Ubiquitin-dependent Turnover of Adenosine Deaminase Acting on RNA 1 (ADAR1) Is Required for Efficient Antiviral Activity of Type I Interferon*

Received for publication, May 7, 2016, and in revised form, September 12, 2016. Published, JBC Papers in Press, October 11, 2016, DOI 10.1074/jbc.M116.737098

Lemin Li1†, Guanghui Qian2†, Yibo Zuo3, Yukang Yuan3, Qiao Cheng3, Tingting Guo1, Jin Liu3, Chang Liu4, Liting Zhang3, and Hui Zheng1,2

From the 1Institutes of Biology and Medical Sciences, Jiangsu Key Laboratory of Infection and Immunity, Soochow University, Suzhou 215123, China and the 2Institutes of Pediatric Research, Children’s Hospital of Soochow University, Suzhou, Jiangsu Province 215025, China

Edited by George DeMartino

Adenosine deaminase acting on RNA 1 (ADAR1) catalyzes RNA editing of cellular and viral RNAs. Besides RNA editing, ADAR1 has recently been shown to play important roles in maintaining the body balance, including tissue homeostasis, organ development, and autoimmune regulations, by inhibiting both IFN production and subsequent IFN-activated pathways. Accordingly, the question was raised how IFN signaling induced by viral infections overcomes the inhibitory effect of constitutively expressed ADAR1 (ADAR1-P110) to execute efficient antiviral activity. Here we unexpectedly found that IFN signaling promoted Lys48-linked ubiquitination and degradation of ADAR1-P110. Furthermore, we identified the E3 ligase β transducin repeat-containing protein responsible for IFN-mediated ADAR1-P110 down-regulation. IFN signaling promoted the interaction between β transducin repeat-containing protein and ADAR1-P110 as well as protein turnover of ADAR1-P110. Moreover, we found that both lysine 574 and 576 are essential for ADAR1-P110 ubiquitination. Critically, we demonstrated that down-regulation of ADAR1-P110 is required for IFN signaling to execute efficient antiviral activity during viral infections. These findings renew the understanding of the mechanisms by which IFN signaling acts to achieve antiviral functions and may provide potential targets for IFN-based antiviral therapy.

The IFN family of cytokines plays critical roles in regulating antiviral innate immunity, cell proliferation, and immunomodulatory responses (1, 2). So far, three classes of IFNs (type I, II, and III) have been identified. Among them, the type I IFN (IFN-I or IFNα/β) family has been extensively studied and well characterized. IFN-I family members trigger their signaling through activation of IFN-I receptors (IFNAR1 and IFNAR2), which is followed by tyrosine phosphorylation and activation of the Janus kinase family (JAK1 and Tyk2). Activated JAK1 and Tyk2 subsequently induce tyrosine phosphorylation of STAT1 and STAT2, which form a transcriptional complex. This complex translocates into the nucleus to induce the expression of interferon-stimulated genes (ISGs)3 (3). Among these ISGs, the RNA-activated protein kinase (PKR) is well established as an important IFN-inducible antiviral component (4) that is a RNA virus sensor and inhibits viral replication by activating the α subunit of the translation initiation factor (eIF-2α) to block protein translation (3).

One of the most important functions of IFN-I is the protection of cells from viral infections (2, 3). Based on this function, IFN-I has been used to treat some viral infection diseases, including chronic hepatitis C virus infection (5). However, recent studies have demonstrated that IFN-I signaling could play deleterious roles in some normal biological functions, especially when cells undergo excessive activated IFN-I signaling. It has been reported that uncontrolled activated IFN-I signaling contributes to autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and Aicardi-Goutières syndrome (6, 7). IFN-I also has important effects on lethality in diverse models of sepsis or endotoxic shock (8). In addition, numerous studies have suggested that excessive IFN-I signaling could be involved in impairing normal organismal homeostasis, including the maintenance of both fetal and adult hematopoietic stem cells (9), intestinal homeostasis (10), and liver morphological and functional integrity (11).

Based on the complex biological activity of IFN-I, the maintenance of normal organismal functions requires balanced IFN-I signaling. Sufficient IFN-I signaling must be maintained in cells to produce the protective effects for antipathogen responses, and, at the same time, IFN-I signaling must be restricted to prevent harmful effects on tissue homeostasis.

*This work was supported in part by Program of 1000 Young Talents; National Natural Science Foundation of China Grants 31370873, 31570865, and 31600695; Jiangsu Provincial Distinguished Young Scholars Grant BK20130004; Jiangsu Provincial Innovative Research Team; Changjiang Scholars and Innovative Research Team, University of Ministry of Education of China Grant PCSIRT-IRT1075; and the Priority Academic Program Development of Jiangsu Higher Education Institutions. The authors declare that they have no conflicts of interest with the contents of this article.

†Both authors contributed equally to this work.

‡To whom correspondence should be addressed: Institutes of Biology and Medical Sciences, Soochow University, 199 Ren’ai Rd., Suzhou, Jiangsu Province 215123, China. Tel.: 86-512-65883505; Fax: 86-512-65882135; E-mail: huizheng@suda.edu.cn.

3The abbreviations used are: ISG, interferon-stimulated gene; PKR, RNA-activated protein kinase; β-TrCP, β transducin repeat-containing protein; Z-VAL, benzoyloxycarbonyl-VAL; Ub, ubiquitin; CHX, cycloheximide; MOI, multiplicity of infection; ADAR, adenosine deaminase acting on RNA.
Therefore, some intracellular mechanisms have evolved to limit excessive activated IFN-I signaling. For example, suppressor of cytokine signaling proteins 1 (SOCS1) inhibits cytokine-induced activation of the JAK family (12), protein kinase D2 (PKD2) promotes IFNAR1 ubiquitination and degradation to limit IFN-I signaling (13–15), and some of ISG-encoded proteins inhibit the recruitment of JAK to IFN-I receptors (16). Recently, adenosine deaminase acting on RNA 1 (ADAR1) has attracted much attention because of its important negative regulatory effect on IFN-I production and subsequent IFN-I-activated pathways (9, 17–19).

