoxicity of lymphocytes, and enhances the phagocytic activity of macrophages (11). Type I IFNs strongly activate the production of ISG15 and the formation of ISG15 conjugates. Konzi and Pitha reported that IFN-ω is a stronger inducer of ISG15 expression in PHA-stimulated human PBMC than IFN-α2 and IFN-ω is more efficient in blocking human immunodeficiency virus (HIV) replication than IFN-α2 (155). Therefore, they analyzed whether ISG15 has any effect on HIV replication. NL4-3 HIV-1 provirus DNA was transfected into the CEM X 174 human lymphoblastic cell line either in the presence or absence of a plasmid expressing human ISG15. Nuclear and cytoplasmic RNAs were harvested 48 hours posttransfection. Northern blot analysis revealed that a noticeable increase of nuclear 9.2 k HIV-1 RNA signal and a dramatic decrease of cytoplasmic HIV-1 RNA, including 9.2 k unspliced form and 4.2 k and 2.0 k mature forms, in cells transfected with the ISG15 expressing plasmid (155). This is the first report that linked ISG15 directly to cellular anti-viral responses. It will be interesting to see how significant the increased level of ISG15 protein and whether there is also an increase of protein ISGylation in these cells transfected with the ISG15 expression plasmid.

Several interesting observations, of particular immunologic interest, have been made with the UBP43 deficient mice. Mice lacking expression of UBP43 are resistant to intracerebral (i.c.) injection of lymphocytic choriomeningitis virus (LCMV) (156). In contrast, wild-type mice die between six to eight days post-injection with LCMV. UBP43 knockout mice have high levels of basal ISGylation within their meningeal cells, ependymal cells, and the cells of the choroid plexus, which have been demonstrated to express UBP43 and are targeted by LCMV infection (132;156). UBP43 knockout mice have much lower viral loads than wild type mice after i.c. LCMV injection.

MEFs from the knockout mice also show enhanced resistance to infection by Sindbis virus (156). No differences in viral titers were observed between wild-type and UBP43 knockout MEF cells 30 hours following Sindbis virus infection. However, titers taken 4 days after infection showed that ubp43$^{-/-}$ mice had significantly lower viral titers. Moreover, less IFN was required to inhibit viral replication in UBP43 knockout mice. Resistance to vesicular stomatitis virus infection is also observed in both UBP43 deficient mice and MEFs (156).

Based on a set of observation of ISG15 localization, a mechanism for a putative anti-viral activity of ISGylation was proposed in 1994. Loab and Haas report examined the localization of ISG15 and its conjugates in A549 lung epithelial carcinoma cells (157). The immunostaining using an ISG15-specific antibody yielded a pattern that was cytosolic and which appeared primarily perinuclear. Moreover, the immunostaining patterns also suggested that these proteins associated with the cellular cytoskeleton. In a set of cellular extractions, actin, tubulin, and soluble cellular proteins were removed, leaving an intermediate filament network of vimentin and cytokeratin. ISG15 signals were observed to remain associated with this network, and it was concluded that ISG15 proteins associate with the intermediate filaments of cells but also that these cytoskeletal proteins were not direct targets of ISGylation. The cytoskeletal network provides viral proteins with sites for virus particle assembly (158). Many viruses also induce morphological changes in cytoskeletal architecture (159). It was hypothesized that the adsorption of ISG15 proteins to the intermediate filament network inhibits the binding of viral proteins to these sites (157).

The observations that influenza viruses can modulate the regulation of the ISG15 system also points to a potential anti-viral role for ISG15 conjugation (85). Influenza A virus does not activate ISG15 synthesis in A549 cells due to a block in the IFN response (85). This is in contrast to the influenza B virus which does show ISG15 upregulation following infection. However, although ISG15 is expressed, its conjugation is blocked by the influenza B viral protein NS1B, which directly interacts with the ISG15 protein. It is hypothesized that the direct interaction between these two proteins prevents the binding of ISG15 to its E1 activating enzyme UBE1L and results in the lack of protein ISGylation upon influenza B infection (85).

Most recently, we analyzed the effect of lacking UBP43 expression on LPS signaling and cellular immune responses against Gram negative bacteria infection (Kim *et al.* manuscript submitted). UBP43 deficient mice are hypersensitive to LPS treatment and more efficient in restricting the growth of *Salmonella typhimurium* than wild-type mice.

9.4. Possible role in pregnancy

The role of ISG15 in pregnancy has been best described in ruminant species. In ruminants, expression of IFN-τ is activated and responsible for the establishment of early pregnancy (160-162). In primates, it is chorionic gonadotropin which is responsible for establishment of pregnancy (163). IFN-τ has a 45-70% homology to mammalian type I IFNs. ISG15 expression is upregulated in the endometrium of humans (164;165), baboons (165), cows (44;166-169), sheep (170-173), swine, and mice (174) in response to IFNs produced by embryos. Moreover, the placenta is enriched with macrophages which produce IFN when activated. The expression of bovine ISG15 is increased in the endometrium during early pregnancy (44). Invasion of receptive uterine epithelium by the conceptus during the initiation of pregnancy induces the decidual response, and this event is characterized by the activation of angiogenesis and inflammation. ISG15 is present in decidual cells of pregnant tissue during the first and second trimesters. The observation that ISG15 expression increases during pregnancy is interesting, but its involvement in the process is not yet clear.

9.5. Possible role in cancer

The possible relationships between ISG15 and cancer are intriguing. As mentioned in a previous section, the ISG15 activating enzyme UBE1L is not present in many lung cancers and other solid tumors and, an IFN insensitive leukemia cell line lacks functional UBE1L (89;94). Furthermore, the increased expression of ISG15 protease UBP43 was observed in mice expressing AML1-ETO (120). The AML1-ETO fusion protein expression is specifically related to acute myeloid leukemia characterized by the 8;21 chromosome translocation (175). These reports indicate a possible role for protein ISG15 modification system in cancer development. However, molecular mechanisms, including particular targets, related to cancer development are not known.

A possible link between extracellular ISG15 and presentation of tumor antigens has been made. The melanoma cell lines Me76.3 and Me67.9 secrete type I IFN and extracellular ISG15. More than 60 units/ml of type I IFN and 200 pg/ml of ISG15 have been detected in the media of cultured Me76.3 and Me67.9 cells. Extracellular ISG15 was responsible for the upregulation of E-cadherin in co-cultured immature dendritic cells (176). E-cadherin is a catenin-associated cell surface adhesion molecule whose expression increases cellular adhesion, and E-cadherin has been associated with decreased metastasis of tumor cells (177). The increase of E-cadherin expression in dendritic cell has been proposed to decrease the ability of these dendritic cells to present tumor-specific antigens by inhibiting their migration from tumor sites.

ISG15 expression and conjugation has been shown to be induced in NB4 cells, a human acute promyelogenous leukemia (APL) cell line, following treatment with all-trans retinoic acid (ATRA) (178). ATRA is used to treat APL patients expressing the promyelocytic leukemia/retinoic acid receptor α (PML-RARα) fusion protein. ATRA triggers myeloid differentiation and treatment with ATRA terminally differentiates APL cells (179-181). ATRA treatment has been reported to induce IFN secretion in NB4 cells (182). We also observed IFN release in ATRA-stimulated NB4 cells and observed that this IFN secretion is not solely, but largely responsible for ATRA-induced ISGylation (Dao & Zhang, paper in preparation).

