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mHERC6 is the essential ISG15 E3 ligase in the murine system

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ABSTRACT

Posttranslational protein modification by ubiquitin and ubiquitin-like modifiers (UBLs) is mediated by a hierarchical cascade of conjugating enzymes and affects multiple biological processes within the cell. Interferon-stimulated gene 15 (ISG15) is an UBL, which is strongly induced by type I Interferon and ISG15 modification was shown to play an essential role in antiviral defense. While hHERC5 is the major E3 ligase for ISG15 modification in humans, ISGylation in the murine systems at the level of E3 ligases was weakly characterized as rodent genomes lack a direct homologue of hHERC5.

Here, we show that mHERC6 is strongly induced by different pathogen-associated molecular patterns (PAMPs) in a type I Interferon receptor (IFNAR1) dependent manner. We demonstrate that mHERC6 is essential for endogenous murine ISGylation and thus represents the dominant ISG15 E3 ligase in mice. In contrast to its human homologue, mHERC6 is also capable to mediate conjugation of human ISG15.

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1. Introduction

Various processes within a cell are controlled by posttranslational protein modification systems which allow adaptation to external stimuli, mediate intracellular signaling events, control protein stability and allow the defense against pathogens. Beside phosphorylation, covalent linkage to ubiquitin represents one of the major posttranslational modification systems. Ubiquitylation controls protein degradation but also other processes like protein interaction, signaling, endocytosis of cell surface receptors or DNA repair [1]. In addition, so called ubiquitin-like proteins (UBLs) sharing structural similarities with ubiquitin and using similar molecular machineries for conjugation to substrates further extend the repertoire of controlling protein function [2].

Interferon-stimulated gene 15 (ISG15) was the first ubiquitin-like modifier identified and is strongly induced by type I Interferons [3,4]. Beside PKR, MX and RnaseL, ISG15 modification (ISGylation) represents one of the major antiviral Interferon effector systems [5]. ISG15-deficient mice exhibit normal antiviral responses upon vesicular stomatitis virus and lymphocytic choriomeningitis virus infections [6], but are more susceptible to Influenza A, Influenza B, Herpes simplex virus type 1, murine gamma-herpesvirus and Sindbis virus infections [7]. Thus, loss of ISGylation can in certain cases be compensated, but ISG15 conjugation is inevitable for the defense of multiple viruses *in vivo*. Inefficiency of ISGylation to antagonize specific virus infections was also shown to originate from various viral strategies interfering with the ISG15 modification process [8–10]. Beside antiviral defense, recent work showed that ISG15 deficient animals exhibit reduced bone density and impaired osteoblast function uncovering an essential role of ISG15 in bone formation [11].

Multiple substrates within the cell were identified to be ISG15 conjugated [12,13] and ISG15 modification of cellular and viral proteins were reported to mediate the antiviral activity. However, effector mechanisms of ISGylation are just beginning to emerge [14]. Strong experimental evidence was provided that, at least in human cells, ISG15 modification is closely linked to the translation process [15]. An appealing theory suggests that viral infection via Interferon induction initiates the ISGylation machinery, which subsequently modifies newly synthesized viral proteins representing the major translation products in an infected cell. Using a HPV pseudovirus system, evidence was provided that L1capsid-ISGylation reduced infectivity in a dominant fashion presumably by disturbing virion assembly [15]. In human cells ISG15 modification inhibits Influenza A replication and ISGylation has been shown to interfere with NS1A function by inhibiting nuclear translocation [16,17].

In analogy to ubiquitin or other UBLs, ISG15 is conjugated to a wide variety of target proteins mediated by the activity of E1, E2 and E3 ligases. ISG15 is activated by the E1 enzyme Ube1L [8]. The E2 for ISG15 is UbcH8 [18,19] which also serves as an ubiquitin E2 [20].

