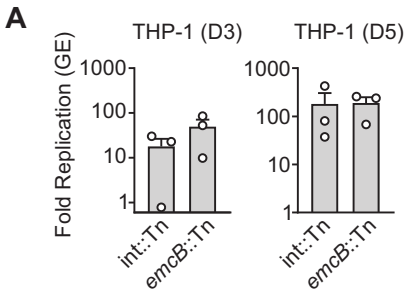
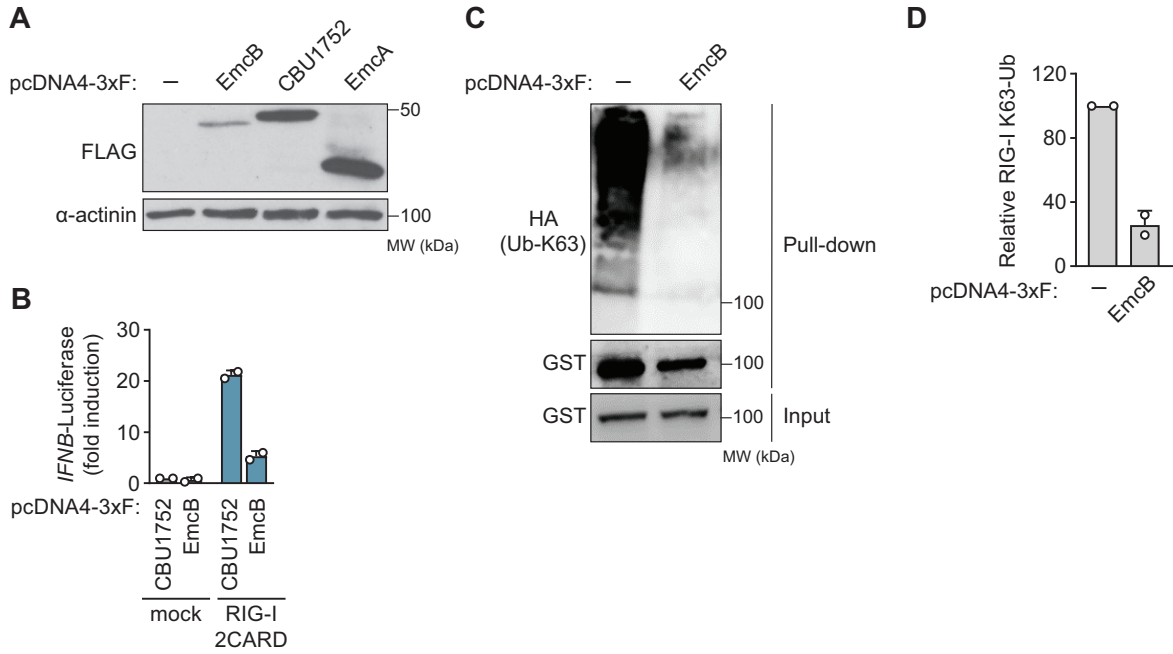
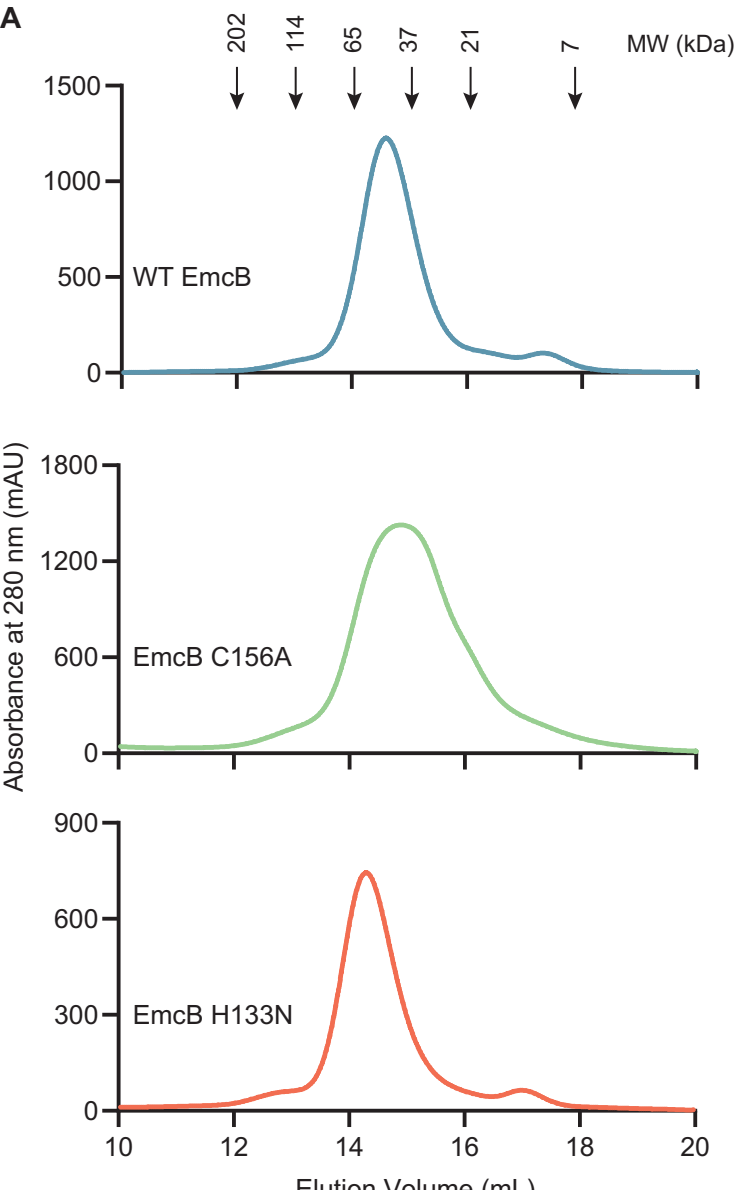
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