Expression of UBE1L is upregulated following ATRA treatment in NB4 cells (178). Promoter sequences of UBE1L have been reported to possess retinoic acid receptor element half-sites. Whether these half-sites are sufficient for direct activation by retinoic acid is not known. It is interesting to point out that the UBE1L promoter contains IFN stimulating response element (ISRE) and it is an IFN inducible gene (26;40). In co-transfection experiments it was shown that the co-transfection of UBE1L with PML/RARα could result in the enhanced degradation of the oncogenic fusion protein (178). Degradation of the fusion protein could be inhibited with proteasome inhibitors and degradation of PML/RARα was directly related to expression of UBE1L. Overexpression of UBE1L in NB4 cells using a retroviral transduction system induced apoptosis of these cells (178).

9.6. Possible role in protein turnover

Due to the similarities between ubiquitin and ISG15, a possible role for ISG15 in protein turnover has been explored by several groups. The involvement of ubiquitin-like modifiers in protein turnover is not unprecedented. Proteins modified by NEDD8 are degraded by the proteasome facilitated by the adaptor protein Nedd8 ultimate buster 1 (NUB1) (183). The mechanism does not occur directly through Nedd8 conjugation but rather indirectly through the recruitment of a ubiquitin conjugating protein complex.

It was observed that the inhibition of cellular proteasomes resulted in increased levels of ISG15

conjugates, while addition of lysosomal protease inhibitors did not appear to significantly increase ISG15 conjugates (135). In these experiments, cells were first treated with 1000 unit/ml IFN-α for 24 hours then incubated with proteasome inhibitors, such as 20 μM MG132 or lactacystin, for an additional 6 hours. Yet, in the presence of ATP depleting reagents, treatment with proteasome inhibitor MG132 did not significantly enhance the levels of ISG15 conjugation. Levels of ubiquitin conjugates were enhanced following ATP depletion and inhibition of proteasomal degradation. These observations suggested that the effect of MG132 in increasing ISG15 conjugate levels required *de novo* ISG15 conjugation. The expectation was that ATP-depletion would abrogate ATP-dependent ISG15 and ubiquitin conjugation. In the absence of *de novo* formation of these conjugates, the block of proteasome activity should increase the level of total conjugates if the conjugates are normally degraded via the proteasomal pathway. Since no increase of ISG15 conjugates was observed upon blocking both de novo ISG15 conjugation and proteasomal degradation, the authors conclude that ISG15 conjugates are not degraded through the proteasome pathway, however, the proteasome somehow modulates protein ISGylation (135).

Using a set of different ISG15 specific antibodies and MEF cells from wild-type and UBP43 deficient mice, it was reported by a second group that a brief treatment (three hours) with the proteasome inhibitors MG132 (10 μM) and lactacystin (5 μM) did not affect the levels of protein ISGylation in the absence or presence of IFN stimulation (200 unit/ml IFN-β for 18 hours) (138). Using these conditions, inhibition of proteasomal degradation increased levels of ubiquitylated proteins but had no significant effect on the levels of ISGylated proteins. Moreover, when a specific ISG15 target was examined, there was no appreciable difference in ISG15-modified PLCγ1 in the presence or absence of the proteasome inhibitor (138).

In summary, although two groups showed opposite effects of proteasome inhibitors on the level of cellular protein ISGylation, both groups had concluded that protein ISGylation does not target proteins for degradation through the proteasomal pathway. However, the possibility that ISG15 modification affects protein degradation via other pathways could not be excluded. Furthermore, the different effects of proteasome inhibitors on the level of total cellular ISGylated proteins observed by the two groups may be related to the different concentration of IFNs and proteasome inhibitors used in the assay and the different lengths of time in which the cells were treated. It is well known that IFN responses can be elicited by various cellular stresses, including the toxicity of various drugs.

10. PERSPECTIVES

There are indications that ISG15 may be an important component in the innate immune response to viral and bacterial infections, IFN signaling, protein turnover, pregnancy, and cancer biology. The role of protein ISGylation in these very important biologic processes is not entirely clear and requires further investigation. Certainly, generation and characterization of ISG15 knockout mice and UBE1L knockout mice are a priority and reports about the phenotypes of these mice are eagerly awaited. How will these mice respond to viral and bacterial infections? How will they respond to IFN or LPS treatments? Will they show neurological abnormalities? Will they be more or less susceptible to certain types of cancer? These mice may ultimately reveal the function for ISG15 and ISG15 conjugation. Also of high priority is the identification of ISG15 modification site(s) in its target proteins. Two important questions are: 1) Are ISG15 targets modified via a common structural feature or sequence motif, and 2) What are the consequences of eliminating the modification site(s)?

There are also a number of other pressing questions. What other targets are modified by ISG15? Do ISG15 targets fall into discernable families or categories? Does ISG15 modify a particular set of proteins analogous to Nedd8 modification of cullin family members? What is the half-life of a protein which becomes ISG15-modified? Does ISG15-modification increase or decrease the half-life of the modified protein?

The observation that ISG15 conjugates may associate with the cellular cytoskeleton is also an interesting observation. What are the consequences of this localization? How do ISG15 conjugates localize to the cytoskeleton and what structural properties impart ISG15 conjugates with this localization?

The localization of UBCH8 has not yet been reported in the literature. Localization of ISG15 E1, E2, and E3 molecules may be important pieces of information as they may give insight into the potential sites of ISG15 modification. Identification of additional ISG15 targets may also help to answer questions about sites of ISG15 modification. Knowledge of the target proteins and the cellular compartments through which they traffic and reside may identify the cellular sites of modification and deconjugation. Interestingly, the localization for UBP43 has been reported to be mainly nuclear when a GFP-UBP43 fusion protein is expressed in transiently transfected cells (121). The consequence of this is not known, but the observation poses a couple of interesting questions. Do conjugates traffick into the nucleus where ISG15 is cleaved? Can ISGylation occur within the nucleus? A recent publication reported ISG15 and/or its conjugates within multiple cellular compartments, including the cytosol, nucleus, mitochondria, endoplasmic reticulum, Golgi, and perinuclear space (184). This report does not necessarily contradict the original ISG15 localization studies, as there may be cell type differences and as the antibodies were generated differently and probably recognized differing epitopes. It does however suggest that the localization studies warrant further examination.

The identification of UBC8 as a major ISG15 E2 poses the question: Is UBC8 more efficient at ubiquitylation or ISGylation? The identification of an E2 molecule serving both ubiquitin and ISG15 conjugation raises the possibility that other ubiquitin E2 molecules may

also be capable of mediating ISGylation. It would also be interesting to know whether E3 molecules identified for UBC8 also act as E3 molecules for ISG15.

ISG15 and its associated enzymes could prove to be molecules with high therapeutic potential. ISG15 and UBP43 appear to play important roles in IFN signaling, although the mechanism of the action is not known. It remains to be seen if the isopeptidase activity of UBP43 is directly related to the heightened IFN responses seen in UBP43 knockout mice and cells or, whether there is another function of UBP43 that is responsible for the observed phenotypes. If the prolonged presence of ISG15 conjugates results in prolonged and increased IFN signaling, one can envision the value of UBP43 as a therapeutic target. By targeting the activity of UBP43, it may be possible to modulate IFN responses.

