DUBA: A Deubiquitinase That Regulates Type I Interferon Production

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Science **318**, 1628 (2007);
DOI: 10.1126/science.1145918

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

ical role in maintaining pilus integrity in the face of severe mechanical and chemical stress while bound to host cells. GAS pili show considerable antigenic variation, indicating an important role in virulence, and the pilin subunits are T antigens that are used for serotyping (*13*). The presence of several conserved regions on a highly variable background (Fig. 2B) suggests that the structure could help provide an effective pilus-based vaccine against GAS.

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25. Coordinates and structure factor amplitudes have been deposited in the Protein Data Bank with accession number 3B2M. We thank T. Caradoc-Davies for help with refinement and M. Middleditch for help with mass spectrometry. Supported by the Health Research Council (HRC) of New Zealand, the Foundation for Research, Science and Technology (FRST) of New Zealand, and the Maurice Wilkins Centre for Molecular Biodiscovery. H.J.K. received an FRST Bright Futures Scholarship, and T.P. is an HRC Hercus Fellow.

Supporting Online Material

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29 May 2007; accepted 23 October 2007
10.1126/science.1145806

DUBA: A Deubiquitinase That Regulates Type I Interferon Production

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Production of type I interferon (IFN-I) is a critical host defense triggered by pattern-recognition receptors (PRRs) of the innate immune system. Deubiquitinating enzyme A (DUBA), an ovarian tumor domain-containing deubiquitinating enzyme, was discovered in a small interfering RNA–based screen as a regulator of IFN-I production. Reduction of DUBA augmented the PRR-induced IFN-I response, whereas ectopic expression of DUBA had the converse effect. DUBA bound tumor necrosis factor receptor–associated factor 3 (TRAF3), an adaptor protein essential for the IFN-I response. TRAF3 is an E3 ubiquitin ligase that preferentially assembled lysine-63–linked polyubiquitin chains. DUBA selectively cleaved the lysine-63–linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream signaling complex containing TANK-binding kinase 1. A discrete ubiquitin interaction motif within DUBA was required for efficient deubiquitination of TRAF3 and optimal suppression of IFN-I. Our data identify DUBA as a negative regulator of innate immune responses.

Innate immune responses are initiated when host cellular PRRs encounter pathogen-associated molecular patterns (PAMPs) (*1*). Double- and single-stranded RNAs are virus-derived PAMPs that trigger the intracellular PRRs Toll-like receptor 3 (TLR3), retinoic acid–inducible protein I (RIG-I), and melanoma differentiation–associated gene 5 (MDA5) (*2*–*4*). Activation of these intracellular sensors leads to the recruitment of adaptor proteins for interferon-α (IFN-α) and IFN-β production. Toll-interleukin 1 receptor domain–containing adaptor inducing IFN-β (TRIF) interacts with TLR3, whereas IFN-β promoter stimulator 1 [(IPS-1), also called Cardif, MAVS, and VISA] is recruited by RIG-I and MDA5. These adaptors mediate the assembly of a signaling complex composed of the ubiquitin ligase TRAF3 and the kinases TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor κB kinase ε [(IKKε), also called IKK*i* (*1*, *5*–*7*)]. This complex activates the downstream transcription factors, IFN regulatory factors 3 and 7 (IRF3 and IRF7), to switch on IFN-I expression, which is an essential aspect

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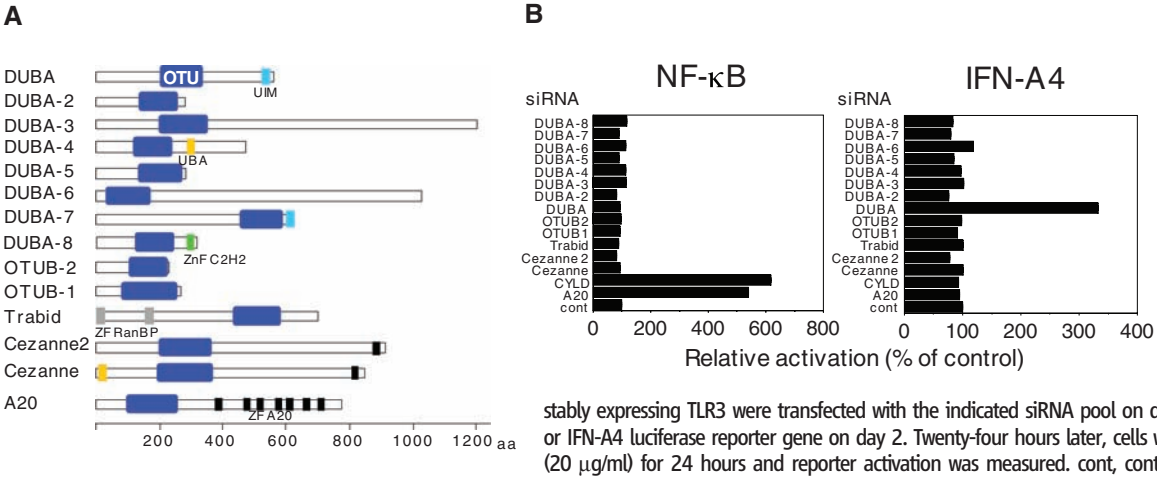

