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UBXN3B positively regulates STING-mediated antiviral immune responses

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The ubiquitin regulatory X domain-containing proteins (UBXNs) are likely involved in diverse biological processes. Their physiological functions, however, remain largely unknown. Here we present physiological evidence that UBXN3B positively regulates stimulator-of-interferon genes (STING) signaling. We employ a tamoxifen-inducible Cre-LoxP approach to generate systemic Ubxn3b knockout in adult mice as the Ubxn3b-null mutation is embryonically lethal. Ubxn3b−/−, like Sting−/− mice, are highly susceptible to lethal herpes simplex virus 1 (HSV-1) and vesicular stomatitis virus (VSV) infection, which is correlated with deficient immune responses when compared to Ubxn3b+/+ littermates. HSV-1 and STING agonist-induced immune responses are also reduced in several mouse and human Ubxn3b−/− primary cells. Mechanistic studies demonstrate that UBXN3B interacts with both STING and its E3 ligase TRIM56, and facilitates STING ubiquitination, dimerization, trafficking, and consequent recruitment and phosphorylation of TBK1. These results provide physiological evidence that links the UBXN family with antiviral immune responses.
mediated phosphorylation of STING provides a mechanism for TBK1 to phosphorylate IRF3 and induction of IFN-I. Interestingly, among all the UBXNs tested, UBXN3B most significantly potentiated a STING-induced IFN-I response, which was ~150-fold relative to vector alone (Supplementary Fig. 1a). The screening results, we used cGAMP, a second messenger that binds and stimulates STING signaling. cGAMP was used to prime HEK293T cells stably expressing FLAG-STING (designated HEK293T-STING cell line) following transfection with a UBXN3B expression plasmid. We found that UBXN3B synergized with cGAMP to induce an IFN-I response in a dose-dependent manner (Supplementary Fig. 1b and 1c). To confirm that the enhancing effect on STING signaling was specific to UBXN3B, we included two UBXN members, UBXN3A which is the closest sibling of UBXN3B and UBXN9 which is more dissimilar from UBXN3B, in terms of domain structure. Indeed, only UBXN3B, but not UBXN3A or UBXN9, enhanced the cGAMP-induced IFN-I response (Supplementary Fig. 1d). In addition, we tested if UBXN3B has a modulatory effect on other important antiviral signaling pathways. The results demonstrate that UBXN3B failed to synergize with retinoic acid-inducible gene 1 (RIG-I)-like receptor (RLR)-initiated and Toll-like receptor (TLR)-mediated IFN-I induction (Supplementary Fig. 2). These data suggest that UBXN3B specifically targets STING signaling.

UBXN3B is critical for anti-DNA virus immune responses. UBXN3B, also known as Fas-associated factor 2 and UBXD8, might play a role in regulating lipid metabolism. However, its physiological function remains largely unclear. We therefore attempted to generate general Ubxn3b−/− mice using the CRISPR-Cas9 technology and unfortunately failed, suggesting that Ubxn3b deletion is embryonically lethal, consistent with a recent publication. We therefore planned to generate an inducible conditional knockout strain so as to systemically ablate UBXN3B gene expression. We obtained Ubxn3blox/lox (exon 1, homologous recombination) from our collaborator and crossed it with a tamoxifen-inducible Cre mouse line. To generate a Cre+/*-Ubxn3blox/lox mouse. The tamoxifen-inducible Cre model has been successfully applied to many gene deletions including essential genes. Indeed, Ubxn3b protein expression was abolished in various tissues of tamoxifen-treated Cre+/*-Ubxn3blox/lox mice (Fig. 1a). However, no discernible developmental defects or behavioral abnormality were observed in these mice. We then infected tamoxifen-pretreated Cre+/*-Ubxn3blox/lox (referred to as Ubxn3b−/−) littermates with a DNA virus, herpes simplex virus 1 (HSV-1), which effectively induces STING-signaling. To exclude any potential side effects of tamoxifen on HSV-1 pathogenesis and/or antiviral immunity, we included tamoxifen-treated Cre+/*-Ubxn3blox/lox littermates as controls. As shown in Fig. 1b, Ubxn3b−/− mice (N = 8) died of HSV-1 infection earlier than Ubxn3b+/+ mice (N = 8) and the other control mice (N = 9). The overall survival rate of Ubxn3b−/− mice was also much lower (P < 0.01, log-rank test). In addition, all infected Ubxn3b−/− mice developed severe neurological symptoms, as well as hunched posture, decreased movement, and labored breathing on day 3 after infection. These symptoms were completely absent from Ubxn3b+/+ animals (Supplementary Movie 1). Compared to Sting−/*- mice (N = 8), Ubxn3b−/− mice (N = 9) were a bit less sensitive to HSV-1 infection (Fig. 1c). In agreement with the survival data, the serum IFN-I abundance was markedly decreased at 4 and 8 h after HSV-1 inoculation in Ubxn3b−/− (N = 6) compared to Ubxn3b+/+ (N = 10) mice (non-parametric Mann–Whitney test, P < 0.05), demonstrating that UBXN3B is critical for HSV-1-induced IFN response (Fig. 1d). In agreement with this, the mRNA levels of Ifnb1, Tifa, and...
UBXN3B is critical for IFN-I induction by and control of HSV-1 infection in vivo. a Immunoblots showing Ubxn3b knockout efficiency in various tissues without/with tamoxifen (TMX) treatment in Cre+/− Ubxn3bflx/flx mice. Tubulin is a housekeeping protein control. b, c The survival curves of tamoxifen (TMX)-treated Ubxn3bflx/flx, TMX-treated Cre+/−, mock-treated Cre+/− Ubxn3bflx/flx (designated Ubxn3b+/−) and TMX-treated Cre+/− Ubxn3bflx/flx (designated Ubxn3b−/−) littermate and Sting−/− mice challenged with 1 × 107 plaque-forming units (PFU) per mouse of HSV-1 i.v. N = 8–9 mice/ genotype. ***P < 0.001 (log-rank test). d The serum IFN-I concentrations (mean ± s.e.m) of mice challenged with 2 × 106 PFU per mouse of HSV-1 i.v. **P < 0.01, *P < 0.05 (non-parametric Mann–Whitney test), (an IFN-I-induced antiviral effector) in leukocytes of fl/fl mice were also lower than those in Ubxn3b+/+ littermates (N = 5 each genotype, P < 0.05, non-parametric Mann–Whitney test) (Supplementary Fig. 3). Given that HSV-1 can penetrate the brain and elicit fatal encephalitis in mice, we then quantified HSV-1 titers in the brain by plaque-forming assay. Ubxn3b−/− mice had a significantly increased number of viral particles compared to Ubxn3b+/+ mice on day 3 after infection (N = 5 each genotype, P < 0.01, non-parametric Mann–Whitney test) (Fig. 1e).

