Stratified ubiquitination of RIG-I creates robust immune response and induces selective gene expression

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The activation of retinoic acid–inducible gene I (RIG-I), an indispensable viral RNA sensor in mammals, is subtly regulated by ubiquitination. Although multiple ubiquitination sites at the amino terminus of RIG-I have been identified, their functional allocations in RIG-I activation remain elusive. We identified a stratified model for RIG-I amino-terminal ubiquitination, in which initiation at either Lys164 or Lys172 allows subsequent ubiquitination at other lysines, to trigger and amplify RIG-I activation. Experimental and mathematical modeling showed that multisite ubiquitination provides robustness in RIG-I–mediated type I interferon (IFN) signaling. Furthermore, the flexibly controlled ultrasensitivity and IFN activation intensity determine the specificity of the IFN-stimulated gene transcription and manipulate cell fate in antiviral immune response. Our work demonstrates that tunable type I IFN signaling can be regulated through multisite RIG-I ubiquitination and elucidates a new paradigm for dynamic regulation in RIG-I–mediated antiviral signaling.

INTRODUCTION

Upon viral infection, efficient antiviral responses are primarily initiated from the recognition of specific microbial components by retinoic acid–inducible gene I (RIG-I), melanoma differentiation–associated gene 5 (MDA5), cyclic guanosine monophosphate synthase (cGAS), and other sensors (1–3). RIG-I specifically recognizes short double-stranded RNA (dsRNA) to activate type I interferon (IFN) signaling and promotes IFN-stimulated gene (ISG) transcription (1, 4). After viral recognition, RIG-I undergoes multiple modifications, including conformational change, dephosphorylation, ubiquitination, and oligomerization, to unleash its full activity (5).

The N terminus of RIG-I, which contains two caspase activation and recruitment domains (CARDs), transmits upstream signaling to the mitochondrial antiviral signaling (MAVS) protein for type I IFN activation (6). K63-linked ubiquitination plays a critical role in RIG-I activation (7, 8). Gack et al. (9) first demonstrated that K63-linked ubiquitination of RIG-I at Lys172 (K172) is required for type I IFN activation. Other 3 lysines, such as MEX3C and TRIM4, were then found to play parallel roles in RIG-I activation (10, 11). Furthermore, unanchored ubiquitin chains are also reported to augment RIG-I activation signaling (12–14). Recently, we found that optimal RIG-I activation requires conjugated K63-linked ubiquitination as a prerequisite to initiate its binding with unanchored ubiquitin chains in cell systems (13). Although the importance of K63-linked ubiquitination of RIG-I is widely appreciated, the physiological significance of multisite ubiquitination in RIG-I has not been addressed. How multiple ubiquitination sites are coordinated during RIG-I activation remains unclear.

Here, we identified a stratified mechanism for RIG-I N-terminal ubiquitination. The K63-linked ubiquitin chains attached to either K164 or K172 are indispensable for initiating RIG-I ubiquitination. Subsequently, random ubiquitination upon the remaining ubiquitination sites further triggers and amplifies downstream type I IFN signaling. Moreover, we found that ubiquitination upon one initiation site (K164 or K172) together with one additional site is the minimum requirement for the activation of RIG-I–induced type I IFN signaling. Mathematical and experimental analyses further revealed that increasing ubiquitination site numbers decreases the ultrasensitivity of RIG-I activation but increases the intensity and robustness of RIG-I–mediated antiviral signaling. Furthermore, the intensity and the tunable ultrasensitivity of IFN signaling activation mediated by diverse E3 ligases determine the specific patterns of ISG expression by manipulating diverse activation states of multiple downstream signaling pathways. Collectively, our findings revealed a novel strategy underlying RIG-I multisite ubiquitination, which provides a robust and optimal control over innate antiviral responses.

RESULTS

The function of RIG-I N-terminal ubiquitination in antiviral responses

K63-linked ubiquitination on RIG-I N terminus (RIG-I–N) plays an essential role in RIG-I activation (fig. S1A), as previously described (9–11). Although there are 18 lysine residues in RIG-I–N, only 10 of them were reported to be conjugated by K63-linked polyubiquitin chains during viral infection (9–11). We summarized eight important ubiquitination sites on RIG-I–N according to previous functional experiments (Fig. 1A) (9–11). Unanchored ubiquitin chains have been reported to enhance the stability of RIG-I tetramers after the conjugated K63-linked ubiquitination upon RIG-I (13, 14). Thus, we mainly investigated conjugated RIG-I ubiquitination. We generated RIG-I8KR cells by introducing a RIG-I 8KR mutant (all eight lysines are substituted with arginine) into RIG-I knockout (KO) (RIG-I–null) human embryonic kidney (HEK) 293T and A549 cell lines (fig. S1, B to E). The results indicated that K63-linked ubiquitination of RIG-I–N and IFN-β activation after intracellular (IC) polyinosinic-polycytidylic acid [poly(IC)] stimulation were totally abrogated in RIG-I–null cells (fig. 1B, D, and S2A). To evaluate RIG-I–N ubiquitination in antiviral immune response, we infected A549 wild-type (WT), RIG-I–null, and RIG-I–8KR cells with vesicular stomatitis virus (VSV)–enhanced green fluorescent protein (eGFP) and observed a markedly enhanced viral load in RIG-I–null A549 cells (fig. S2B). We also observed diminished antiviral capability (fig. S2B) and enhanced virus amplification in RIG-I–null cells (fig. S2C), suggesting that RIG-I–N ubiquitination plays an indispensable role in antiviral responses.