Remarkably, ISGylation at the level of E3 ligase usage differs between human and mouse. Using RNA interference (RNAi) it was shown clearly that human HERC5 (hHERC5) is the major ISG15 E3 ligase in human cells, essential for the conjugation of the vast majority of substrates [21,22]. hHERC5 belongs to the family of HERC E3 ligases which are characterized by a C-terminal HECT domain (homologous to E6-AP C-terminus) [23] and a RCC1 (regulator of

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chromosome condensation 1)-like domain [24]. In concordance with the function of ISGylation as a cotranslational process, hHERC5 is associated with polyribosomes [15]. However, while HERC5 is present in the genome of most mammals, a direct homologue in rodents is missing [25]. The closest related protein in the mouse is murine HERC6 (mHERC6) which exhibits 43% identity with hHERC5 on the level of amino acids and represents the homologue of hHERC6. hHERC6 however was shown not to posses ISG15 conjugating activity [21]. It was shown previously that mHERC6 can mediate murine ISG15 conjugation when overexpressed together with murine Ube1L and UbCH8 [26]. However, it was unclear to what extend mHERC6 contributes to the endogenous ISGylation process, whether rodents employ one essential or multiple redundant ISG15 E3 ligases and what signals induce ISGylation at the level of E3.

2. Materials and methods

2.1. Plasmid construction

Full length cDNA of mHERC6 was amplified from Interferon beta stimulated ES cells and cloned into pTriEx-2 vector in frame with the S-Tag (Novagen). Plasmids encoding hHERC5 and hHERC6 were provided by Jon Huibregtse and used to construct pTriEx-2 based expression vectors. Catalytic inactive mutants with cysteine to alanine substitutions within the HECT domain for mHERC6 (C970A), hHerc5 (C994A) and hHerc6 (C985A) were generated with the QuikChange II kit (Stratagene). DNA encoding the isolated HECT domain of mHERC6 (aminoacids (aa) 658–1003) and the RCC1-like domain truncation mutant (aminoacids (aa) 371–1003) were amplified via PCR and cloned into the pTriEx-2 vector. Plasmids encoding human Ube1l and human UbcH8 were described previously [21]. Murine and human ISG15 were expressed using pcDNA3.1 (Invitrogen).

2.2. Cell culture

HEK293T and NIH/3T3 cells were originally derived from American Type Culture Collection (ATCC), and cultivated in DMEM containing 10% FCS, Penicillin/Streptomycin and L-Glutamine (PAA) at 37 °C and 5% CO_2.

2.3. Overexpression experiments

HEK293T or NIH/3T3 cells were seeded in 6-well plates the day before transfection at 90% confluence. A total amount of 2 μg plasmid DNA was transfected using FuGENE HD (Roche) according to manufacturers' specifications. Twenty-four hours after transfection, NIH/3T3 cells were stimulated with 250 U/ml murine IFN-β (Sigma) and harvested after additional 24 h. HEK293T cells were harvested 48 h after transfection.

2.4. Knockdown of mHERC6 in NIH/3T3 cells

Cells in 6-well plates were transfected with silencer select siRNA specific for mHERC6 (5′-CACCAUACCUUAUACUGAAtt) or non-specific scrambled siRNA (Ambion) using Xtreme GENE siRNA (Roche). Cells were stimulated 24 h after transfection with 250 U/ml IFN-β and harvested 24 h later.

2.5. Preparation and stimulation of bone marrow-derived macrophages (BMMs)

BMMs were generated as previously described [6], seeded in 6-well plates and treated with IFN-α (Calbiochem), IFN-β, LPS (Sigma), poly(I:C) (Invivogen), Zymosan A (Sigma) or CpG (ODN1668 Invivogen). Complexed poly(I:C) was generated by incubating poly(I:C) (Invivogen) with Lipofectamine RNAiMAX as described in the manufacturers' protocol (Invitrogen).

2.6. Protein preparation, Western blotting and antibodies

Cells were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40) supplemented with complete protease inhibitor (Roche). Forty micrograms protein of total cell lysates were separated by SDS–PAGE, blotted and probed with the following antibodies: GAPDH (6C5, Millipore), β-actin (I-19, Santa Cruz), S-Tag (Novagen), mISG15 [6] and hISG15 (rabbit antiserum raised against full length hISG15 kindly provided by Peter Kloetzels lab).

