Elevated Expression of ISG15 in Tumor Cells Interferes with the Ubiquitin/26S Proteasome Pathway

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Abstract

IFN-stimulatory gene factor 15 (ISG15) is a ubiquitin-like protein, which is conjugated to many cellular proteins. However, its role in protein degradation is unclear. Here, we show that ISG15 is highly elevated and extensively conjugated to cellular proteins in many tumors and tumor cell lines. The increased levels of ISG15 in tumor cells were found to be associated with decreased levels of polyubiquitinated proteins. Specific knockdown of ISG15 expression using ISG15-specific small interfering RNA (siRNA) was shown to increase the levels of polyubiquitinated proteins, suggesting an antagonistic role of ISG15 in regulating ubiquitin-mediated protein turnover. Moreover, siRNA-mediated down-regulation of the major E2 for ISG15 (UbcH8), which blocked the formation of ISG15 protein conjugates, also increased the levels of polyubiquitinated proteins. Together, our results suggest that the ISG15 pathway, which is deregulated during tumorigenesis, negatively regulates the ubiquitin/proteasome pathway by interfering with protein polyubiquitination/degradation. (Cancer Res 2006; 66(2): 921-8)

Introduction

IFN-stimulated gene factor 15 (ISG15) is a 15-kDa protein that is induced by type I IFNs (IFN-α and IFN-β; refs. 1–3) and is a member of the ubiquitin-like protein (UBL) superfamily of proteins (4, 5). It was first identified as a ubiquitin cross-reactive protein (UCRP) using antibodies specific to ubiquitin (1, 6). ISG15 is composed of two ubiquitin-like domains, each of which bears striking similarity to ubiquitin. The COOH terminus of ISG15 retains the canonical LRLRGG ubiquitin sequence required for its conjugation to intracellular targets (1, 6, 7). Like ubiquitin, ISG15 is conjugated to cellular proteins by a mechanism similar to that of ubiquitin (6). UBE1L, an E1-like protein whose expression is elevated by type I IFNs, has been identified as the activating enzyme for ISG15 (4, 8). UbcH8, a ubiquitin E2 enzyme whose expression is also elevated by type I IFNs, forms an obligate ISG15 thioester catalyzed by UBE1L, suggesting it also to be a conjugating enzyme (E2) for ISG15 (9, 10). UBP43 has been identified as an enzyme responsible for deconjugation of ISG15 from target substrates (4, 11).

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ISG15 is elevated during early pregnancy in response to conceptus-derived IFN-τ in ungulates (12–14). ISG15 expression is also induced during viral infection and upon nephrotoxic damage (1, 15) and elevated in ataxia telangiectasia cells defective in ataxia telangiectasia mutated kinase (16). Several ISG15 target proteins have been identified, including Serpin 2a, signal transducers and activators of transcription 1 (STAT1), some key regulators of signal transduction [e.g., phospholipase Cγ1, Janus-activated kinase 1 (JAK1), and extracellular signal-regulated kinase 1], and IFN-α/β-induced antiviral proteins (12, 13). It has been suggested that ISG15 overexpression stabilizes its target proteins by forming ISG15-protein conjugates (17). However, direct evidence for such a function is still lacking. Surprisingly, ISG15$^{-/-}$ mice are viable and fertile and display no obvious abnormalities (18). On the other hand, mice lacking UBP43 develop a severe phenotype with brain injuries and lethal hypersensitivity to poly(deoxyinosinic-deoxycytidylic acid) (19).

In the current study, we show that ISG15 and its protein conjugates are overexpressed in many tumor cell lines, oncogene-transformed cells, and tumor biopsies compared with their normal counterparts. Overexpression of ISG15 is also associated with decreased protein polyubiquitination and their turnover in tumor cells. Specific knockdown of either ISG15 or its conjugating enzyme (E2), UbcH8, using their respective small interfering RNAs (siRNA) restored protein polyubiquitination and the turnover of polyubiquitinated proteins via the 26S proteasome. These results suggest that elevated expression of ISG15 and its conjugates antagonize protein polyubiquitination and turnover and may contribute to the deregulation of the ubiquitin/26S proteasome pathway in tumors.

Materials and Methods

Cells. All cells were cultured in RPMI supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin (100 units/mL), and streptomycin (100 μg/mL) in a 37°C incubator with 5% CO_2.

Immunoblotting. Cell lysates were analyzed by 15% SDS-PAGE (unless indicated otherwise) followed by immunoblotting with the appropriate antibody. Visualization of bands was done using the enhanced chemiluminescence Western procedure (Pierce, Rockford, IL) and the Kodak Image Station 2000R. For immunoblotting of tumor tissue biopsies (discarded specimens that were obtained as part of a protocol approved by the Robert Wood Johnson Medical School Institutional Review Board), frozen tissue specimens (about 0.1 g each) were homogenized and then analyzed by immunoblotting with the appropriate antibody.

siRNA knockdown of ISG15 in breast cancer ZR-75-1 cells. A 21-nucleotide duplex siRNA-targeting ISG15 and a control siRNA (siControl nontargeting siRNA 1) were purchased from Dharmacon Research, Inc. (Lafayette, CO). The siRNA-targeting ISG15 corresponds to the region 232-250 (accession no. AY168648). Breast cancer ZR-75-1 cells were cultured to semiconfluency and transfected with ISG15 siRNA using Oligofectamine (Invitrogen, Carlsbad, CA). Seventy-two hours after siRNA transfection, cells

were further transfected with a hemagglutinin (HA)-ubiquitin plasmid (20) using the PolyFect transfection reagent (Qiagen, Valencia, CA) for another 24 hours.

siRNA knockdown of UbcH8 in breast cancer ZR-75-1 cells. A 21-nucleotide duplex siRNA-targeting UbcH8 siRNA was purchased from Dharmacon Research. The siRNA-targeting UbcH8 corresponds to the region 237-255 (accession no. AF031141). Breast cancer ZR-75-1 cells were sequentially transfected with the UbcH8 siRNA and HA-ubiquitin cDNA as described above.