Fig. 1. An siRNA-based screen for OTU DUB family members. (**A**) Schematic of OTU family members. Gene accession numbers are listed in table S1. ZnF C2H2, zinc finger domain (C2H2-type); ZF RanBP, zinc finger domain (Ran-binding protein and others); ZF A20, zinc finger domain (A20-like); aa, amino acids. (**B**) HEK293 cells stably expressing TLR3 were transfected with the indicated siRNA pool on day 0 and then with a NF-κB or IFN-A4 luciferase reporter gene on day 2. Twenty-four hours later, cells were stimulated with poly(I:C) (20 μg/ml) for 24 hours and reporter activation was measured. cont, control.

of the innate immune response to invading viruses (*1*, *5*, *8*–*10*).

Ubiquitin is a protein found in all eukaryotic cells that gets covalently linked to various proteins by ubiquitin ligase enzymes (*11*). Lysines within ubiquitin can be modified so that polyubiquitin chains are formed; chains formed through linkages at Lys^{48} often mark target proteins for proteasome-mediated proteolytic destruction, whereas those linked through Lys^{63} can direct other outcomes. These chains have been implicated in the activation of signaling pathways that affect DNA repair or the transcription factor nuclear factor κB (NF-κB) (*11*, *12*). Deubiquitinating enzymes (DUBs) are proteases that specifically cleave ubiquitin linkages, negating the action of ubiquitin ligases (*13*). The DUB A20, for example, acts as a negative regulator of the classical NF-κB activation pathway (*14*). A20 belongs to a subfamily of 14 DUBs characterized by an ovarian tumor (OTU) domain (Fig. 1A). The physiological function of only a few members is known.

To identify potential roles of OTU subfamily DUBs, we used small interfering RNA oligonucleotides (siRNA oligos) to deplete various DUBs in TLR3-expressing human embryonic kidney (HEK) 293 cells. The impact on NF-κB activation or IFN-I production in response to the TLR3 ligand polyinosine:polycytidine acid [poly(I:C)] (which mimics viral RNA) was determined by luciferase reporter assay. Decreased A20 expression resulted in an increase in TLR3-induced NF-κB–dependent gene transcription (Fig. 1B). Activated TLR3 simultaneously promotes an IFN-I response, but this outcome is executed by the transcription factors IRF3 and IRF7 (*8*, *10*). The impact of A20 was limited to NF-κB activation because A20 reduction had a negligible effect on TLR3-induced activation of an IFN-A4 promoter (Fig. 1B). CYLD, a tumor suppressor belonging to the ubiquitin-specific protease subfamily of DUBs, is reported to be a negative regulator of NF-κB signaling (*12*, *13*, *15*, *16*). Depletion of CYLD resulted in increased TLR3-induced NF-κB–dependent gene transcription but did not increase activation of an IFN-A4 promoter (Fig. 1B). Similar to the classical NF-κB signaling