To verify the relationship between K63-linked ubiquitination and ISG transcription, we performed transcriptome sequencing, and the
Fig. 1. A stratified mechanism of RIG-I–N multisite ubiquitination. (A) Schematic map for reported conjugated ubiquitination sites on RIG-I–N. (B) Luciferase activity in RIG-I KO 293T cells (RIG-I(N)) transfected with an interferon-stimulated response element (ISRE) luciferase (ISRE-luc) reporter, together with an empty vector (EV), RIG-I(WT), or RIG-I(P), following by treatment with or without IC poly(I:C) (low molecular weight (LMW); 5 mg/ml); the same dosage will be used if there is no special note. Immunoblot was performed using anti-Flag antibodies to measure the expression of RIG-I in RIG-I(WT)– and RIG-I(P)–transfected RIG-I(N) 293T cells. (C) Immunoblot (IB) analysis of extracts of RIG-I(N) cells transfected with RIG-I(WT) or its mutants and treated with or without IC poly(I:C) for 12 hours. (D) Re-coimmunoprecipitation (Re-IP) and immunoblot analysis of extracts of 293T cells transfected with RIG-I(WT) or 8KR, together with hemagglutinin (HA)–K63 ubiquitin (Ub), with or without IC poly(I:C) treatment. Note: Samples after one-time immunoprecipitation (IP) were boiled for 5 min with 10% SDS. The supernatant of samples was then diluted 10 times and used for second-time immunoprecipitation with anti-Flag beads. WCL, whole-cell lysate. (E) Transcriptome sequencing analysis of WT, RIG-INull, and RIG-I8KRA549 cells treated with SeV for indicated time points. The fragments per kilobase million (FPKM) values of the top 200 up-regulated genes were shown. (F) Luciferase activity in RIG-I(N) cells transfected with ISRE-luc, together with EV, RIG-I(WT), or its mutants, following by treatment with or without IC poly(I:C). Immunoblot was performed using anti-Flag antibody to quantify the expression of RIG-I(WT) and mutants. (G) Immunoblot analysis of extracts of RIG-I(N) cells transfected with RIG-I(WT) or its mutants and treated with or without IC poly(I:C) for 12 hours. (H) Re-coimmunoprecipitation and immunoblot analysis of extracts of 293T cells transfected with RIG-I(WT), DM, or 6KR, together with HA-K63 ubiquitin, with or without IC poly(I:C) treatment for 12 hours. (I) Luciferase activity in RIG-I(N) cells transfected with ISRE-luc, together with EV, RIG-I(WT), DM, or 6KR, followed by treatment with or without IC poly(I:C). Immunoblot was performed using anti-Flag antibody to quantify the expression of RIG-I(WT) and mutants. (J) Immunoblot analysis of extracts of RIG-I(N) cells transfected with RIG-I(WT) or its mutants and treated with or without IC poly(I:C) for 12 hours. (K) Re-coimmunoprecipitation and immunoblot analysis of extracts of 293T cells transfected with RIG-I(WT) or its mutants, together with HA-K63 ubiquitin, with or without IC poly(I:C) treatment for 12 hours. (L) Illustration of stratified RIG-I ubiquitination process. RIG-I in green, inactivated state; RIG-I in red, active state. Data in (B), (F), and (I) are means ± SD of three independent experiments. ***p < 0.001 versus RIG-I(WT) cells.
results showed that the expression of IFNβ1 and most ISGs, such as CCL5 (which encodes RANTES), IFIT1 (which encodes ISG56), and IFIT2 (which encodes ISG54), was inhibited in both RIG-I WT null and RIG-I 6KR cells (Fig. 1E). Real-time polymerase chain reaction (PCR) validation consistently showed that the expression of IFNβ1 and ISGs was totally abrogated without RIG-I–N ubiquitination (Fig. S2D). The minimal antiviral capability in RIG-I 6KR cells (Fig. S2, B and C) might possibly result from viral suppression in an IFN-independent manner (15). Thus, these data suggest that the eight selected ubiquitination sites on RIG-I–N are required for RIG-I–mediated IFN-β induction and antiviral capacity.

Functional allocations of the ubiquitination sites on RIG-I–N
To investigate the functional allocations of the ubiquitination sites on RIG-I–N, we generated a series of RIG-I mutants on RIG-I–N (Fig. 1A and fig. S3A) and assessed the type I IFN signaling activation and antiviral capabilities mediated by WT RIG-I and its eight single mutants (Figs. S3, B to D, and S4). Consistent with the results we have reported, none of these mutants were found to abolish RIG-I–mediated activation of type I IFN signaling (13). However, it should be noted that recoinmunchoprecipitation samples were treated with 10% SDS to exclude the linkage of unanchored ubiquitin chains. We found that K172R mutation of RIG-I cannot affect the conjugated K63-linked ubiquitination of RIG-I but can reduce its binding to unanchored ubiquitin chains (fig. S5, A and B), and USP3 (ubiquitin-specific peptidase 3) can only cleave the unanchored K63-linked ubiquitin chains on it (fig. SSC), which is consistent with our previous data (16). Furthermore, the seeming incongruity about the RIG-I ubiquitination with Chen’s group may be ascribed to the differences between in vivo and in vitro experimental system (17), the structural distinction between the functional domain and full-length RIG-I (13), or the different types of K63 ubiquitination, including conjugated and unanchored ones (12).

Because K164 and K172 are important sites for RIG-I–N ubiquitination (9, 11, 13), RIG-I DM (in which both K164 and K172 were mutated), but not the other mutants, failed to activate type I IFN signaling (Fig. 1, F and G, and fig. S3E). This was further supported by the ubiquitination assay, which showed that, compared to RIG-I WT or RIG-I 6KR, RIG-I DM can barely be ubiquitinated (Fig. 1H). Although RIG-I 6KR can still respond to IC poly(I:C) stimulation, the maximum activation of type I IFN mediated by RIG-I 6KR was about 50 to 60% of that mediated by WT RIG-I (Fig. 1F and fig. S3E), which implies that K164 and K172 of RIG-I might be the initiation sites for RIG-I–N ubiquitination, whereas the remaining sites may further enhance RIG-I activity. We found that IFN-β and ISRE-luc activities gradually subsided with decreasing lysine residues (Fig. 1, I and J, and fig. S3F), which confirms that the ubiquitination of the other six lysines amplified RIG-I activation (Fig. 1K).