Results

Overexpression of a 15-kDa UCRP in tumor cells. A 15-kDa protein, which cross-reacted with anti-ubiquitin antibody as revealed by immunoblotting, was shown to exhibit highly variable expression in two different breast cancer cell lines (Fig. 1*A*). As shown in Fig. 1*A*, this 15-kDa UCRP was highly expressed in breast cancer ZR-75-1 but was barely detectable in breast cancer BT474 cells. Similarly, this 15-kDa protein was prominently detected in colorectal cancer cells (HCT116 and HT29) but not KM12 (data not shown). A high level of this 15-kDa UCRP was also detected in ovarian carcinoma 2774, prostate carcinoma DU145, and Bro melanoma cells but not in two other normal nontransformed human skin fibroblasts (data not shown). Similar to tumor cells, T-antigen-transformed 2RA cells but not their parental WI38 cells expressed a high level of this 15-kDa UCRP (Fig. 1*B*). In contrast to the variable expression of this 15-kDa UCRP in different cells, the expression of the 8-kDa ubiquitin seemed relatively constant in all these cells.

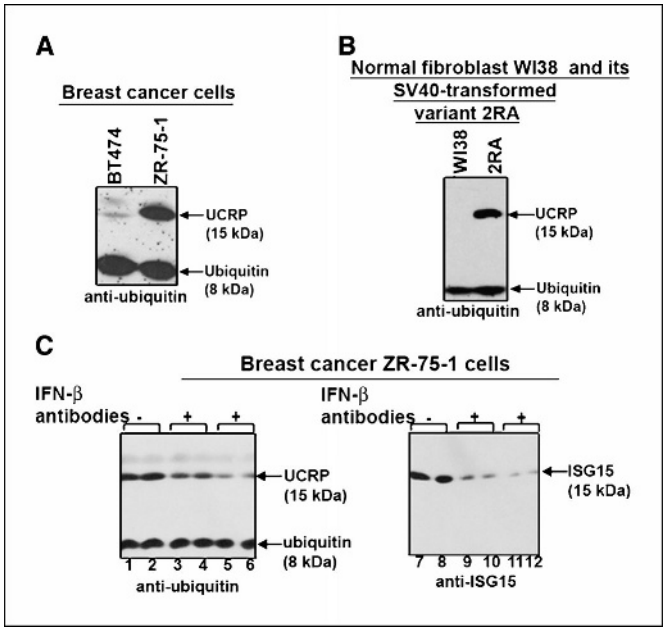

Figure 1. Elevated expression of a 15-kDa UCRP in tumor cell lines. *A,* varied expression of a 15-kDa UCRP in breast cancer cells. Lysates from semiconfluent breast cancer BT474 and ZR-75-1 cells (5×10^5 each) were analyzed by immunoblotting with anti-ubiquitin antibodies. *B,* elevated expression of a 15-kDa UCRP in SV40 T-antigen-transformed 2RA cells. Levels of the 15-kDa UCRP in human primary lung fibroblast WI38 cells and 2RA (SV40 T-antigen-transformed WI38) cells were determined by immunoblotting using anti-ubiquitin antibodies. *C,* IFN-β regulates the expression of ISG15 in breast cancer cells. The breast cancer ZR-75-1 cells were cultured for 4 days in the presence of human IFN-β antibodies raised in either rabbits (*lanes 3, 4, 9*, and *10*; 100 NU/mL), or sheep (*lanes 5, 6, 11*, and *12*; 100 NU/mL). Cell lysates were immunoblotted with either anti-ISG15 antibodies (*right*) or anti-ubiquitin antibodies (*left*).

The molecular weight of this 15-kDa UCRP suggested that it could be either diubiquitin or ISG15 (21). ISG15 but not diubiquitin expression is known to be induced by IFN (6, 16, 21). Neutralization of IFN-β by the addition of anti-IFN-β antibodies to the culture medium has been shown to suppress ISG15 expression in ataxia telangiectasia cells (16). To determine the identity of this 15-kDa UCRP, ZR-75-1 cells were treated with anti-IFN-β antibodies. As shown in Fig. 1*C* (*left*), the level of this 15-kDa protein was greatly reduced in ZR-75-1 cells after 4 days of treatment with anti-IFN-β antibodies raised in either rabbit (Fig. 1*C, lanes 3* and *4*) or sheep (Fig. 1*C, lanes 5* and *6*). The expression of ubiquitin (8 kDa) was not affected under these conditions (*left, lanes 1-6*).

To further identify this 15-kDa protein as ISG15, we turned to the use of ISG15-specific antibodies. As shown in Fig. 1*C* (*right*), a 15-kDa protein in ZR-75-1 cells was immunoreactive with the anti-ISG15 antibodies. In addition, the level of this immunoreactive protein was similarly reduced in ZR-75-1 cells treated with anti-IFN-β antibodies (Fig. 1*C*, compare *lanes 9-12* with *lanes 7-8*). Moreover, the expression of ISG15, as detected by the use of anti-ISG15 antibodies, was shown to be highly elevated in ZR-75-1 cells compared with that in BT474 cells (Fig. 2*A*). These results further suggest that this 15-kDa UCRP, which is highly elevated in many tumor cells, is ISG15.

Elevated but heterogeneous expression of ISG15 and its conjugates in tumors. In addition to the variable expression of free ISG15, variable expression of ISG15-protein conjugates was also detected in a panel of breast cancer cells (Fig. 2*A*). As shown in Fig. 2*A*, the expression level of ISG15-protein conjugates was low in BT474 cells but high in MDA-MB-231, MDA-MB-435, and ZR-75-1 cells. The same membrane used in Fig. 2*A* (*left*) was stained with Ponceau S to confirm equal protein loading (Fig. 2*A, right*).