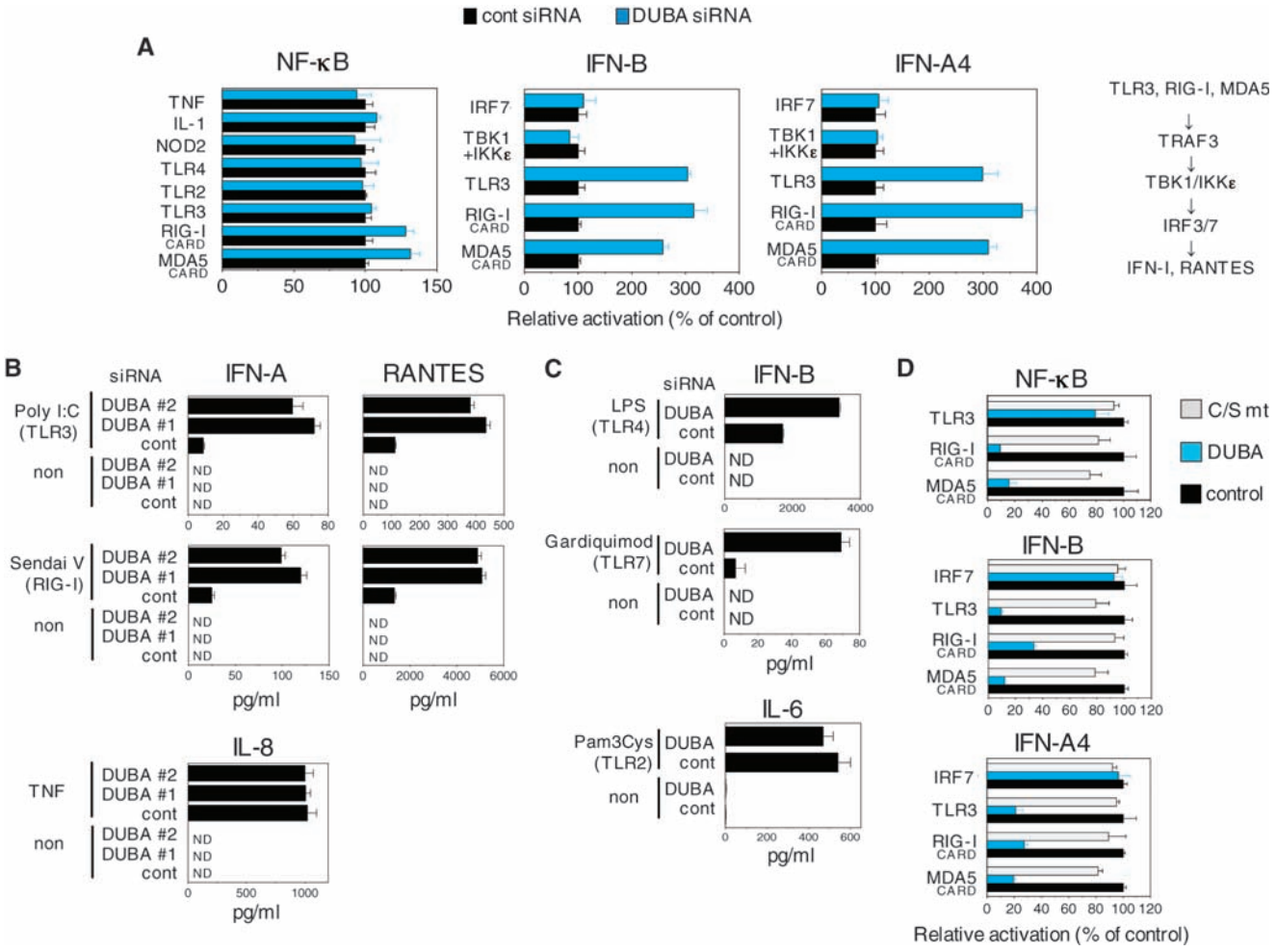

Fig. 2. Regulation of RIG-I and MDA5 signaling by DUBA. (**A**) HEK293 cells transfected with a DUBA siRNA (#1 in table S1) or a control siRNA were assayed for NF-κB activation or IFN-I (IFN-B and IFN-A4 promoter) activity. HEK293 cells were transfected with control or DUBA siRNA (20 nM) on day 0 and then with a NF-κB, IFN-B, or IFN-A4 luciferase reporter gene, together with indicated activators, on day 2. After 24 hours, ligands Pam3CSK4 (10 μg/ml), poly(I:C) (20 μg/ml), LPS (10 μg/ml), muramyldipeptide (10 μg/ml), IL-1 (50 ng/ml), or TNF-α (50 ng/ml) were added to the culture for 24 hours, and then reporter gene activation was measured. Data represent the mean ± SD of triplicate samples. (**B**) Parental HEK293 cells (middle and bottom panels) or those stably expressing TLR3 (top panels) were transfected with control or DUBA siRNAs. After 72 hours, cells were treated with poly(I:C) (20 μg/ml), Sendai virus [20 units (U)/ml], or TNF-α (50 ng/ml). Secreted IL-8, IFN-A, and RANTES were measured 24 hours later. Data represent the mean ± SD of triplicate samples. ND, not detectable. (**C**) DUBA function in macrophages. The macrophage cell line RAW264.3 was transfected with either a control siRNA or mouse DUBA siRNA and, 3 days later, cells were stimulated with the TLR ligands indicated. Cytokines secreted into the supernatant over the next 24 hours were measured. Data represent the mean ± SD of triplicate samples. (**D**) Effects of overexpressed DUBA. HEK293 cells were transfected with the indicated reporter gene and activators, together with either wild-type DUBA or the predicted catalytic active site mutant DUBA C224S (C/S mt). NF-κB or IFN-I reporter activity was measured after 36 hours. Data represent the mean ± SD of triplicate samples.

cascade, the IFN-I response is thought to require ubiquitination (*17*). We speculated that another DUB might negatively regulate IFN-I expression. Depletion of OTU subfamily member DUBA augmented TLR3-induced activation of an IFN-A4 promoter without altering activation of a NF-κB–dependent reporter (Fig. 1B). DUBA is predicted to be 571 amino acids long, and the catalytic residues (Asp^{221}, Cys^{224}, and His^{334}) essential for cysteine protease activity are conserved (fig. S1A). Northern blot analysis identified DUBA mRNA transcripts in various organs, including the liver and placenta, as well as in peripheral blood leukocytes (fig. S1B).