To further investigate RIG-I multisite ubiquitination, we assessed the role of K164 or K172 in combination with other lysines. We found that K164 or K172 alone was insufficient to activate type I IFN signaling, whereas one initiation site (K164 or K172) in combination with at least another one lysine residue was enough to trigger RIG-I signaling (fig. S3G). To verify the randomness in the ubiquitination of the remaining six nonessential sites during RIG-I activation, we performed a similar experiment using a variety of RIG-I mutants (for example, 164+1K-2, 172+1K-2, 2KR-2, and 4KR-2; fig. S3A). The luciferase assay showed that, once initiated, random ubiquitination of one additional site can induce significant RIG-I activation (fig. S3, H and I). Thus, we put forward a stratified model of RIG-I–N multisite ubiquitination (Fig. 1L). After initiation through ubiquitin ligation at either RIG-I K164 or K172, the remaining lysine residues are randomly ubiquitinated to amplify the downstream signaling (Fig. 1L).

To further ascertain the distinction between K164 and K172, we infected the RIG-I WT, RIG-I K164R, or RIG-I 4KR A549 cells with Sendai virus (SeV) and conducted the transcriptome sequencing (fig. S6A). However, we could not observe marked differences in global gene expression patterns under viral infection among RIG-I WT, RIG-I K164R, and RIG-I K172R A549 cells (fig. S6A). Single mutation of neither K164 nor K172 could substantially affect IFN-β expression as well as downstream ISGs (fig. S6B). Hence, our results indicated that it may be functionally redundant for ubiquitination initiation at either K164 or K172 site in RIG-I–mediated signaling.

Stratified ubiquitination of RIG-I manipulates the ultrasensitivity of RIG-I–mediated type I IFN signaling activation
Because activated RIG-I can form tetramers (14), we quantified the fluorescence bright spots (RIG-I oligomers) after IC poly(I:C) stimulation in HEK 293T cells transfected with RIG-I–eGFP to reflect RIG-I activation (Fig. 2A and fig. S7A). We stimulated WT or mutated RIG-I–eGFP–transfected cells with IC poly(I:C) for the indicated duration and visualized the activated cell numbers (fig. S7B). Coimmunoprecipitation results also showed a similar oligomerization tendency for RIG-I activation (fig. S7C), validating the reliability of our system. Furthermore, RIG-I mutants showed similar subcellular localization with WT RIG-I (fig. S7D).

To assess the ultrasensitivity of RIG-I activation, we first evaluated the steady state of RIG-I activation. IC poly(I:C) stimulation for 8 hours was enough to reach the steady state (fig. S7E). We then checked the ultrasensitivity in RIG-I WT and various RIG-I mutant–transfected cells (Fig. 2B). Reducing ubiquitination sites on RIG-I–N can increase the ultrasensitivity of RIG-I activation [Hill coefficient \((n_H)\): WT, 1.075; 6KR, 2.661] (Fig. 2B). A similar strategy was used to evaluate multisite ubiquitination on type I IFN activation at population levels. We noticed that the steady state of RIG-I–induced ISRE activation was reached roughly 36 hours after stimulation (fig. S8A). We further performed luciferase reporter assay (fig. S8B) and found that RIG-I WT cells did not generate the switch-like behavior of type I IFN activation, although the switch-like behavior became more evident when RIG-I can only be ubiquitinated at fewer lysine sites \((n_H;\) WT, 0.5610; 6KR, 1.531) (Fig. 2C). These results suggested that the ultrasensitive responses can be modulated by varying the ubiquitination sites.

Mathematical modeling of RIG-I stratified ubiquitination
To rigorously analyze the observed dynamics, we constructed a mathematical model that depicts the “stratified ubiquitination” of RIG-I (Fig. 2D; for the model construction, see Supplementary Methods and fig. S9A). The parameters were either experimentally determined or assigned reasonable initial values so that the complete model can produce a ubiquitination profile comparable to experimental observation (Figs. 2I and 2, B and C), as previously described (see the Supplementary Materials) (18). Furthermore, to exclude the possibility that the dynamic profiles were produced by a specific parameter set, we generated 1000 random parameter sets from a log-normal distribution. Simulation results showed that the ISRE activities exhibited high variations (fig. S9B). Reducing the number of modification sites would result in decreased randomness and increased ultrasensitivity in dynamic signaling, as previously described (19, 20). Simulation results suggested that the ultrasensitivity in the RIG-I tetramer assembly substantially increased with more lysine