ADAR1 is a double-stranded RNA binding protein that catalyzes RNA editing (converting adenosine to inosine) of cellular and viral RNAs (20). There are two protein size forms of ADAR1 (P110 and P150). ADAR1-P110 is constitutively expressed in ubiquitous types of cells, whereas P150 is only expressed when cells are stimulated by some activators (20, 21). The adenosine-to-inosine editing is of broad physiological function. Therefore, ADAR1 executes complex biological activities, including proviral or antiviral effects dependent on different virus types and the interaction between hosts and viruses (20). Despite the complexity, ADAR1 has been shown to promote the infection of numerous types of viruses, including VSV; measles virus; EBV; influenza virus; dengue virus; HIV, type 1; human respiratory syncytial virus; and human herpesviruses 8 (20, 21). An increasing number of studies have demonstrated that ADAR1-mediated enhancement of viral infections is mainly through RNA editing or inhibition of IFN activity (including both IFN-I production and IFN-activated PKR antiviral activity) (21).

The inhibitory effect of constitutively expressed ADAR1 on IFN-I signaling maintains the IFN-I signaling balance and tissue homeostasis. However, it is also of vital importance that the balance of IFN-I signaling should be able to be broken and regulated under some pathological conditions, such as viral infections. Virus-activated IFN-I signaling must act to overcome the ADAR1-mediated inhibitory effect to achieve efficient antiviral activity. However, the action mechanisms still remain unexplored.

Here we discovered that IFN-I signaling down-regulated the level of constitutively expressed ADAR1-P110, which is dependent on Lys 48- or Lys 63-linked polyubiquitination and degradation of ADAR1-P110 mediated by IFN-I signaling. Furthermore, we identified the E3 ligase β-TrCP, which interacts with ADAR1-P110 and is responsible for IFN-mediated ADAR1-P110 down-regulation. Moreover, we found that both lysine 574 and lysine 576 are essential for ADAR1-P110 ubiquitination. Critically, we demonstrated that down-regulation of ADAR1-P110 is required for IFN signaling to achieve efficient antiviral activity during viral infections. Our findings reveal the action mechanisms by which IFN signaling achieves efficient antiviral functions and may provide potential targets for IFN-based antiviral therapy.

Results

ADAR1-P110 Levels Are Down-regulated in IFN Signaling—To determine the possible effect of IFN signaling on ADAR1-P110, we first analyzed the change of ADAR1-P110 protein levels in cells under conditions of IFN treatment. To this end, HEK293T cells were treated with IFNα for various times. Using a specific antibody against ADAR1-P110, we found that endogenous ADAR1-P110 levels were gradually down-regulated with IFNα (1000 IU/ml) treatment (Fig. 1A). ADAR1-P110 levels were reduced by around 80% when cells were treated with IFNα for 6 h, although its levels went back up slightly with 12-h IFNα treatment (Fig. 1A). To further confirm that IFNα signaling is capable of promoting the down-regulation of ADAR1-P110, FLAG-ADAR1-P110 was overexpressed in cells that were subsequently treated with IFNα. The result showed that exogenous FLAG-ADAR1-P110 levels were also substantially down-regulated with IFNα treatment (Fig. 1B and C). When cells were treated with increased amounts of IFNα, we found that endogenous ADAR1-P110 was down-regulated by IFNα treatment in a dose-dependent manner (Fig. 1D). Furthermore, IFN-mediated ADAR1-P110 down-regulation was significantly inhibited by the proteasome inhibitor MG132 (Fig. 1E). An interesting report showed that ADAR1-P150 can be down-regulated in a caspase-dependent manner (30). To analyze the role of caspase in ADAR1-P110 down-regulation, we utilized a caspase inhibitor, Z-VAD, to block caspase activity. Our data showed that inhibition of caspase activity by Z-VAD did not block IFN-induced ADAR1-P110 down-regulation (Fig. 1F). These data strongly suggest that IFNα signaling negatively regulates cellular levels of ADAR1-P110 in a proteasome-dependent manner.

IFN Signaling Promotes Lys 48-linked Polyubiquitination of ADAR1-P110—Given that IFN signaling was able to down-regulate ADAR1-P110 levels, we questioned whether IFN treatment promotes ADAR1-P110 ubiquitination. Cells were treated with IFNα, and then the ubiquitination of ADAR1-P110 was analyzed. As shown in Fig. 2A, IFNα treatment noticeably increased the polyubiquitination of endogenous ADAR1-P110 (Fig. 2A). Similarly, we confirmed that IFNα treatment promoted exogenous ADAR1-P110 ubiquitination (Fig. 2B). Furthermore, cells were treated with increased amounts of IFNα. The result showed that ADAR1-P110 polyubiquitination was up-regulated by IFNα treatment in a dose-dependent manner (Fig. 2C).

Two lysine residues of ubiquitin, Lys 48 and Lys 63, have been extensively studied and are most commonly used for the analysis of protein polyubiquitination. We sought to determine whether IFN signaling affects the Lys 48-linked or Lys 63-linked ubiquitination of ADAR1-P110. We transfected cells with FLAG-ADAR1-P110 together with HA-tagged Ub-WT or HA-Ub-Lys 48 only (R48K) or HA-Ub-Lys 63 only (R63K). Then cells were treated with IFNα. We found that IFNα treatment remarkably promoted the Lys 48-linked ubiquitination of ADAR1-P110 (Fig. 2D) but did not obviously affect IFNα-induced Lys 63-linked ubiquitination of ADAR1-P110 (Fig. 2D). Collectively, our data demonstrated that IFN signaling promotes Lys 48-linked poly-ubiquitination of ADAR1-P110.