We next asked if the antiviral function of UBXN3B was cell-type specific. We isolated bone marrow cells from Cre+/− Ubxn3bflx/flx mice pretreated without (Ubxn3b+/+) or with tamoxifen (Ubxn3b−/−) and differentiated them into macrophages (bone marrow–derived macrophage (BMDMs)) with L929 cell-conditioned medium, conventional dendritic cells (cDCs) with granulocyte–macrophage colony-stimulating factor (GM-CSF) and plasmacytoid DC (pDCs) with Flt3L. Induction of IFN-I protein by HSV-1, STING-stimulating immunostimulatory DNA (ISD) and cGAMP was remarkably compromised in knockout cells (Fig. 2a). Correspondingly, induction of Oas1a protein, an IFN-stimulated gene (ISG), was also impaired in knockout cDC cells (Fig. 2b). The mRNA levels of Ifi1b and Tnfa were decreased in Ubxn3b−/− cells at 6 h after infection, which was accompanied by increased viral load (Fig. 2c, d). The above-mentioned immune cells are not very permissive to HSV-1 Infection. We next isolated mouse embryonic fibroblasts (MEFs), which are highly permissive to HSV-1 in contrast to the above-mentioned immune cells we induced in vitro, from untreated Cre+/− Ubxn3bflx/flx E14 embryos and induced Ubxn3b deletion with 4-hydroxyl tamoxifen in vitro. Ubxn3b protein expression was almost completely depleted after 4-hydroxyl tamoxifen treatment in Cre+/− Ubxn3bflx/flx (Ubxn3b−/−) cells (Fig. 2e). In agreement with the aforementioned results, the viral titers produced by Ubxn3b−/− cells were higher than Ubxn3b+/− (Fig. 2f). Ifi1b and its ISG mRNA levels induced by HSV-1 were consistently down-regulated in Ubxn3b−/− cells (Fig. 2g).