Consistent with the observations in tumor cell lines, we have also observed highly elevated expression of ISG15 in human tumor biopsies. As shown in Fig. 2*B*, ISG15 was extensively conjugated to cellular proteins in endometrium (Fig. 2*B, top, lanes 4-6*) tissue specimens but not in their respective normal tissues (Fig. 2*B, top, lanes 1-3*). The extent of ISG15 conjugation to cellular proteins in endometrial tumors (Fig. 2*B, top*) was shown to be quite variable as observed in tumor cell lines (Fig. 2*A*). In addition to elevated expression of ISG15-protein conjugates, free ISG15 was also shown to be elevated in tumors compared with normal tissues (Fig. 2*B, top*, compare *lanes 3-6* with *lanes 1-3*). Protein loading was generally higher in normal tissue samples (Fig. 2*B, bottom*). Similar to endometrial tissues, ISG15 conjugates were highly elevated in colon tumor biopsies obtained from two different patients diagnosed with colon cancer but not in normal colon tissues obtained from two normal individuals (data not shown).

Overexpression of ISG15 is associated with reduced levels of polyubiquitinated proteins in tumor cells. Our previous studies have shown that the anticancer drug camptothecin induces 26S proteasome–dependent degradation of topoisomerase I in BT474 but not in ZR-75-1 breast cancer cells (22). Here, we have observed that ISG15 is overexpressed in ZR-75-1 but not in BT474 cells (see Figs. 1 and 2). This correlation could suggest that ISG15, which is a ubiquitin-like protein, is responsible for the defective polyubiquitination and consequently degradation of topoisomerase I in ZR-75-1 cells. Indeed, ubiquitin-like proteins are known to modulate ubiquitin-mediated degradation of cellular proteins in some cases (23, 24). Therefore, we examined the status of polyubiquitinated proteins in cells overexpressing ISG15. As shown in Fig. 3*A* (compare *lanes 1* and *2*), the level of polyubiquitinated proteins

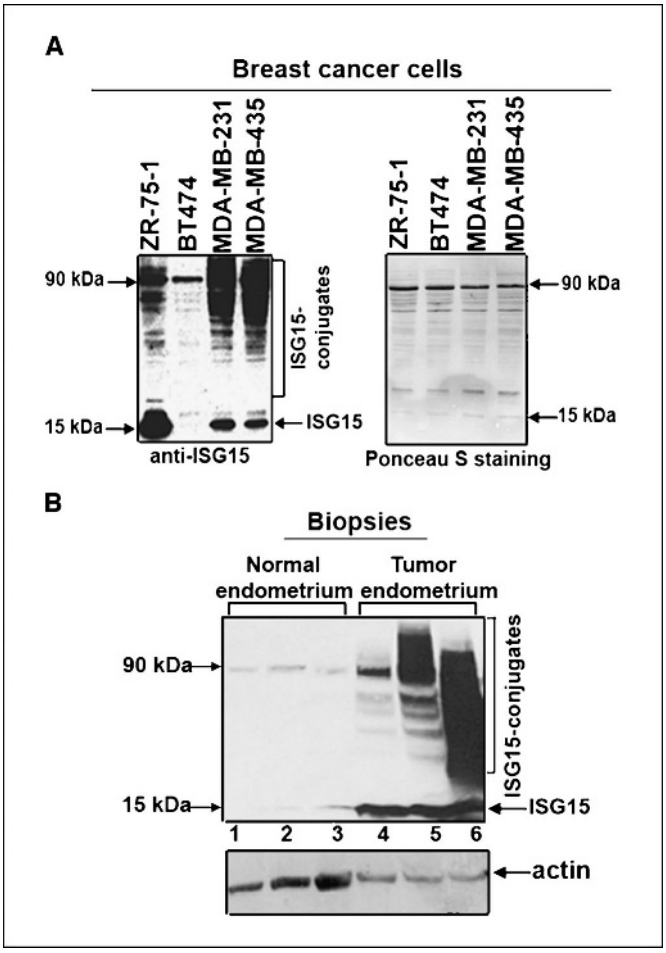

Figure 2. Elevated but heterogeneous expression of ISG15 and its conjugates in tumors. *A,* elevated expression of ISG15 and its conjugates in breast cancer cell lines. Breast cancer ZR-75-1, BT474, MDA-MB-231, and MDA-MB-435 cells (5×10^5 each) were cultured to semiconfluency. Cell lysates were analyzed by immunoblotting with anti-ISG15 antibodies (*left*). The same membrane filter was stained with Ponceau S to ascertain equal protein loading (*right*). *B,* overexpression of ISG15 and its conjugates in human endometrial tumors. Frozen endometrium tissue specimens obtained from three different patients diagnosed with endometrium tumors (*lanes 4-6*) and three normal individuals (*lanes 1-3*) were analyzed by immunoblotting with anti-ISG15 antibodies. The same membrane filter (*top*) was stripped and reprobed with anti-actin antibodies to assess equal protein loading (*bottom*).

(see protein species marked by *) was higher in BT474 cells than ZR-75-1 cells despite the protein loading being relatively lower in BT474 than in ZR-75-1 cells (Fig. 3*A, bottom*). This result indicates that the overall protein polyubiquitination is reduced in ZR-75-1 cells compared with BT474 cells, which could explain the defective degradation of topoisomerase I observed in camptothecin-treated ZR-75-1 cells (22).

The ubiquitin antibody used in the above experiment is known to cross-react with ISG15 (6). Consequently, we could not be certain whether the polyubiquitinated proteins (see species marked by *) identified in Fig. 3*A* also contained ISG15-protein conjugates and/or other UBL-protein conjugates. To rule out these other possibilities, HA-tagged ubiquitin cDNA was transfected into ZR-75-1 and BT474 breast cancer cells, and polyubiquitinated proteins were determined by immunoblotting with anti-HA rather than anti-ubiquitin antibodies. Indeed, consistent with the results shown in Fig. 3*A*, a higher level of polyubiquitinated proteins (HA-ubiquitin conjugated proteins marked by *) was observed in BT474 cells than in ZR-75-1 cells (Fig. 3*B*, compare *lanes 3* and *7*). The polyubiquitinated proteins migrated as a smear in Fig. 3*A* and a compressed band in Fig. 3*B*, which was due to the different gel systems used in these experiments (5% and 15% discontinuous gel versus 15% gel). The presence of a strong nonspecific band in Fig. 3*B* is due to the cross-reactivity of the HA antibody to an unknown cellular protein.