β-TrCP Contributes to ADAR1-P110 Polyubiquitination in IFN Signaling—To find the ubiquitinligase of ADAR1-P110, we first analyzed the amino acid sequences of ADAR1-P110. We noticed that there is a putative DSG(X)2+5 motif in ADAR1-P110 conserved across various species, including Homo sapien...
ens, Mus musculus, and Rattus norvegicus (Fig. 3A). The DSG(X)2+nS motif has been shown to be an important characteristic of substrates for β-TrCP E3 ubiquitin ligase, and the serine mutation in this motif of a substrate results in its inability to interact with β-TrCP (22). We therefore speculated that β-TrCP is a potential ubiquitin ligase of ADAR1-P110. To provide evidence for this hypothesis, the Ser578 of human ADAR1-P110 was mutated to alanine (SA). Furthermore, both Ser578 and Ser585 were mutated to alanine (AA). Cells transfected with FLAG-ADAR1-P110 (WT, SA, or AA) were treated with IFNα for 30 min, and then ADAR1-P110 ubiquitination levels were analyzed. We found that the ubiquitination of ADAR1-P110 mutants, especially the AA mutant, was significantly weaker than that of ADAR1-P110-WT (Fig. 3B). To further demonstrate the effect of β-TrCP on the specific motif of ADAR1-P110, cells were transfected with FLAG-ADAR1-P110-WT/SA/AA together with HA-tagged β-TrCP (HA-β-TrCP). As expected, β-TrCP induced much higher levels of ADAR1-P110-WT ubiquitination compared with the ubiquitination of ADAR1-P110 mutants (Fig. 3C). These results imply that the interaction between β-TrCP and ADAR1-P110 contributes to ADAR1-P110 ubiquitination.

To delineate the possible effect of β-TrCP on ADAR1-P110 ubiquitination, cells were transfected with FLAG-ADAR1-P110 together with or without HA-β-TrCP. Our data showed that overexpression of β-TrCP substantially promoted ADAR1-P110 ubiquitination (Fig. 3D). Then the shRNAs against β-TrCP (shBTR) were used to knock down β-TrCP. However, knockdown of β-TrCP significantly decreased the ubiquitination levels of ADAR1-P110 (Fig. 3E). To functionally demonstrate that β-TrCP is responsible for ADAR1-P110 ubiquitination in IFN signaling, cells were treated with IFNα. The result showed that IFNα-mediated ADAR1-P110 ubiquitination was significantly inhibited by knockdown of β-TrCP (Fig. 3F). Collectively, these results suggest that the ubiquitin ligase β-TrCP promotes ADAR1-P110 ubiquitination in IFN signaling.

IFN Signaling Promotes the Interaction between β-TrCP and ADAR1-P110—To further understand the effect of β-TrCP on ADAR1-P110 ubiquitination, we analyzed the interaction...
between β-TrCP and ADAR1-P110. Cells were co-transfected with FLAG-ADAR1-P110 and HA-β-TrCP. The result confirmed the interaction between FLAG-ADAR1-P110 and HA-β-TrCP (Fig. 4A). Also, FLAG-ADAR1-P110 can interact with endogenous β-TrCP (Fig. 4B). Moreover, we found that endogenous β-TrCP can interact with endogenous ADAR1-P110 (Fig. 4C). Given that the serine mutations in the DSG(X)²⁺ⁿS motif of protein substrates block the recognition of β-TrCP by its substrates, we detected the interaction between β-TrCP and ADAR1-P110-SA/AA mutants. The result was consistent with our hypothesis that the DSG(X)²⁺ⁿS motif of ADAR1-P110 is important for β-TrCP binding (Fig. 4D). Next we sought to determine whether IFN signaling upregulated β-TrCP levels. Cells were treated with IFNα for 2 or 6 h. The result showed that IFN signaling did not obviously affect β-TrCP levels within 6 h (Fig. 4E). However, we found that IFN treatment promoted the interaction between β-TrCP and ADAR1-P110 (Fig. 4F). All of these data suggest that β-TrCP can interact with ADAR1-P110 and that this interaction is promoted by IFN signaling.

β-TrCP Promotes Protein Turnover of ADAR1-P110—It has been clarified that Lys⁴⁸-linked ubiquitination of a protein substrate usually leads to its degradation (23). Given that IFN signaling is able to promote the Lys⁴⁸-linked ubiquitination of ADAR1-P110, we speculated that the protein stability of ADAR1-P110 could be regulated by IFN signaling. To address this hypothesis, we conducted cycloheximide (CHX) pulse-chase analyses on ADAR1-P110 protein stability. To address this hypothesis, we conducted cycloheximide (CHX) pulse-chase analyses on ADAR1-P110 protein stability. Cells transfected with FLAG-ADAR1-P110 were treated with or without IFNα together with the protein synthesis inhibitor CHX. The results showed that IFNα treatment accelerated the degradation of ADAR1-P110 (Fig. 5A). Given that β-TrCP was able to promote ADAR1-P110 ubiquitination and degradation, we speculated that β-TrCP could affect cellular levels of ADAR1-P110. Cells were transfected with increased amounts of β-TrCP, and then the level of endogenous ADAR1-P110 was analyzed by immunoblotting. The result showed that overexpression of β-TrCP substantially blocked ADAR1-P110 protein degradation (Fig. 5C). Furthermore, the protein stability of ADAR1-P110-S578A (SA) or ADAR1-P110-S585A (AA) was analyzed. Compared with ADAR1-P110-WT, the degradation rate of ADAR1-P110-SA and -AA was much lower (Fig. 5D), suggesting that the binding of β-TrCP to ADAR1-P110 contributes to the degradation of ADAR1-P110. Taken together,
we conclude that β-TrCP promotes protein turnover of ADAR1-P110.

