SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
TARGETED THERAPIES
ANTIBODY THERAPY

Received
23 October 2013

Accepted
28 April 2014

Published
15 May 2014

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USP8 modulates ubiquitination of LRIG1 for Met degradation

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The Met receptor tyrosine kinase is an attractive target for cancer therapy as it promotes invasive tumor growth. SAIT301 is a novel anti-Met antibody, which induces LRIG1-mediated Met degradation and inhibits tumor growth. However, detailed downstream mechanism by which LRIG1 mediates target protein down-regulation is unknown. In the present study, we discovered that SAIT301 induces ubiquitination of LRIG1, which in turn promotes recruitment of Met and LRIG1 complex to the lysosome through its interaction with Hrs, resulting in concomitant degradation of both LRIG1 and Met. We also identified USP8 as a LRIG1-specific deubiquitinating enzyme, reporting the interaction between USP8 and LRIG1 for the first time. SAIT301 triggers degradation of LRIG1 by inhibiting the interaction of LRIG1 and USP8, which regulates ubiquitin modification and stability of LRIG1. In summary, SAIT301 employs ubiquitination of LRIG1 for its highly effective Met degradation. This unique feature of SAIT301 enables it to function as a fully antagonistic antibody without Met activation. We found that USP8 is involved in deubiquitination of LRIG1, influencing the efficiency of Met degradation. The relation of Met, LRIG1 and USP8 strongly supports the potential clinical benefit of a combination treatment of a USP8 inhibitor and a Met inhibitor, such as SAIT301.

Met is a product of the met proto-oncogene and a receptor for its physiological ligand, hepatocyte growth factor/scatter factor (HGF/SF)[1,2]. Upon HGF binding, the C-terminal tail of Met gets phosphorylated and numerous downstream signaling pathways become activated through the binding of several adaptor proteins[3–5]. In many cancers, aberrant activation of Met signaling has been implicated in aggressive tumor growth, invasion as well as resistance to other targeted therapies[6–8], making Met as an attractive target for cancer therapy[9–13].

Cbl, a key E3 ubiquitin ligase for Receptor Tyrosine Kinase (RTK), is an important negative regulator of RTKs[14]. Upon activation of RTKs, Cbl protein interacts with a phosphorylated tyrosine on the RTK leading to its down-regulation through ubiquitination[14–16]. LRIG1 is another negative regulator of RTKs including Met and works in a Cbl-independent manner. While Cbl-dependent destabilization of Met is dictated by receptor activation[14–16], LRIG1 pathway does not require receptor activation and ubiquitination for its function, decoupling Met signaling from its down-regulation mechanism. Met receptor interacts with the transmembrane protein LRIG1, independently of HGF stimulation[17–19]. However, detailed downstream mechanism by which LRIG1 mediates target protein down-regulation is unknown.

Endocytosis is important for the function of many plasma membrane receptors[20], and conjugation of ubiquitin to these membrane proteins is the major component of the regulatory mechanism for their internalization and lysosomal degradation[21–24]. Deubiquitination, the opposite process, is also critically involved in regulating the degradation of several RTKs by removing monoubiqutin and polyubiquitin chains from ubiquitin-conjugated proteins, resulting in inhibition of protein degradation[25–27]. Therefore, a balance between ubiquitination and deubiquitination rules the fate of internalized receptors and their downstream signaling.

Recently, we developed a novel anti-Met antibody, SAIT301, which promotes a Cbl-independent, LRIG1-mediated Met degradation pathway and the internalization of both Met and LRIG1 without Met ubiquitination[28]. Here, we investigated the molecular mechanism of LRIG1-mediated Met down-regulation by a Met-targeting therapeutic antibody, SAIT301. The present study delineates, for the first time, 1) the ubiquitination of LRIG1 and its role as a trigger for lysosomal degradation of LRIG1 or LRIG1-Met complex, and 2) the importance of ubiquitin specific protease 8 (USP8)-dependent deubiquitination in regulation of LRIG1 stability. These results suggest that simultaneous blockage of USP8 may further enhance LRIG1-dependent Met degradation and

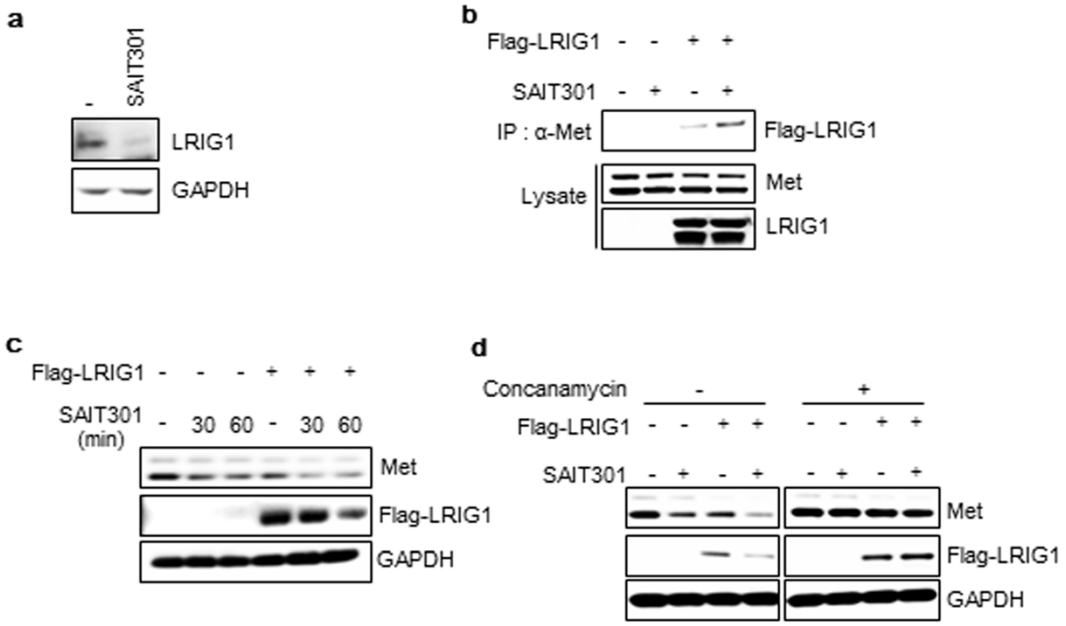

Figure 1 | SAIT301 induces degradation of LRIG1. (a) After SAIT301 (5 μg/ml) treatment for 1 h in EBC1 cells, cell lysates were subjected to immunoblot with anti-LRIG1 antibody. (b) After SAIT301 (5 μg/ml) treatment, MKN45 cells over-expressing Flag-tagged LRIG1 were incubated for 1 h. Cell lysates were subjected to immunoprecipitation with anti-Met antibody. (c) Flag-tagged LRIG1 was expressed in MKN45 cells. After SAIT301 (5 μg/ml) treatment, cells were incubated for indicated time. Cell lysates were subjected to immunoblot with anti-LRIG1 or anti-Met antibody. (d) MKN45 cells were transfected with Flag-LRIG1. After incubation with 100 nM concanamycin for 4 h and SAIT301 for 1 h, cell lysates were subjected to immunoblot with anti-LRIG1 or anti-Met antibody.

subsequent tumor growth inhibition by SAIT301 and other Met targeting drugs that have a similar mechanism of action.