We also monitored the fate of HA-ubiquitin–conjugated proteins in BT474 and ZR-75-1 cells in the presence of the protein synthesis inhibitor cycloheximide (10 μg/mL). The levels of these polyubiquitinated proteins were reduced significantly in BT474 cells upon treatment with cycloheximide for 6 hours, suggesting the turnover of these polyubiquitinated proteins (Fig. 3*B, lanes 7* and *8*). The level of polyubiquitinated proteins in ZR-75-1 cells was also reduced, albeit to a lesser extent, upon cycloheximide treatment (Fig. 3*B, lanes 3* and *4*). These results suggest that overexpression of ISG15 is correlated with decreased protein polyubiquitination and degradation.

Knockdown of ISG15 expression increases the level of polyubiquitinated proteins in tumor cells. To confirm that overexpression of ISG15 in tumor cells is causally linked to the decreased level of polyubiquitinated proteins, ISG15 siRNA was employed to knock down the expression of ISG15 in ZR-75-1 cells. As shown in Fig. 4*A* (*top*), ISG15 siRNA significantly reduced ISG15 expression (about 80%) as evidenced by immunoblotting using anti-ubiquitin antisera (see the reduction in the intensities of the 15-kDa protein band marked ISG15). Interestingly, the amount of high molecular weight polyubiquitinated proteins (marked by *) was significantly elevated in ZR-75-1 cells treated with ISG15 siRNA compared with mock-transfected ZR-75-1 cells (compare *lanes 1* and *4* in Fig. 4*A, top*). The turnover of polyubiquitinated proteins in ISG15 siRNA-transfected ZR-75-1 cells was measured in the presence of cycloheximide (see Fig. 4*A, top*). About 60% of polyubiquitinated proteins (see species marked by *) in ISG15 siRNA-transfected ZR-75-1 cells were turned over in 6 hours. The turnover of polyubiquitinated proteins in the presence of cycloheximide was blocked by the proteasome inhibitor MG132 (10 μmol/L; data not shown), suggesting that the turnover of polyubiquitnated proteins was mediated by the 26S proteasome in ZR-75-1 cells transfected with ISG15 siRNA. We observed minimal turnover of the polyubiquitinated proteins in mock-transfected ZR-75-1 cells. However, as discussed before, these polyubiquitinated proteins may contain other proteins (e.g., ISG15 protein conjugates), which are cross-reactive with the ubiquitin antibody.

We also monitored the turnover of HA-ubiquitinated proteins in ZR-75-1 cells treated with ISG15 siRNA. Seventy-two hours after ISG15 siRNA transfection, cells were further transfected with HA-ubiquitin cDNA for 24 hours. As shown in Fig. 4*B* (*middle*), ISG15 siRNA significantly reduced ISG15 expression (about 70%) as evidenced by immunoblotting using anti-ISG15 antisera (compare *lanes 1-3* with *lanes 4-6*, respectively). However, under the same conditions, the amount of HA-ubiquitinated high molecular weight proteins as judged by immunoblotting with anti-HA antibody was significantly increased in cells treated with ISG15 siRNA compared with the cells treated with control siRNA (Fig. 4*B, top*, compare *lanes 1* and *4*). The turnover of HA-tagged ubiquitinated proteins was then monitored in the presence of cycloheximide. About 60% of HA-tagged polyubiquitinated proteins in ZR-75-1 cells transfected with ISG15 siRNA were degraded (Fig. 4*B, top*) after 6 hours of cycloheximide treatment. However, under the same conditions,

minimal turnover of the polyubiquitinated proteins in control siRNA-treated ZR-75-1 cells was observed (Fig. 4*B, top*, compare *lanes 1* and *3*). The same membrane filter as shown in Fig. 4*B* (*top*) was stripped and reprobed with anti-tubulin antibody for assessing protein loading (Fig. 4*B, bottom*). The turnover of polyubiquitinated proteins in the presence of cycloheximide was blocked by the proteasome inhibitor MG132 (10 μmol/L; data not shown), suggesting that the turnover of polyubiquitinated proteins was mediated by the 26S proteasome in ZR-75-1 cells transfected with ISG15 siRNA.

To test if indeed overexpression of ISG15 inhibits proteasome-mediated degradation of proteins, we monitored the effect of ISG15 siRNA on the steady-state level of p53 in ZR-75-1 cell. p53 is known to be a rapid turnover protein degraded by 26S proteasome (25). As shown in Fig. 4*C*, p53 was expressed at a high level in ZR-75-1 cells (expressing a high level of ISG15) but was significantly reduced in ZR-75-1 cells transfected with ISG15 siRNA (Fig. 4*C, top*). In addition to p53, we have also observed that the steady-state level of STAT3, another proteasome-degraded protein, is elevated in ISG15-overexpressing cells (data not shown). Similarly, 26S proteasome–mediated degradation of TOP1 in camptothecin-treated cells was also shown to be blocked in ISG15-overexpressing cells (Fig. 1*A*). These results suggest that ISG15 negatively regulates the level of polyubiquitinated proteins and their turnover.

Protein ISGylation interferes with protein polyubiquitination. The interfering effect of ISG15 on protein polyubiquitination could involve conjugation of ISG15 to its target proteins. Indeed, we have observed an inverse correlation between the level of ubiquitin-protein conjugates and the ISG15-protein conjugates in a number of tumor cells. As shown in Fig. 5*A* (*left*), the level of polyubiquitinated proteins (based on the detection of HA-tagged polyubiquitinated protein in HA-ubiquitin–transfected cells) was significantly higher in SW620 than in SW480 colorectal cancer cells. The reverse was true for the expression levels of ISG15-protein conjugates in these cells (see Fig. 5*A, right*). A similar inverse correlation between the level of ubiquitin-protein conjugates and ISG15-protein conjugates was also observed in MDA-MB-231 and MDA-MB-435 breast cancer cells (data not shown). This correlation suggests that ISGylation of cellular proteins could be responsible for reduced protein polyubiquitination in ISG15-overexpressing tumor cells.