Fig. 2. RIG-I–N multisite ubiquitination controls the ultrasensitivity of RIG-I–mediated type I IFN signaling activation. (A) Fluorescence microscope analyses of 293T cells transfected with RIG-I–eGFP with or without IC poly(I:C) for 6 hours. Bright spots were measured by ImageJ. Scale bars, 10 μm. (B) Dose-response curves [10⁻⁴ (−4), 10⁻³ (−3), 10⁻² (−2), 10⁻¹ (−1), 10² (0), and 10³ (1) μg/ml] in indicated cell lines followed by treatment with or without IC poly(I:C). Each point represents the mean value of the bright spot area from 50 cells. (C) Dose responses [10⁻⁴ (−4), 10⁻³ (−3), 10⁻² (–2), 10⁻¹ (−1), 10² (0), and 10³ (1) μg/ml] in indicated cell lines transfected with ISRE-luc, followed by treatment with or without IC poly(I:C). Each point represents four independent experiments. ISRE activity was normalized by the maximum activation level. (D) Mathematical model of RIG-I ubiquitination. The subscripts in R denote the ubiquitination state, where R represents RIG-I. In the initiation phase, either K164 or K172 was randomly ubiquitinated. After initiation, the ubiquitin chains were further randomly ligated on RIG-I and resulted in ubiquitination amplification. “0”: without ubiquitin; “1”: ubiquitinated. (E) Violin plots for Hill coefficients of RIG-I oligomerization (top) and ISRE activation (bottom) through computational simulation. The violin plots were based on kernel density estimation using the ksdensity function in MATLAB. n = 1000 sets for each condition. The green circles represent the experimental data in (B) and (C). (F) Simulation of ISRE-luc activity. (Top) Statistical significance can be detected for any two groups except that between the K164R and K172R groups (P = 0.7462). (Bottom) Function of RIG-I can be awakened by either of the two initiation sites cooperating with the other six noncore sites. a.u., arbitrary unit. (G) Partial rank correlation coefficient (PRCC) for parameters and nonzero initial conditions. Data in (B) and (C) are means ± SD of at least three independent experiments.
residues mutated (Fig. 2E, top), which is consistent with the experimental results (Fig. 2B). However, there seemed to be a non-negligible discrepancy in Hill coefficients for ISRE responses between simulation and experimental data (Fig. 2C and fig. S9C). We selected parameter sets with ISRE Hill coefficients <1 under the WT condition and then investigated the distribution of four parameters (r, n', K, and δ) responsible for ISRE induction (eq. S8 and table S1). Only the cooperativity (n') deviated significantly from the original distribution, suggesting that the cooperativity might be smaller than one (fig. 9D). The selected parameter sets with n' < 1 for ISRE activities substantially improved the fitting [Fig. 2, E (bottom) and C]. We argued that the trend of increased ultrasensitivity with fewer nonessential ubiquitination sites (Fig. 2, B, C, and E) was conserved irrespective of the differential expression levels and activities of the E3 ubiquitin ligases/deubiquitinases (DUBs) responsible for RIG-I ubiquitination/deubiquitination (see Supplementary Methods and fig. S10). The model simulation further showed that mutating more lysine sites in RIG-I–N can result in a gradual decrease in ISRE activities (Fig. 2F, top). The monotonic decreasing pattern in ISRE activation was conserved irrespective of sampling time points (fig. S9E). Furthermore, mutating one of the initiation sites (K164 or K172) did not markedly affect ISRE activities (Fig. 2F, top). However, when only one initiation site was retained (K164 only and K172 only), there was no detectable ISRE activity (Fig. 2F, bottom). Adding even one more site for ubiquitination can evoke significant ISRE activation (Fig. 2F, bottom). Sensitivity analysis based on PRCC (21) showed that basal RIG-I induction, RIG-I oligomerization, and cooperativity were among the most influential factors (Fig. 2G). The deubiquitination rate and the amount of DUBs negatively regulated and cooperativity were among the most influential factors (Fig. 2G). The deubiquitination rate and the amount of DUBs negatively regulated and cooperativity were among the most influential factors (Fig. 2G).

Collectively, these results demonstrated that stratified ubiquitination of RIG-I can shape the dynamic response in RIG-I signaling.

**Multisite ubiquitination of RIG-I orchestrates the robustness and ultrasensitivity of type I IFN signaling activation**

Because WT RIG-I induced the highest IFN intensity—although RIG-I6KR led to the highest ultrasensitivity (Fig. 2) among the RIG-I mutant–transfected cells—we wonder whether they can specifically dictate different ISG expression. We then conducted transcriptome sequencing in RIG-IWT, RIG-IK164R, RIG-IK172R, and RIG-I6KR A549 cells. By comparing the top 2000 up-regulated genes in each group, many ISGs were generally up-regulated in four cell lines (fig. S11A). However, the fold change [log2(FC) as the index] in cell lines may provide further information (fig. S11B). We found that global gene transcriptions in RIG-IK164R and RIG-IK172R cells showed similar expression patterns (fig. S11B). By contrast, gene transcription in RIG-IWT and RIG-I6KR was markedly different (fig. S11B).

We then investigated a simple scenario to simulate the underlying specificity in gene transcription (eq. S8 in Supplementary Methods). Three thousand cases were generated by randomly sampling the parameters from a log-normal distribution with respect to the reference values (table S1). The normalized differences were subsequently investigated (see eq. S9). The dendrogram showed highly variable profiles, and the transcription of a fraction of genes was not sensitive to lysine mutation (fig. S12A). Furthermore, a specific set of genes was up-regulated in RIG-IWT compared with RIG-I6KR cells (fig. S12A, red). Accordingly, we could also detect down-regulated genes under the WT condition (fig. S12A, green). Notably, the general expression pattern was relatively robust to parametric sampling (fig. S12B). By clustering, we could identify both up-regulated and down-regulated genes under the WT condition compared to the 6KR condition (see eq. S9 for quantifying relative expression; Fig. 3A). We further performed pairwise comparison for log2FC of the top 2000 up-regulated genes (Fig. 3B), which were induced by type I IFN signaling (Fig. 1E and fig. S11C). We noticed a relatively higher concordance in the gene transcriptional pattern (third quadrant) between RIG-IK164R and RIG-IK172R cells, whereas large variations can be observed in other cases (Fig. 3B). We then categorized the transcriptional patterns into seven groups (Fig. 3C and fig. S13). A fraction of genes showed a similar transcription pattern among RIG-IWT, RIG-IK164R, RIG-IK172R, and RIG-I6KR cells (n = 51; Fig. 3C). These genes were supposed to be efficiently up-regulated with a low dose of IFNs. Furthermore, cells with a higher IFN activation intensity (RIG-IWT) can up-regulate a specific set of genes (n = 12 + 55; Fig. 3C, groups 2 and 3). Cells with higher ultrasensitivity and lower IFN intensity (RIG-I6KR) also elevated the expression of particular genes (n = 12 + 21; Fig. 3C). The remaining genes were considered to be regulated with a trade-off between IFN intensity and ultrasensitivity (n = 19 and n = 30, respectively; Fig. 3C). These results were further confirmed by real-time PCR (Fig. 3, D to F). Together, these results indicated that IFN intensity and ultrasensitivity can selectively manipulate ISG expression.