2.7. Animals and in vivo poly(I :C) injections

All animal experiments were performed under conditions specified by the german animal protection law and approved (X09/10H and G0154/04). Eight- to 12-week-old mice raised under specific pathogen free conditions were injected intraperitoneally with poly(I:C) (5 μg/g bodyweight) (Invivogen). Animals treated for 6 or 24 h or untreated control mice were sacrificed and organs were removed for RNA isolation. Interferon type I receptor deficient animals were previously described [27].

2.8. RNA isolation and reverse transcription

Total RNA from BMM or NIH/3T3 cells was isolated using the RNeasy Mini Kit (Qiagen). RNA from homogenized mouse organs was isolated with peqGOLD RNAPure (Peqlab). Before cDNA synthesis, samples were treated with DNase I (Roche). Reverse transcription was performed with Superscript II (Invitrogen) according to the manufacturers' protocol.

2.9. Real-time PCR

Real-time PCR was carried out with the LightCycler 480 SYBR Green Mastermix (Roche). The following primer pairs were used for amplification: mHERC6 forward primer 5′-AGGCAATTCCAGCC CGAAGAACT-3′ and reverse primer 5′-TGTAGGCCTTTCACATGCA GCCT-3′, GAPDH forward primer 5′-TCCTGCACCACCAACTGCTTA GCC-3′ and reverse primer 5′-GTTCAGCTCTGGGATGACCTTGCC-3′. The relative mHERC6 expression was calculated with the ΔΔCt method.

3. Results

3.1. mHERC6 expression is strongly induced by pathogen associated molecular patterns (PAMPs) and induction depends on type I Interferon receptor signaling

mHERC6 was shown previously to efficiently mediate ISGylation when overexpressed with the ISG15 E1 activating and E2 conjugating enzymes. However, the regulation of endogenous mHERC6 and its relevance for the ISGylation process *in vivo* is still unclear.

ISGylation plays an important role in pathogen defence and in line with this is induced by type I Interferons and different pathogen-associated molecular patterns [4,28]. As depicted in Fig. 1A, stimulation with type I Interferons, different PAMPs engaging TLR3 (polyI:C), TLR4 (LPS), RIG-I/MDA-5 (polyI:C complexed to liposomes), TLR2/TLR6 (Zymosan) and TLR9 (CpG) induced strong ISGylation in primary bone marrow-derived macrophages (BMMs). Stimulation of different pattern recognition receptors activates