Results

Degradation of LRIG1 by a Met-targeting antibody. LRIG1 destabilizes the Met receptor in HGF- and Cbl-independent manners, however its detailed mechanism is not fully elucidated yet. Previously, we have demonstrated the implication of LRIG1 in Met degradation by a Met–targeting antibody, SAIT301[28]. To investigate the underlying molecular mechanism of LRIG1-mediated Met degradation, we first examined the change in cellular level of LRIG1 after SAIT301 treatment. Upon treatment with SAIT301 for 1 hour, total protein level of LRIG1 decreased in EBC1 cells (Figure 1a). Next, we over-expressed Flag-LRIG1 in MKN45 cells which have a low level of inherent LRIG1. As shown in Figure 1b, SAIT301 strongly induced interaction of Met and LRIG1. In parallel, the levels of both Met and LRIG1 were markedly decreased following SAIT301 treatment (Figure 1c), suggesting that SAIT301 induces interaction of Met and LRIG1, and simultaneous degradation of both molecules. This concomitant degradation of LRIG1 and Met induced by SAIT301 was completely prevented by treatment of concanamycin (Figure 1d), a specific inhibitor of lysosomal degradation pathway. Taken together, these results suggest that SAIT301 induces interaction of Met and LRIG1 and subsequent lysosomal degradation of two proteins.

Ubiquitination of LRIG1 promotes lysosomal degradation of LRIG1 and Met. We previously demonstrated that SAIT301-induced Met degradation is mediated by LRIG1 through a lysosomal pathway[28]. It is well known that ubiquitination is important in the lysosomal degradation pathway[21]. SAIT301, however, did not induce significant ubiquitination of Met in EBC1 cells while 5D5, another bivalent anti-Met antibody that is known to utilize Cbl for degradation of Met, strongly induced Met ubiquitination[28]. Therefore, we first examined whether LRIG1 could be modified by ubiquitin and whether this modification is related with SAIT301-induced degradation of Met. Flag-LRIG1 overexpressing MKN45 cell line was pre-treated with concanamycin before SAIT301 treatment to maintain consistent levels of LRIG1 through the duration of the experiment. In this experiment, the level of ubiquitinated LRIG1 was markedly increased after SAIT301 treatment (Figure 2a). Similar results were obtained by *in vitro* ubiquitination assay using purified Flag-LRIG1 protein (Figure 2b).

Hepatocyte growth factor-regulated Tyr-kinase substrate (Hrs) functions by binding to ubiquitin via ubiquitin-interacting motif (UIM), which is essential for efficient sorting of ubiquitinated membrane proteins[22,23]. Thus, Hrs is proposed to be a sorting receptor that recognizes the ubiquitin moieties of membrane proteins and introduces them into multivesicular bodies in mammalian cells[24]. Since SAIT301 induces both ubiquitination and reduction of LRIG1, we hypothesized that ubiquitinated LRIG1 in turn binds to Hrs, resulting in lysosomal degradation of the LRIG1 (and thus the Met-LRIG1 complex). Therefore, we investigated that the effect of LRIG1 ubiquitination on the interaction with Hrs. We first checked whether SAIT301 or 5D5 binding results in ubiquitination of different substrates. When Flag-LRIG1 was over-expressed in MKN45 cells, SAIT301 induced dramatic ubiquitination of LRIG1 while 5D5 induced ubiquitination of Met and not LRIG1 (Figure 2c). Furthermore, SAIT301 treatment significantly increased the level of LRIG1 co-immunoprecipitated with Hrs while 5D5 had no such effect, which actually induced the interaction of Met and Hrs (Figure 2d). The amount of Met that co-immunoprecipitated with LRIG1 and Hrs by SAIT301 treatment is only a minor pool of total Met. The interaction of LRIG1 and Hrs was further confirmed by immunofluorescence assay where SAIT301 induced colocalization of LRIG1 and Hrs (Figure 2e). Subsequent lysosomal degradation of Met/LRIG1 complex was verified by co-localization of LRIG1 and Met with a lysosomal marker (Lamp1) (Figure 2f). This mechanism differentiates SAIT301 from other agonistic Met-targeting antibodies that engage Cbl-dependent direct ubiquitination of Met without inducing the interaction of LRIG1 and Met. Actually, SAIT301 blocked phosphorylation of Met (Y1234/5)