A substantial part of ISGs, however, retained a quantitatively similar transcription (n = 51; Fig. 3C) or kept the transcriptional tendency in line with IFN intensity (n = 55; Fig. 3C). This indicated robustness in gene induction. Furthermore, our model argued that the number of ubiquitination sites can impart noises upon antiviral signaling. We roughly evaluated the level of noises by calculating the coefficient of variation (CV) of temporally integrated RIG-I oligomers over different numbers of ubiquitination sites and found that the temporal integral of RIG-I oligomers with more ubiquitinated sites exhibited lower cell-to-cell variability and therefore more robust responses (Fig. 3G). This property might be reminiscent of the pattern in NFKBIA expression and timing of nuclear factor κB (NF-κB) signaling (22). Because RIG-I signaling also converges to NF-κB activation, whether there exists potential linkage between them needs further investigation. Thus, these data suggested that stratified ubiquitination of RIG-I generated by multisite ubiquitination orchestrated the trade-off between robustness and ultrasensitivity in RIG-I–induced type I IFN signaling.

**E3 ligases of RIG-I regulate IFN intensity and ultrasensitivity to determine specific ISG expression**

Because several E3 ligases (TRIM4, TRIM25, and MEX3C) contribute significantly to RIG–mediated signaling (Fig. 4A and fig. S14A), the partial overlap in the ubiquitination site specificity may provide a “fail-safe” mechanism (Fig. 4A). The antiviral capacity will not be completely diminished unless all essential sites are removed or inhibited for ubiquitination (Figs. 1 and 2). This may help explain why cells with singular knockdown of TRIM4 or TRIM25 can retain partial but significant IFN-β promoter activity (11). We wonder whether these E3 ligases may also affect signaling ultrasensitivity. We used our mathematical model to investigate the dynamics of TRIM4, TRIM25, or MEX3C depletion. In total, 1000 random parameter sets were generated, and Hill coefficients were quantified accordingly (Fig. 4B). The model simulation argued that the ultrasensitivity of RIG–induced signaling was increased by TRIM4, TRIM25, or MEX3C depletion (for E3 depletion, the corresponding initial value was set to 0; see Fig. 4B and table S1). There is also a minor but significant elevation of ultrasensitivity in TRIM25 KO cells compared with TRIM4 or MEX3C KO cells (P = 0.0029 and P < 0.0001, respectively; Fig. 4B). On the basis of the model prediction, we silenced TRIM4, TRIM25, or MEX3C and observed higher ultrasensitive responses in RIG-I signaling (n1; NC, 0.5414; siTRIM25, 1.332; siMEX3C, 1.127; siTRIM4, 0.8168) (Fig. 4C and fig. S14B), consistent
with the notion that fewer ubiquitination sites lead to higher ultrasensitivity (Figs. 2E and 4B). To relate to a physiological system with endogenous RIG-I and viral infection, we repeated the ultrasensitivity detection with SeV infection in WT 293T cells (Fig. 4D and fig. S15). The systematic ultrasensitivity was increased after silencing TRIM4, TRIM25, or MEX3C (Fig. 4D), indicating that the multisite ubiquitination of RIG-I mediated by E3 ligases can flexibly regulate ultrasensitivity in type I IFN signaling under viral infection in a physiologically relevant system. Moreover, real-time PCR validation showed a similar pattern of specific gene expression in TRIM25 knockdown and RIG-I6KR cells because they displayed both reduced IFN intensity and increased ultrasensitivity to dictate specific ISG induction (Fig. 3): XAF1 and IFIT1 were specifically up-regulated by higher IFN intensity; PLAU and CD83 showed higher induction in A549 RIG-I6KR cells with higher ultrasensitivity (Fig. 3, D and E, and Fig. 4E). These data supported the notion that selective gene expression can be elaborately regulated by RIG-I ubiquitination states via different E3 ligases.

Ubiquitination states of RIG-I determine combinatorial activation of multiple downstream signaling pathways
To figure out the inherent mechanism of selective gene induction, we monitored the activation of several critical signaling pathways downstream of RIG-I [for example, IRF3 (IFN regulatory factor 3), NF-κB, and MAPKs (mitogen-activated protein kinases)] involved in antiviral responses. We found that the activation of these pathways displayed an impulse response after SeV infection (Fig. 5A). In RIG-IWT cells, IRF3, ERK (extracellular signal–regulated kinase), and p38 were gradually activated within 12 hours after viral infection. However, these signaling proteins were activated more abruptly with lower activation levels in RIG-I6KR A549 cells during the early stage of infection (Fig. 5, A and B, and fig. S16). There is no significant difference in p65 and c-Jun N-terminal kinase (JNK) activation between RIG-IWT and RIG-I6KR cells after SeV infection (Fig. 5B). Notably, temporal control of kinases or transcription factors determines the specificity in gene expression (23, 24). Furthermore, transcriptional responses can also be modulated by the fold change of transcription factors (25). Therefore, differences in the induction pattern of antiviral regulators may contribute to selective gene expression. These results suggested that differences in ubiquitination states of RIG-I may specifically modulate combinatorial activation of multiple pathways in antiviral signaling.