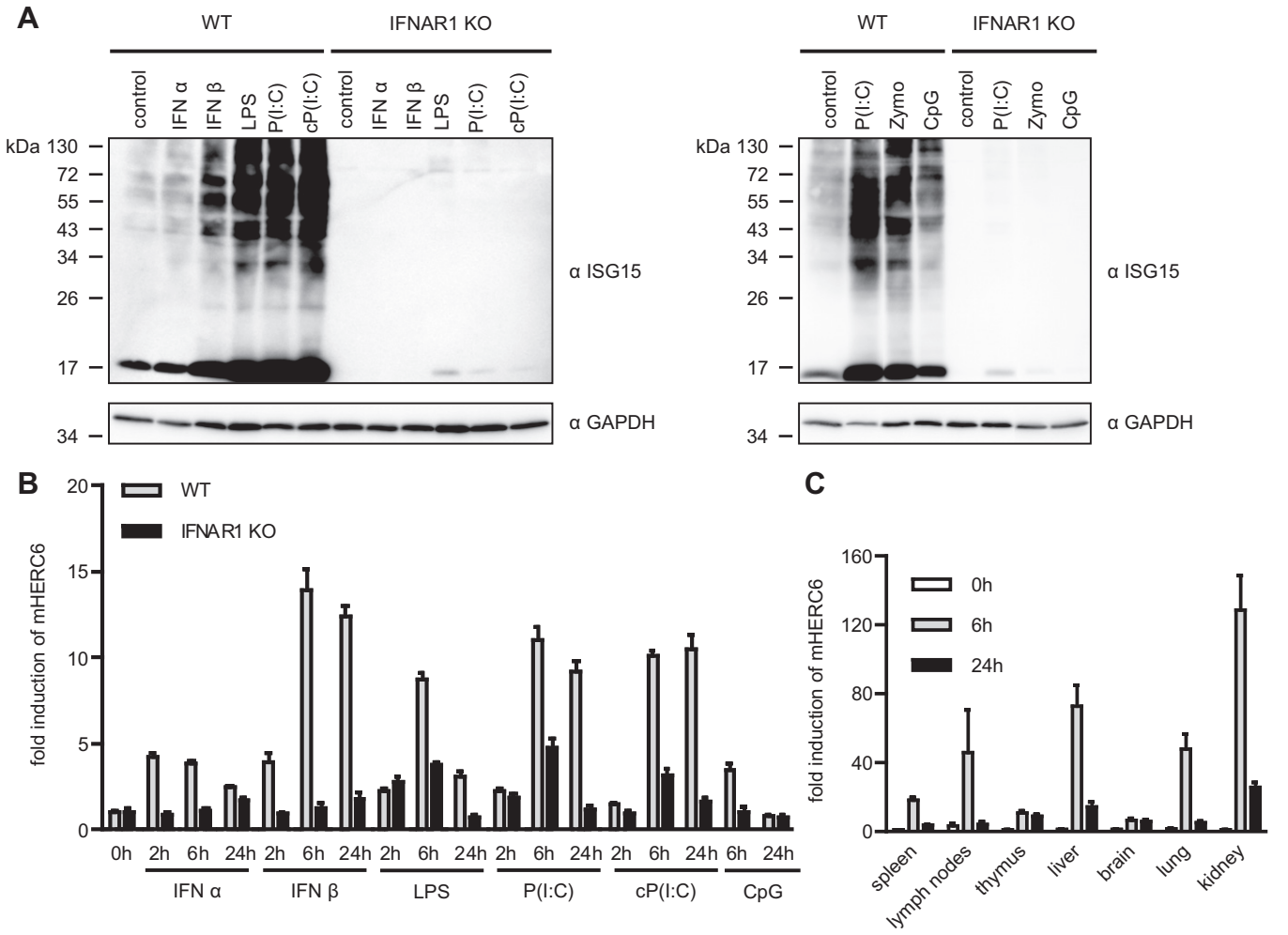

Fig. 1. ISGylation and mHERC6 are strongly induced by different PAMPs in an IFNAR1-dependent manner. (A) Western blot analysis of ISGylation in wildtype (WT) and IFNAR1$^{-/-}$ bone marrow-derived macrophages (BMM) stimulated for the indicated time points with either lipopolysaccharide (LPS) (100 ng/ml), Interferon alpha (IFN-α) (250 U/ml), Interferon beta (IFN-β) (250 U/ml), poly(I:C) (P(I:C)) (25 μg/ml), poly(I:C) complexed (cP(I:C)) (200 ng/ml), Zymosan (Zymo) (100 μg/ml) or CpG (5 μg/ml). (B) BMMs were stimulated as described in (A). mHERC6 mRNA expression was analyzed by real-time RT-PCR and is depicted as fold increase relative to levels in unstimulated WT cells. Error bars indicate standard deviation ($n = 3$). (C) Induction of mHERC6 *in vivo*. Mice ($n = 4$) were stimulated with poly(I:C) (5 μg/g body weight) and cDNA from different organs was subjected to real-time PCR using mHERC6 specific primers. Expression levels are depicted as fold induction relative to expression in unstimulated wildtype (WT) organs. Error bars indicate standard error of mean (SEM).

NF-κB signaling leading to the expression of target genes such as proinflammatory cytokines. As one effector system, type I Interferon is secreted, binds to the type I Interferon receptor (IFNAR1) and activates IFN target genes in an autocrine or paracrine manner [29]. When bone marrow-derived macrophages were generated from IFNAR1 deficient mice (IFNAR1$^{-/-}$) ISGylation could not be induced by either stimuli clearly demonstrating that ISG15 modification strictly depends on IFNAR1 signaling (Fig. 1A).

To evaluate whether mHERC6 plays a role in the ISG15-mediated immune response we analyzed whether the stimuli responsible for ISGylation also induce mHERC6 expression. Thus, mHERC6 mRNA levels were analyzed by real-time PCR after stimulation with the PAMPs mentioned above. As shown in Fig. 1B, WT BMMs exhibited strong induction of mHERC6 upon stimulation with IFN-β, LPS, complexed or uncomplexed poly(I:C) and CpG. In contrast, mHERC6 was not or only weakly induced in the absence of IFNAR1, clearly showing that induction is also largely dependent on type I Interferon signaling.