Two individual DUBA siRNA oligos increased TLR3-induced activation of an IFN-A4 promoter (fig. S2A), and depletion of endogenous DUBA protein was confirmed by Western blotting (fig. S2B). In the absence of TLR3 stimulation by poly(I:C), DUBA depletion did not influence responses to basal NF-κB or IFN-A4 (fig. S2A). Only double-stranded DUBA siRNA oligos increased IFN-A4 promoter activity (fig. S2C), thus excluding nonspecific engagement of the IFN-I and NF-κB pathways by the siRNAs (*18*).

RIG-I and MDA5 are cytosolic DExD/H-box RNA helicases that have a caspase activation and recruitment domain (CARD). These helicases detect invading RNA viruses and engage the IFN-I response through the adaptor protein IPS-1 (*1*, *4*, *5*). We expressed the CARD domains of RIG-I and MDA5 (RIG-I_{CARD} and $MDA5_{CARD}$) to limit possible nonspecific engagement of the helicase domains by the siRNA oligos. Depletion of DUBA increased activation of IFN-B and IFN-A4 promoters in response to RIG-I_{CARD} and $MDA5_{CARD}$ expression, and the magnitude of the effect was similar to that seen when poly(I:C) was used to stimulate TLR3 (Fig. 2A). The increased transcriptional response to poly(I:C) after DUBA depletion was concomitant with increased secretion of the cytokines IFN-A and RANTES, both of which are under the control of IRF3 and IRF7 (*8*, *10*) (Fig. 2B). Although depletion of DUBA led to a slight increase in the transcription of a NF-κB–dependent reporter gene in response to RIG-I_{CARD} and $MDA5_{CARD}$ overexpression, it did not alter NF-κB activation by tumor necrosis factor (TNF), interleukin-1 (IL-1), nucleotide-binding oligomerization domain 2 (NOD2), or TLRs 2 to 4 (Fig. 2A), nor did depletion of DUBA alter NF-κB–dependent TNF-induced secretion of IL-8 (Fig. 2B). Thus,