Multisite ubiquitination of RIG-I provides a trade-off between antiviral capability and survival of the cells
We next investigated whether different ISG expression patterns affect viral restriction and cell fate. The results showed a comparable virus

![Figure 3](http://advances.sciencemag.org/)

**Fig. 3.** Multisite ubiquitination of RIG-I orchestrates the robustness and ultrasensitivity of type I IFN signaling activation. (A) The significantly up-regulated (Up) and down-regulated (Down) groups were marked with dark blue points. The expression with \( P < 0.05 \) and at least twofold changes was chosen. Horizontal dashed line indicates \( P = 0.05 \), whereas the vertical dashed lines represent the expression with twofold change. Ratio, WT/6KR. (B) Pairwise comparison among the log2FC of the top 2000 up-regulated genes in the indicated four cell lines. (C) Gene clusters in four cell lines. \( n \), number of genes in the indicated pattern. (D to F) Real-time PCR analysis of IFNB1 and indicated ISGs in A549 RIG-IWT, RIG-I6KR, RIG-I164R, and RIG-I6KR cells treated with SeV for indicated time points. (D) Genes specifically up-regulated by high intensity of type I IFNs. (E) Genes specifically up-regulated by high ultrasensitivity of type I IFN signaling. (F) IFNB1 mRNA level and genes not affected by IFN intensity and ultrasensitivity. (G) CV for RIG-I oligomerization under different conditions under viral stimulation. Data in (D) to (F) are means ± SD of three independent experiments. NS, not significant. *\( P < 0.05 \) and **\( P < 0.001 \) versus RIG-IWT cells.
restriction in RIG-I<sup>WT</sup> and RIG-I<sup>6KR</sup> cells under low-dose VSV-eGFP infection (MOI, 0.1; Fig. 5C). However, viral clearance is significantly elevated in RIG-I<sup>WT</sup> cells compared with RIG-I<sup>6KR</sup> cells under high-dose VSV-eGFP infection (MOI, 1; Fig. 5D). In addition, the cell mortality is markedly lower in RIG-I<sup>6KR</sup> cells under either high or low dose of VSV-eGFP infection (Fig. 5, C and D, right). These results suggested that RIG-I<sup>WT</sup> cells induce more IFN-β expression (Fig. 3F) at the expense of elevated cell mortality compared with RIG-I<sup>6KR</sup> cells, implying a trade-off between antiviral capability and cell survival.

**DISCUSSION**

The type I IFN pathway plays a crucial role in host defense against viral infection. Over the past decade, RIG-I was demonstrated to be an indispensable sensor to recognize a variety of RNA viruses (1, 5). After viral recognition, RIG-I undergoes a conformational change, leading to RIG-I activation to trigger type I IFN signaling. Multiple posttranslational modifications of RIG-I, especially ubiquitination, are essential for RIG-I activation to trigger type I IFN signaling. Multiple posttranslational modifications of RIG-I, especially ubiquitination, are essential for RIG-I activation to trigger type I IFN signaling. Multiple posttranslational modifications of RIG-I, especially ubiquitination, are essential for RIG-I activation to trigger type I IFN signaling.
in response to viral infection (9–11). Unanchored K63 ubiquitin chains can also be linked after ligation of conjugated ubiquitin chains to stabilize RIG-I tetramers (13, 14). However, one unresolved question in this field is that how RIG-I is dynamically ubiquitinated at multiple lysine residues by partially redundant E3 ligases. Our studies provided evidence that multisite K63-linked ubiquitination of RIG-I–N generates a stratified ubiquitination mode and incrementally promotes RIG-I activation. The stratified ubiquitination of RIG-I orchestrates the robustness and ultrasensitivity of RIG-I–mediated type I IFN signaling activation to dictate a specific ISG transcription.

We found that K63-linked ubiquitination of RIG-I–N on eight lysine residues is indispensable in RIG-I–induced type I IFN activation.
derreactivity. Analog response upstream enables a broad and authentic environment of stimuli (for example, viral loads) to prevent over- or un-

In the context of decisive responses, immediate activation only occurs in the downstream signaling.

The multisite ubiquitination pattern in RIG-I tetramerization and activation may provide robustness in antiviral signaling. The partial overlap in the ubiquitination site specificity of TRIM25, TRIM4, and MEX3C may provide a fail-safe mechanism (Fig. 4A). The antiviral capacity will not be completely diminished unless all essential sites are removed or inhibited for ubiquitination (Fig. 1). This may help explain why cells with singular knockdown of 2CARDs were characterized by large regions of hydrophobic residues, which are prone to aggregation (28). Ubiquitin chains are also reported as polymers favoring multivalent stochastic binding and microstate sampling on an intermolecular basis (29). Consequently, ubiquitination and tetramerization might stabilize CARDs to further augment RIG-I activation (28). This may partially explain why the number of ubiquitination sites determines RIG-I activation.

Another possibility argues that the number of sites can impart on the noises in antiviral activity. Using the model inferred from the experimental data, we roughly evaluated the level of noises by calculating the CV of temporally integrated RIG-I oligomers over the number of feasible lysine sites (Fig. 3G). We found that the temporal integral of RIG-I oligomers with more ubiquitinated sites exhibited more robust responses relevant to the lower cell-to-cell variability (Fig. 3G). This dynamic property might be reminiscent of the pattern in NFKBIA expression and timing of NF-κB signaling (22). Because RIG-I signaling also converges to NF-κB activation, whether there exists potential linkage between these two dynamic patterns demands further investigation.