To analyze inducibility of mHERC6 *in vivo,* mice were injected intraperitoneally with poly(I:C) and mHERC6 expression in different organs was analyzed by real-time PCR before and 6 or 24 h after poly(I:C) administration. As shown in Fig. 1C, mHERC6 expression was readily induced in all organs examined (spleen, lymph nodes, thymus, liver, brain, lung and kidney) reaching highest expression levels at 6 h.

These results reveal that mHERC6 expression coincides with the induction of ISGylation and is also strictly dependent on IFNAR1 signaling.

3.2. mHERC6 strongly induces ISG15 conjugation in murine cells

As reported previously and shown in Fig. 2A, cotransfection of mHERC6 with expression constructs for Ube1L (E1), UbcH8 (E2) and mISG15 efficiently catalyzed ISGylation of multiple substrates in the absence of IFN-β stimulation. Thus, ISGylation can be reconstituted with these proteins without further Interferon stimulation. In contrast, transfection of Ube1L (E1), UbcH8 (E2) and ISG15 together with a catalytic inactive mHERC6 mutant, where the active site cysteine at amino acid position 970 of the HECT domain was replaced by alanine, did not lead to ISG15 substrate conjugation.

To evaluate the contribution of the RCC1-like domain within mHERC6 and to test whether the isolated HECT domain on its