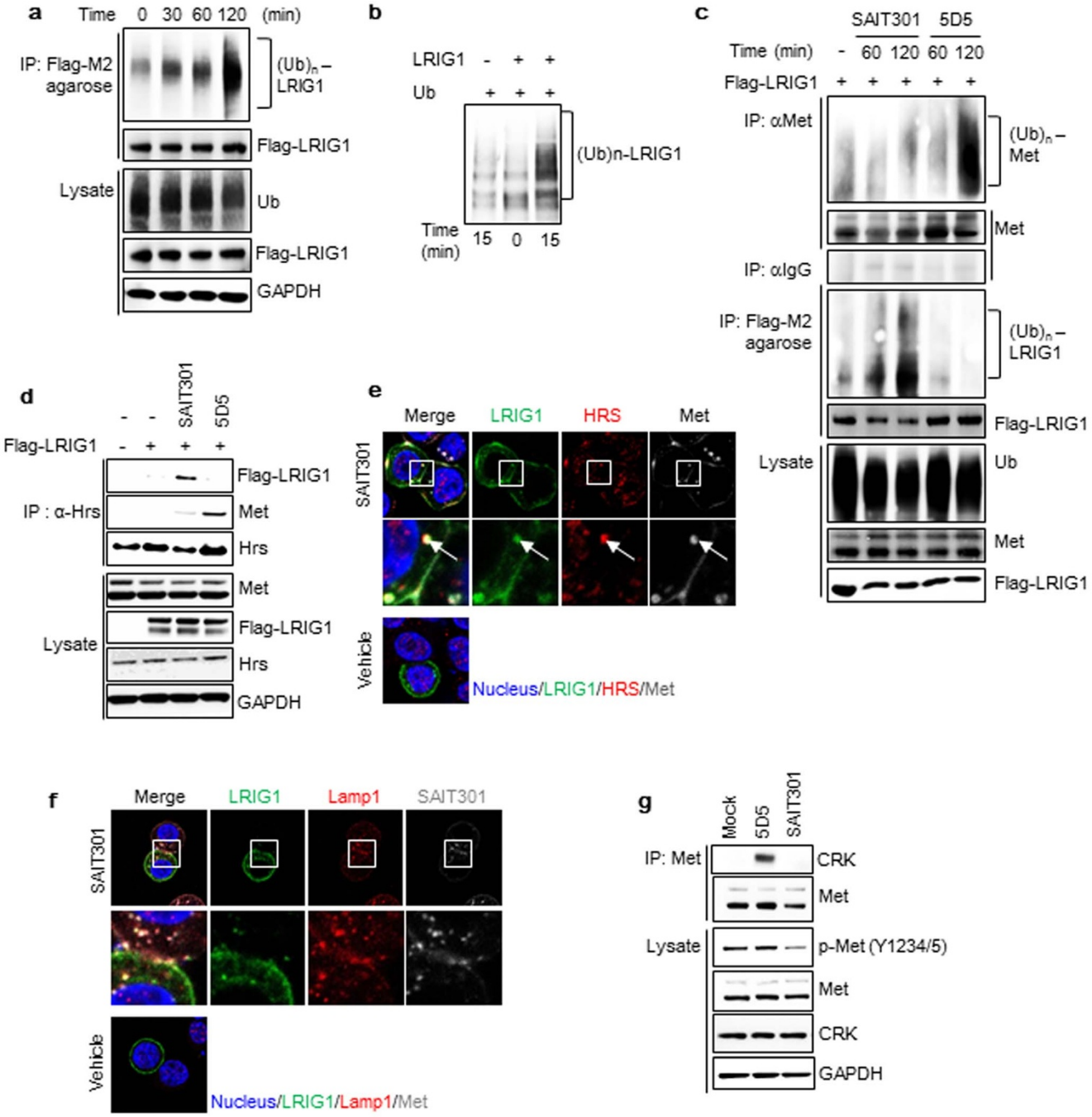

Figure 2 | Ubiquitination of LRIG1 promotes lysosomal degradation of LRIG1 and Met. (a) MKN45 cells expressing Flag-tagged LRIG1 were incubated with 100 nM concanamycin for 4 h and SAIT301 for the indicated periods. Cell lysates were then subjected to immunoprecipitation with Flag-M2 agarose followed by immunoblot with anti-Ubiquitin or anti-Flag antibody. (b) LRIG1 could be ubiquitinated *in vitro*. Ubiquitination assay was performed using purified proteins as described under "Materials and methods." (c) MKN45 cells expressing Flag-tagged LRIG1 were incubated with 100 nM concanamycin for 4 h and SAIT301 for the indicated periods. Cell lysates were then subjected to immunoprecipitation with Flag-M2 agarose or anti-Met antibody followed by immunoblot with anti-Met, anti-Flag or anti-Ubiquitin antibody. (d) MKN45 cells expressing Flag-tagged LRIG1 were incubated with 100 nM concanamycin for 4 h and SAIT301 for 1 hr. Cell lysates immunoprecipitated with anti-Hrs antibody were followed by immunoblot with indicated antibodies. (e and f) Co-localization of (e) Hrs or (f) Lamp1 with LRIG1 and Met in LRIG1 overexpressing MKN45. Cells were treated with SAIT301 for 1 h. (g) EBC1 cells were incubated with 5 μg/ml of SAIT301 for 1 h. Cell lysates were subjected to immunoprecipitation with anti-Met antibody followed by immunoblot with anti-Crk or anti-Met antibodies.

for signaling activation, while 5D5, an agonistic antibody, did not (Figure 2g). This is further substantiated by 5D5-induced interaction of Met and Crk (Figure 2g), one of the pivotal adaptor proteins responsible for recycling of many RTKs at early endosome[29]. In contrast, such association was not observed with SAIT301 (Figure 2g). These results suggest that SAIT301-induced ubiquitination of LRIG1 promotes recruitment of Met-LRIG1 complex to the lysosome through its interaction with Hrs, resulting in lysosomal degradation of Met and LRIG1. Furthermore, by circumventing the Cbl-dependent Met ubiquitination and recycling via Crk, SAIT301 avoids activation of Met that may result in agonism during the Met degradation process.