Although much recent progress has been made to deduce ISG15 function and the function of its associated enzymes, a complete cellular role is still lacking. Certainly, much more work remains to be done.

11. ACKNOWLDGMENTS

This work was supported by NIH grants R01GM066955 and R01CA079849 to D.E.Z. C.T.D. is a Ruth L. Kirschstein NRSA fellow and supported by NIH grant F32GM071191. The authors would like to thank members of the Zhang lab for their valuable suggestions. This is manuscript 17110-MEM from The Scripps Research Institute.

12.REFERENCES

1. Levy, D E & A. Garcia-Sastre: The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine Growth Factor Rev* 12**,** 143-156 (2001)

2. Tannenbaum, C S & T. A. Hamilton: Immune-inflammatory mechanisms in IFN gamma-mediated anti-tumor activity. *Seminars in Cancer Biology* 10**,** 113-123 (2000)

3. Stark, G R, I. M. Kerr, B. R. Williams, R. H. Silverman, & R. D. Schreiber: How cells respond to interferons. Annu Rev Biochem 67, 227-264 (1998)

4. Pestka, S, C. D. Krause, & M. R. Walter: Interferons, interferon-like cytokines, and their receptors. Immunol Rev 202, 8-32 (2004)

5. Fields, B. N., Knipe, D. M., and Howley, P. M. (1996) Fundamental Virology, 3rd Ed., Lippincott-Raven, Philadelphia

6. Havell, E A, T. G. Hayes, & J. Vilcek: Synthesis of two distinct interferons by human fibroblasts. Virology 89, 330-334 (1978)

7. De Maeyer, E. and De Maeyer-Guignard, J. *Interferons and other regulatory cytokines*, John Wiley & Sons, New York (1988)

8. Taniguchi, T, N. Mantei, M. Schwarzstein, S. Nagata, M. Muramatsu, & C. Weissmann: Human leukocyte and fibroblast interferons are structurally related. *Nature* 285**,** 547-549 (1980)

9. Stewart, H J, S. H. McCann, & A. P. Flint: Structure of an interferon-alpha 2 gene expressed in the bovine conceptus early in gestation. *J Mol Endocrinol* 4**,** 275-282 (1990)

10. LaFleur, D W, B. Nardelli, T. Tsareva, D. Mather, P. Feng, M. Semenuk, K. Taylor, M. Buergin, D. Chinchilla, V. Roshke, G. Chen, S. M. Ruben, P. M. Pitha, T. A. Coleman, & P. A. Moore: Interferon-kappa, a novel type I interferon expressed in human keratinocytes. *J Biol Chem* 276**,** 39765-39771 (2001)

11. Janeway, C. A., Travers, P., Walport, M., and Capra, J. D. (1999) *Immunobiology: The immune system in health and disease*, 4th Ed., Elsevier Science/Garland Publishing, New York

12. Biron, C A: Role of early cytokines, including alpha and beta interferons (IFN-alpha/beta), in innate and adaptive immune responses to viral infections. *Semin Immunol* 10**,** 383-390 (1998)

13. Alexopoulou, L, A. C. Holt, R. Medzhitov, & R. A. Flavell: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413**,** 732-738 (2001)

14. Matsumoto, M & T. Seya: Recognition of dsRNA by Toll-like receptor 3. *Uirusu* 51**,** 209-214 (2001)

15. Palsson-McDermott, E M & L. A. O'Neill: Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113**,** 153-162 (2004)

16. Weinstock-Guttman, B & L. D. Jacobs: What is new in the treatment of multiple sclerosis? *Drugs* 59**,** 401-410 (2000)

17. Goodkin, D E: Interferon beta-1b. *Lancet* 344**,** 1057-1060 (1994)

18. Baron, S, S. K. Tyring, W. R. Fleischmann, Jr., D. H. Coppenhaver, D. W. Niesel, G. R. Klimpel, G. J. Stanton, & T. K. Hughes: The interferons. Mechanisms of action and clinical applications. *JAMA* 266**,** 1375-1383 (1991)

19. Wiegand, J, E. Jackel, M. Cornberg, H. Hinrichsen, M. Dietrich, J. Kroeger, W. P. Fritsch, A. Kubitschke, N. Aslan, H. L. Tillmann, M. P. Manns, & H. Wedemeyer: Long-term follow-up after successful interferon therapy of acute hepatitis C. *Hepatology* 40**,** 98-107 (2004)

20. Karayiannis, P: Current therapies for chronic hepatitis B virus infection. *Expert Rev Anti Infect Ther* 2**,** 745-760 (2004)

21. Billiau, A, B. C. Kieseier, & H. P. Hartung: Biologic role of interferon beta in multiple sclerosis. *J Neurol* 251 Suppl 2**,** II10-II14 (2004)

22. Calabresi, P A, L. A. Stone, C. N. Bash, J. A. Frank, & H. F. McFarland: Interferon beta results in immediate reduction of contrast-enhanced MRI lesions in multiple sclerosis patients followed by weekly MRI. *Neurology* 48**,** 1446-1448 (1997)

23. Gutterman, J U: Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci U S A* 91**,** 1198-1205 (1994)

24. Borden, E C, D. Lindner, R. Dreicer, M. Hussein, & D. Peereboom: Second-generation interferons for cancer: clinical targets. *Semin Cancer Biol* 10**,** 125-144 (2000)

25. Martensen, P M & J. Justesen: Small ISGs coming forward. *J Interferon Cytokine Res* 24**,** 1-19 (2004)

26. Leaman, D W, M. Chawla-Sarkar, B. Jacobs, K. Vyas, Y. Sun, A. Ozdemir, T. Yi, B. R. Williams, & E. C. Borden: Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. *J Interferon Cytokine Res* 23**,** 745-756 (2003)

27. Farrell, P J, R. J. Broeze, & P. Lengyel: Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells. *Nature* 279**,** 523-525 (1979)

28. Der, S D, A. Zhou, B. R. Williams, & R. H. Silverman: Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A* 95**,** 15623-15628 (1998)

29. Schwartz, D C & M. Hochstrasser: A superfamily of protein tags: ubiquitin, SUMO and related modifiers. *Trends Biochem Sci* 28**,** 321-328 (2003)

30. Jentsch, S & G. Pyrowolakis: Ubiquitin and its kin: how close are the family ties? *Trends Cell Biol* 10**,** 335-342 (2000)

31. Korant, B D, D. C. Blomstrom, G. J. Jonak, & Knight E Jr: Interferon-induced proteins. Purification and characterization of a 15,000-dalton protein from human and bovine cells induced by interferon. *J Biol Chem* 259**,** 14835-14839 (1984)

32. Blomstrom, D C, D. Fahey, R. Kutny, B. D. Korant, & Knight E Jr: Molecular characterization of the interferon-induced 15-kDa protein. Molecular cloning and nucleotide and amino acid sequence. *J Biol Chem* 261**,** 8811-8816 (1986)

33. Reich, N, B. Evans, D. Levy, D. Fahey, Knight E Jr, & J. E. Darnell, Jr.: Interferon-induced transcription of a gene encoding a 15-kDa protein depends on an upstream enhancer element. *Proc Natl Acad Sci U S A* 84**,** 6394-6398 (1987)