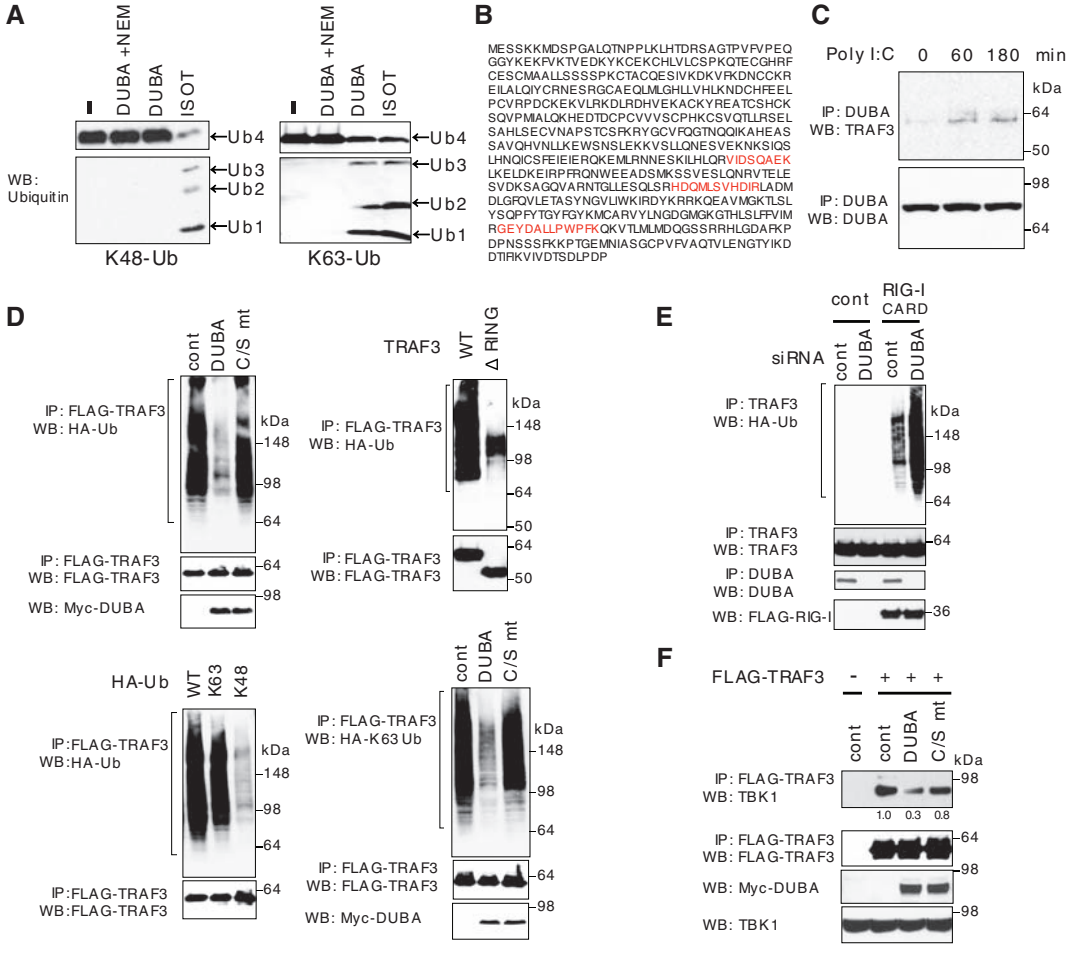

Fig. 3. Interaction of DUBA with TRAF3 and deubiquitination of TRAF3. (**A**) Ubiquitin isopeptidase activity of DUBA. Recombinant DUBA (1 μg) purified from *E. coli* or IsoT (positive control) was incubated with Lys^{48} (K48)–linked or Lys^{63} (K63)–linked tetra-ubiquitin chains (0.5 μg) at 37°C for 16 hours. Reactions were analyzed by Western blotting (WB) with ubiquitin antibody. Cysteine protease activity was blocked with 20 μM NEM in the reactions indicated. The top and bottom lanes in each panel are from different film exposure time points of the same membrane. (**B**) Sequence of TRAF3 and the matching endogenous peptides (highlighted in red) identified by mass spectrometry. The purified DUBA complex was analyzed by means of one-dimensional gel electrophoresis in combination with the nano liquid-chromatography tandem mass spectrometry. (**C**) Interaction of endogenous DUBA and TRAF3. HEK293 cells stably expressing TLR3 were stimulated with 20 μg/ml poly(I:C), and lysates were prepared at the time points indicated. Immunoprecipitation (IP) was performed with a DUBA antibody, and coprecipitating endogenous TRAF3 was detected by Western blotting. (**D**) Effects of exogenous DUBA on transfected TRAF3. HEK293 cells were cotransfected with FLAG-TRAF3, HA-tagged ubiquitin (HA-Ub), and with either empty vector, Myc-tagged wild-type (WT) DUBA, or DUBA C/S mt. Twenty-four hours later, cell lysate was subjected to heat denaturing in 1% SDS, TRAF3 was immunoprecipitated with FLAG antibody, and Western blotting was done with HA or FLAG antibodies. Wild-type TRAF3 or ΔRING TRAF3 mutant was used in the top right panel. Wild-type ubiquitin or mutants retaining only a single lysine (K48 or K63) were used in the bottom panels. 1% of input lysate was subjected to Western blotting with Myc antibody. (**E**) Ubiquitination of endogenous TRAF3. HEK293 cells were transfected with 20 nM control or DUBA #1 siRNA. Thirty-six hours later, cells were cotransfected with control or FLAG-tagged RIG-I_{CARD} and HA-Ub and were incubated for 24 hours. After heat denaturing in 1% SDS, endogenous TRAF3 was immunoprecipitated with anti-TRAF3 and subjected to immunoblotting with anti-HA or anti-TRAF3. 1% of input lysate was subjected to Western blotting with FLAG antibody. 50% of input lysate was subjected to immunoblotting for DUBA, as in fig. S2B. (**F**) DUBA dissociates TRAF3 from TBK1. HEK293 cells were transfected with FLAG-TRAF3 together with control plasmid, Myc-tagged DUBA, or DUBA C/S mt. After 48 hours, the TRAF3 complex was immunoprecipitated with anti-FLAG beads and subjected to immunoblotting with anti-TBK1 and anti-FLAG. 1% of input lysate was subjected to Western blotting with Myc and TBK1 antibodies. TBK1 was quantified by densitometry.

the impact of DUBA on NF-κB signaling appears to be confined to that triggered by RIG-I and MDA5.

In response to Sendai virus infection, which activates endogenous, cytosolic RIG-I (*2*), depletion of DUBA augmented secretion of IFN-A and RANTES in virus-infected HEK293 cells (Fig. 2B). DUBA depletion increased IFN-B secretion from the RAW264.3 macrophage cell line after treatment with ligands for TLR4 or TLR7 (Fig. 2C and fig. S3A) but did not affect TLR2-induced NF-κB–dependent production of IL-6. We used enforced expression of adaptors TRIF, IPS-1, and IL-1 receptor–associated kinase 4 (IRAK-4) to bypass activation of TLR3, RIG-I/MDA5, and TLRs 7 to 9, respectively, and to induce IFN-I expression. In each instance, depletion of DUBA enhanced activation of an IFN-A4 promoter (fig. S3B), supporting the notion that DUBA is a negative regulator of IFN-I expression downstream of multiple PRRs.