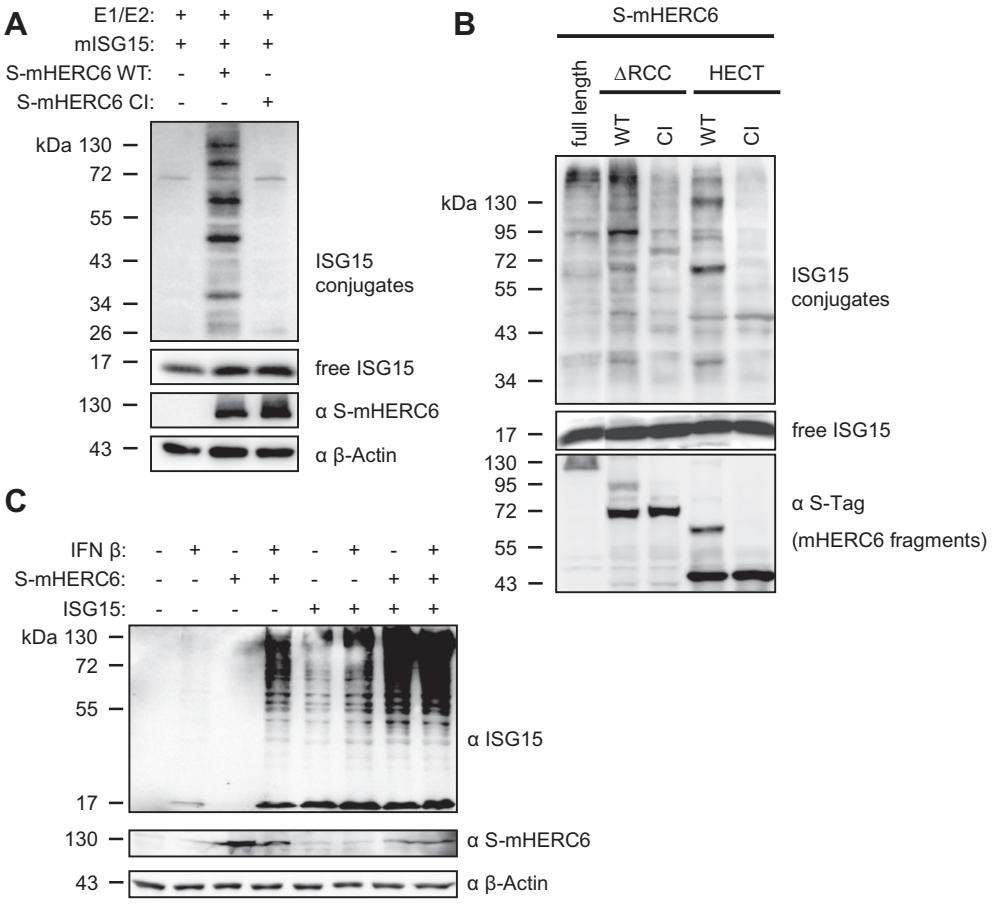

Fig. 2. mHERC6 overexpression enhances ISGylation in IFN-β-stimulated cells and together with Ube1L and UbcH8 reconstitutes ISGylation. (A) HEK293T cells were transfected with expression vectors (500 ng each) for Ube1L, UbcH8, mISG15 together with wildtype mHERC6 (mHERC6 WT) or catalytic inactive mHERC6 (mHERC6 CI). ISGylation was monitored 48 h after transfection by immunoblotting. Protein levels of mHERC6 and beta-actin (β-actin) were monitored using S-Tag and β-actin specific antibodies, respectively. (B) HEK293T cells were transfected with expression vectors for Ube1L (E1), UbcH8 (E2) and murine ISG15. In addition, expression vectors encoding full length, a truncation mutant lacking the RCC1-like domain (ΔRCC: amino acids 371–1003) and the isolated HECT domain of murine HERC6 (HECT: amino acids 658–1003) carrying a N-terminal S-Tag were used for cotransfection experiments. Immunoblots were probed using anti-ISG15 antiserum and antibodies directed against the S-Tag. (C) NIH/3T3 cells in 6-well plates were either left untransfected (lanes 1, 2) or transfected with 1 μg of an expression vector encoding S-tagged mHERC6 (pTriEx-mHERC6) (lanes 3, 4). Cells were stimulated with 250 U/ml recombinant IFN-β where indicated. In addition, cells were transfected with an expression vector for murine ISG15 (lanes 5–8) together with either empty pTriEx vector (lanes 5, 6) or the pTriEx-mHERC6 plasmid (lanes 7, 8) and stimulated with recombinant IFN-β (250 U/ml) where indicated. ISGylation was monitored by immunoblotting. Protein levels of mHERC6 and beta-actin (β-actin) were monitored using S-Tag and β-actin specific antibodies, respectively.

own has the potential to mediate ISGylation, expression vectors encoding the HECT domain or a mHERC6 truncation mutant lacking the RCC1-like domain (ΔRCC) were used in transfection experiments together with E1, E2 and mISG15. As depicted in Fig. 2B, the mutant lacking the RCC1-like domain efficiently catalyzed ISG15 conjugation while ISGylation was severely reduced when only the HECT domain of mHERC6 was expressed. Remarkably, expression of the ΔRCC mutant as well as the HECT domain of mHERC6 exhibited an additional band of higher molecular weight detected by the anti S-Tag antibody even under reducing conditions. As these signals were also detected by the anti ISG15 antiserum, these bands most likely represent autoISGylated mHERC6 variants.

To test whether mHERC6 overexpression alone can boost ISG15 conjugation, NIH/3T3 cells were transfected with an expression construct encoding S-tagged mHERC6 and subsequently (24 h after transfection) stimulated with IFN-β. As shown in Fig. 2C, ISGylation levels upon IFN-β stimulation were dramatically increased in cells overexpressing mHERC6 compared to mock transfected cells. When mHERC6 was cotransfected together with an expression plasmid encoding mISG15, high levels of ISGylation were observed, which could not further be enhanced by IFN-β administration, showing that mHERC6 expression is critical for the ISGylation process.

3.3. mHERC6 is essential for ISG15 conjugation

Despite the fact that the overexpression experiments point to an important role of mHERC6 in ISGylation, it was entirely unclear whether endogenous mHERC6 like hHERC5 is essential for the ISGylation process or whether lack of mHERC6 can be compensated by redundant pathways.