Both Lysine 574 and Lysine 576 Are Essential for ADAR1-P110 Ubiquitination—Given that β-TrCP promoted the ubiquitination of ADAR1-P110, next try to identify the ubiquitin acceptor lysine residues in ADAR1-P110. Previous studies of the substrates of β-TrCP ubiquitin ligase showed that the lysine residues for ubiquitin conjugation mostly locate in positions 9–13 upstream of this DSG(X)2+nS motif in protein substrates (22). Accordingly, we generated the ADAR1-P110-K564R mutant, which harbors a lysine mutation upstream of the DSG(X)2+nS motif of ADAR1-P110. Cells were transfected with either FLAG-ADAR1-P110-WT or -K564R. We found that the ubiquitination level of the ADAR1-P110-K564R mutant was similar as that of ADAR1-P110-WT (Fig. 6A). This result suggests that lysine 564 could not be the ubiquitin acceptor residue of ADAR1-P110.

We further generated the ADAR1-P110-K574R.K576R mutant, which harbors two mutation sites (lysine 574 and lysine 576) very close to the DSG(X)2+nS motif of ADAR1-P110. Of note, the ubiquitination level of ADAR1-P110-K574R.K576R mutant was much lower than that of ADAR1-P110-WT (Fig. 6B). To further identify which lysine residue, lysine 574 or lysine 576, is essential for ADAR1-P110 ubiquitination, we generated two mutants, ADAR1-P110-K574R and ADAR1-P110-K576R (Fig. 6C). Cells were transfected with either the ADAR1-
Compared with ADAR1-P110-WT, both mutants had lower levels of ubiquitination (Fig. 6C), suggesting that both of these two lysine residues are the ubiquitin acceptor residues of ADAR1-P110. Taken together, our data demonstrate that both lysine 574 and lysine 576 are essential for ADAR1-P110 ubiquitination.

Down-regulation of ADAR1-P110 Is Required for Efficient Antiviral Activity of Type I IFN—Given that type I IFN promoted the ubiquitination and protein turnover of ADAR1-P110, we sought to determine the pathophysiological significance of ADAR1-P110 down-regulation in IFN signaling. To assess IFN-mediated signaling and antiviral activity, we chose human fibroblast-derived cell line 2fTGH. 2fTGH cells are widely used for IFN studies because of their high sensitivity to IFN-I and excellent ability to produce IFN-I during virus infections (24). VSV is a very sensitive virus model that is usually used to assess IFN antiviral functions (25, 26). Firstly, 2fTGH cells were infected with VSV for various times, and then ADAR1-P110 levels were analyzed by immunoblotting. We found that ADAR1-P110 levels were also gradually down-regulated during VSV infections (Fig. 7A), which was consistent with the observation that IFNα promoted ADAR1-P110 down-regulation (Fig. 1A). These data suggest that ADAR1-P110 down-regulation occurs during actual viral infections.

Next we analyzed the effect of ADAR1-P110 on IFN-mediated antiviral activity in our infection model, although a previous report has demonstrated that ADAR1 knockdown promoted the effect of IFN on the down-regulation of viral titers (27). We pretreated cells with IFNα and then infected them with VSV. The VSV-encoded protein VSV-G was analyzed by immunoblotting. As expected, IFNα treatment significantly decreased the content of VSV-G (Fig. 7B, lanes 1 and 2). We found that knockdown of endogenous ADAR1-P110 remarkably decreased VSV replication (Fig. 7B, lanes 1 and 3). This result suggests that ADAR1-P110 inhibits cellular antiviral ability in a basal IFN-secreting environment. Furthermore, when exogenous IFN was involved in the antiviral response, we observed that IFNα-mediated anti-VSV ability was significantly enhanced by knockdown of ADAR1-P110 (Fig. 7B, lane 3).
versus lane 4 and lane 5 versus lane 6). Taken together, our data suggest that endogenous ADAR-P110 inhibits IFN-mediated antiviral activity.

Based on the above results, we speculated that, during viral infections, IFN production and subsequent IFN signaling would encounter the inhibitory effect of ADAR1-P110. In conjunction with our previous data, which showed that IFN signaling actually down-regulated endogenous ADAR1-P110 levels through ubiquitin-dependent degradation, we hypothesized that IFN-mediated ADAR1-P110 down-regulation could be important for IFN antiviral activity. Given that we have demonstrated that the ADAR1-P110-S578A,S585A mutant (AA) impaired ADAR1-P110 ubiquitination and down-regulation (Fig. 3, B and C), we transfected cells with ADAR1-P110-AA, after which cells were infected with VSV-GFP, a VSV construct with the GFP gene. As expected, IFN treatment significantly inhibited VSV infection, as shown by a decreased VSV-GFP signal (Fig. 7C). Overexpression of ADAR1-P110-WT inhibited IFN-mediated antiviral activity (Fig. 7C). Importantly, the inhibitory effect of ADAR1-P110-AA on IFNs-mediated antiviral function was much more significant than that of ADAR1-P110-WT (Fig. 7C). Furthermore, cells were collected for the FACS analyses. The result confirmed that ADAR1-P110-AA had a stronger inhibitory effect on IFN antiviral activity than ADAR1-P110-WT (Fig. 7D, top panel), which can be explained by the enhanced stability of ADAR1-P110-AA under conditions of IFN treatment compared with ADAR1-P110-WT (Fig. 7D, bottom panel). Collectively, we demonstrated that inhibition of ADAR1-P110 down-regulation restricted IFN-mediated antiviral activity.