In HEK293 cells, ectopic expression of DUBA suppressed activation of IFN-A and IFN-B promoters but not a NF-κB–dependent promoter in cells treated with poly(I:C) to stimulate TLR3 (Fig. 2D). When coexpressed with RIG-I_{CARD} or $MDA5_{CARD}$, DUBA inhibited activation of a NF-κB–dependent promoter and the IFN-A and IFN-B promoters (Fig. 2D). All inhibitory effects of DUBA required its cysteine protease activity because the catalytic site mutant Cys^{224}→Ser^{224} (C224S) was inactive (*19*).

The downstream signaling machinery common to multiple PRRs that induce IFN-I expression includes the ubiquitin ligase TRAF3, the kinases TBK1 and IKKε, and the transcription factors IRF3 and IRF7 (*1*, *5*–*8*, *10*). DUBA reduction did not affect activation of IFN-B or IFN-A4 promoters in response to overexpressed IRF7 and IRF3, or to coexpressed TBK1 and IKKε (Fig. 2A and fig. S3C), indicating that DUBA must act upstream of TBK1 and IKKε. Consistent with these observations, overexpression of DUBA failed to inhibit IFN-A4 and IFN-B promoter activation by overexpressed IRF7 (Fig. 2D).

The conserved catalytic triad within the OTU domain of DUBA suggests that DUBA has deubiquitinating activity. Recombinant DUBA purified from *Escherichia coli* was incubated with Lys^{48}- or Lys^{63}-linked tetra-ubiquitin chains. DUBA degraded Lys^{63}-linked tetra-ubiquitin chains as effectively as did the positive control isopeptidase T (IsoT), but DUBA was not active against Lys^{48}-linked chains (Fig. 3A). The cysteine protease inhibitor *N*-ethylmaleimide (NEM) inhibited DUBA's ability to degrade Lys^{63}-linked ubiquitin chains, which is consistent with DUBA being a cysteine protease.

We immunoprecipitated FLAG epitope–tagged DUBA from HEK293 cells and identified associated proteins by mass spectrometry. Three peptides were from TRAF3 (Fig. 3B), which is a RING finger–type ubiquitin ligase that is essential for IFN-I expression downstream of multiple TLRs and the helicase receptors RIG-I and MDA5 (*6*, *7*). Increased binding of endogenous DUBA to endogenous TRAF3 was observed after poly(I:C) stimulation of TLR3 (Fig. 3C).

We hypothesized that DUBA might switch off IFN-I expression by deubiquitinating TRAF3. When overexpressed FLAG-TRAF3 was immunoprecipitated from lysates (after boiling in 1% SDS to remove associated proteins), smears corresponding to ubiquitinated TRAF3 were detected with hemagglutinin (HA)–tagged ubiquitin (Fig. 3D) and anti-TRAF3 (fig. S4). Ubiquitination of TRAF3 was also confirmed by the existence of ubiquitinated TRAF3 peptides in our mass spectrometry analysis. Wild-type DUBA, but not the catalytic site mutant DUBA C224S, reduced the amount of ubiquitinated TRAF3 detected. Sendai virus infection induced endogenous TRAF3 ubiquitination in fibroblasts, as evidenced by an anti-ubiquitin blot and slower migrating TRAF3 bands (fig. S5).

TRAF3 lacking the domain (ΔRING) required for E3 ligase activity and IFN-I production (*20*) exhibited less ubiquitin modification (Fig. 3D), which is consistent with an autoubiquitination mechanism. With the use of HA-tagged ubiquitin mutants in which only Lys^{48} or Lys^{63} was available to form polyubiquitin chains, TRAF3 mainly acquired Lys^{63}-linked polyubiquitin chains, and this modification was reduced by coexpression of DUBA (Fig. 3D). Ubiquitination of endogenous TRAF3 was detected upon overexpression of RIG-I_{CARD}, and this was increased by depletion of DUBA (Fig. 3E and fig. S6). Just as A20 is a DUB of TRAF6 in the classical NF-κB signaling pathway (*12*, *14*, *16*, *21*), DUBA appears to promote the removal of Lys^{63}-linked polyubiquitin chains from TRAF3. One possibility is that TRAF3 ubiquitination facilitates the recruitment of downstream signaling components. In support of this notion, DUBA overexpression partially reduced TRAF3-TBK1 interaction (Fig. 3F). Because TRAF3 is dispensable for RIG-I–mediated activation of NF-κB (*6*, *7*), the mechanism of how DUBA controls the NF-κB signaling remains to be elucidated.