The UBXN3B protein is evolutionarily conserved, with 97% identity between human and rodent. We then asked if its antiviral function is also evolutionarily conserved. We employed the CRISPR-Cas9 technology to generate UBXN3b−/− in H1975 cell, a human lung epithelial cell line (Supplementary Fig. 4a). Consistent with the results from mouse cells, IFN1 induction by ISD was much lower in UBXN3b−/− cells than wild type (WT) cells. But the dsRNA analog polyinosinic:polycytidylic acid...
(polyIC)-induced IFNB1 expression was not significantly impaired (Supplementary Fig. 4b). The HSV-1 titers (Supplementary Fig. 4c) and intracellular HSV-1 protein levels (Supplementary Fig. 4d) were very much increased in UBXN3B−/− cells compared to WT cells, suggesting a critical antiviral role for UBXN3B. The immune responses to HSV-1 including IFN-I (IFNB1) and inflammatory response (TNFA) were reduced in the absence of UBXN3B expression (Supplementary Fig. 4e). To extend this further, we next examined the UBXN3B antiviral function in primary human trophoblasts. These cells (1) may be...
Fig. 3 UBXN3B is critical for type I IFN responses to SeV and VSV, but not EMCV infection. a The survival curves of Ubx3b−/− (N = 6) and Ubx3b−/− (N = 7) mice infected with 1 × 10³ PFU of VSV i.v. The results are pooled from two experiments. *P < 0.05 (log-rank test). b, c qPCR analysis of (Ifnb1) expression in cDCs infected with b VSV (MOI = 5) or c SeV (200 hemagglutination units per 10⁵ cells). d ELISA of IFN-α in the cell culture supernatants of cDCs infected with SeV and VSV as in b, c. N = 3 per genotype per time point. *P < 0.05 (unpaired Student’s t test). e The survival curves of Ubx3b+/+ (N = 7) and Ubx3b−/− (N = 8) mice infected with 200 PFU of EMCV i.p. P = 0.53 (log-rank test). The results are pooled from two experiments. f qPCR analysis of (Ifnb1) expression in cDCs infected with EMCV (MOI = 5) for the indicated time. Bars/data points: mean ± s.e.m. Two biological replicates were pooled for qPCR (N = 2 per genotype per time point). *P < 0.05 (unpaired Student’s t test). The results are representative of two independent experiments.

phylogenically related to vertical transmission of HSV-1; (2) can be passaged in vitro for up to 12–15 generations⁴, which allows us to knockout the genes of interest with CRISPR-Cas9 and examine their functions; and (3) can be obtained in a large quantity. In parallel, we included STING−/− as a positive control. As shown in Fig. 2h, STING and UBXN3B protein expression was depleted in knockout cells. Both STING−/− and UBXN3B−/− cells were more permissive to HSV-1 infection than WT (Fig. 2i); the

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**Fig. 2** UBXN3B is critical for STING-dependent IFN-I induction in mouse primary cells. a ELISA of IFN-α in the cell culture supernatants of Ubx3b+/+, Ubx3b−/− bone marrow-derived macrophages (MΦs), Flt3-induced pDCs, and GM-CSF-induced cDCs (pooled from five litters) 20 h after the indicated treatments. N = 3 per genotype. *P < 0.05 (unpaired Student’s t test). b Immunoblot of an interferon-stimulated gene Oas1a, Sting, and Ubx3b expression in cDCs 20 h after the indicated treatments. Tubulin is a housekeeping protein control. qPCR analysis of (c) Ifnb1 and Tnfa mRNA expression and d cellular HSV-1 genome loads in cDCs infected with HSV-1 (MOI = 10) for the indicated time. e Immunoblot showing Ubx3b and housekeeping Gapdh protein expression in mock (Ubx3b+/+) or 4-hydroxy tamoxifen-treated Cre−/− Ubx3bfl/fl (Ubx3b−/−) MEFs. f Viral titers (plaque-forming units/ml) in the supernatant of MEFs infected with HSV-1 (MOI = 0.1). N = 3 per genotype per time point. *P < 0.05; **P < 0.01 (unpaired Student’s t test). g qPCR analysis of selected immune gene mRNA expression in MEFs infected with HSV-1 as in h. The immunoblots show knockout efficacy of STING and UBXN3B by CRISPR-Cas9 in human primary trophoblasts. Actin is a housekeeping protein control. i Fluorescent microscopic images of human primary trophoblasts infected with HSV-1-GFP (MOI = 0.3) for 18 h. Objective, ×5. Scale bar, 10 μm. j qPCR analysis of Ifnb1 mRNA expression in human primary trophoblasts infected with HSV-1-GFP for the indicated time. Bars/data points: mean ± s.e.m. Two biological replicates were pooled for qPCR (N = 2 per genotype per time point). *P < 0.05; **P < 0.01 (unpaired Student’s t test). The results are representative of two independent experiments.
4-hydroxyl tamoxifen-induced Cre (MOI = 2 or presence of 40 µM of chloroquine (CQ) for 48 h). The results show that Ifnb1 and Tnfa mRNA upregulation by CpG DNA (TLR9), FLS-1 (Pam2CysKKKPSF, TLR2/6), lipopolysaccharide (LPS, TLR4), high-molecular-weight polyIC (TLR3, MDAS), and single-stranded poly-uridine (polyU, TLR7) in Ubxn3b−/− was not significantly different from Ubxn3b+/+ cDCs (Supplementary Fig. 5a–e). These data demonstrate that UBXN3B is not essential for TLRI/TLR-dependent signaling28. We next asked if UBXN3B regulated the IFN-JAK-STAT pathway, which induces expression of a large number of antiviral effectors. As shown in Supplementary Fig. 5f, the Isg15 and Oas1a mRNA expression levels were similar between Ubxn3b+/+ and Ubxn3b−/− cDCs, suggesting that UBXN3B is dispensable for the JAK-STAT signaling pathway. Furthermore, we observed that Ubxn3b expression was induced by IFN-α in both cDCs and MEFs, which was completely dependent on the IFN-I receptor signaling (Supplementary Fig. 6). Intriguingly, we noted that Ubxn3b was gradually and constantly upregulated throughout IFN-α treatment (Supplementary Fig. 6a). These data suggest that UBXN3B is an ISG.