Furthermore, we established the ADAR1-P110 knockdown stable cell line (shADAR1-stable) using 2fTGH cells (Fig. 7E).
Then we re-expressed either resistant ADAR1-P110-WT (res-WT) or resistant ADAR1-P110-K574R,K576R (res-2KR) into shADAR1-stable cells. These two resistant expression constructs were generated by mutating the corresponding sites of ADAR1-P110 targeted by ADAR1-P110 shRNAs so that they are unable to be targeted and silenced by ADAR1-P110 shRNAs in shADAR1-stable cells. After that, cells were infected with VSV. As shown in Fig. 7F, re-expression of ADAR1-P110-WT (res-WT) promoted VSV infection in ADAR1 knockdown stable cells. Importantly, re-expression of ADAR-P110–2KR (res-2KR) more significantly enhanced VSV infection compared with that of ADAR1-P110-WT (Fig. 7F). Consistently, when exogenous IFN was added to cells to inhibit viral infection, we found that the inhibitory effect of ADAR1-P110–2KR on IFN-mediated antiviral activity was much more significant than that of ADAR1-P110-WT (Fig. 7F). Consistently, when exogenous IFN was added to cells to inhibit viral infection, we found that the inhibitory effect of ADAR1-P110–2KR on IFN-mediated antiviral activity was much more significant than that of ADAR1-P110-WT (Fig. 7F), indicating that inhibition of ADAR1-P110 ubiquitination and degradation blocked normal antiviral activity mediated by IFN. Taken together, our data suggest that, during viral infection, down-regulation of ADAR1-P110 is required for IFN-I to execute efficient antiviral activity.

Discussion

The common understanding of IFN action is that IFN activity is achieved by activated JAK/STAT signaling pathways (31). However, activated JAK/STAT signaling could be not enough for IFN signaling to exert efficient antiviral functions. IFN signaling first has to overcome the inhibitory effects mediated by some negative regulatory factors in cells such as ADAR1. ADAR1 has been shown to be a critical suppressor of IFN responses (including IFN production and IFN signaling), which protects cells from the harmful effects of excessively activated IFN signaling and, therefore, provides an important guarantee of tissue homeostasis (9, 10, 17). Here we discovered that IFN-I signaling promoted H9252-TrCP-mediated Lys 48-linked ubiquitination and degradation of constitutively expressed ADAR1-P110. More importantly, we demonstrated that down-regulation of ADAR1-P110 is very important for efficient IFN-I activity during viral infections (Fig. 7, A–G). Thus, our studies renew the understanding of the mechanisms of IFN-I action and reveal that IFN-I signaling utilizes the E3 ligase β-TrCP to down-regulate constitutively expressed ADAR1-P110 to achieve efficient antiviral activity.

As mentioned above, ADAR1 has two forms: P110 and P150. Both ADAR1-P110 and -P150 have RNA-editing activity (20). In addition, ADAR1-P150 has been demonstrated to be an essential negative regulator of the mitochondrial antiviral signaling protein MAVS-mediated RNA-sensing pathway (17). Although ADAR1-P150 is IFN-inducible, the level of ADAR1-P150 is
P150 during the early stages (a couple of hours) of IFN signaling is very low and hardly detectable. However, ADAR1-P110 levels were obviously down-regulated during several hours (about 2–8 h) of treatment with IFN-I (Fig. 1A). Given that the ADAR1-P150 form does not exist in cells at the initial stage of IFN-I treatment, we speculated that ADAR1-P110 is the predominant form to suppress IFN-I signaling. Therefore, this study focused on constitutively expressed ADAR1-P110. Our data clearly showed that IFN-I signaling induced ADAR1-P110 ubiquitination and promoted protein turnover of ADAR1-P110 (Figs. 2, 3, and 5). It is also interesting but out of the scope of this study to explore the effect of
IFN-I signaling on ADAR1-P150, which could be associated with the regulation of virus-sensing pathways.

ADAR1 deficiency has recently been shown to be associated with aberrant organ development and severe autoimmune diseases. Mouse embryos with an editing-deficient knockin mutation of ADAR1 died at embryonic day 13.5 (18). Loss of ADAR1 results in increased IFNα/β production and global up-regulation of IFN-inducible transcripts and therefore leads to rapid apoptosis of hematopoietic stem cells (9). Genetic mutations of ADAR1 result in the autoimmune disorder Aicardi-Goutières syndrome, which is associated with excessive activated IFN-I signaling (17). All of these studies demonstrated that ADAR1 is an important suppressor of IFN signaling and functions. This conclusion is consistent with many recent reports that showed that ADAR1 isoforms control autoimmunity and multiorgan development syndrome, which is associated with excessive activated IFN-I signaling. Another interesting result observed is that ADAR1-P110 levels were slowly raised after 12-h treatment with IFN-I (Fig. 1A). It is not surprising. Given that balanced IFN-I signaling is important, lack of ADAR1-P110 in cells could be harmful to cells because of uncontrolled, excessively activated IFN-I signaling. Another interesting result showed that IFN-I signaling promoted the binding of β-TrCP to ADAR1-P110 (Fig. 4F). Similarly, a previous study reported that IFN-I signaling promoted the binding of β-TrCP to IFNAR1, which resulted in IFNAR1 ubiquitination and degradation (28, 29). Therefore, we speculated that IFN-I signaling is able to promote the β-TrCP action, although the detailed mechanisms remain unknown. It could be interesting to explore β-TrCP action mechanisms in the future. Here the interaction between β-TrCP and ADAR1-P110 promotes IFN signaling, whereas the interaction between β-TrCP and IFNAR1 restricts the IFN-mediated signaling pathway. This opposite effect of β-TrCP on IFN responses could maintain another balance to hold IFN-I signaling in check. However, more evidence is needed to completely address this “balance” hypothesis.