To test if protein ISGylation is important for ISG15 interference of the ubiquitin pathway, the major ISG15 E2 (UbcH8) was knocked down by the use of UbcH8 siRNA in ZR-75-1 cells. As shown in Fig. 5*B* (*right*), UbcH8 siRNA significantly reduced the amount of high molecular weight ISG15 conjugates (marked as *) without altering the expression level of free ISG15 as evidenced by immunoblotting using ISG15 antisera. Interestingly, the amount of HA-ubiquitinated proteins was again significantly increased in cells treated with UbcH8 siRNA compared with the cells treated with control siRNA (Fig. 5*B, left*, compare *lanes 1* and *4*). The turnover rate of HA-tagged polyubiquitinated proteins was then examined in the presence of cycloheximide. Consistent to the results obtained with ISG15 siRNA transfections, about 60% of HA-tagged polyubiquitinated proteins was degraded within 6 hours of cycloheximide treatment in ZR-75-1 cells transfected with UbcH8 siRNA (Fig. 5*B, top left*, compare *lanes 4* and *6*). However, minimal turnover of HA-tagged ubiquitinated proteins was observed in ZR-75-1 cells transfected with control siRNA (Fig. 5*B, top left*, compare *lanes 1* and *3*). The same membrane filter (*top*) was stripped and reprobed with anti-tubulin antibody for assessing protein loading (Fig. 5*B, bottom left*). These results suggest that the ISG15 conjugation pathway interferes with protein polyubiquitination.

Forced expression of ISG15 decreases protein polyubiquitination. To further support the role of ISG15 in negative regulation of the ubiquitin pathway, the effect of ISG15 overexpression on the status of endogenous polyubiquitinated proteins in ZR-75-1 cells

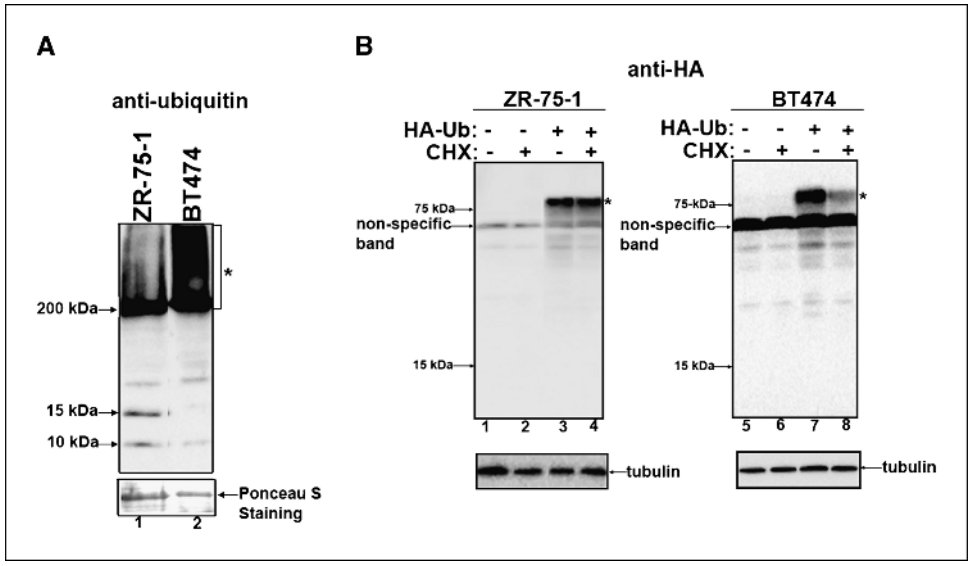

Figure 3. Reduced protein polyubiquitination in tumor cells overexpressing ISG15. *A,* varied levels of polyubiquitinated proteins in breast cancer cells. The breast cancer BT474 and ZR-75-1 cells (5×10^5 each) were lysed using 2× SDS sample buffer. Lysates were then analyzed using discontinuous (5%/15%) SDS-PAGE followed by immunoblotting with anti-ubiquitin antibodies (*top*). *, position of immunostained, high molecular weight protein species. The same membrane (*top*) was stained with Ponceau S stain. Top portion of the membrane (*bottom*). *B,* varied levels of HA-ubiquitin–conjugated proteins in breast cancer cells. The breast cancer ZR-75-1 (*left*) and BT474 (*right*) cells were transiently transfected with a HA-ubiquitin cDNA expression plasmid as described in Materials and Methods. Forty-eight hours after transfection, cells were treated with cycloheximide (*CHX*) for 6 hours and then analyzed by immunoblotting with anti-HA antibodies (*top*). Cycloheximide treatment (*top of lane*). *, position of immunostained, high molecular weight protein species. The same membranes (*top*) were reprobed with anti-tubulin antibodies to assess equal protein loading (*bottom*).