34. Knight, E Jr, D. Fahey, B. Cordova, M. Hillman, R. Kutny, N. Reich, & D. Blomstrom: A 15-kDa interferon-induced protein is derived by COOH-terminal processing of a 17-kDa precursor. *J Biol Chem* 263**,** 4520-4522 (1988)

35. Haas, A L, P. Ahrens, P. M. Bright, & H. Ankel: Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. *J Biol Chem* 262**,** 11315-11323 (1987)

36. Bayer, P, A. Arndt, S. Metzger, R. Mahajan, F. Melchior, R. Jaenicke, & J. Becker: Structure determination of the small ubiquitin-related modifier SUMO-1. *J Mol Biol* 280**,** 275-286 (1998)

37. Whitby, F G, G. Xia, C. M. Pickart, & C. P. Hill: Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes. *J Biol Chem* 273**,** 34983-34991 (1998)

38. Deshaies, R J: SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* 15**,** 435-467 (1999)

39. Liu, M, R. Reimschuessel, & B. A. Hassel: Molecular cloning of the fish interferon stimulated gene, 15 kDa (ISG15) orthologue: a ubiquitin-like gene induced by nephrotoxic damage. *Gene* 298**,** 129-139 (2002)

40. Ritchie, K J & D. E. Zhang: ISG15: the immunological kin of ubiquitin. *Semin Cell Dev Biol* 15**,** 237-246 (2004)

41. Knight, E Jr & B. Cordova: IFN-induced 15-kDa protein is released from human lymphocytes and monocytes. *J Immunol* 146**,** 2280-2284 (1991)

42. D'Cunha, J, Knight E Jr, A. L. Haas, R. L. Truitt, & E. C. Borden: Immunoregulatory properties of ISG15, an interferon-induced cytokine. *Proc Natl Acad Sci U S A* 93**,** 211-215 (1996)

43. D'Cunha, J, S. Ramanujam, R. J. Wagner, P. L. Witt, E. Jr. Knight, & E. C. Borden: *In vitro* and *in vivo* secretion of human ISG15, an IFN-induced immunomodulatory cytokine. *J Immunol* 157**,** 4100-4108 (1996)

44. Austin, K J, S. K. Ward, M. G. Teixeira, V. C. Dean, D. W. Moore, & T. R. Hansen: Ubiquitin cross-reactive protein is released by the bovine uterus in response to interferon during early pregnancy. *Biol Reprod* 54**,** 600-606 (1996)

45. Kelso, A: Cytokines: principles and prospects. *Immunol Cell Biol* 76**,** 300-317 (1998)

46. Prudovsky, I, A. Mandinova, R. Soldi, C. Bagala, I. Graziani, M. Landriscina, F. Tarantini, M. Duarte, S. Bellum, H. Doherty, & T. Maciag: The non-classical export

routes: FGF1 and IL-1alpha point the way. *J Cell Sci* 116**,** 4871-4881 (2003)

47. Nickel, W: The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem* 270**,** 2109-2119 (2003)

48. Landriscina, M, C. Bagala, A. Mandinova, R. Soldi, I. Micucci, S. Bellum, I. Prudovsky, & T. Maciag: Copper induces the assembly of a multiprotein aggregate implicated in the release of fibroblast growth factor 1 in response to stress. *J Biol Chem* 276**,** 25549-25557 (2001)

49. Mandinova, A, R. Soldi, I. Graziani, C. Bagala, S. Bellum, M. Landriscina, F. Tarantini, I. Prudovsky, & T. Maciag: S100A13 mediates the copper-dependent stress-induced release of IL-1alpha from both human U937 and murine NIH 3T3 cells. *J Cell Sci* 116**,** 2687-2696 (2003)

50. Jackson, A, S. Friedman, X. Zhan, K. A. Engleka, R. Forough, & T. Maciag: Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells. *Proc Natl Acad Sci U S A* 89**,** 10691-10695 (1992)

51. Mouta, C C, M. Landriscina, S. Bellum, I. Prudovsky, & T. Maciag: The comparative release of FGF1 by hypoxia and temperature stress. *Growth Factors* 18**,** 277-285 (2001)

52. Shin, J T, S. R. Opalenik, J. N. Wehby, V. K. Mahesh, A. Jackson, F. Tarantini, T. Maciag, & J. A. Thompson: Serum-starvation induces the extracellular appearance of FGF-1. *Biochim Biophys Acta* 1312**,** 27-38 (1996)

53. Ananyeva, N M, A. V. Tjurmin, J. A. Berliner, G. M. Chisolm, G. Liau, J. A. Winkles, & C. C. Haudenschild: Oxidized LDL mediates the release of fibroblast growth factor-1. *Arterioscler Thromb Vasc Biol* 17**,** 445-453 (1997)

54. Tarantini, F, I. Micucci, S. Bellum, M. Landriscina, S. Garfinkel, I. Prudovsky, & T. Maciag: The precursor but not the mature form of IL1alpha blocks the release of FGF1 in response to heat shock. *J Biol Chem* 276**,** 5147-5151 (2001)

55. Rubartelli, A, F. Cozzolino, M. Talio, & R. Sitia: A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J* 9**,** 1503-1510 (1990)

56. Loeb, K R & A. L. Haas: The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. *J Biol Chem* 267**,** 7806-7813 (1992)

57. Kessler, D S, D. E. Levy, & J. E. Darnell, Jr.: Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. *Proc Natl Acad Sci U S A* 85**,** 8521-8525 (1988)

58. Tanaka, N & T. Taniguchi: The interferon regulatory factors and oncogenesis. *Semin Cancer Biol* 10**,** 73-81 (2000)

59. Au, W C, P. A. Moore, W. Lowther, Y. T. Juang, & P. M. Pitha: Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. *Proc Natl Acad Sci U S A* 92**,** 11657-11661 (1995)

60. Hiscott, J, P. Pitha, P. Genin, H. Nguyen, C. Heylbroeck, Y. Mamane, M. Algarte, & R. Lin: Triggering the interferon response: the role of IRF-3 transcription factor. *J Interferon Cytokine Res* 19**,** 1-13 (1999)

61. Meraro, D, M. Gleit-Kielmanowicz, H. Hauser, & B. Z. Levi: IFN-stimulated gene 15 is synergistically activated through interactions between the myelocyte/lymphocyte-specific transcription factors, PU.1, IFN regulatory factor-8/IFN consensus sequence binding protein, and IFN regulatory factor-4: characterization of a new subtype of IFN-stimulated response element. *J Immunol* 168**,** 6224-6231 (2002)

62. Tenen, D G, R. Hromas, J. D. Licht, & D. E. Zhang: Transcription factors, normal myeloid development, and leukemia. *Blood* 90**,** 489-519 (1997)

63. McKercher, S R, B. E. Torbett, K. L. Anderson, G. W. Henkel, D. J. Vestal, H. Baribault, M. Klemsz, A. J. Feeney, G. E. Wu, C. J. Paige, & R. A. Maki: Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J* 15**,** 5647-5658 (1996)

64. Scott, E W, M. C. Simon, J. Anastasi, & H. Singh: Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265**,** 1573-1577 (1994)