Given that IFN-I signaling leads to ADAR1-P110 down-regulation and that viral infections can induce IFN-I production, we presumed that viral infections can also down-regulate ADAR1-P110. Our data clearly showed that ADAR1-P110 levels were significantly down-regulated during VSV infection (Fig. 7A). Based on this result, we wanted to know what happens to IFN-I antiviral activity when cellular ADAR1-P110 is unable to be ubiquitinated and down-regulated. Thus, we next sought to determine the pathophysiological significance of ADAR1-P110 down-regulation during viral infections. In this study, we have clarified the mechanisms of ADAR1-P110 down-regulation and identified the key serine and lysine residues (including Ser578 and Ser585 as well as Lys574 and Lys576) for ADAR1-P110 ubiquitination and degradation (Figs. 3 and 6). Therefore, we mutated these key residues (AA and 2KR) to limit ubiquitin-dependent down-regulation of ADAR1-P110. Our data demonstrated that ADAR1-P110-AA significantly inhibited IFN-I-mediated antiviral activity (Fig. 7, C and D). Furthermore, we established ADAR1-P110 knockdown cells and then re-expressed either ADAR1-P110-WT or the ADAR1-P110–2KR mutant. This result showed that re-expression of ADAR1-P110–2KR more significantly inhibited IFN-I signaling and therefore promoted viral infections compared with ADAR1-P110-WT (Fig. 7, F and G). Thus, using this well-established model of ADAR1-P110 mutants, we clarified that, during viral infections, inhibition of ADAR1-P110 ubiquitin-dependent down-regulation significantly restricts IFN-I antiviral efficiency.

This study uncovered the detailed mechanism by which our body breaks the balance of IFN-I signaling to establish efficient antiviral responses by ubiquitin-dependent down-regulation of constitutively expressed ADAR1 during viral infections (Fig. 8). These findings promote a better understanding of the action mechanisms of IFN-I (or other cytokine) activation and therefore may provide potential therapeutic targets for IFN-related diseases.

**Experimental Procedures**

**Cells, Constructs, and Reagents**—The 293T and 2fTGH cell lines were obtained from the ATCC. All cells were cultured at 37 °C under 5% CO2 in DMEM (HyClone) supplemented with 10% FBS (Gibco, Life Technologies), 100 units/ml penicillin, and 100 μg/ml streptomycin. FLAG-ADAR1-P110 (ADAR1) was a gift from Dr. A. D. J. Scadden (University of Cambridge). HA-UB, HA-UB-R48K, and HA-UB-R63K were gifts from Dr. Lingqiang Zhang (State Key Laboratory of Proteomics). The

**FIGURE 7.** Down-regulation of ADAR1-P110 is required for efficient antiviral activity of type I IFN. A, 2fTGH cells were infected with VSV (MOI = 0.5) for different times. Whole cell extracts were subjected to immunoblotting (IB) using the indicated antibodies. B, 2fTGH cells were transfected with either control shRNAs (shCON) or shADAR1-P110 (shADAR1). 48 h after transfection, cells were pretreated with or without IFNα (50 IU/ml) for 20 h. Then cells were challenged by VSV (MOI = 0.5) for 20 h. Whole cell extracts were subjected to immunoblotting using the indicated antibodies. C, 2fTGH cells transfected with FLAG-ADAR1-P110 (WT or AA) were pretreated with or without IFNα (50 IU/ml) for 20 h. Then cells were challenged by VSV-GFP (MOI = 0.5) for 20 h. VSV-GFP levels were observed by fluorescence. D, 2fTGH-shADAR1 stable cells were transfected with either resistant ADAR1-P110-WT (res-WT) or resistant ADAR1-P110-K574A,576R (res-2KR) as indicated. Then cells were challenged by VSV (MOI = 1.0) for 18 h and VSV viral RNA levels were analyzed by quantitative RT-PCR. E, 2fTGH-shADAR1 cells were transfected as in F. 48 h after transfection, cells were treated with IFNα (5 IU/ml) overnight and then were challenged by VSV (MOI = 1.0) for 18 h. VSV viral RNA levels were analyzed by quantitative RT-PCR. *, p < 0.05; ***, p < 0.001.
HA-tagged β-TrCP plasmid was a gift from Dr. Serge Y. Fuchs (University of Pennsylvania). Both shRNAs against β-TrCP (shBTR) and shRNAs against ADAR1 constructs (shADAR1) were purchased from Genechem (Shanghai, China). The shBTR target sequences were as follows: 5′-GTGGAATT-TGTGGAACATC-3′ and 5′-TTCCCTCAGAGAGAGAGACTG-3′. The shADAR1 target sequences were as follows: 5′-CCACTTGTATCTTCATTT-3′ and 5′-ATGGGTTTCACAGAGGTAA-3′. All mutants, including ADAR1-P110-S578A(SA), ADAR1-P110-S578A,S585A (AA), ADAR1-P110-K564R, and ADAR1-P110-K574R,K576R (2KR), were generated by the QuikChange site-directed mutagenesis kit (Stratagene). All plasmids were confirmed by DNA sequencing. Recombinant human IFNα was purchased from PBL Interferon (University of Pennsylvania). Both shRNAs against β-TrCP and ADAR1 constructs (shBTR) and shRNAs against ADAR1 were purchased from Genechem (Shanghai, China). The following antibodies were used: antibodies against PKR (Z-VAD (Bachem) was a kind gift from Dr. Sudan He (Soochow University). Cycloheximide and other chemicals were from Sigma.

**Transfection, Immunoblotting, and Immunoprecipitation**—All transient transfections were carried out using either Lipofectamine Plus (Invitrogen) or LongTrans (Ucallm) according to the instructions of the manufacturer. All cells were harvested using lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.5 mM EDTA, PMSF (50 μg/ml), and protease inhibitor mixtures (Sigma). N-ethylmaleimide (10 mM) was added into the above lysis buffer when protein ubiquitination was analyzed. Immunoprecipitation was performed using specific antibodies overnight on a rotor at 4 °C. Protein G-agarose beads (Millipore, 16-266) were added to samples and incubated for an additional 3 h on a rotor at 4 °C. After washing five times with lysis buffer, the immunoprecipitates were eluted by boiling with loading buffer containing β-mercaptoethanol for 10 min and analyzed by SDS-PAGE, followed by transfer to PVDF membranes. The membranes were then blocked andprobed with the primary antibodies, followed by the respective HRP-conjugated goat anti-mouse or goat anti-rabbit (Bio-world) secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura extended kits (Thermo Scientific). The following antibodies were used: antibodies against FLAG (Sigma, F7425), ADAR1 (Cell Signaling Technology, 12317), β-TrCP (Cell Signaling Technology, 11984), VSV-G (Santa Cruz Biotechnology, sc-66180), HA (Santa Cruz Biotechnology, sc-7392), ubiquitin (Santa Cruz Biotechnology, sc-8017), tubulin (Proteintech, 66031-1-lg), caspase3 (Cell Signaling Technology, 9665) and β-actin (Proteintech, 66009-1-lg). The ImageJ program (http://rsbweb.nih.gov/ij/download.html) was used for densitometric analyses of Western blots.