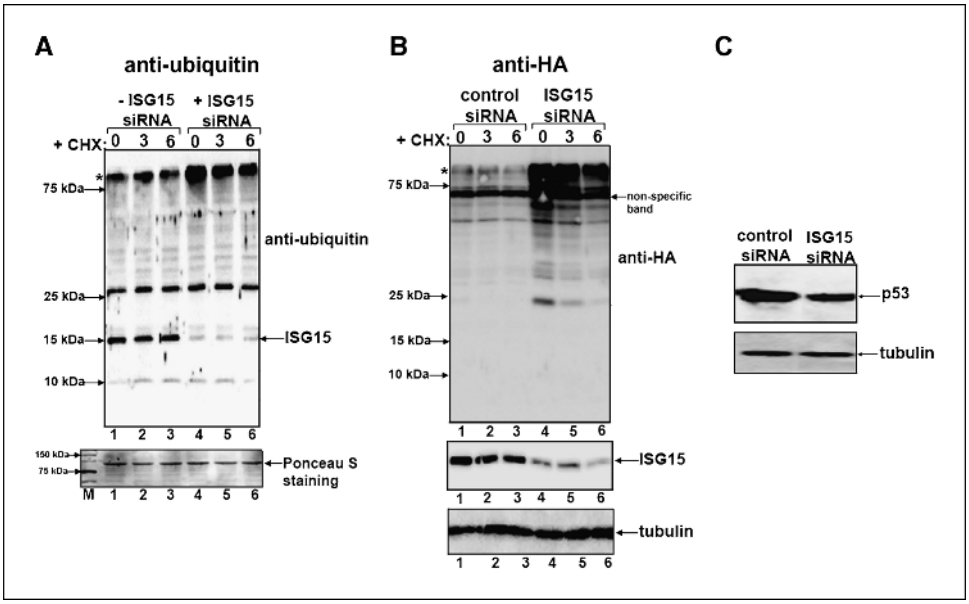

Figure 4. siRNA-mediated knockdown of ISG15 elevates the level of protein polyubiquitination in tumor cells. *A,* siRNA-mediated knockdown of ISG15 elevates the levels of polyubiquitinated proteins in ZR-75-1 breast cancer cells. ZR-75-1 cells were treated with ISG15 siRNA for 72 hours. Cells were then treated with cycloheximide (*CHX*; 10 μg/mL) for 0, 3, and 6 hours. Following cycloheximide treatment, all cells were lysed using SDS-sample buffer. Cell lysates were then analyzed by immunoblotting using anti-ubiquitin antibodies (*top*). *, position of the high molecular weight protein species (compressed due to the gel electrophoresis conditions). The same membrane (*top*) was stained with Ponceau S stain. Top portion of the membrane (*bottom*). *B,* the ISG15 siRNA increases conjugation of the ectopically expressed HA-tagged ubiquitin to the cellular proteins in ZR-75-1 cells. ZR-75-1 cells were treated with ISG15 siRNA for 72 hours followed by transfection with HA-ubiquitin expression vector for 24 hours. Cells were then treated with cycloheximide (10 μg/mL) for 0, 3, and 6 hours. Cell lysates were then analyzed by immunoblotting using anti-HA antibodies. *, position of immunostained, high molecular weight protein species (compressed due to the gel electrophoresis conditions). Bottom portion of the same membrane filter (*top*) was immunostained successively with anti-ISG15 (*middle*) and anti-tubulin (*bottom*) antibodies. *C,* the reduced turnover of p53 in ZR-75-1 cells. The same samples as in Fig. 3*B* (*lanes 1* and *4*) were reloaded on 10% SDS-polyacrylamide gels. Proteins were then transferred onto a nitrocellulose membrane and then immunoblotted with anti-p53 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; *top*). The filter used for immunoblotting was reprobed with anti-tubulin to assure equal protein loading (*bottom*).

was studied. As shown in Fig. 6*A*, the level of polyubiquitinated proteins (anti-ubiquitin cross-reactive material marked by *) was significantly diminished upon forced expression of ISG15 in ZR-75-1 cells. The elevated expression of Flag-ISG15 in transfected ZR-75-1 cells was evidenced by reprobing the same membrane filter with anti-ISG15 antibodies (Fig. 6*A, bottom*).

We also tested the effect of forced expression of ISG15 on the conjugation of HA-ubiquitin to the cellular proteins (based on detection of HA-tagged polyubiquitinated protein) in both BT474 and ZR-75-1 cells transiently transfected with Flag-ISG15. As shown in Fig. 6*B*, the amount of high molecular weight polyubiquitinated proteins (marked by *) was significantly reduced in both BT474 and ZR-75-1 cells transfected with Flag-ISG15 (Fig. 6*B, top*). The same membrane filter was successively reprobed with anti-ISG15 (to verify the ectopic expression of Flag-ISG15; Fig. 6*B, middle*) and then anti-tubulin antibodies (for assessing protein loading; Fig. 6*B, bottom*). These results again show that the expression level of ISG15 has a major effect on protein polyubiquitination.

Discussion

We have shown that the levels of both free ISG15 and ISG15 conjugates are greatly elevated in many tumor cell lines and tumor biopsy samples. The reason for the tumor-specific ISG15 overexpression in different tumors is unclear. One possibility is that the elevated expression of ISG15 may be due to constitutively activated nuclear factor-κB (NF-κB) in many tumor cells (16, 26). ISG15 is known to be under the transcriptional control of the NF-κB/IFN pathway (16). It seems plausible that constitutively activated NF-κB in tumor cells elevates the expression of IFN, which in turn transcriptionally up-regulates the expression of ISG15. Consistent with this explanation, we have shown that NF-κB activity is greater in ISG15-overexpressing ZR-75-1 than in ISG15-underexpressing BT474 breast cancer cells.[5]

The reason for the difference in the extent of ISG15 conjugation in various tumors is unclear. However, UBE1L (the ISG15-activating E1 enzyme; ref. 27) and UBP43 (an ISG15-specific deconjugating enzyme; ref. 28) are known to be deregulated in certain tumors, which could contribute to the varied expression levels of ISG15 conjugates in tumor biopsy samples.

The biological function of ISG15 is still unclear. Recent studies have suggested that ISG15, like ubiquitin, is involved in proteasomal degradation of selected proteins (29). In support of this notion, transfection of UBE1L has been shown to stimulate promyelocytic leukemia (PML)/retinoic acid receptor α (RARα) degradation in NB4 APL cells treated with retinoic acid (30). However, it is not known whether PML/RARα is directly conjugated to ISG15. On the other hand, ISG15 conjugation to Serpin 2a, JAK, or STAT1 does not increase their respective rate of degradation (31, 32). Despite this controversy, our current studies have suggested for the first time that the ISG15 pathway negatively regulates protein polyubiquitination and degradation. This conclusion is based on the following observations. First, the elevated expression of ISG15 and its protein conjugates is

[5] Unpublished results.

inversely correlated with the expression levels of polyubiquitinated proteins among many tumor cell lines (e.g., ZR-75-1/BT474 breast cancer cells and SW620/SW480 colorectal cancer cells). Second, knock down of the expression of ISG15 with siRNA specifically elevated the level of polyubiquitinated proteins in ZR-75-1 cells. Third, knockdown of UbcH8, the major E2 for ISG15, similarly elevated the level of polyubiquitinated proteins in ZR-75-1 cells. Fourth, overexpression of ISG15 in either ZR-75-1 or BT474 cells significantly reduced the level of polyubiquitinated proteins. Together, these results strongly suggest that the ISG15 pathway negatively regulates the ubiquitin pathway, possibly at the level of protein polyubiquitination.