We observed a damped ultrasensitivity response in RIG-I oligomerization with increasing ubiquitination sites (Fig. 2). The ultrasensitivity behavior is relatively robust to site-to-site variations (30) or the sampling time (31), excluding the possibility of a specific parametric effect. Why does innate immune signaling mediated by RIG-I activation with multisite ubiquitination favor an analog but not a highly switch-like response? In the context of decisive responses, immediate activation only occurs during the transition between alternative sates, and therefore, they are usually restrained with a narrow range of environmental stimuli. However, cells must faithfully reflect the continuous spectrum or strength of environmental stimuli (for example, viral loads) to prevent over- or underreactivity. Analog response upstream enables a broad and authentic record of environmental information and may possibly lead to switch-like or decisive responses by coupling with the on/off biological “switch” in the downstream signaling.

We have demonstrated that there is a trade-off between viral clearance and mortality in RIG-IWT and RIG-I6KR cells (Fig. 5, C and D). Notably, some genes were selectively up-regulated by RIG-I6KR, such as PLAU, CD83, and PTX3. The urokinase plasminogen activator (encoded by PLAU) can inhibit virion release by maintenance of intracytoplasmic vesicles that actively accumulate virions (32). CD83 has been shown to exert costimulatory functions on myeloid dendritic cells (mDCs) and the down-regulation of CD83 represents a viral escape mechanism (33). PTX3 can also potentely restrict viral infection in a previous study (34). Therefore, although the IFN induction in RIG-I6KR cells is markedly lower than that in RIG-IWT cells (Fig. 5F), RIG-I6KR cells can also induce the expression of other antiviral proteins as exemplified above and limit viral infection. This may explain why the antiviral capacity in RIG-IWT and RIG-I6KR cells was comparable under a low dose of VSV infection (Fig. 5C). However, cells may use a different strategy under a higher dose of VSV infection by inducing massive IFN expression (Fig. 3F). The IFN-mediated innate immune response is hardwired to provide a robust defense against invading pathogens, as previously reviewed (4). However, high-level type I IFNs are toxic to hosts and can promote apoptosis to eliminate infected cells (35). For example, XAF1 (specifically up-regulated by RIG-IWT) is strongly proapoptotic (36) by promoting autophagic cell death via the up-regulation of beclin-1 and inhibition of the Akt pathway (37). Moreover, ISG54 (encoded by IFT12), together with ISG56 (encoded by IFT11, specifically up-regulated by RIG-IWT) and ISG69 (encoded by IFT33), was reported to promote apoptosis (38). As a result, RIG-IWT cells support higher IFN-β production and more efficient viral clearance at the expense of increased cell mortality compared with RIG-I6KR A549 cells when cells are confronted with severe viral invasion. Therefore, we argued that following low-dose virus infection, RIG-I is less ubiquitinated and markedly up-regulates certain antiviral factors (for example, PLAU, CD83, and PTX3) in an IFN-independent manner to eliminate viral infection and protect cells from death. However, a high-dose viral infection requires more efficient antiviral program, and therefore, RIG-I is fully ubiquitinated to favor massive IFN induction and provide a robust line of defense. To restrict viral spread under high-dose virus infection, the cell death program might be facilitated by IFNs in infected cells to provide protection to surrounding uninfected cells with minimum damage to the host. Therefore, ubiquitin editing of RIG-I may dictate specific ISG expression to exterminate invading viruses confronted with different doses of viral infection and determine cell fate (Fig. 5E).

Collectively, we have identified a stratified ubiquitination mode for RIG-I activation through multisite ubiquitination. The stratified ubiquitination mechanism modulated by different E3 ligases may serve as a “rheostat” to guarantee robust antiviral immune response and selective ISG expression. Thus, our findings provide a novel paradigm for multisite ubiquitination to dynamically shape innate immune responses.

MATERIALS AND METHODS

Cell culture and transfection

Cells were cultivated in Dulbecco’s modified Eagle’s medium (HyClone) supplemented with 10% fetal bovine serum (GenStar) and 1% l-glutamine (Gibco) at 37°C in 5% CO2. Overexpression plasmids were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Poly(LC) (LMW) was purchased from Invivogen.

Antibodies and reagents

Monoclonal anti-Flag M2-peroxidase (A8592), monoclonal anti-β-actin antibody produced in mouse AC-74 (A2228), anti-HA affinity gel (E6779-5X), and anti-Flag M2 affinity gel (A2220) were purchased.
from Sigma. Anti–c-myc–horseradish peroxidase (11814150001) and anti–HA–peroxidase (high affinity from rat immunoglobulin G1) (12013819001) were purchased from Roche. RIG-I (D14G6) rabbit monoclonal antibody (mAb) (37345), phospho-IRF3 (Ser396, D4 G) rabbit mAb (49785), NF-kB p65 (L8F6) mouse mAb (6956), phospho–NF-kB p65 (Ser536, 93H1) rabbit mAb (3033), p44/42 MAPK (ERK1/2) rabbit mAb (9102), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) rabbit mAb, stress-activated protein kinase (SAPK)/JNK rabbit mAb (9251), phospho–SAPK/JNK (Thr183/Tyr185) rabbit mAb (9252), phospho–SAPK/JNK (Thr183/Tyr185) rabbit mAb (9251), p38 MAPK rabbit mAb (9212), phospho–p38 MAPK (Thr180/Tyr182) rabbit mAb (9211), and TRIM25 (D9T7G) rabbit mAb (13773) were purchased from Cell Signaling Technology. IRF3 (FL-425) (sc-9082) monoclonal antibody (mAb) (3743S), phospho-IRF3 (Ser396, D4 G) (12013819001) were purchased from Roche. RIG-I (D14G6) rabbit mAb (9252), phospho–SAPK/JNK (Thr183/Tyr185) rabbit mAb (9251), p38 MAPK rabbit mAb (9212), phospho–p38 MAPK (Thr180/Tyr182) rabbit mAb (9211), and TRIM25 (D9T7G) rabbit mAb (13773) were purchased from Cell Signaling Technology. IRF3 (FL-425) (sc-9082) was purchased from Santa Cruz Biotechnology.