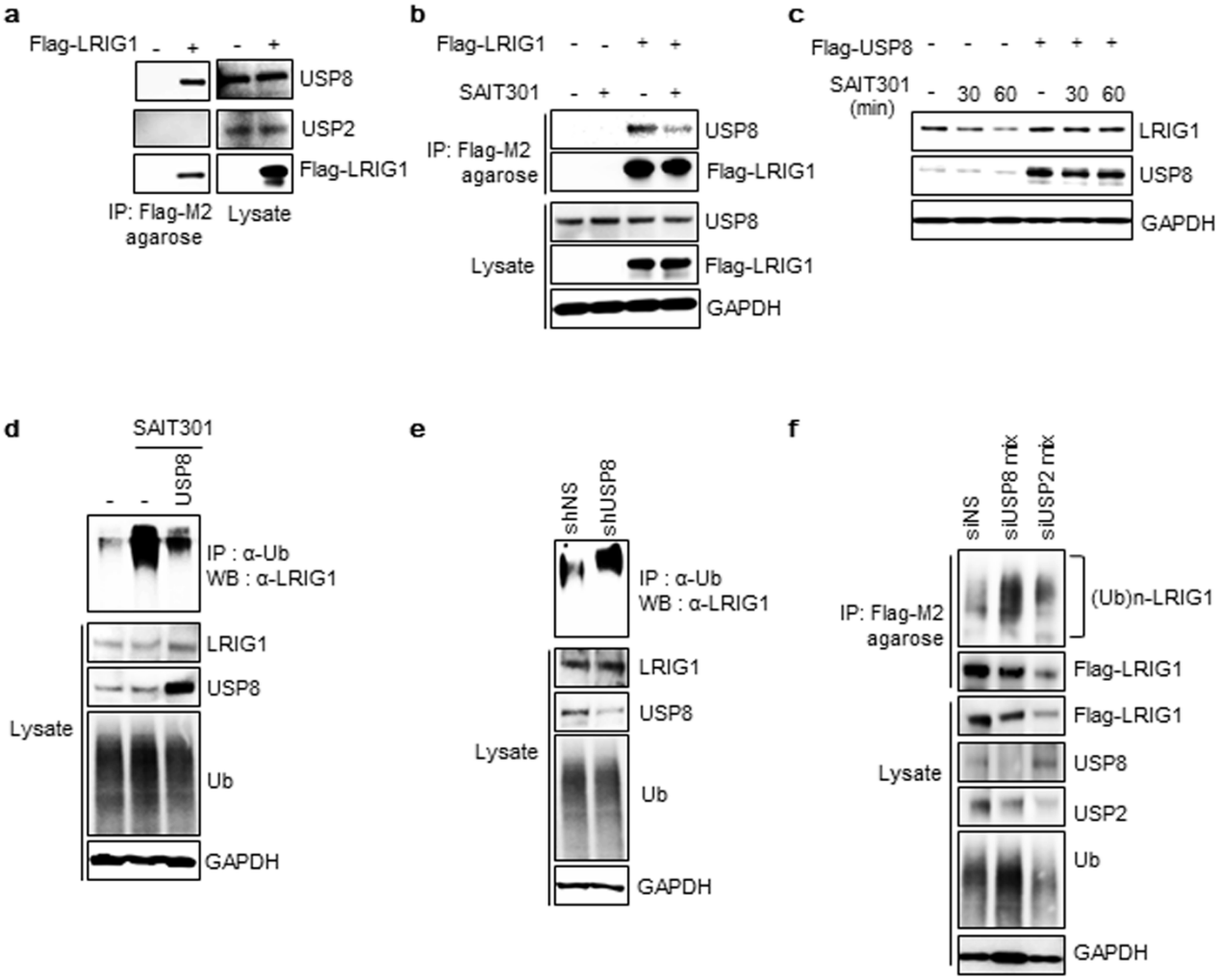

Figure 3 | USP8 regulates LRIG1 ubiquitination. (a) Flag-LRIG1 was over-expressed in MKN45 cells with SAIT301 treatment for 1 h. Cell lysates were subjected to immunoprecipitation with Flag-M2 agarose followed by immunoblot with anti-USP8 or anti-USP2 antibody. (b) MKN45 cells were transfected with Flag-LRIG1. After SAIT301 treatment for 1 h, cell lysates subjected to immunoprecipitation with Flag-M2 agarose followed by immunoblot with anti-USP8 or anti-Flag antibody. (c) MKN45 cells over-expressing USP8 and LRIG1 were treated with SAIT301 for the indicated periods. Cell lysates were subjected to immunoblot with indicated antibodies. (d) EBC1 cells overexpressing Flag-USP8 were incubated with SAIT301 for 1 h. Cell lysates were subjected to immunoprecipitation with anti-Ubiquitin antibody followed by immunoblot with anti-LRIG1 or anti-Ubiquitin antibody. (e) EBC1 cells transfected with shNS (control vector) or shUSP8 were treated with SAIT301 for 1 h. Cell lysates were then processed as in (d). (f) MKN45 cells were co-transfected with Flag-LRIG1 and following siRNAs; siNS (non-specific siRNAs), mixture of 3 individual USP8 specific siRNAs, or mixture of 2 individual USP2 specific siRNAs. After SAIT301 treatment for 1 h, cell lysates subjected to immunoprecipitation with Flag-M2 agarose followed by immunoblot with anti- Ubiquitin or anti-Flag antibody. Note that 100 nM concanamycin was treated for 4 h before cell lysis for the experiments of figures 3b, 3d, 3e, and 3f.

USP8 counteracts on ubiquitination of LRIG1. Ubiquitination is a reversible post-transcriptional modification, which triggers internalization and lysosomal degradation of cell-surface receptors. The stability of many ubiquitinated membrane proteins and receptors is controlled by deubiquitinating enzymes such as USP2 and USP8[25–27]. To investigate whether these two enzymes are also responsible for deubiquitination of LRIG1, we performed immunoprecipitation assay. As shown in Figure 3a, USP8, but not USP2, indeed interacted with LRIG1. Interestingly, SAIT301 treatment markedly decreased the interaction of LRIG1 and USP8 (Figure 3b). This result suggests that SAIT301 perturbs USP8-mediated modulation of LRIG1, resulting in the degradation of LRIG1. It also suggests that sufficient amount of USP8 may counteract SAIT301-induced degradation of LRIG1. To confirm this observation, USP8 was ectopically over-expressed and the protein levels of LRIG1 were measured. Over-expression of USP8 substantially reduced the LRIG1 degradation (Figure 3c).

As the ubiquitination of LRIG1 induced by SAIT301 has important role in the degradation of LRIG1, we investigated the effect of USP8 on the level of ubiquitinated LRIG1. SAIT301 treatment significantly enhanced the ubiquitination of LRIG1, whereas over-expression of USP8 markedly diminished the ubiquitination of LRIG1 (Figure 3d). To determine whether endogenous USP8 deubiquitinates LRIG1, we used shUSP8 to decrease the level of endogenous USP8 in EBC1 cells. Knock-down by shUSP8, but not the non-targeting control shRNA, significantly enhanced ubiquitination of LRIG1 (Figure 3e). In addition, we observed that the treatment of the mixture of three USP8 siRNAs, not the mixture of USP2 siRNAs, significantly enhanced SAIT301-mediated ubiquitination of LRIG1, which supports further the specificity of USP8 in the LRIG1 deubiquitination (Figure 3f; a densitometric analysis revealed the relative band intensities of ubiquitinated LRIG1/total LRIG1 in cell lysate as following. siNS: 100.0 $\pm$ 6.7, siUSP8 mix: 381.3 $\pm$ 28.1, and siUSP2 mix: 243.3 $\pm$ 14.9). Collectively, these results suggest that SAIT301