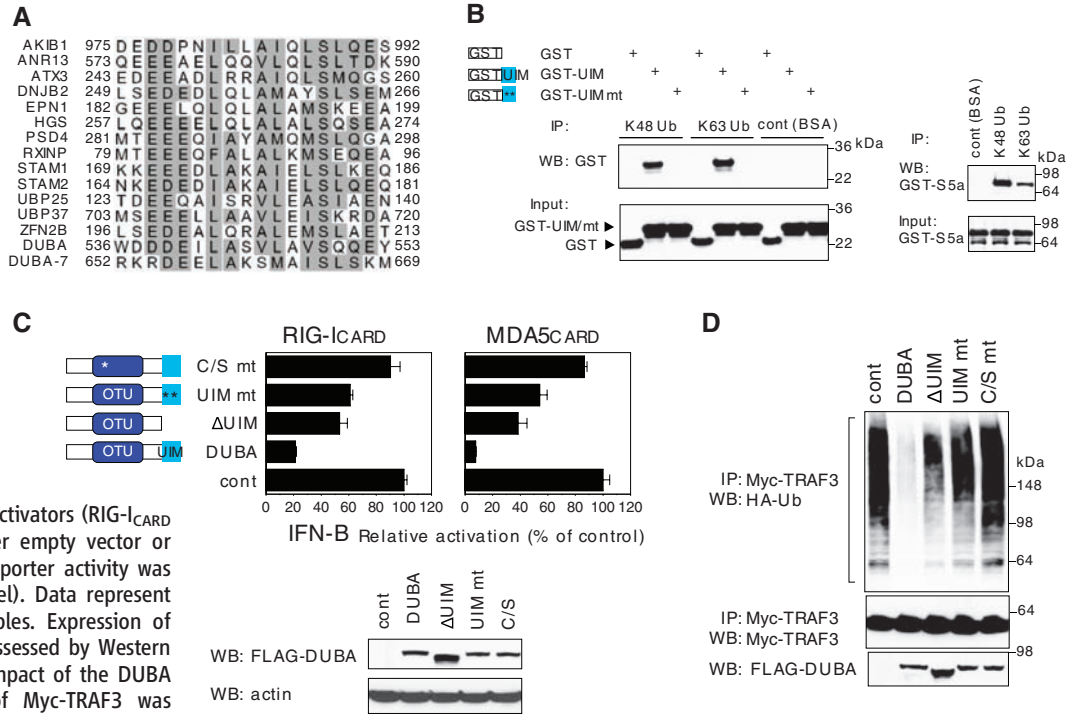

Fig. 4. Role for the UIM domain of DUBA. (**A**) Protein sequence alignment of UIM domain. Conserved amino acid residues are highlighted. (**B**) Interaction of the DUBA UIM domain with ubiquitin chains. GST, GST-UIM, and GST-UIM mt (L542A/S549A) proteins were incubated with agarose beads coated with either K48-Ub polyubiquitin-chains, K63-Ub polyubiquitin-chains, or bovine serum albumin (BSA). Bound material was analyzed by Western blotting with GST antibody (left panels). GST-S5a was used as a positive control (right panels). (**C**) Function of DUBA UIM. HEK293 cells were transfected with IFN-B reporter and indicated activators (RIG-I_{CARD} or $MDA5_{CARD}$), together with either empty vector or the version of DUBA indicated. Reporter activity was measured after 36 hours (top panel). Data represent the mean ± SD of triplicate samples. Expression of wild-type and mutant DUBA was assessed by Western blotting (bottom panel). (**D**) The impact of the DUBA mutants on the ubiquitination of Myc-TRAF3 was determined as in Fig. 3D.

Ubiquitin-binding domains (UBDs), such as the ubiquitin-associated domain (UBA) and ubiquitin-interacting motif (UIM), can influence various cellular events through binding to ubiquitinated proteins (*22*, *23*), but the function of UBDs in the deubiquitination reaction remains to be elucidated. DUBA has a putative UIM embedded in a conserved C-terminal helix (Fig. 4A). Both Lys^{48}- and Lys^{63}-linked polyubiquitin chains could bind glutathione *S*-transferase (GST) fused to the DUBA UIM in pull-down studies (Fig. 4B). Mutation of conserved residues in the DUBA UIM domain (L542A/S549A) prevented this binding. These results indicate that the DUBA UIM is capable of interacting with polyubiquitin chains. DUBA UIM mutants DUBAΔUIM and DUBA L542A/S549A were used to determine the role of the UIM in the negative regulation of IFN-I expression. Both mutants retained some ability to attenuate $RIG\text{-}I_{CARD}$–induced or $MDA5_{CARD}$-induced activation of an IFN-B promoter, although they were less effective than wild-type DUBA (Fig. 4C). As compared with wild-type DUBA, both UIM mutants were also consistently less effective at reducing the level of ubiquitinated TRAF3 in cells (Fig. 4D). These results suggest that there is an important role for the DUBA UIM in DUBA function but that the UIM may not be the sole substrate-recognition site. The binding affinities between ubiquitin and UIMs are typically very low (dissociation constant $K_d > 100$ μM) (*22*, *23*), so the DUBA UIM is predicted to play a supportive but dispensable role by capturing and presenting ubiquitinated substrates to the catalytic domain.