We next explored the molecular mechanism by which UBXN3B acts on STING-dependent signaling. Lysine (K) 63-linked ubiquitination and dimerization of STING is a prerequisite for STING trafficking out of the ER to perinuclear vesicles where it recruits the antiviral kinase TBK1 to induce IFN-17,9,10,29. STING is then phosphorylated and degraded via autophagy29, and proteasomes30. UBXN3B proteins are likely involved in regulation of E3 ubiquitin ligase29. We first asked whether UBXN3B played a role in STING dimerization, trafficking, and phosphorylation. Indeed, STING dimerization took places 8 h after HSV-1 infection in Ubxn3b−/− MEFs, but this was obviously reduced in Ubxn3b−/− cells (Fig. 4a). We observed a similar phenotype in UBXN3B regulates STING signaling. All the aforementioned in vivo and in vitro results clearly indicate an essential role of UBXN3B in the STING signaling pathway. To see whether UBXN3B also plays a role in other pathogen pattern recognition receptors such as RLR and TLR signaling pathways, we examined immune response induction in cDCs by several well-established RLR/TLR agonists. The results show that Ifnb1 and Tnfa mRNA expression was, however, much lower in knockout cells (Fig. 2). These results demonstrate that the STING-regulating function of UBXN3B is evolutionarily conserved.

UBXN3B regulates immune responses to some RNA viruses. STING signaling is not only essential for induction of immune responses to DNA viruses but also important for antiviral immunity to certain RNA viruses such as VSV and Sendai virus (SeV). We then investigated the physiological role of UBXN3B during RNA virus infection. Consistent with the phenotype of Sting−/− infected with VSV5, Ubxn3b−/− mice (N = 6) were also more susceptible to lethal VSV infection than its Ubxn3b+/+ littermates (N = 7 each genotype, P < 0.05, log-rank test) (Fig. 3a). The Ifnb1 mRNA expression was modestly decreased in knockout cDCs at 12 h after infection (Fig. 3b). Similarly the Ifnb1 transcripts were also reduced in knockout cells infected with SeV (Fig. 3c). Consistent with this, the IFN-α protein concentration in the knockout cell supernatants was modestly lower than WT (Fig. 3d). However, Ubxn3b was dispensable for the control of a non-enveloped (+) single-stranded RNA virus, encephalomyocarditis virus (EMCV) infection in vivo (Fig. 3e), and for innate immune responses in cDCs (Fig. 3f). This is also consistent with the phenotype of Sting−/− mice25.

IFNB1 mRNA expression was, however, much lower in knockout IFNB1−/−/mice (N = 2 per genotype per time point). *P < 0.05 (unpaired Student’s t test). The results are representative of two to three independent experiments.
cDCs (Fig. 4b). Moreover, HSV-1-induced phosphorylation of TBK1 and IRF3 was inhibited in Ubxn3b−/− cDCs (Fig. 4b). Consequently, Ifnb1 and Tnfa mRNA transcripts were also decreased in Ubxn3b−/− (Fig. 4c). STING is degraded after HSV-1 infection in permissive cells like MEFs. We extended the HSV-1 infection and indeed observed overt Sting degradation in Ubxn3b−/− cells at 12 h and this was inhibited in knockout cells (Fig. 4d). A similar result was noted in H1975 cells (Supplementary Fig. 7). ISD also stimulated STING degradation, which was partially blocked in Ubxn3b−/− (Fig. 4e). These data suggest that STING translocation from the ER to perinuclear vesicles is influenced by UBXN3B deficiency too. We investigated this in H1975 cells by immunofluorescence microscopy. In unstimulated cells, STING co-localized with an ER-resident protein calreticulin very well. STING was localized to some punctate structures that were away from calreticulin-stained ER 3 h after ISD stimulation in WT cells, but remained co-localized with calreticulin in ISD-treated UBXN3B−/− cells (Supplementary Fig. 8). Following translocation, STING is phosphorylated and degraded partially by autophagy. We did not readily detect phospho-Sting in ISD-treated MEFs, likely because of its rapid degradation following phosphorylation in these cells. We thus added chloroquine to block autophagy-mediated Sting degradation in order to capture phospho-Sting. Indeed, chloroquine treatment delayed Sting degradation and Sting phosphorylation was observed at 4 h after ISD treatment in Ubxn3b+/+ cells but clearly absent in Ubxn3b−/− MEFs (Fig. 4e). Interestingly, STING phosphorylation was much readily detected in human trophoblasts. Using a specific antibody against only the phosphorylated human STING, we noted rapid phosphorylation of STING at 4 h after cGAMP treatment. STING phosphorylation was obviously reduced, so was TBK1; while STING degradation was inhibited in UBXN3B−/− cells (Fig. 4f). As a result, IFNB1 and Tnfa mRNA upregulation by cGAMP was repressed in UBXN3B−/− cells (Fig. 4g).