Luciferase reporter assays
Cells were plated in 24-well plates and transfected with plasmids encoding the ISRE/IFN-β luciferase reporter (firefly luciferase; 20 ng) and pRL-TK (Renilla luciferase plasmid; 8 ng) together with different plasmids (100 ng). Cells were harvested after IC poly(I:C) stimulation for the indicated times in passive lysis buffer (Promega). Enzyme activity was normalized by the efficiency of transfection on the basis of Renilla luciferase activity levels. Fold induction relative to the basal level was measured in cells. The values were means ± SD of three independent transfections performed in parallel.

Immunoprecipitation and immunoblot analysis
Cells were extracted in an ice-cold low-salt lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 1% Triton X-100] supplemented with a protease inhibitor cocktail (5 mg/ml; Roche). A 20-μl aliquot of each sample was subjected to SDS–polyacrylamide gel electrophoresis. For immunoprecipitation experiments, whole-cell extracts were incubated with anti-Flag agarose gels (Sigma) overnight. The beads were washed three times with low-salt lysis buffer. As for the re-immunoprecipitation experiments, samples after one-time immunoprecipitation were boiled for 5 min with 10% SDS. The supernatant of samples was then diluted 10 times and used for second-time immunoprecipitation with anti-Flag beads. The immunoprecipitates were resuspended in 3× SDS loading buffer (FD Biotechnology) and boiled for 5 min. The released proteins were electrophoresed on 8 to 12% SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes, with subsequent blocking using 5% skim milk. The membranes were incubated with the indicated antibodies and detected using enhanced chemiluminescence (Millipore).

KO of RIG-I by the CRISPR-Cas9 system and rescued cell line generation
We analyzed guide RNA (gRNA) in the website http://crispr.mit.edu/ and chose the gRNA sequence with the highest score to design primers: RIG-I guide, 5‘-AGATCAGAATAAGTATCGGT-3’. Annealing products were annealed and then linked to the pCRISPR-V2 vector. For the generation of rescued cell lines, stable overexpression plasmids of RIG-I WT and mutants were conducted the synonymous mutation at the single-guide RNA targeting sequence.

RNA extraction, RNA sequencing, and real-time PCR analysis
After total cellular RNA was isolated by TRIzol reagent (Invitrogen), reverse transcription was performed using a reverse transcription kit (Vazyme). RNA extracts were sequenced on an Illumina HiSeq platform by HONOR TECH. Real-time PCR was performed with the SYBR Green qPCR Mix (GenStar). Data were normalized to the GAPDH gene, and the relative abundance of transcripts was calculated by the C_{i} models. The primers used for real-time PCR are listed in Supplementary Methods.

Knockdown of E3 ligase by RNA interference
Lipofectamine RNAiMAX (Invitrogen) was used for the transfection of small interfering RNA (siRNA) into A549 cells according to the manufacturer’s protocols. The siRNA sequences are as follows: NC, UUCUCCGAACGUGUCAGUTT (sense) and ACGUGACACGUGUGGAGAATT (antisense); siTRIM4, GGAUUACUAGUCGUUCAATT (sense) and UUGAAGCUACUGUAUUCCTT (antisense); siTRIM25, AGGUCCACCCUGUGUAUATTT (sense) and UUAUAUCAGUGGAGCUATT (antisense); siMEX3C, GAAACUAUAGAGGCUCUATT (sense) and UUGAGCUCCUAUUGUUCCTT (antisense). All siRNAs were obtained from TransheepBio-Tech.

The mathematical modeling of multisite ubiquitination of RIG-I in innate antiviral responses
We used ordinary differential equations to evaluate the ubiquitination patterns of RIG-I upon viral invasion. Then, RIG-I is subject to ubiquitination by multiple E3 ligases (fig. S1A) and deubiquitinated by DUBs. The specific ubiquitin-ligation states of the “primed” RIG-I depend on the “flows” from both upstream (hypoubiquitinated) and downstream (hyperubiquitinated) ubiquitin-editing states. Each possible state is governed by “in-out” flows generated by upstream/downstream reactions. Furthermore, ubiquitinated RIG-I with at least two lysine sites can form tetramers and induce RIG-I activation. To model ISRE activity and different gene expression, we used a Hill function to approximate the relation between total RIG-I tetramers and the production rates. Detailed description was displayed in the Supplementary Materials.

Stochastic parameter variation
The stochastic kinetic parameters and nonzero initial conditions were chosen from a log-normal distribution. The cooperativity coefficient λ followed a uniform distribution in [1.5 3]. The dendrogram for the “pseudo-“gene expression data was obtained using the “clustergram” function of MATLAB (R2011a, v7.12.0.635; MathWorks). To identify differentially expressed “genes” in silico, we used “volcano plot” (39) and selected those genes up-regulated or down-regulated significantly by at least twofold for further analysis. The volcano plot was drawn using the “mavolcanoplot” function of MATLAB.

Sensitivity analysis using PRCC
Sensitivity analysis based on PRCC quantifies the correlation between each parameter and systematic responses; for a detailed definition, please refer to the study of Marino et al. (21). The systematic output used to measure the sensitivity was $R_{	ext{edg}}^T$, which denotes total RIG-I tetramers. The PRCC provides a robust metric to parametric sensitivities for nonlinear relation between input and output. $P$ represents either kinetic parameters or nonzero initial conditions.

Statistical analysis
Student’s t test was used for functional luciferase statistical analyses with GraphPad Prism 5.0 software. Hill slopes were calculated with nonlinear regression analysis in GraphPad Prism 5.0 software.
REFERENCES AND NOTES


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Stratified ubiquitination of RIG-I creates robust immune response and induces selective gene expression
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