**Cycloheximide Chase Assay**—The half-life of FLAG-ADAR1-P110 was determined by CHX pulse-chase assay. Cells were transfected with FLAG-ADAR1-P110. 48 h after transfection, cells were treated with DMSO or CHX (50 μg/ml) together with or without IFNα for the indicated times. Cells were then collected, and the equal amounts of boiled lysates were analyzed by Western blotting. Each experiment was carried out in triplicate.

**RNA Isolation and Real-time PCR**—Total RNAs were isolated from cells using TRIzol reagent (Invitrogen). cDNA was synthesized by reverse transcription using oligo(dT) and then subjected to quantitative real-time PCR with VSV RNA primers and β-actin primers in the presence of SYBR Green (Bio-Rad). The primer sequences were as following: VSV (5′-ACGGCGTACTTCCAGATGG-3′) and 5′-CTCGGTTCAAGTCGGACTGAGTTAGCCTGGAGAA-3′ and 5′-ATAGCA CAGCCTGGATAGAAAA-3′ and 5′-ATAGCA CAGCCTGGACTAGCCACAC-3′. The relative expression of the target genes was normalized to β-actin mRNA. The results were analyzed from three independent experiments and shown as the average mean ± S.D.

**Flow Cytometry Analysis and Immunofluorescence Microscopy**—Cells infected with VSV-GFP (MOI = 0.5) were subjected to analyses by either immunofluorescence microscopy or flow cytometry. Briefly, for immunofluorescence microscopy analysis, VSV-GFP viruses were pictured with an upright fluorescence microscope. Magnification was ×200. For flow cytometry analysis, cells were collected with cold 1× PBS and acquired in a FACSCalibur (BD Biosciences) equipped with a 488-nm argon laser and a 635-nm red diode laser. Data were analyzed by FlowJo software (FlowJo, Ashland, OR).

**Virus and Viral Infection**—VSV was a gift from Dr. Chen Wang (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). VSV-GFP was a gift from Dr. Chunsheng Dong (Soochow University). The antiviral effect of IFNα...
was determined by pretreating cells with IFNα overnight prior to infection with viruses. Briefly, cells were transfected with FLAG-ADAR1-P110-WT or mutants. 48 h after transfection, cells were pretreated with IFNα (60 IU/ml) overnight. After washing twice, cells were challenged by VSV or VSV-GFP at a multiplicity of infection (MOI) of 0.5 for 1.5 h. The infection medium was removed by washing twice. Then cells were fed with fresh medium and incubated for an additional 20 h. Cells were analyzed by immunofluorescence or FACS or by Western blotting using a VSV-G antibody.

Statistical Analysis—Student’s t test was used for comparison between different groups. All data are presented as mean ± S.E. All differences were considered statistically significant at p < 0.05.

Author Contributions—H. Z. conceived and designed the project. L. L. and G. Q. performed most of the experiments and data analysis. Y. Z., Y. Y., and Q. C. contributed to the cellular experiments and plasmid purification. T. G., J. L., C. L., and L. Z. provided technical support. H. Z. and L. Z. wrote the manuscript.

Acknowledgments—We thank Dr. A. D. J. Scadden (University of Cambridge), Dr. Serge Fuchs (University of Pennsylvania), Dr. Lingjiang Zhang (State Key Laboratory of proteomics), Dr. Chen Wang (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences), and Dr. Chunsheng Dong (Soochow University) for their kind permission.