The aforementioned data clearly demonstrate that UBXN3B regulates STING signaling and its downstream immune responses. To test if UBXN3B directly participated in STING signaling complex, we employed a co-immunoprecipitation assay in HEK293T cells with simultaneous expression of FLAG-tagged UBXNs and HA-tagged STING. UBXN3B but not its sibling UBXN3A co-immunoprecipitated with STING (Fig. 5a), suggesting that UBXN3B–STING binding is specific and that UBXN3B is involved in STING signaling. By fluorescence microscopy, we observed that UBXN3B co-localized with STING to the ER in unstimulated cells (Mock), and partly translocated together with STING to punctate structures after ISD treatment (Fig. 5b).
further showed that the UAS domain of UBXN3B was required for UBXN3B–STING interaction (Fig. 5c, d; Supplementary Fig. 9a, b).

Ubiquitination of STING is essential for its downstream signaling and induction of antiviral immune responses. Several UBXNs including UBXN3B with the UBA-UBX domain are putative adaptor molecules that interact with E3 ligases and their substrates29,30. They likely also determine substrate specificity under different physiological conditions. We hypothesized that UBXN3B might serve as an adaptor that bridges STING and its E3 ligase. Three E3 ligases TRIM32, TRIM56, and AMFR have recently been shown to mediate STING ubiquitination during viral infection7,8,15. In particular, TRIM56 mediates both covalent and non-covalent ubiquitination of STING7. We first evaluated the ability of each of the E3 ligases to activate STING-dependent IFN-I responses. Results show that overexpression of TRIM56, but not AMFR, dramatically activated and synergized with cGAMP to induce IFN-I responses. Results show that overexpression of TRIM56, but not AMFR, dramatically activated and synergized with cGAMP to induce IFN-I, while TRIM32 modestly activated and synergized with cGAMP to induce IFN-I (Fig. 5e). The coil-coiled domain of UBXN3B mediated its interaction with TRIM56 (Fig. 5f; Supplementary Fig. 9c).

Very recently, another E3 ligase MUL1 was shown to be essential for HSV-1–induced STING ubiquitination on K224 and IRF3 activation31. We performed co-immunoprecipitation with MUL1 and UBXN3B overexpression in HEK293 cells to see if UBXN3B also regulated MUL1–STING interaction. We however observed no interaction between MUL1 and UBXN3B. These data suggest that UBXN3B specifically regulates STING–TRIM56 interaction. Indeed, HSV-1 infection induced endogenous Sting binding to Trim56 and Ubxn3b in MEFs (Fig. 6a). Furthermore, viral infection-induced Sting–Trim56 binding was almost abrogated in Ubxn3b−/− cells but was restored in UBXN3B-reconstituted knockout cells (Fig. 6b). These data overall demonstrate that UBXN3B is critical for HSV-1-induced STING–TRIM56 interaction. We next examined the role of UBXN3B in TRIM56-mediated STING ubiquitination. Overexpression of TRIM56 enhanced endogenous K63-linked ubiquitination of STING in WT cells (HEK293T–STING cell line), but this effect was reduced in UBXN3B−/− cells (Fig. 6d) (UBXN3B−/− was generated on HEK293T–STING background (see Fig. 6c). As previously described7, K150R mutation decreased STING K63 ubiquitination by TRIM56 (Supplementary Fig. 11a). Trans-complementation of UBXN3B in knockout cells restored normal STING ubiquitination (Fig. 6d).
To validate the aforementioned overexpression results, we examined endogenous K63 ubiquitination of Sting in cDCs. HSV-1 infection induced K63 ubiquitination of Sting at 4 and 8 h after infection in Ubxn3b−/−, but this was obviously impaired in Ubxn3b+/− cDCs (Fig. 6c). We recapitulated this in primary human trophoblasts treated with cGAMP (Supplementary Fig. 11b). Of note, co-immunoprecipitation of STING with TBK1 was observed in WT cells, but not in UBXN3B−/− cells, after cGAMP stimulation (Supplementary Fig. 11b). These data suggest that UBXN3B modulates TRIM56-mediated K63-linked ubiquitination of STING and activation of TBK1.