65. Rosenbauer, F, J. F. Waring, J. Foerster, M. Wietstruk, D. Philipp, & I. Horak: Interferon consensus sequence binding protein and interferon regulatory factor-4/Pip form a complex that represses the expression of the interferon-stimulated gene-15 in macrophages. *Blood* 94**,** 4274-4281 (1999)

66. Nicholl, M J, L. H. Robinson, & C. M. Preston: Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1. *J Gen Virol* 81**,** 2215-2218 (2000)

67. Mossman, K L, P. F. Macgregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards, & J. R. Smiley: Herpes simplex virus triggers and then disarms a host antiviral response. *J Virol* 75**,** 750-758 (2001)

68. Hermeking, H, C. Lengauer, K. Polyak, T. C. He, L. Zhang, S. Thiagalingam, K. W. Kinzler, & B. Vogelstein: 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* 1**,** 3-11 (1997)

69. Lock, C, G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, P. Klonowski, A. Austin, N. Lad, N. Kaminski, S. J. Galli, J. R. Oksenberg, C. S. Raine, R. Heller, & L. Steinman: Gene-microarray analysis of multiple sclerosis

lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* 8**,** 500-508 (2002)

70. Desai, S D, Y. Mao, M. Sun, T. K. Li, J. Wu, & L. F. Liu: Ubiquitin, SUMO-1, and UCRP in camptothecin sensitivity and resistance. *Ann N Y Acad Sci* 922**,** 306-308 (2000)

71. Siddoo-Atwal, C, A. L. Haas, & M. P. Rosin: Elevation of interferon beta-inducible proteins in ataxia telangiectasia cells. *Cancer Res* 56**,** 443-447 (1996)

72. Han, S Y, S. H. Kim, & L. E. Heasley: Differential gene regulation by specific gain-of-function JNK1 proteins expressed in Swiss 3T3 fibroblasts. *J Biol Chem* 277**,** 47167-47174 (2002)

73. Ruckes, T, D. Saul, J. Van Snick, O. Hermine, & R. Grassmann: Autocrine antiapoptotic stimulation of cultured adult T-cell leukemia cells by overexpression of the chemokine I-309. *Blood* 98**,** 1150-1159 (2001)

74. Liu, M, B. T. Hummer, X. Li, & B. A. Hassel: Camptothecin Induces the Ubiquitin-like Protein, ISG15, and Enhances ISG15 Conjugation in Response to Interferon. *Journal of Interferon & Cytokine Research* 24**,** 647-654 (2004)

75. Pickart, C M: Mechanisms underlying ubiquitylation. *Annu Rev Biochem* 70**,** 503-533 (2001)

76. Huang, D T, D. W. Miller, R. Mathew, R. Cassell, J. M. Holton, M. F. Roussel, & B. A. Schulman: A unique E1-E2 interaction required for optimal conjugation of the ubiquitin-like protein NEDD8. *Nat Struct Mol Biol* (2004)

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

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

79. Laney, J D & M. Hochstrasser: Substrate targeting in the ubiquitin system. *Cell* 97**,** 427-430 (1999)

80. Balakirev, M Y, S. O. Tcherniuk, M. Jaquinod, & J. Chroboczek: Otubains: a new family of cysteine proteases in the ubiquitin pathway. *EMBO Rep* 4**,** 517-522 (2003)

81. Borodovsky, A, H. Ovaa, N. Kolli, T. Gan-Erdene, K. D. Wilkinson, H. L. Ploegh, & B. M. Kessler: Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem Biol* 9**,** 1149-1159 (2002)

82. Evans, P C, T. S. Smith, M. J. Lai, M. G. Williams, D. F. Burke, K. Heyninck, M. M. Kreike, R. Beyaert, T. L. Blundell, & P. J. Kilshaw: A novel type of deubiquitinating enzyme. *J Biol Chem* 278**,** 23180-23186 (2003)

83. Verma, R, L. Aravind, R. Oania, W. H. McDonald, J. R. Yates, III, E. V. Koonin, & R. J. Deshaies: Role of Rpn11 metalloprotease in deubiquitylation and degradation by the 26S proteasome. *Science* 298**,** 611-615 (2002)

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

85. Yuan, W & R. M. Krug: Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. *EMBO J* 20**,** 362-371 (2001)

86. Zhao, C, S. L. Beaudenon, M. L. Kelley, M. B. Waddell, W. Yuan, B. A. Schulman, J. M. Huibregtse, & R. M. Krug: The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN-alpha/beta-induced ubiquitin-like protein. *Proc Natl Acad Sci U S A* 101**,** 7578-7582 (2004)

87. Kim, K I, N. V. Giannakopoulos, H. W. Virgin, & D. E. Zhang: Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation. *Mol Cell Biol* 24**,** 9592-9600 (2004)

88. Malakhov, M P, O. A. Malakhova, K. I. Kim, K. J. Ritchie, & D. E. Zhang: UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *J Biol Chem* 277**,** 9976-9981 (2002)

89. Kok, K, R. Hofstra, A. Pilz, B. A. van den, P. Terpstra, C. H. Buys, & B. Carritt: A gene in the chromosomal region 3p21 with greatly reduced expression in lung cancer is similar to the gene for ubiquitin-activating enzyme. *Proc Natl Acad Sci U S A* 90**,** 6071-6075 (1993)

90. Kok, K, J. Osinga, B. Carritt, M. B. Davis, A. H. van der Hout, A. Y. van der Veen, R. M. Landsvater, L. F. de Leij, H. H. Berendsen, P. E. Postmus, *et al.*: Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. *Nature* 330**,** 578-581 (1987)

91. Brauch, H, B. Johnson, J. Hovis, T. Yano, A. Gazdar, O. S. Pettengill, S. Graziano, G. D. Sorenson, B. J. Poiesz, J. Minna, *et al.*.: Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinoma of the lung. *N Engl J Med* 317**,** 1109-1113 (1987)

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

93. Carritt, B, K. Kok, B. A. van den, J. Osinga, A. Pilz, R. M. Hofstra, M. B. Davis, A. Y. van der Veen, P. H. Rabbitts, K. Gulati, *et al.*: A gene from human chromosome region 3p21 with reduced expression in small cell lung cancer. *Cancer Res* 52**,** 1536-1541 (1992)

94. Malakhova, O A, M. Yan, M. P. Malakhov, K. J. Ritchie, K. I. Kim, L. F. Peterson, K. Shuai, & D. E.

Zhang: Protein ISGylation modulates the JAK-STAT signaling pathway. *Genes Dev* 17**,** 455-460 (2003)

95. McLaughlin, P M, W. Helfrich, K. Kok, M. Mulder, S. W. Hu, M. G. Brinker, M. H. Ruiters, L. F. de Leij, & C. H. Buys: The ubiquitin-activating enzyme E1-like protein in lung cancer cell lines. *Int J Cancer* 85**,** 871-876 (2000)

96. Grenfell, S J, J. S. Trausch-Azar, P. M. Handley-Gearhart, A. Ciechanover, & A. L. Schwartz: Nuclear localization of the ubiquitin-activating enzyme, E1, is cell-cycle-dependent. *Biochem J* 300 (Pt 3)**,** 701-708 (1994)

97. de Veer, M J, M. Holko, M. Frevel, E. Walker, S. Der, J. M. Paranjape, R. H. Silverman, & B. R. Williams: Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol* 69**,** 912-920 (2001)