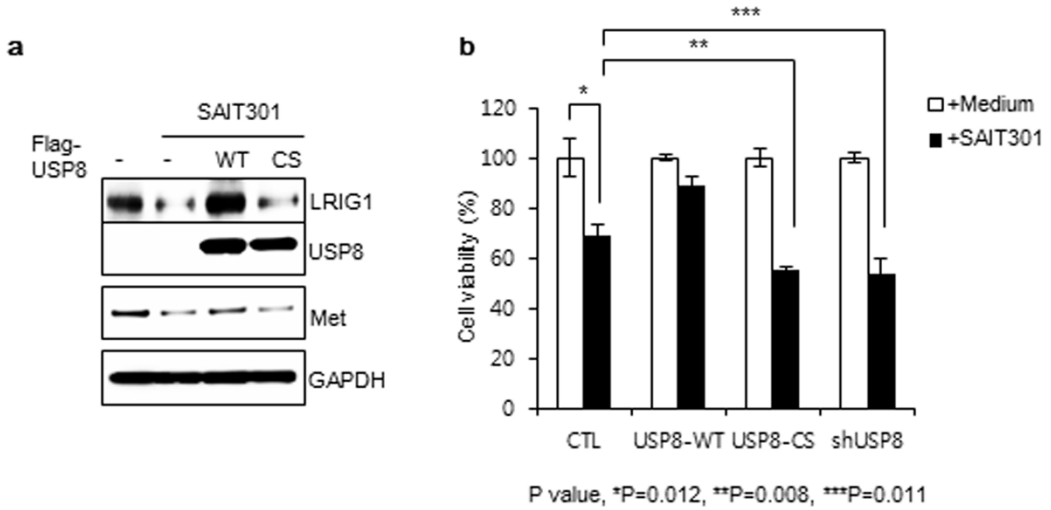

Figure 4 | USP8 regulates LRIG1-mediated Met degradation. (a) EBC1 cells transfected with Flag-tagged USP8 (WT) or its catalytically inactive form (C786S) were treated with SAIT301 and then incubated for 1 h. Cell lysates were subjected to immunoblot with indicated antibodies. (b) The viability of EBC1 cells which had been transfected with Flag-tagged USP8 (WT), its catalytically inactive form (C786S) or shUSP8 was measured by CTG assay after treatment with 2 μg/ml of SAIT301 for 72 h (mean ± s.d., n = 3). P values were calculated by student's t-test (two-tailed distribution & paired t-test).

triggers degradation of LRIG1 by interfering the interaction of USP8 and LRIG1, and USP8 regulates ubiquitin modification and stability of LRIG1.

USP8 regulates LRIG1-mediated Met degradation. To elucidate the protective effect of USP8 on the LRIG1-mediated Met degradation, EBC1 cells were transfected with plasmid encoding wild-type USP8 or mutant USP8-CS, a dominant negative mutant in which a highly conserved Cys residue at the core domain was replaced by Ser (C786S)[25,26]. Over-expression of USP8 significantly reversed SAIT301-mediated degradation of both LRIG1 and Met while USP8-CS had no such impact (Figure 4a). These results implicate that deubiquitination activity of USP8 is crucial for the regulation of the protein levels of both LRIG1 and Met through deubiquitination of LRIG1.

To investigate the role of USP8 on SAIT301-induced cancer cell growth inhibition, we transfected EBC1 cells with USP8, USP8-CS or shUSP8 expression constructs. Over-expression of USP8 almost completely reversed SAIT301-induced growth inhibition of EBC1 cells. In contrast, decreasing the functional level of USP8 by transfecting USP8-CS or shUSP8 substantially enhanced the anti-tumor effect of SAIT301 (Figure 4b). Taken together, these data suggest that USP8 works as a regulator for LRIG1-mediated Met degradation by controlling the level of ubiquitination of LRIG1. It is plausible that concomitant treatment of a USP8 inhibitor will lead to an enhanced antitumor efficacy of Met targeting antibodies such as SAIT301.

USP8 determines the sensitivity of cancer cells to a Met-targeting antibody. Based on the result that the presence of USP8 can negatively influence the LRIG1 ubiquitination and degradation of Met-LRIG1 complex, we assessed the correlation between the level of USP8 in tumors and anti-tumor effect of SAIT301 using tumor samples from patient-derived non-small cell lung cancer (NSCLC) xenograft models. Among the 6 tumors with high Met mRNA levels, two tumors (lung tumor #1 and #2) had high LRIG1 protein level measured by ELISA (Figure 5a). Lung tumor #1 had decent level of USP8, whereas lung tumor #2 had barely detectable level of USP8 (Figure 5b).

Interestingly, in lung tumor #2 with low levels of USP8, Met protein level was significantly reduced by SAIT301 treatment, compared to vehicle (PBS) treatment, which was not seen in lung tumor #1 (Figure 5c). It should be noted that very efficient Met degradation by SAIT301 is observed in almost all Met positive cancer cells, even with very low Met expression level. The fact that SAIT301 did not induce Met degradation in tumor #1 strongly implicates the potential role of USP8 in the antibody-mediated Met degradation. Moreover, consistent with reduction of Met protein level, SAIT301 demonstrated a strong tumor growth inhibition only in lung tumor #2, resulting in the reduction of tumor volume by 76% (Figure 5d). These results support the hypothesis that the presence of USP8 may affect the potency of anti-tumor efficacy of SAIT301, and SAIT301 may benefit from concomitant inhibition of USP8 for improved efficacy.

Discussion

In the present study, we found that SAIT301, a Met targeting antibody, promotes degradation of both LRIG1 and Met through ubiquitination of LRIG while it has no significant effect on ubiquitination of Met. Our experimental evidence suggests that SAIT301-induced ubiquitination of LRIG1 promotes recruitment of Met-LRIG1 complex to the lysosome through its interaction with Hrs, leading to lysosomal degradation of Met and LRIG1 (Figure 2c and 2d). In contrast, 5D5, an agonistic Met antibody, induced strong interaction of Met and Crk, one of the pivotal adaptor proteins responsible for recycling of many RTKs at early endosome[29] (Figure 2g). This result suggests that agonistic antibodies, such as 5D5, can prolong the activity of Met signaling by promoting Met ubiquitination in a Cbl-dependent manner and recycling of Met, while SAIT301 induces more efficient lysosomal degradation by avoiding recycling of Met. It is plausible that the low or non-agonistic feature of SAIT301 is attributed to its unique mechanism of Met degradation through ubiquitination of LRIG1.

Ubiquitin-specific proteases (USPs) are a subclass of deubiquitinating enzymes with specific targets of therapeutic importance[30]. Due to their highly-specific activity and involvement in several human pathologies including cancer, USPs are rapidly emerging as promising targets for drug design[31,32]. USP8 interacts with a number of clinically relevant cancer targets, including EGFR and ERBB2[25,26]. Here, we report for the first time that LRIG1 is another substrate of USP8. We demonstrated that SAIT301 inhibits the interaction of LRIG1 and USP8. Also, when over-expressed, USP8 decreases LRIG1 ubiquitination by SAIT301 treatment (Figure 3d). Knockdown of USP8 either by shRNA or siRNA, in contrast, substantially increased SAIT301-mediated ubiquitination of LRIG1 (Figures 3e and 3f). Thus, USP8 appears to regulate ubiquitination and stability of LRIG1 and counteracts LRIG1-mediated Met degradation.