Discussion

Being a central molecule of the anti-DNA virus immune signaling pathway, STING is subjected to complex regulation by cellular factors and also many viruses. We here present physiological and biochemical evidence that UBXN3B controls DNA virus infection by regulating STING-mediated antiviral immune responses. First, ectopic expression of UBXN3B potentiates specifically STING-dependent IFN-I induction. Second, Ubxn3b−/− mice and cells are deficient in IFN-I induction by HSV-1, ISD, and cGAMP that stimulate STING signaling. Third, Ubxn3b+/− mice are highly susceptible to HSV-1 and VSV infection, but not to EMCV infection, recapitulating the Sting−/− phenotypes.

Fourth, UBXN3B interacts with both STING and TRIM56 simultaneously, and HSV-1-induced STING–TRIM56 interaction is compromised in UBXN3B−/− cells. Fifth, UBXN3B deficiency leads to decreased K63 ubiquitination and dimerization of STING, and consequent phosphorylation of TBK1 and IRF3. STING translocation out of the ER to the ER–Golgi intermediate compartment, followed by phosphorylation and degradation after HSV-1/ISD/cGAMP stimulation, is also partly blocked in UBXN3B−/− cells. Lastly, UBXN3B can be induced by IFN-I, likely to form a positive feedback loop to stimulate antiviral immune responses. All these observations strongly suggest that UBXN3B directly regulate STING signaling. However, STING-mediated immune responses are not completely abolished in UBXN3B−/− cells and mice, suggesting that UBXN3B-dependent regulation of STING and IFN-I-inducing pathways exist. Several E3 ligases that may not be directly relevant to UBXN3B have been shown to positively regulate STING signal, such as TRIM32 and TRIM56 enhanced STING-dependent IFN-I induction and significantly potentiated the cGAMP effects on STING. However, overexpression of a K27-E3 ligase AMFR failed to induce IFN-Is by itself or together with cGAMP, suggesting a different mechanism of AMFR action than TRIM32 and TRIM56.

A similar phenomenon has been observed for TRAF3, a K63- or E3 ligase critical for TLR-dependent and RLR-dependent IFN-I induction, and K63-linked polyubiquitination activates STING. However, overexpression of a K27-E3 ligase AMFR failed to induce IFN-Is by itself or together with cGAMP, suggesting a different mechanism of AMFR action than TRIM32 and TRIM56.

Methods

Mouse models. Mice with the exon 1 of Ubxn3b flanked by two LoxP sites (Ubxn3bfl/fl) were generated via homologous recombination by Dr. Fujimoto at Nagoya University. The homozygous Ubxn3bfl/fl were then crossed with homozygous tamoxifen-inducible Cre recombinase–estrogen receptor T2 mice (The Jackson Laboratory, Stock # 008463) to generate male and female Cre+/- mice. The homozygous tamoxifen-inducible Cre recombinase–estrogen receptor T2 mice (The Jackson Laboratory, Stock # 008463) to generate male and female Cre+/- mice. The homozygous tamoxifen-inducible Cre recombinase–estrogen receptor T2 mice (The Jackson Laboratory, Stock # 008463) to generate male and female Cre+/- mice. The homozygous tamoxifen-inducible Cre recombinase–estrogen receptor T2 mice (The Jackson Laboratory, Stock # 008463) to generate male and female Cre+/- mice. 

Surprisingly, adult mice depleted of Ubxn3b survive and develop as normally as WT mice. This interesting finding suggests that some of the genes indispensable for embryonic development may become non-essential at later stages of development. Our results provide proof of principle for in-depth study of the physiological functions of essential genes like UBXN3B in adult systemic knockout mice.

Of note, Ubxn3b protein expression is still abundant and ubiquitous in adult mice, suggesting that it plays different physiological roles than that at the embryonic stage. Previous studies demonstrated that UBXN3B regulated triglyceride synthesis and lipid droplet turnover in vitro cell cultures, and promoted very low-density lipoprotein secretion out of hepatocytes in mice fed with a high-fat diet. More recently, UBXN3B was shown to regulate de novo sterol biosynthesis by targeting the rate-limiting enzyme of the mevalonate pathway–HMG-CoA reductase to proteosomal degradation. In the current study, we show that under viral infection conditions, UBXN3B bridges the ER protein STING and its cytoplasmic E3 ligase TRIM56 to initiate antiviral immune responses. The multifaceted nature of UBXN3B is likely attributable to its capability as an adaptor to interface different E3 ligases and substrates under different physiological conditions. The involvement of an adaptor in an E3 ligase action could greatly expand the substrate spectrum of the E3 ligase in response to various physiological and environmental cues.

Following DNA virus infection, STING undergoes four forms of polyubiquitination, K11-linked22, K27-linked15, K48-linked16,17, and K63-linked8,31, resulting in contrasting effects on STING-mediated immune signaling. Particularly, K27-linked and K63-linked polyubiquitination activates STING. In agreement with published data, forced expression of K63- or E3 ligases TRIM32 and TRIM56 enhanced STING-dependent IFN-I induction and significantly potentiated the cGAMP effects on STING. However, overexpression of a K27-E3 ligase AMFR failed to induce IFN-Is by itself or together with cGAMP, suggesting a different mechanism of AMFR action than TRIM32 and TRIM56.