98. Pru, J K, K. J. Austin, A. L. Haas, & T. R. Hansen: Pregnancy and interferon-tau upregulate gene expression of members of the 1-8 family in the bovine uterus. *Biol Reprod* 65**,** 1471-1480 (2001)

99. Friedman, R L, S. P. Manly, M. McMahon, I. M. Kerr, & G. R. Stark: Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. *Cell* 38**,** 745-755 (1984)

100. Brem, R, K. Oraszlan-Szovik, S. Foser, B. Bohrmann, & U. Certa: Inhibition of proliferation by 1-8U in interferon-alpha-responsive and non-responsive cell lines. *Cell Mol Life Sci* 60**,** 1235-1248 (2003)

101. Krepler, C, U. Certa, V. Wacheck, B. Jansen, K. Wolff, & H. Pehamberger: Pegylated and conventional interferon-alpha induce comparable transcriptional responses and inhibition of tumor growth in a human melanoma SCID mouse xenotransplantation model. *J Invest Dermatol* 123**,** 664-669 (2004)

102. Bradbury, L E, G. S. Kansas, S. Levy, R. L. Evans, & T. F. Tedder: The CD19/CD21 signal transducing complex of human B lymphocytes includes the target of antiproliferative antibody-1 and Leu-13 molecules. *J Immunol* 149**,** 2841-2850 (1992)

103. Sato, S, A. S. Miller, M. C. Howard, & T. F. Tedder: Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. *J Immunol* 159**,** 3278-3287 (1997)

104. Nyman, T A, S. Matikainen, T. Sareneva, I. Julkunen, & N. Kalkkinen: Proteome analysis reveals ubiquitin-conjugating enzymes to be a new family of interferon-alpha-regulated genes. *Eur J Biochem* 267**,** 4011-4019 (2000)

105. Kumar, S, W. H. Kao, & P. M. Howley: Physical interaction between specific E2 and Hect E3 enzymes determines functional cooperativity. *J Biol Chem* 272**,** 13548-13554 (1997)

106. Nuber, U, S. Schwarz, P. Kaiser, R. Schneider, & M. Scheffner: Cloning of human ubiquitin-conjugating enzymes UbcH6 and UbcH7 (E2-F1) and characterization of their interaction with E6-AP and RSP5. *J Biol Chem* 271**,** 2795-2800 (1996)

107. Ciechanover, A, D. Shkedy, M. Oren, & B. Bercovich: Degradation of the tumor suppressor protein p53 by the ubiquitin-mediated proteolytic system requires a novel species of ubiquitin-carrier protein, E2. *J Biol Chem* 269**,** 9582-9589 (1994)

108. Blumenfeld, N, H. Gonen, A. Mayer, C. E. Smith, N. R. Siegel, A. L. Schwartz, & A. Ciechanover: Purification and characterization of a novel species of ubiquitin-carrier protein, E2, that is involved in degradation of non-"N-end rule" protein substrates. *J Biol Chem* 269**,** 9574-9581 (1994)

109. Walden, H, M. S. Podgorski, D. T. Huang, D. W. Miller, R. J. Howard, D. L. Minor, Jr., J. M. Holton, & B. A. Schulman: The structure of the APPBP1-UBA3-NEDD8-ATP complex reveals the basis for selective ubiquitin-like protein activation by an E1. *Mol Cell* 12**,** 1427-1437 (2003)

110. Huibregtse, J M, M. Scheffner, S. Beaudenon, & P. M. Howley: A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A* 92**,** 2563-2567 (1995)

111. Joazeiro, C A & A. M. Weissman: RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102**,** 549-552 (2000)

112. Hatakeyama, S & K. I. Nakayama: U-box proteins as a new family of ubiquitin ligases. *Biochem Biophys Res Commun* 302**,** 635-645 (2003)

113. Scheffner, M, U. Nuber, & J. M. Huibregtse: Protein ubiquitylation involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* 373**,** 81-83 (1995)

114. Weissman, A M: Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2**,** 169-178 (2001)

115. Bellon, S F, K. K. Rodgers, D. G. Schatz, J. E. Coleman, & T. A. Steitz: Crystal structure of the RAG1 dimerization domain reveals multiple zinc-binding motifs including a novel zinc binuclear cluster. *Nat Struct Biol* 4**,** 586-591 (1997)

116. Chin, L S, J. P. Vavalle, & L. Li: Staring, a novel E3 ubiquitin-protein ligase that targets syntaxin 1 for degradation. *J Biol Chem* 277**,** 35071-35079 (2002)

117. Tanaka, K, T. Suzuki, T. Chiba, H. Shimura, N. Hattori, & Y. Mizuno: Parkin is linked to the ubiquitin pathway. *J Mol Med* 79**,** 482-494 (2001)

118. Niwa, J, S. Ishigaki, M. Doyu, T. Suzuki, K. Tanaka, & G. Sobue: A novel centrosomal ring-finger protein, dorfin, mediates ubiquitin ligase activity. *Biochem Biophys Res Commun* 281**,** 706-713 (2001)

119. Moynihan, T P, H. C. Ardley, U. Nuber, S. A. Rose, P. F. Jones, A. F. Markham, M. Scheffner, & P. A. Robinson: The ubiquitin-conjugating enzymes UbcH7 and UbcH8 interact with RING finger/IBR motif-containing domains of HHARI and H7-AP1. *J Biol Chem* 274**,** 30963-30968 (1999)

120. Liu, L Q, R. Ilaria, Jr., P. D. Kingsley, A. Iwama, R. A. van Etten, J. Palis, & D. E. Zhang: A novel ubiquitin-specific protease, UBP43, cloned from leukemia fusion protein AML1-ETO-expressing mice, functions in hematopoietic cell differentiation. *Mol Cell Biol* 19**,** 3029-3038 (1999)

121. Schwer, H, L. Q. Liu, L. Zhou, M. T. Little, Z. Pan, C. J. Hetherington, & D. E. Zhang: Cloning and characterization of a novel human ubiquitin-specific protease, a homologue of murine UBP43 (Usp18). *Genomics* 65**,** 44-52 (2000)

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

123. Yergeau, D A, C. J. Heatherington, Q. Wang, P. Zhang, A. H. Sharpe, M. Binder, M. Marin-Padilla, D. . Tenen, N. A. Speck, & D. E. Zhang: Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. *Nat Genet* 15, 303-306 (1997)

124. Zhang, X, J. Shin, T. W. Molitor, L. B. Schook, & M. S. Rutherford: Molecular responses of macrophages to porcine reproductive and respiratory syndrome virus infection. *Virology* 262**,** 152-162 (1999)

125. Li, X L, J. A. Blackford, C. S. Judge, M. Liu, W. Xiao, D. V. Kalvakolanu, & B. A. Hassel: RNase-L-dependent destabilization of interferon-induced mRNAs. A role for the 2-5A system in attenuation of the interferon response. *J Biol Chem* 275**,** 8880-8888 (2000)

126. Kang, D, H. Jiang, Q. Wu, S. Pestka, & P. B. Fisher: Cloning and characterization of human ubiquitin-processing protease-43 from terminally differentiated human melanoma cells using a rapid subtraction hybridization protocol RaSH. *Gene* 267**,** 233-242 (2001)