USP8 is known to enhance cell growth as its expression increases in cancer cell[33]. We demonstrate here that transient over-expression of USP8 reverses growth inhibition of lung cancer cells by SAIT301 through prevention of the down-regulation of both LRIG1 and Met, while knock-down of USP8 enhanced SAIT301-induced growth inhibition. These observations imply that the level of USP8 in cancer can determine the sensitivity of cancer cells to Met targeting antibodies such as SAIT301. Consistent with these data, we observed a correlation between the levels of USP8 and anti-tumor effect of SAIT301 in human patient-derived lung tumors. SAIT301 treatment resulted in more efficient Met degradation and substantial tumor growth inhibition in tumor with lower levels of USP8 (Figures 5c and 5d). With respect to Met protein levels between two samples in figure 5, tumor #2 demonstrated higher levels of Met than that of tumor #1. It is plausible therefore to state that tumor #2 was more responsive to SAIT301 just due to its higher Met expression level. Alternatively, other factors than USP8 expression, such as mutation(s) on particular genes, might have caused tumor #1 less dependent upon Met receptor for growth. Although we cannot exclude those possibilities at this moment, it should be also noted that efficient Met degradation by SAIT301 was not observed in tumor #1 (Figure 5c), whereas very efficient Met degradation by SAIT301 was usually observed in almost all Met positive cancer cells even with very low Met expression level. This strongly implicates the potential role of USP8 in the antibody-mediated Met degradation. Moreover, we showed that suppression of USP8 activity, by over-expression of USP8-CS, led to enhancement of the anti-tumor activity of Met-targeting therapeutic antibody by promoting degradation of both Met and LRIG1. This result is reminiscent of a recent report showing that inhibition of USP8 overcomes gefitinib-resistance in NSCLC cells through down-regulating the levels of RTKs including Met[30]. It is therefore speculated that USP8 may play a role in the resistance mechanism to Met-targeting therapies. As such, importance for simultaneous inhibition of USP8 may be more widely applied for other RTK-targeting agents as well, to enhance their anti-tumor efficacy. Further research is warranted for exploring the effect of combination treatments with RTK-targeting agents and USP8 inhibitors.

In summary, the findings in the current study highlight LRIG1-mediated Met degradation and the implication of USP8 in the regulation of LRIG1 ubiquitination by a Met-targeting antibody. Further studies are needed to elucidate the clinical benefit of combination treatment of a USP8 inhibitor and Met-targeting antibodies.

Methods

Antibodies, reagents and cell lines. The following commercially available antibodies were used: mouse IgG (eBiosciences, San Diego, CA, USA), Met, LRIG1 (Abcam, Cambridge, UK), Hrs, LAMP1, Ubiquitin (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) and GAPDH (14C10) antibodies (Cell Signaling, Danvers, MA, USA).