A similar phenomenon has been observed for TRAF3, a K63- or E3 ligase critical for TLR-dependent and RLR-dependent IFN-I induction, and K63-linked polyubiquitination activates STING. However, overexpression of a K27-E3 ligase AMFR failed to induce IFN-Is by itself or together with cGAMP, suggesting a different mechanism of AMFR action than TRIM32 and TRIM56. A similar phenomenon has been observed for TRAF3, a K63- or E3 ligase critical for TLR-dependent and RLR-dependent IFN-I induction, and K63-linked polyubiquitination activates STING. However, overexpression of a K27-E3 ligase AMFR failed to induce IFN-Is by itself or together with cGAMP, suggesting a different mechanism of AMFR action than TRIM32 and TRIM56.
transfected into HEK293T cells using Lipofectamine 2000. FLAG-STING-expressing cells were selected with 200 µg/ml zoxin.

Ubxn3b deletion in primary MEFs. Pregnant Cre<sup>+/−</sup> Ubxn3b<sup>fl/fl</sup> females (mated to Cre<sup>+/−</sup> Ubxn3b<sup>fl/fl</sup> male) were euthanized on day 14 of gestation. Embryos were deacylated and enucleated, and then digested with trypsin for 10 min at 37°C rotating. Fibroblasts were filtered through a 100 µm filter, cultured in RPMI 1640 medium (Life Technologies, NY, USA), supplemented with 10% FBS and antibiotics/antimycotics, propagated for two passages, and then frozen. Cre<sup>−/−</sup> Ubxn3b<sup>fl/fl</sup> MEFs were identified by genotyping. The Cre<sup>−/−</sup> Ubxn3b<sup>fl/fl</sup> MEFs with MEFS were treated with 4-hydroxyl tamoxifen at 0.01 mg/ml for 3 days to generate Ubxn3b<sup>−/−</sup> cells. After induction, the cells were further passage two times in RPMI medium. Non-treated Cre<sup>−/−</sup> Ubxn3b<sup>fl/fl</sup> MEFs were considered Ubxn3b<sup>+/+</sup>.

Dissertation of BMDMs and DCS. BMDMs were differentiated using a published method. Briefly, bone marrow was isolated from mock-pretreated and tamoxifen-pretreated Cre<sup>−/−</sup> Ubxn3b<sup>fl/fl</sup> mice and then differentiated into macrophages in L929-conditioned medium (RPMI 1640, 20% FBS, 30% L929 culture medium, 1x antibiotics/antimycotics) in 10-cm Petri dishes at 37°C, 5% CO<sub>2</sub> for 5 days. The culture medium was replaced by fresh L929-conditioned medium every 2 days. The attached BMDMs were dislodged by pipetting and counted for plating in 12-well or 6-well cell culture plates. Bone marrow-derived cDCs and pDCs were induced from bone marrow cells with 10 ng/ml GM-CSF and 100 ng/ml Flt3L (PeproTech, respectively), each for 6–8 days. BMDMs/DCs were cultured in RPMI 1640 medium containing 10% (volume/volume) FBS (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) and maintained at 37°C and 5% CO<sub>2</sub> overnight, and then washed once with pre-warmed fresh medium.

Isolation of human trophoblasts. Human trophoblasts were isolated from first trimester or term placenta using published methods. Briefly, a piece of placenta was minced with a razor blade and then digested with 0.23% trypsin and 0.2 mg/ml DNAse I in Hank’s balanced salt solution to release cells. The dispersed cells were passed through a 0.4 µm cell strainer, and trophoblasts were isolated through Percoll gradient (10–70% v/v) centrifugation. Cells were maintained in complete RPMI 1640 medium with 10% FBS and antibiotics. The IRB of Yale University and New York Medical College approved the study and determined the human subject study. The study used de-identified leftover specimens that were otherwise discarded. The investigators did not have any contact with the subjects and had no access to the patient information and did not link the results to the subjects. The isolation was irrelevant to clinical care and uses random specimens. The research did not investigate the health/disease status/genetic information of the subjects.

Ligand treatment and viral infection conditions. PRR ligands were transfected into cells with Lipofectamine 2000 using standard procedures. For hard-to-transfect cells such as trophoblasts, H1975, MEFS, and DCs, transfection was carried out in cell suspension. Briefly, cells were dislodged by trypsin digestion and pelleted by brief centrifugation. The cell pellet was then suspended in the transfection mix (DNA + Lipofectamine 2000 prepared as above) for 20 min with intermittent gentle agitation. Pre-warmed RPMI 1640 complete medium was then added and plated for further culture. For viral infection, viruses were allowed to attach and infect cells for 2 h, the cells were then washed with pre-warmed 1x phosphate-buffered saline (PBS) once and incubated with fresh medium. The MOI and infection time were specified for each experiment in the figure legend.