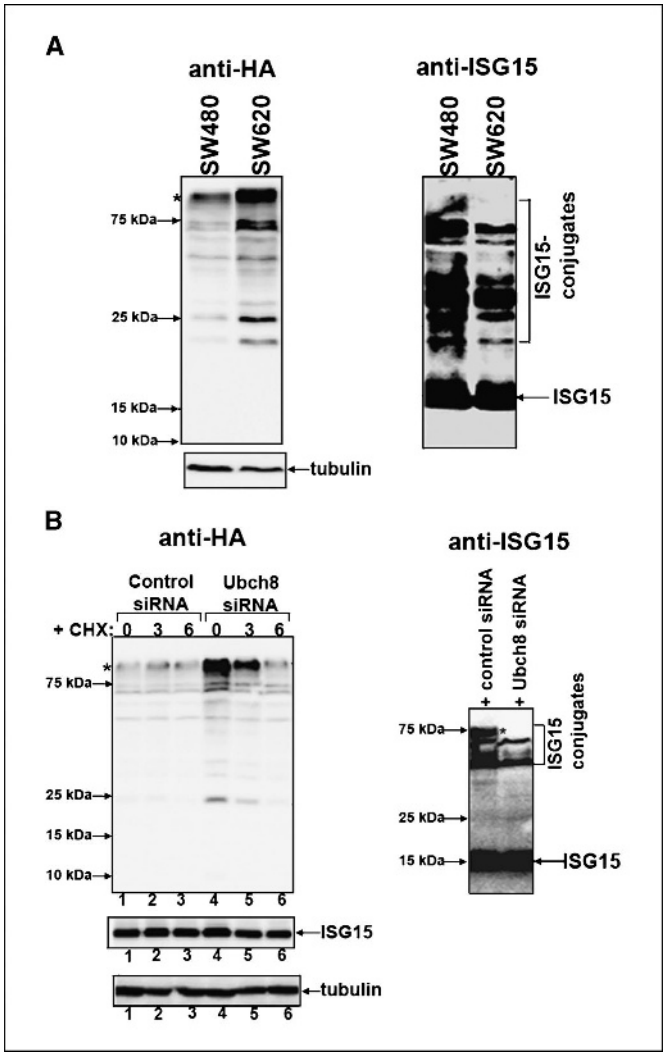

Figure 5. siRNA-mediated knockdown of UbcH8 elevates the level of protein polyubiquitination in tumor cells. *A,* an inverse correlation between the levels of polyubiquitinated proteins and ISG15 conjugates. Colorectal cancer SW480 and SW620 cells were transfected with an HA-ubiquitin cDNA plasmid using the PolyFect transfection reagent as described in Materials and Methods. Twenty-four hours after transfection, cells were lysed using 2× SDS sample buffer. Cell lysates were then analyzed by Western blotting using anti-HA antibodies (*top left*). *, position of immunostained, high molecular weight protein species (compressed due to the gel electrophoresis conditions). The same membrane (*top*) was stripped and reprobed with anti-tubulin antibody (Santa Cruz Biotechnology) to assess equal protein loading (*bottom left*). The same membrane (*top*) was restripped and probed with anti-ISG15 antibody to detect ISG15 conjugates (*right*). *B,* UbcH8 siRNA reduces the level of ISG15 conjugates but increases the level of polyubiquitinated proteins in ZR-75-1 cells. ZR-75-1 cells were treated with UbcH8 siRNA for 72 hours followed by transfection with an HA-ubiquitin cDNA expression plasmid for 24 hours as described in Materials and Methods. Cell lysates were then immunoblotted with anti-HA antibodies. The same membrane filter (*top*) was immunostained with anti-ISG15. Bottom portion of the membrane (*middle left*). The same membrane was stripped and reprobed with anti-tubulin (Santa Cruz Biotechnology) antibodies to assess protein loading (*bottom left*). The same samples as shown in *lanes 1* and *4* (*top left*) were reloaded on another gel and immunoblotted with anti-ISG15 antibodies to monitor the levels of ISG15 conjugates (*right*).

How ISG15 inhibits polyubiquitination of the cellular proteins is unclear. ISG15 could potentially interfere with the ubiquitin pathway at the level of E1, E2, and E3. Indeed, ISGylation of Ubc13 (ubiquitin E2) is shown to disrupt its ability to form the thioester bond with ubiquitin (33, 34). Whether other ubiquitin E2 functions are similarly inhibited by ISG15 through E2 ISGylation is not known. Analogous to ubiquitin E2, ubiquitin E1 has also been shown to be conjugated to ISG15 (35). However, it is unclear whether ISGlyation of ubiquitin E1 disrupts its ability to form the thioester bond with ubiquitin. It has also been reported that the ISG15 pathway converges with the ubiquitin pathway at E2/E3 (10). Thus far, only one E2 (UbcH8) has been identified for ISG15 (9, 10). This ISG15 E2 is also a ubiquitin E2 (36, 37). Consequently, it is possible that elevated expression of ISG15 may negatively interfere with the ubiquitin pathway by directly competing at a common site on this ISG15/ubiquitin E2 (UbcH8). However, although only one E2 has been identified for ISG15, there are many ubiquitin E2s (38). It is not clear whether other ubiquitin E2s can also function as ISG15 E2s. It has been shown that ISG15/ubiquitin E2 (UbcH8) interacts with many ubiquitin E3s (e.g., Rsp5 E3 ligase and members of the HECT and single-subunit RING E3 families; refs. 10, 36, 39). It is also possible that these ISG15 E2-interacting ubiquitin E3s are dual function E3s, which could conjugate both ubiquitin and ISG15 to their respective substrates. Indeed, the ubiquitin E3 ligase (Rsp5), which interacts with UbcH8, has been shown to be a dual function E3 ligase capable of conjugating ISG15 to a specific target *in vitro* (10). Consequently, elevated expression of ISG15 in tumor cells may switch these ubiquitin E3s to ISG15 E3s, leading to decreased levels of polyubiquitinated proteins. In addition to reduced levels of polyubiquitinated proteins, the turnover rate of polyubiquitinated proteins also seems reduced in ISG15 overexpressing tumor cells. It seems possible that the ISG15 pathway may interfere with the ubiquitin/26S proteasome pathway at multiple steps. A schematic diagram of this model is shown in Fig. 6*C*.