To address this experimentally, NIH/3T3 cells were transfected with either siRNA suitable to knockdown mHERC6 or scrambled siRNA as a control. Twenty-four hours after transfection, cells were stimulated with IFN-β and ISGylation was analyzed by immunoblotting 24 h after IFN-β administration. As shown in Fig. 3A, the transfected siRNA efficiently knocked down mHERC6 expression and almost completely abrogates IFN-β induced ISGylation (Fig. 3B), while application of unspecific scrambled siRNA did not affect ISGylation levels. These findings clearly show that mHERC6

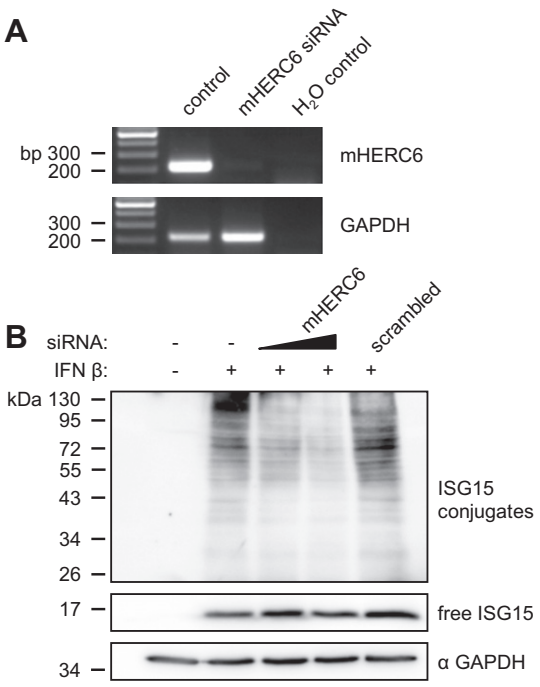

Fig. 3. Knockdown of mHERC6 abrogates ISGylation in IFN-β-induced murine cells. (A) NIH/3T3 cells were transfected with 2 μg (180 pMol) siRNA directed against mHERC6 and stimulated with IFN-β (250 U/ml) 24 h after transfection. Twenty-four hours after induction, RNA was isolated and PCR was performed employing mHERC6 or GAPDH specific primers. (B) NIH/3T3 cells were transfected with either 2 μg (180 pMol) (lane 3) or 5 μg (400 pMol) (lane 4) mHERC6 siRNA. Alternatively, cells were transfected with 2 μg of non-specific scrambled siRNA. Twenty-four hours after transfection, cells were stimulated with recombinant IFN-β (250 U/ml) for additional 24 h where indicated. ISGylation and GAPDH (serving as a loading control) were monitored by immunoblotting.

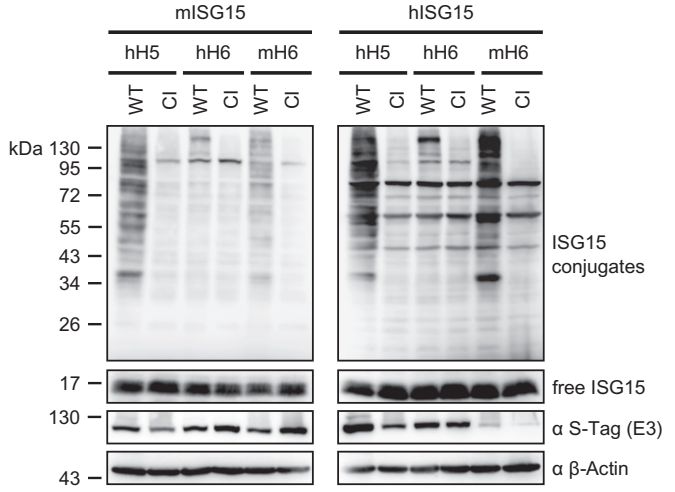

Fig. 4. mHERC6 mediates conjugation of murine and human ISG15. HEK293T cells were transfected with expression vectors for Ube1L (E1), UbcH8 (E2) and where indicated with murine ISG15 (mISG15) (left panel) or human ISG15 (hISG15) (right panel). As depicted, either wildtype (WT) or catalytic inactive (CI) mutant versions of S-tagged human HERC5 (hH5), human HERC6 (hH6) or murine HERC6 (mH6) proteins were expressed using pTriEx vector based constructs. Protein expression was visualized by immunoblotting.

is essential for efficient ISGylation and that its absence cannot be rescued by other ligases or restored by alternative pathways.

Given results demonstrate that mHERC6 is the major ISG15 E3 ligase in the murine system.