To determine whether DUBA, like A20, may function as part of a negative feedback loop, we asked whether DUBA was up-regulated in macrophages upon stimulation. When bone marrow–derived macrophages were exposed to lipopolysaccharide (LPS), there was substantial induction of DUBA protein (fig. S7). TRAF3 controls the nonclassical NF-κB/NF-κB2 pathway by regulating the NF-κB–inducing kinase–IKKα kinase cascade that results in the phosphorylation of NF-κB2/p100 and its subsequent proteasomal processing to transcriptionally competent p52 (*24*–*26*). Reduction of DUBA did not affect NF-κB2/p100 processing, either in the presence or absence of BAFF (B cell–activating factor belonging to the TNF family)–receptor engagement by B lymphocyte stimulator ligand (fig. S8). This finding suggests that DUBA affects only one aspect of TRAF3 signaling: namely, that which is required for IFN-I expression. Diseases such as systemic lupus erythematosus, where excess IFN-I production substantially contributes to pathology (*27*), stress the importance of negative regulation of IFN-I. Indeed, by suppressing IFN-I production, DUBA may function to inhibit the emergence of lupus-like autoimmune disorders.

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19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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28. We thank members of the Dixit laboratory, I. Wertz, and Y.-M. Loo for discussion, K. Newton for proofreading, R. J. Deshaies for S5a, and M. Vasser, J.-A. Hongo, M. Hazen, P. Chang, A. P. Martinez, D. Davis, and J. Lee for technical assistance.

Supporting Online Material

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31 May 2007; accepted 30 October 2007
Published online 8 November 2007;
10.1126/science.1145918
Include this information when citing this paper.

Sensing X Chromosome Pairs Before X Inactivation via a Novel X-Pairing Region of the *Xic*

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Mammalian dosage compensation involves silencing of one of the two X chromosomes in females and is controlled by the X-inactivation center (*Xic*). The *Xic*, which includes *Xist* and its antisense transcription unit *Tsix/Xite*, somehow senses the number of X chromosomes and triggers *Xist* up-regulation from one of the two X chromosomes in females. We found that a segment of the mouse *Xic* lying several hundred kilobases upstream of *Xist* brings the two *Xic*s together before the onset of X inactivation. This region can autonomously drive *Xic* trans-interactions even as an ectopic single-copy transgene. Its introduction into male embryonic stem cells is strongly selected against, consistent with a possible role in trans-activating *Xist*. We propose that homologous associations driven by this novel X-pairing region (*Xpr*) of the *Xic* enable a cell to sense that more than one X chromosome is present and coordinate reciprocal *Xist/Tsix* expression.

In female mammals, random X-chromosome inactivation (XCI) is triggered during early development. The *Xic*, which contains the *Xist* gene and its antisense unit *Tsix/Xite*, controls the initial steps of XCI. These steps include sensing the number of X chromosomes, counting the X:autosome ratio, and choosing the X to inactivate. The *Xic* was originally defined as a region of 680 kb to 1.2 Mb that was essential for XCI [reviewed in (*1*)], but so far no single-copy *Xist*-containing transgene has been found to recapitulate the random XCI process in a manner equivalent to that found in X-autosome translocations (*2*). Complete *Xic* function therefore requires long-range elements in addition to *Xist/Tsix/Xite*.

At the onset of XCI, *Xist* becomes up-regulated and *Tsix* down-regulated on the future inactive X (*3*). Although antisense transcription negatively regulates *Xist* in cis (*4*–*6*), there must also be some form of trans-regulation between the two *Xist/Tsix* loci, given the tight coordination of their mutually exclusive expression patterns. Recent insight into this comes from the finding that the two *Xic*s associate transiently at around the time that *Xist* is monoallelically up-regulated (*7*, *8*). Pairing is disrupted when *Tsix* or *Xite* are deleted (*7*, *8*). CTCF and transcription are thought to participate in pairing (*9*). However, although ectopic multicopy arrays of *Tsix* and *Xite* can associate in trans with the endogenous *Xic* (*8*), single-copy *Xist/Tsix/Xite* transgenes such

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