ISG15 is an IFN-inducible protein (6). The diverse biological functions of IFNs are based on the rapid expression of many genes, including *ISG15* (40). It is plausible that the defective protein turnover in tumor cells is not solely due the overexpression of ISG15 but is a consequence of overexpression/down-regulation of genes involved in proteasome function in response to IFNs. Indeed, previous studies have shown that the expression of the subunits of 19S regulatory cap of the 26S proteasome as well as some ubiquitin-conjugating enzymes (E2s) are induced in response to IFN treatment (41, 42). However, in the current study, we have shown that the siRNA-mediated knockdown of either ISG15 or ISG15 conjugates restores protein polyubiquitination and degradation in tumor cells. Our results thus strongly suggest that the defective turnover of polyubiquitinated proteins is primarily due to the overexpression of ISG15 conjugates in tumor cells. Indeed, aberrant degradation of cellular proteins during tumorigenesis is well known (43). However, it is possible that expression of ISG15, by itself,

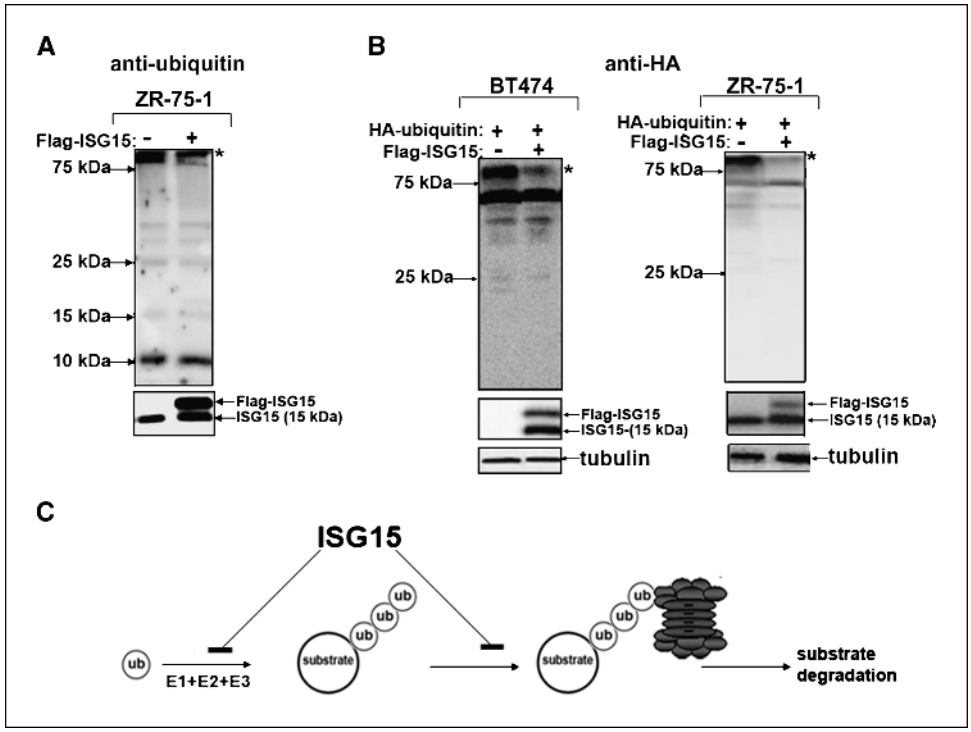

Figure 6. Overexpression of ISG15 reduces protein polyubiquitination in breast cancer cells. *A,* ISG15 overexpression decreases the level of polyubiquitinated proteins in ZR-75-1 cells. The breast cancer ZR-75-1 cells were transfected with Flag-ISG15 expression vector using PolyFect transfection reagent as described in Materials and Methods. Twenty-four hours after transfection, cells were lysed using 2× SDS-sample buffer. Cell lysates were then analyzed by immunoblotting using anti-ubiquitin antibodies (*top*). *, position of immunostained, high molecular weight protein species (compressed due to the gel electrophoresis conditions). The same membrane (*top*) was stripped and reprobed with anti-ISG15 antibodies to verify ectopic expression of Flag-ISG15. Bottom portion of the membrane (*bottom*). *B,* ISG15 overexpression decreases the level of proteins conjugated to HA-tagged ubiquitin in breast cancer cells. The breast cancer ZR-75-1 and BT474 cells were cotransfected with HA-ubiquitin and Flag-ISG15 expression vectors using the PolyFect transfection reagent as described in Materials and Methods. Twenty-four hours after transfection, cells were lysed with 2× SDS-sample buffer. Cell lysates were then analyzed by immunoblotting using anti-HA antibodies (*top*). *, position of immunostained, high molecular weight protein species. The same membranes (*top*) were stripped and reprobed with anti-ISG15 antibodies to verify ectopic expression of Flag-ISG15. Bottom portions of the membranes (*middle*). The same membranes (*top*) were stripped and reprobed with anti-tubulin antibody (Santa Cruz Biotechnology) to assess equal protein loading (*bottom*). *C,* a schematic illustrating the interference of ISG15 with the ubiquitin/26S proteasome pathway. In this model, the ISG15 pathway negatively regulates protein polyubiuqitination at multiple steps. The ISG15 pathway could interfere with protein ubiquitination at the level of E1, E2, and/or E3. This negative interference could result from either ISGylation of ubiquitin E1/E2/E3 or competition for dual functional E2/E3. In addition to interference with protein polyubiquitination, the ISG15 pathway may also interfere with proteasomal degradation of polyubiquitinated proteins.

also contributes to tumorigenesis. Indeed, elevated expression of ISG15 but not its conjugates is seen in human tumor endometrial biopsy samples (see Fig. 2*B*). In addition, deletion of UBE1L is highly associated with small and non–small cell lung cancers and other solid tumors (27, 44). Clearly, further studies are necessary to establish if the elevated expression of ISG15 in tumors also contributes to tumorigenesis.

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