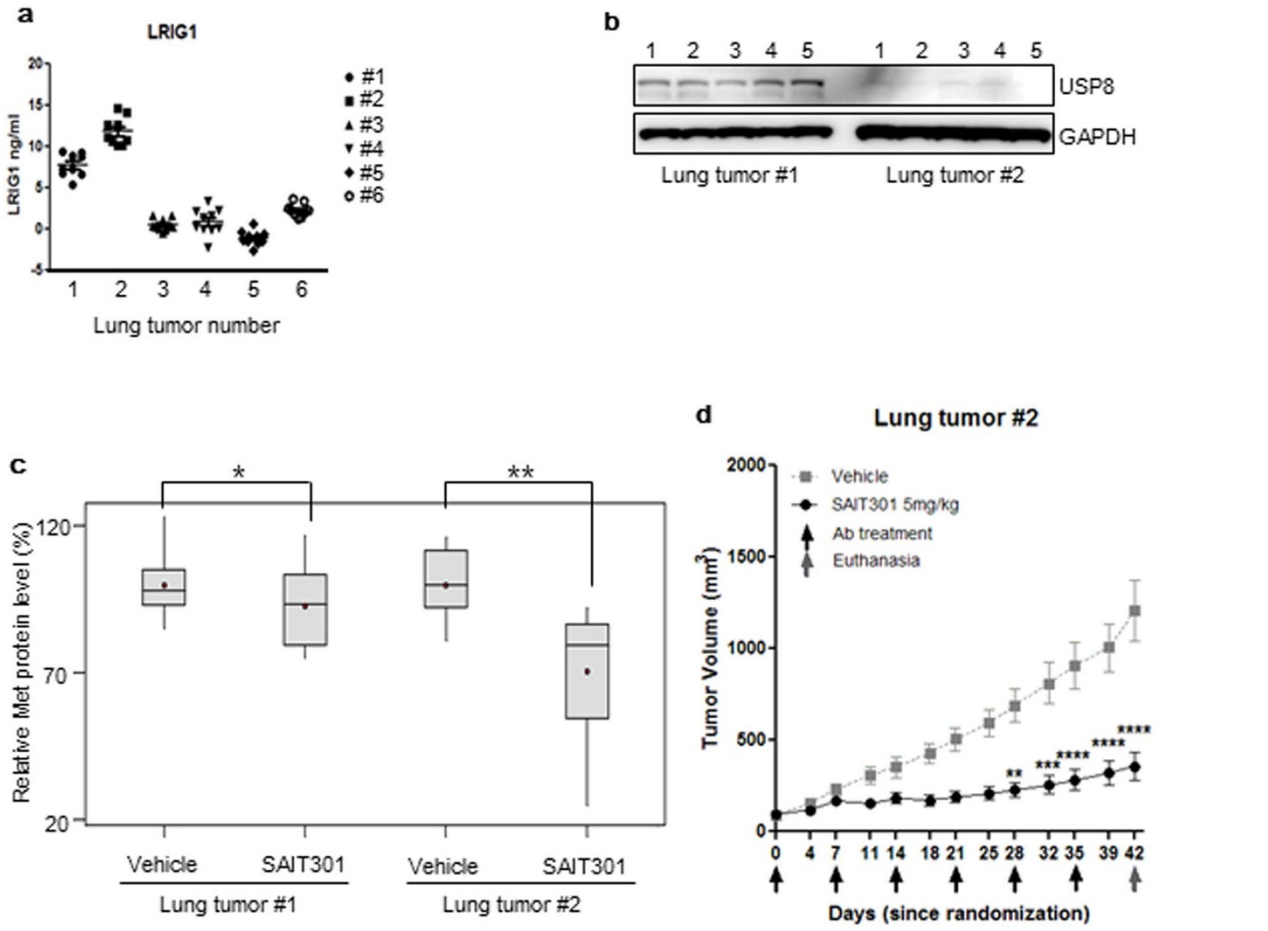

Figure 5 | USP8 determines sensitivity of cancer cells to a Met-targeting antibody. (a) LRIG1 protein levels in patient-derived lung tumor xenograft samples were measured by ELISA. Each symbol represents one tumor sample. (b) USP8 protein levels were measured by immunoblot in patient-derived lung tumor xenograft samples. (c) Met protein levels in patient-derived lung tumor xenograft samples were measured by ELISA, and expressed as a percentage (%) relative to the amount of Met in vehicle-treated tumor. Asterisks (*) represent P-values according to Student's t test, *$P < 0.02$, **$P < 0.0001$. (d) Patient-derived lung tumor growth over time. Each group consists of 10 mice. Tumor volumes measured on indicated days are plotted (mean and s.e.m.) for treatment groups (SAIT301) and vehicle (negative control: PBS) group. Asterisks (*) represent P-values versus vehicle group according to repeated measures ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

For Immunofluorescence assay, the following antibody was used: Met (Invitrogen, Carlsbad, CA, USA), LRIG1 (R&D systems, Minneapolis, MN, USA). siRNAs were purchased from Qiagen (Courtaboeuf, France) and 5D5 was derived from HB-11895 purchased from ATCC (Rockville, MD, USA). Human gastric carcinoma MKN45 (JCRB0254) and human NSCLC cell line EBC1 (JCRB0820) were purchased from the Health Science Research Resource Bank (Osaka, Japan).

Cell proliferation assay. The Celltiter Glo (CTG) luminescent assay was used to assess tumor cell proliferation in response to antibody treatment *in vitro*. EBC1 cells were plated at a density of $5*10^3$ cells per well in FBS 10% (v/v) RPMI 1640 medium onto a 96-well plate (BD Biosciences, Palo Alto, CA, USA). After 24 h incubation, 100 μL of SAIT301 diluted in 10% FBS (v/v) RPMI medium were added. After 72 h incubation, 100 μL of the CTG solution (Promega, Fitchburg, WI, USA) was added to each well followed by incubation at 37°C for 30 min. The luminescence signal was recorded using Envision 2104 Multi-label Reader (Perkin Elmer, Foster City, CA, USA).

Immunofluorescence and confocal microscopy. In preparing the fixed cell image, cells were fixed with 4% formaldehyde, then permeabilized and blocked with 0.05% Triton X-100 and 5% Donkey serum (Jackson immune, West Grove, PA, USA) in PBS. Slides were subjected to primary then fluorescently labeled secondary antibodies (Invitrogen, Carlsbad, CA, USA) in 5% Donkey serum in PBS.

Assays for protein-protein interaction. For immunoprecipitation, cells were solubilized in lysis buffer containing protease inhibitors and phosphatase inhibitors (Roche, Switzerland). Cell lysates were incubated with indicated antibody for 12 h at 4°C. The antigen-antibody complex was added to protein A/G resins (Thermo Fisher Scientific, Basingstoke, UK) and incubated reaction for 2 h at 4°C. In case of using Flag-M2 agarose, cell lysates were directly incubated with Flag-M2 agarose (Sigma Aldrich, St. Louis, MO, USA) for 12 h at 4°C. The beads were collected by centrifugation, and then washed three times with lysis buffer and twice with buffer A, which consisted of 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1 mM EDTA. Bound proteins were eluted by 0.2% SDS and separated by SDS–PAGE, followed by immunoblotting with the appropriate antibodies.

***In vitro* ubiquitination assay.** Ubiquitination assay was carried out with purified Flag-LRIG1 protein and Ubiquitin-Protein conjugation kit (BostonBiochem, Cambridge, MA, USA). Flag-LRIG1 was produced in HEK293F cells and was purified using anti-Flag M2 affinity gel (Sigma Aldrich, St. Louis, MO, USA). Flag-LRIG1 protein bound to the affinity gel was mixed with the enzymes, energy solution, and ubiquitin protein of the conjugation kit and incubated for the indicated times at 30°C. Ubiquitination of Flag-LRIG1 was confirmed with immunoblotting using anti-Ubiquitin antibody (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA).

ELISA for Met degradation assay. Total Met was analyzed by a sandwich ELISA. For the sandwich ELISA, total Met levels in homogenized tumor lysates were determined using human total HGF R/c-Met ELISA kit (R&D systems). All assays were performed as recommended by the manufacturers.

Tumor xenograft study. To study the effect of Met antibodies on tumor growth in vivo, tumor xenograft studies using two separate patient-derived tumors (NSCLC) were performed using 5–7 weeks old male NRMI nu/nu mice. For both models, the tumor fragments passaged in vivo in donor mice were collected, made into equally-sized fragments, and implanted subcutaneously into the flank region of the recipient mice under anesthesia. When the average tumor size was 50–250 mm^3, mice were randomized into either SAIT301 (5 mg/kg I.V. once a week) treatment group, or vehicle (PBS I.V. once a week) treatment group. Each group consisted of 10 mice. Tumor volumes and body weights were measured two to three times a week for total study period about 6 weeks. The tumor volume (V) was calculated as follows: $V\ (mm^3) = [\text{long axis length (mm)} \times (\text{short axis length (mm)})^2]/2$. At the end of the in vivo phase, the mice were euthanized; tumors were extracted and fixed in 10% formaldehyde or frozen for further analysis. All experiments were approved by the local authorities, and were conducted according to the guidelines of the German Animal Welfare Act (Tierschutzgesetz).

ELISA for LRIG1. Homogenized tumor tissue samples were quantitated for LRIG1 using sandwich ELISA (MBS908302, MyBioSoure, CA, USA). All procedures were performed as recommended by the manufacture's protocol. In short, tumor lysate (0.1 μg) was loaded into pre-coated microplate and biotin-conjugated LRIG1 antibody was applied for detection. After washing 3 times, avidin conjugated Horseradish Peroxidase (HRP) was added and the absorbance was measured at 450 nm using ELISA plate reader.