127. Malakhova, O, M. Malakhov, C. Hetherington, & D. E. Zhang: Lipopolysaccharide activates the expression of ISG15-specific protease UBP43 via interferon regulatory factor 3. *J Biol Chem* 277**,** 14703-14711 (2002)

128. Hemelaar, J, A. Borodovsky, B. M. Kessler, D. Reverter, J. Cook, N. Kolli, T. Gan-Erdene, K. D. Wilkinson, G. Gill, C. D. Lima, H. L. Ploegh, & H. Ovaa: Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. *Mol Cell Biol* 24**,** 84-95 (2004)

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

130. Stein, R L, Z. Chen, & F. Melandri: Kinetic studies of isopeptidase T: modulation of peptidase activity by ubiquitin. *Biochemistry* 34**,** 12616-12623 (1995)

131. Wilkinson, K D, V. L. Tashayev, L. B. O'Connor, C. N. Larsen, E. Kasperek, & C. M. Pickart: Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochemistry* 34**,** 14535-14546 (1995)

132. Ritchie, K J, M. P. Malakhov, C. J. Hetherington, L. Zhou, M. T. Little, O. A. Malakhova, J. C. Sipe, S. H. Orkin, & D. E. Zhang: Dysregulation of protein modification by ISG15 results in brain cell injury. *Genes Dev* 16**,** 2207-2212 (2002)

133. Tokarz, S, C. Berset, J. La Rue, K. Friedman, K. Nakayama, K. Nakayama, D. E. Zhang, & S. Lanker: The ISG15 isopeptidase UBP43 is regulated by proteolysis via the SCFSkp2 ubiquitin ligase. *J Biol Chem* 279**,** 46424-46430 (2004)

134. Potter, J L, J. Narasimhan, L. Mende-Mueller, & A. L. Haas: Precursor processing of pro-ISG15/UCRP, an interferon-beta-induced ubiquitin-like protein. *J Biol Chem* 274**,** 25061-25068 (1999)

135. Liu, M, X. L. Li, & B. A. Hassel: Proteasomes modulate conjugation to the ubiquitin-like protein, ISG15. *J Biol Chem* 278**,** 1594-1602 (2003)

136. John, J, R. McKendry, S. Pellegrini, D. Flavell, I. M. Kerr, & G. R. Stark: Isolation and characterization of a new mutant human cell line unresponsive to alpha and beta interferons. *Mol Cell Biol* 11**,** 4189-4195 (1991)

137. Kim, K I & D. E. Zhang: ISG15, not just another ubiquitin-like protein. *Biochem Biophys Res Commun* 307**,** 431-434 (2003)

138. Malakhov, M P, K. I. Kim, O. A. Malakhova, B. S. Jacobs, E. C. Borden, & D. E. Zhang: High-throughput immunoblotting. Ubiquitin-like protein ISG15 modifies key regulators of signal transduction. *J Biol Chem* 278**,** 16608-16613 (2003)

139. Hamerman, J A, F. Hayashi, L. A. Schroeder, S. P. Gygi, A. L. Haas, L. Hampson, P. Coughlin, R. Aebersold, & A. Aderem: Serpin 2a is induced in activated macrophages and conjugates to a ubiquitin homolog. *J Immunol* 168**,** 2415-2423 (2002)

140. Morris, E C, T. R. Dafforn, S. L. Forsyth, M. A. Missen, A. J. Horvath, L. Hampson, I. N. Hampson, G. Currie, R. W. Carrell, & P. B. Coughlin: Murine serpin 2A

is a redox-sensitive intracellular protein. *Biochem J* 371**,** 165-173 (2003)

141. Hampson, I N, L. Hampson, M. Pinkoski, M. Cross, C. M. Heyworth, R. C. Bleackley, E. Atkinson, & T. M. Dexter: Identification of a serpin specifically expressed in multipotent and bipotent hematopoietic progenitor cells and in activated T cells. *Blood* 89**,** 108-118 (1997)

142. Aaronson, D S & C. M. Horvath: A road map for those who don't know JAK-STAT. *Science* 296**,** 1653-1655 (2002)

143. Meraz, M A, J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet, & R. D. Schreiber: Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84**,** 431-442 (1996)

144. Durbin, J E, R. Hackenmiller, M. C. Simon, & D. E. Levy: Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84**,** 443-450 (1996)

145. Rodig, S J, M. A. Meraz, J. M. White, P. A. Lampe, J. K. Riley, C. D. Arthur, K. L. King, K. C. Sheehan, L. Yin, D. Pennica, E. M. Johnson, Jr., & R. D. Schreiber: Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell* 93**,** 373-383 (1998)

146. Carpenter, G & Q. Ji: Phospholipase C-gamma as a signal-transducing element. *Exp Cell Res* 253**,** 15-24 (1999)

147. Rana, R S & L. E. Hokin: Role of phosphoinositides in transmembrane signaling. *Physiol Rev* 70**,** 115-164 (1990)

148. Majerus, P W: Inositol phosphate biochemistry. *Annu Rev Biochem* 61**,** 225-250 (1992)

149. Rhee, S G & Y. S. Bae: Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem* 272**,** 15045-15048 (1997)

150. Johnson, G L & R. Lapadat: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298**,** 1911-1912 (2002)

151. Mahajan, R, L. Gerace, & F. Melchior: Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association. *J Cell Biol* 140**,** 259-270 (1998)

152. Recht, M, E. C. Borden, & E. Jr. Knight: A human 15-kDa IFN-induced protein induces the secretion of IFN-gamma. *J Immunol* 147**,** 2617-2623 (1991)

153. Owhashi, M, Y. Taoka, K. Ishii, S. Nakazawa, H. Uemura, & H. Kambara: Identification of a ubiquitin family protein as a novel neutrophil chemotactic factor. *Biochem Biophys Res Commun* 309**,** 533-539 (2003)

154. Majetschak, M, U. Krehmeier, M. Bardenheuer, C. Denz, M. Quintel, G. Voggenreiter, & U. Obertacke: Extracellular ubiquitin inhibits the TNF-alpha response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness. *Blood* 101**,** 1882-1890 (2003)

155. Kunzi, M S & P. M. Pitha: Role of interferon-stimulated gene ISG-15 in the interferon-omega-mediated inhibition of human immunodeficiency virus replication. *J Interferon Cytokine Res* 16**,** 919-927 (1996)

156. Ritchie, K J, C. S. Hahn, K. I. Kim, M. Yan, D. Rosario, L. Li, J. C. de la Torre, & D. E. Zhang: Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection. *Nat Med* 10**,** 1374-1378 (2004)

157. Loeb, K R & A. L. Haas: Conjugates of ubiquitin cross-reactive protein distribute in a cytoskeletal pattern. *Mol Cell Biol* 14**,** 8408-8419 (1994)

158. Murti, K G, R. Goorha, & M. W. Klymkowsky: A functional role for intermediate filaments in the formation of frog virus 3 assembly sites. *Virology* 162**,** 264-269 (1988)

159. Shoeman, R L, E. Mothes, C. Kesselmeier, & P. Traub: Intermediate filament assembly and stability *in vitro*: effect and implications of the removal of head and tail domains of vimentin by the human immunodeficiency virus type 1 protease. *Cell Biol Int Rep* 14**,** 583-594 (1990)