3.4. In contrast to hHERC6, mHERC6 is capable to conjugate hISG15

The finding that mHERC6 is the major ISG15 E3 ligase in murine cells appears in sharp contrast to the reported inability of the human homologue hHERC6 to act as an ISG15 E3 ligase towards human ISG15.

As human and murine ISG15 proteins were shown to differ substantially and show only 66% identity at the amino acid level we asked whether human ISG15 in principle cannot be conjugated by HERC6 orthologues. To further gain insight into species specific differences in ISG15 E3 usage, the cross reactivity of mHERC6 towards hISG15 was analyzed. Therefore, HEK293T cells were transfected with either murine ISG15 (Fig. 4 left panel) or human ISG15 (Fig. 4 right panel) together with Ube1L (E1), UbcH8 (E2) and either human HERC5, human HERC6 or murine HERC6.

Catalytic inactive mutants, where the active site cysteine was replaced by alanine, were used as controls. As shown in Fig. 4, hHERC5 expression caused robust conjugation of either murine or human ISG15. In contrast, expression of hHERC6 neither induced ISGylation of human nor murine ISG15. Most interestingly, overexpression of mouse HERC6 together with human ISG15 induced strong ISGylation levels within the cells, clearly showing that murine HERC6 function is not limited to murine ISG15 and that the enzyme can also catalyze conjugation of human ISG15 to substrates.

As hISG15 and mISG15 can both be conjugated either by mHERC6 or hHERC5, strong evidence is provided that species-specific structural differences within the ISG15 protein do not account for the differences in human and murine E3 usage for ISGylation.

4. Discussion

Within this study it is shown that the murine HECT ligase mHERC6 is strongly induced by different pathogen-associated molecular patterns in a type I Interferon dependent manner. Together with the relatively low inducibility of mHERC6 by IFN-α our observations suggest that mHERC6 and ISGylation are basically induced by IFN-β. As shown in Fig. 2A and reported previously [26], overexpression of mHERC6 together with Ube1L, UbcH8 and ISG15 reconstitutes ISGylation even in unstimulated cells demonstrating that these proteins are sufficient to mediate ISG15 conjugation. As ISGylation in IFN-β stimulated cells was enhanced by mHERC6 overexpression, mHERC6 protein levels appear to strongly influence the murine ISGylation process. However, the most striking evidence that mHERC6 is the major murine ISG15 E3 ligase comes from the observation that knockdown of mHERC6 almost completely abrogates ISGylation. It can however not be excluded that specific target substrates employ distinct E3 ligases for the ISG15 modification process in a similar way to what has been reported for the ISG15 modification of 14-3-3 sigma by EFP [30,31]. Our studies clearly show that in the case of mHERC6 the HECT domain alone is insufficient to mediate ISGylation which is analogous to what has been reported for hHERC5 [21]. However, the RCC1-like domain appears to be dispensable for ISGylation at least in the overexpression experiments performed within this study.

Our studies also confirm previous work which showed that the human homologue hHERC6 is not capable to mediate ISG15 conjugation [21]. It is interesting to speculate whether the use of HERC5 as the major E3 ligase in humans provides additional benefits in this particular posttranslational modification process. Given the high evolutionary pressure in the adaptation of the innate immune system in response to viral escape mechanisms this is a likely scenario. Within this context it will be interesting to see whether murine ISGylation via HERC6 is as tightly coupled to translation

as human ISGylation via hHERC5 and whether hHERC6 has simply lost its ability to conjugate ISG15 or acquired new activity towards specific substrates or other modifiers.

The observed unexpected cross reactivity of mHERC6 towards human ISG15 might also be valuable in the attempt to set up an *in vitro* ISGylation system. So far such a system is not available and one of the major problem is that efforts by us and other labs to express and purify recombinant hHERC5 in an enzymatic active form were not successful [21,22]. Identification of mHERC6 as an effective ISG15 E3 ligase for both human and murine substrates now offers an additional option for these kinds of experiments. It remains to be determined whether ISGylation can be set up *in vitro* simply be employing E1, E2 and E3 ligases or whether the reported coupling to the translation process involving larger protein or polyribosomal complexes is required.

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