1. Crepaldi, T. *et al*. Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells. *J Cell Biol* **125**, 313–320 (1994).
2. Naldini, L. *et al*. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J* **10**, 2867–2878 (1991).
3. Birchmeier, C., Birchmeier, W., Gherardi, E. & Vande Woude, G. F. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* **4**, 915–925 (2003).
4. Comoglio, P. M. & Boccaccio, C. Scatter factors and invasive growth. *Semin Cancer Biol* **11**, 153–165 (2001).
5. Trusolino, L., Bertotti, A. & Comoglio, P. M. MET signalling: principles and functions in development, organ regeneration and cancer. *Nature reviews. Mol Cell Biol* **11**, 834–848 (2010).
6. Trusolino, L. & Comoglio, P. M. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat Rev Cancer* **2**, 289–300 (2002).
7. Comoglio, P. M. & Trusolino, L. Invasive growth: from development to metastasis. *J Clin Invest* **109**, 857–862 (2002).
8. Comoglio, P. M., Giordano, S. & Trusolino, L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat Rev Drug Discov* **7**, 504–516 (2008).
9. Eder, J. P., Vande Woude, G. F., Boerner, S. A. & LoRusso, P. M. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. *Clin Cancer Res* **15**, 2207–2214 (2009).
10. Peruzzi, B. & Bottaro, D. P. Targeting the c-Met signaling pathway in cancer. *Clin Cancer Res* **12**, 3657–3660 (2006).
11. Stellrecht, C. M. & Gandhi, V. MET receptor tyrosine kinase as a therapeutic anticancer target. *Cancer Lett* **280**, 1–14 (2009).
12. Oh, Y. M. *et al*. A new anti-c-Met antibody selected by a mechanism-based dual-screening method: therapeutic potential in cancer. *Mol Cells* **34**, 523–529 (2012).
13. Vigna, E. *et al*. "Active" cancer immunotherapy by anti-Met antibody gene transfer. *Cancer Res* **68**, 9176–9183 (2008).
14. Petrelli, A. *et al*. The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. *Nature* **416**, 187–190 (2002).
15. Qin, S. *et al*. Failure to ubiquitinate c-Met leads to hyperactivation of mTOR signaling in a mouse model of autosomal dominant polycystic kidney disease. *J Clin Invest* **120**, 3617–3628 (2010).
16. Sanada, M. *et al*. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature* **460**, 904–908 (2009).
17. Hedman, H. & Henriksson, R. LRIG inhibitors of growth factor signalling - double-edged swords in human cancer? *Eur J Cancer* **43**, 676–682 (2007).
18. Ordonez-Moran, P. & Huelsken, J. Lrig1: a new master regulator of epithelial stem cells. *EMBO J* **31**, 2064–2066 (2012).
19. Shattuck, D. L. *et al*. LRIG1 is a novel negative regulator of the Met receptor and opposes Met and Her2 synergy. *Mol Cell Biol* **27**, 1934–1946 (2007).
20. Joffre, C. *et al*. A direct role for Met endocytosis in tumorigenesis. *Nat Cell Biol* **13**, 827–837 (2011).
21. Marmor, M. D. & Yarden, Y. Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. *Oncogene* **23**, 2057–2070 (2004).
22. Urbe, S. *et al*. The UIM domain of Hrs couples receptor sorting to vesicle formation. *J Cell Sci* **116**, 4169–4179 (2003).
23. Williams, R. L. & Urbe, S. The emerging shape of the ESCRT machinery. *Nat Rev Mol Cell Biol* **8**, 355–368 (2007).
24. Komada, M. & Kitamura, N. The Hrs/STAM complex in the downregulation of receptor tyrosine kinases. *J Biochem* **137**, 1–8 (2005).
25. Meijer, I. M. & van Leeuwen, J. E. ERBB2 is a target for USP8-mediated deubiquitination. *Cell Signal* **23**, 458–467 (2011).
26. Mizuno, E. *et al*. Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes. *Mol Biol Cell* **16**, 5163–5174 (2005).
27. Berlin, I., Schwartz, H. & Nash, P. D. Regulation of epidermal growth factor receptor ubiquitination and trafficking by the USP8.STAM complex. *J Biol Chem* **285**, 34909–34921 (2010).
28. Lee, J. M. *et al*. Cbl-independent degradation of Met: ways to avoid agonism of bivalent Met-targeting antibody. *Oncogene* **33**, 34–43 (2014).
29. Parachoniak, C. A., Luo, Y., Abella, J. V., Keen, J. H. & Park, M. GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. *Dev Cell* **20**, 751–763 (2011).
30. Byun, S. *et al*. USP8 Is a Novel Target for Overcoming Gefitinib Resistance in Lung Cancer. *Clin Cancer Res* **19**, 3894–3904 (2013).
31. Daviet, L. & Colland, F. Targeting ubiquitin specific proteases for drug discovery. *Biochimie* **90**, 270–283 (2008).
32. Nicholson, B., Marblestone, J. G., Butt, T. R. & Mattern, M. R. Deubiquitinating enzymes as novel anticancer targets. *Future Oncol* **3**, 191–199 (2007).
33. Naviglio, S. *et al*. UBPY: a growth-regulated human ubiquitin isopeptidase. *EMBO J* **17**, 3241–3250 (1998).

Acknowledgments

We thank Dr. Paolo M Comoglio and Dr. Letizia Lanzetti for critical discussion. Flag-USP8, Flag-USP8(C786S) and shUSP8 expression plasmids were kindly provided by Dr. Chin Ha Chung and Dr. Seong Won Lee. Flag-LRIG1 expression plasmid was kindly gifted by Dr. Håkan Hedman.

Author contributions

K.A.K. and Y.M.O. designed research; Y.M.O., S.B.L., J.C., H.S., S.S., Y.J.S., B.K., J.M.L., S.J.O. and Y.J. performed research and analyzed data; K.H.C. and P.H.S. provided idea; K.A.K. and authors wrote the paper.

Additional information

Competing financial interests: The following authors are employed by the Samsung Advanced Institute of Technology: Young Mi Oh, Saet Byoul Lee, Jaehyun Choi, Hye-Young Suh, Seonhui Shim, Yun-Jeong Song, Bogyou Kim, Ji Min Lee, Seung Ja Oh, Yunju Jeong, Kwang Ho Cheong, Paul H Song and Kyung-Ah Kim.

How to cite this article: Oh, Y.M. *et al.* USP8 modulates ubiquitination of LRIG1 for Met degradation. *Sci. Rep.* **4**, 4980; DOI:10.1038/srep04980 (2014).