160. Thatcher, W W, M. D. Meyer, & G. Danet-Desnoyers: Maternal recognition of pregnancy. *J Reprod Fertil Suppl* 49**,** 15-28 (1995)

161. Roberts, R M, J. C. Cross, C. E. Farin, T. R. Hansen, S. W. Klemann, & K. Imakawa: Interferons at the placental interface. *J Reprod Fertil Suppl* 41**,** 63-74 (1990)

162. Bazer, F W & H. M. Johnson: Type I conceptus interferons: maternal recognition of pregnancy signals and potential therapeutic agents. *Am J Reprod Immunol* 26**,** 19-22 (1991)

163. Fishel, S B, R. G. Edwards, & C. J. Evans: Human chorionic gonadotropin secreted by preimplantation embryos cultured *in vitro*. *Science* 223**,** 816-818 (1984)

164. Bebington, C, F. J. Doherty, & S. D. Fleming: Ubiquitin cross-reactive protein gene expression is increased in decidualized endometrial stromal cells at the initiation of pregnancy. *Mol Hum Reprod* 5**,** 966-972 (1999)

165. Bebington, C, S. C. Bell, F. J. Doherty, A. T. Fazleabas, & S. D. Fleming: Localization of ubiquitin and ubiquitin cross-reactive protein in human and baboon endometrium and decidua during the menstrual cycle and early pregnancy. *Biol Reprod* 60**,** 920-928 (1999)

166. Hansen, T R, K. J. Austin, D. J. Perry, J. K. Pru, M. G. Teixeira, & G. A. Johnson: Mechanism of action of interferon-tau in the uterus during early pregnancy. *J Reprod Fertil Suppl* 54**,** 329-339 (1999)

167. Johnson, G A, K. J. Austin, E. A. Van Kirk, & T. R. Hansen: Pregnancy and interferon-tau induce conjugation of bovine ubiquitin cross-reactive protein to cytosolic uterine proteins. *Biol Reprod* 58**,** 898-904 (1998)

168. Johnson, G A, K. J. Austin, A. M. Collins, W. J. Murdoch, & T. R. Hansen: Endometrial ISG17 mRNA and a related mRNA are induced by interferon-tau and localized to glandular epithelial and stromal cells from pregnant cows. *Endocrine* 10**,** 243-252 (1999)

169. Perry, D J, K. J. Austin, & T. R. Hansen: Cloning of interferon-stimulated gene 17: the promoter and nuclear proteins that regulate transcription. *Mol Endocrinol* 13**,** 1197-1206 (1999)

170. Johnson, G A, T. E. Spencer, T. R. Hansen, K. J. Austin, R. C. Burghardt, & F. W. Bazer: Expression of the interferon tau inducible ubiquitin cross-reactive protein in the ovine uterus. *Biol Reprod* 61**,** 312-318 (1999)

171. Johnson, G A, T. E. Spencer, R. C. Burghardt, M. M. Joyce, & F. W. Bazer: Interferon-tau and progesterone regulate ubiquitin cross-reactive protein expression in the ovine uterus. *Biol Reprod* 62**,** 622-627 (2000)

172. Johnson, G A, M. M. Joyce, S. J. Yankey, T. R. Hansen, & T. L. Ott: The Interferon Stimulated Genes (ISG) 17 and Mx have different temporal and spatial expression in the ovine uterus suggesting more complex regulation of the Mx gene. *J Endocrinol* 174**,** R7-R11 (2002)

173. Nighswonger, A M, K. J. Austin, A. D. Ealy, C. S. Han, & T. R. Hansen: Rapid communication: the ovine cDNA encoding interferon-stimulated gene product 17 (ISG17). *J Anim Sci* 78**,** 1393-1394 (2000)

174. Austin, K J, B. M. Bany, E. L. Belden, L. A. Rempel, J. C. Cross, & T. R. Hansen: Interferon-stimulated gene-15 (Isg15) expression is up-regulated in the mouse uterus in response to the implanting conceptus. *Endocrinology* 144**,** 3107-3113 (2003)

175. Licht, J D: AML1 and the AML1-ETO fusion protein in the pathogenesis of t(8;21) AML. *Oncogene* 20**,** 5660-5679 (2001)

176. Padovan, E, L. Terracciano, U. Certa, B. Jacobs, A. Reschner, M. Bolli, G. C. Spagnoli, E. C. Borden, & M. Heberer: Interferon stimulated gene 15 constitutively produced by melanoma cells induces E-cadherin expression on human dendritic cells. *Cancer Res* 62**,** 3453-3458 (2002)

177. Yap, A S & E. M. Kovacs: Direct cadherin-activated cell signaling: a view from the plasma membrane. *J Cell Biol* 160**,** 11-16 (2003)

178. Kitareewan, S, I. Pitha-Rowe, D. Sekula, C. H. Lowrey, M. J. Nemeth, T. R. Golub, S. J. Freemantle, & E. Dmitrovsky: UBE1L is a retinoid target that triggers PML/RARalpha degradation and apoptosis in acute promyelocytic leukemia. *Proc Natl Acad Sci U S A* 99**,** 3806-3811 (2002)

179. Huang, M E, Y. C. Ye, S. R. Chen, J. R. Chai, J. X. Lu, L. Zhoa, L. J. Gu, & Z. Y. Wang: Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 72**,** 567-572 (1988)

180. Castaigne, S, C. Chomienne, M. T. Daniel, R. Berger, J. M. Miclea, P. Ballerini, & L. Degos: Retinoic acids in the treatment of acute promyelocytic leukemia. *Nouv Rev Fr Hematol* 32**,** 36-38 (1990)

181. Warrell, R P, Jr., S. R. Frankel, W. H. Miller, Jr., D. A. Scheinberg, L. M. Itri, W. N. Hittelman, R. Vyas, M. Andreeff, A. Tafuri, & A. Jakubowski: Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N Engl J Med* 324**,** 1385-1393 (1991)

182. Gianni, M, M. Terao, I. Fortino, M. LiCalzi, V. Viggiano, T. Barbui, A. Rambaldi, & E. Garattini: Stat1 is induced and activated by all-trans retinoic acid in acute promyelocytic leukemia cells. *Blood* 89**,** 1001-1012 (1997)

183. Kamitani, T, K. Kito, T. Fukuda-Kamitani, & E. T. Yeh: Targeting of NEDD8 and its conjugates for proteasomal degradation by NUB1. *J Biol Chem* 276**,** 46655-46660 (2001)

184. Austin, K J, A. L. Carr, J. K. Pru, C. E. Hearne, E. L. George, E. L. Belden, & T. R. Hansen: Localization of ISG15 and conjugated proteins in bovine endometrium using immunohistochemistry and electron microscopy. *Endocrinology* 145**,** 967-975 (2004)

185 Thompson, J D, D. G. Higgins, & T. J. Gibson: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22, 4673-4680 (1994)

Key Words: ISG15, Ubiquitin-like modifier, Interferon stimulated gene, UBE1L, UBCM8, UBCH8, UBP43, Review

Send correspondence to: Dr. Dong-Er Zhang, Mail Drop MEM-L51, The Scripps Research Institute, La Jolla, CA 92037, Tel: 858-784-9558, Fax: 858-784-9593,E-mail: dzhang@scripps.edu

http://www.bioscience.org/current/vol10.htm