References

1. Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. (1987) Interferons and their actions. Annu. Rev. Biochem. 56, 727–777
2. Borden, E. C., Sen, G. C., Uze, G., Silverman, R. H., Ransohoff, R. M., Foster, G. R., and Stark, G. R. (2007) Interferons at age 50: past, current and future impact on biomedicine. Nat. Rev. Drug Discov. 6, 975–990
3. Sadler, A. J., and Williams, B. R. (2008) Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8, 559–568
4. Toth, A. M., Zhang, P., Das, S., George, C. X., and Samuel, C. E. (2006) Interferon action and the double-stranded RNA-dependent enzymes ADAR1 adenosine deaminase and PKR protein kinase. Prog. Nucleic Acid Res. Mol. Biol. 81, 369–434
5. Davis, G. L. (2001) Current treatment for chronic hepatitis C. Rev. Gastroenterol. Disord. 1, 59–72
6. Rice, G. I., Forte, G. M., Szyndzielorz, M., Chase, D. S., Aebi, A., Abeldamid, M. S., Ackroyd, R., Allcock, R., Bailey, K. M., Balottin, U., Barone, C., Bernard, G., Bodemer, C., Botella, M. P., Cereda, C., et al. (2013) Assessment of interferon-related biomarkers in Aicardi-Goutières syndrome associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, and ADAR: a case-control study. Lancet Neurol. 12, 1159–1169
7. Rönnblom, L. (2011) The type I interferon system in the etiopathogenesis of autoimmune diseases. Ups. J. Med. Sci. 116, 227–237
8. Trinchieri, G. (2010) Type I interferon: friend or foe? J. Exp. Med. 207, 2053–2063
9. Hartner, J. C., Walkley, C. R., Lu, J., and Orkin, S. H. (2009) ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling. Nat. Immunol. 10, 109–115
10. Qiu, W., Wang, X., Buchanan, M., He, K., Sharma, R., Zhang, L., Wang, Q., and Yu, J. (2013) ADAR1 is essential for intestinal homeostasis and stem cell maintenance. Cell Death Dis. 4, e599
11. Wang, G., Wang, H., Singh, S., Zhou, P., Yang, S., Wang, Y., Zhu, Z., Zhang, J., Chen, A., Billiar, T., Monga, S. P., and Wang, Q. (2015) ADAR1 prevents liver injury from inflammation and suppresses interferon production in hepatocytes. Am. J. Pathol. 185, 3224–3237
12. Coccia, E. M., Usé, G., and Pellegrini, S. (2006) Negative regulation of type I interferon signaling: facts and mechanisms. Cell. Mol. Biol. 52, 77–87
13. Zheng, H., Qian, J., Varghese, B., Baker, D. P., and Fuchs, S. (2011) Ligand-stimulated downregulation of the α interferon receptor: role of protein kinase D2. Mol. Cell. Biol. 31, 710–720
14. Zheng, H., Qian, J., Carbone, C. J., Leu, N. A., Baker, D. P., and Fuchs, S. Y. (2011) Vascular endothelial growth factor-induced elimination of the type 1 interferon receptor is required for efficient angiogenesis. Blood 118, 4003–4006
15. Bhattacharya, S., Zheng, H., Tzimas, C., Carroll, M., Baker, D. P., and Fuchs, S. Y. (1991) Bcr-abl signals to desensitize chronic myeloid leukemia cells to IFNα via accelerating the degradation of its receptor. Blood 118, 4179–4187
16. Malakhova, O. A., Kim, K. I., Luo, J. K., Zou, W., Kumar, K. G., Fuchs, S. Y., Shuai, K., and Zhang, D. E. (2006) UBAP45 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. EMBO J. 25, 2358–2367
17. Pestal, K., Funk, C. C., Snyder, J. M., Price, N. D., Treuting, P. M., and Stetson, D. B. (2015) Isoforms of RNA-editing enzyme ADAR1 independently control nucleic acid sensor MDA5-driven autoimmunity and multi-organ development. Immunity 43, 933–944
18. Liddicoat, B. J., Chalk, A. M., and Walkley, C. R. (2016) ADAR1, inosine and the immune sensing system: distinguishing self from non-self. Wiley Interdiscip. Rev. RNA 7, 175–171
19. Rice, G. I., Kasher, P. R., Forte, G. M., Mannion, N. M., Greenwood, S. M., Szyndzielorz, M., Dickerson, J. E., Bhaskar, S. S., Zampini, M., Briggs, T. A., Jenkinson, E. M., Bacino, C. A., Battini, R., Bertini, E., Brogan, P. A., et al. (2012) Mutations in ADAR1 cause Aicardi-Goutières syndrome associated with a type I interferon signature. Nat. Genet. 44, 1243–1248
20. Samuel, C. E. (2011) Adenosine deaminases acting on RNA (ADARs) are both antiviral and proviral. Virology 411, 180–193
21. Gélas, J. F., Clerzius, G., Shaw, E., and Gatignol, A. (2011) Enhancement of replication of RNA viruses by ADAR1 via RNA editing and inhibition of RNA-activated protein kinase. J. Virol. 85, 8460–8466
22. Fuchs, S. Y., Spiegelman, V. S., and Kumar, K. G. (2004) The many faces of β-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene 23, 2028–2036
23. Sun, L., and Chen, Z. J. (2004) The novel functions of ubiquitination in signaling. Curr. Opin. Cell Biol. 16, 119–126
24. McKendry, R., John, J., Flavell, D., Müller, M., Kerr, I. M., and Stark, G. R. (1991) High-frequency mutagenesis of human cells and characterization of a mutant unresponsive to both α and γ interferons. Proc. Natl. Acad. Sci. U.S.A. 88, 11455–11459
25. Haller, O., Kochs, G., and Weber, F. (2006) The interferon response circuit: induction and suppression by pathogenic viruses. Virology 344, 119–130
26. Randall, R. E., and Goodbourn, S. (2008) Interferons and viruses: an interplaybetweeninduction,signalling,antiviralresponsesandviruscountermeasures. J. Gen. Virol. 89, 1–47
27. Li, Z., Wolff, K. C., and Samuel, C. E. (2010) RNA adenosine deaminase ADAR1 deficiency leads to increased activation of protein kinase PKR and reduced vesicular stomatitis virus growth following interferon treatment. Virology 396, 316–322
28. Kumar, K. G., Tang, W., Ravindranath, A. K., Clark, W. A., Croze, E., and Fuchs, S. Y. (2003) SCF(HOS) ubiquitin ligase mediates the ligand-induced down-regulation of the interferon-α receptor. EMBO J. 22, 5480–5490
29. Zheng, H., Qian, J., Baker, D. P., and Fuchs, S. Y. (2011) Tyrosine phosphorylation of protein kinase D2 mediates ligand-inducible elimination of the type I interferon receptor. J. Biol. Chem. 286, 35733–35741
30. Toth, A. M., Li, Z., Cattaneo, R., and Samuel, C. E. (2009) RNA-specific adenosine deaminase ADAR1 suppresses measles virus-induced apoptosis and activation of protein kinase PKR. J. Biol. Chem. 284, 29350–29356
31. Ren, Y., Zhao, P., Liu, J., Yuan, Y., Cheng, Q., Zuo, Y., Qian, L., Liu, C., Guo, T., Zhang, L., Wang, X., Qian, G., Li, L., Ge, J., Dai, J., et al. (2016) Deubiquitinase USP2a sustains interferons antiviral activity by restricting ubiquitination of activated STAT1 in the nucleus. PLoS Pathog. 12, e1005764