Generation of gene knockout cell lines with CRISPR-Cas9 technology. Two target gene sequences (1-GTGACGTCGAGCACCAGTGCA, 2- TCGGTCCCTGGTGAAGCAGGCGTACC) were common for both mouse and human Ubxn3b) were cloned into lentCRISPR2 vector and co-transfected into HEK293T cells with the human STING ORF into pcDNA3-FLAG vector. The transfected cells were selected with puromycin at 1 µg/ml for trophoblasts, 1 µg/ml for HEK293T cells and 0.5 µg/ml for H1975 cells. Successful knockout clones were confirmed by immunoblotting.

Plaque-forming assay. Quantification of infectious viral particles in tissues/cell culture supernatant was performed on Vero cell monolayer. Briefly, 30–100 µg (total proteins) of tissue lysates triturated in sterile PBS or serial dilutions of supernatant was applied to confluent Vero cells (6-well plate) at 37°C for 2 h. The inoculum was then removed and replaced with 2 ml of DMEM complete medium with 1% SeaPlaque agarose (Cat# 50100, Lonza). Plaques were visualized using Neutral red (Sigma-Aldrich) after 3 days of incubation at 37°C, 5% CO<sub>2</sub>.
Mouse infection and disease monitoring. Six-week-old to eight-week-old sex-matched littermates were infected with 1×10^5 plaque-forming units (PFUs) of HSV-1 in 100 µl of PBS intranasally or 200 PFU of EAMCV intraperitoneally. Morbidity and mortality was monitored twice a day. Neurological symptoms were assessed using an arbitrary scoring criteria from 1 to 5 (where 1 indicated ruffled fur and hunched posture but can easily be made to move around; 2 indicated a hunched posture and slow to move; 3 indicated a hunched posture, some movement, and labored breathing; 4 indicated a hunched posture, labored breathing, and little or no movement; and 5 indicated moribund or dead)31.

Reversible transcription and qPCR. A few grams of animal tissues, 25 µl of whole blood, and up to 1×10^6 culture cells were collected in 350 µl of RLT buffer (QIAgen RNeasy Mini Kit). Soft tissues were homogenized using an electric pestle (Kimble Chase LLC, USA). RNA was extracted following the QIAGEN RNeasy homogenizer instructions. Reverse transcription of RNA into complementary DNA (cDNA) was performed using the BIO-RAD iScript™ cDNA Synthesis Kit. Quantitative PCR (qPCR) was performed with gene-specific primers and 6FAM–TAMRA (6-carboxy-tetramethyl-6-carboxyrhodamine) probes or SYBR Green. Results were calculated using the ΔΔCt method and a housekeeping gene as an internal control. The qPCR primers and probes for immune genes were reported in our previous studies42,52. The primer sequences and 5′-3′ sequences of the primers used for each gene are provided in Supplementary Table 2. The primers for TRIM56 are: 5′-CTTGTCCATTCCTGGA-3′ and 5′-CGACACCTCCCAGAAAAGCA-3′; human PPIA 5′-GTGTCCATACATCACTTCG-3′ and 5′-ACCTCTCTCAAGAGCGCTGT-CTC-3′; mouse PPIA 5′-GGAAAATGTTGGGCACACAC-3′ and 5′-TGTCTGCTTTGCTGTCTCTCCTGC-3′.

Immunofluorescence microscopy. Cells were fixed with 4% paraformaldehyde. The cells were sequentially permeabilized with 0.5% Triton X-100, blocked with 2% serum-free bovine fetal calf serum, and then incubated with Alexa Fluor 488/594-conjugated goat-anti-rabbit/mouse IgGs (1:200, Life Technologies, Cat# A11070) for 1 h at room temperature. Nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). Images were acquired using a Zeiss Axiovert-200 fluorescence microscope (objective, ×40).

Graphing and statistics. The sample size chosen for our animal experiments in this study was estimated based on our prior experience of performing similar sets of experiments and power analysis calculations (http://vassarstats.net/ power_analysis.html). All animal results were included and no method of randomization was applied. For all the bar graphs, data were expressed as mean ± s.e.m. Prism 7 software (GraphPad Software) was used for survival curves, charts, and statistical analyses. Survival curves were analyzed using a log-rank (Mantel–Cox) test. For in vitro results, a standard two-tailed unpaired Student’s t test was used. For animal studies, an unpaired two-tailed non-parametric/parametric Mann–Whitney U test was applied to statistical analysis. P values ≤0.05 were considered significant. The sample sizes (biological replicates), specific statistical tests used, and the main effects of our statistical analyses were detailed in each figure legend.

Data availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

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