SI Figure Legends:

Supplementary Figure 1. EmcB is not require for growth in THP-1 macrophages

(A) qPCR analysis of change in *C. burnetii* genome equivalents (GE) during intracellular growth in PMA-differentiated THP-1 cells infected at an MOI of 30. Data are mean ± SD of three independent experiments. P value at D3 and D5 n.s. by unpaired, two-tailed *t*-test.

Supplementary Figure 2. Production and function of FLAG-tagged EmcA and EmcB

(A) Western blot analysis of FLAG-tagged Dot/Icm effectors in HEK293T cells. Effectors were transfected for 24h and lysates subjected to western blot. Blot is representative of two independent experiments.

(B) IFN-β induction as assessed by human *IFNB* promoter-driven luciferase reporter. HEK293T cells transfected with pGL3-*IFNB* overnight were transfected with indicated effector (100 ng) for 24h, and subsequently transfected with pEBG-RIG-I 2CARD (100 ng) for 24h. Dots are independent replicates. Data are mean ± SD of two independent experiments. P value for EmcB + 2CARD vs. CBU1752 + 2CARD <0.0001, EmcB no tx. vs. CBU1752 no tx n.s., CBU1752 + 2CARD vs. CBU1752 no tx <0.0001, EmcB + 2CARD vs. EmcB no tx <0.05, by two-way ANOVA with Tukey *post hoc* test.

(C) Western blot analysis of full-length RIG-I pulled-down under stimulatory conditions. HEK293T cells transfected with FLAG-EmcB or empty vector, HA-Ub-K63, GST-RIG-I Fl and poly(I:C) (1 ng/μL) were lysed, GST-RIG-I purified and subjected to western blotting with GST and HA antibodies. Blot is representative of two independent experiments.

(D) Quantified densitometry of HA-Ub-K63 to GST-RIG-I in pull-down from (B). Dots are independent replicates. Data are mean ± SD of two independent experiments. P value <0.01 by unpaired, two tailed student's *t*-test.

Supplementary Figure 3.

(A) Size-exclusion chromatography of WT EmcB (blue), EmcB C156A (green), and EmcB H133N (orange) in 20 mM HEPES-KOH pH 7.50, 250 mM KCl, 1 mM DTT after removal of N-terminal 6×his-SUMO2 by overnight incubation with SENP2 protease. Fractions that eluted from 14 to 15 mL contained the majority of EmcB as assessed by SDS-PAGE analysis and were used for biochemical assays.

SI materials and methods:

Bacterial strains

C. burnetii Nine Mile phase II (NMII), strain RSA493 clone 4 and isogenic derivatives were grown in Acidified Citrate Cysteine Medium-2 (Sunrise Science Products) at 5% CO_2, 2.5% O_2 at 37°C (1, 2). The *icmL*::Tn mutant and transposon insertion mutant effector sub-library were described previously (3, 4) and grown in the presence of kanamycin (375 µg/mL) or chloramphenicol (3 µg/mL), respectively. Complemented strains were constructed as previously described (1, 5). In brief, the indicated gene was cloned into the pJB-Kan or pMiniTn7T plasmid under control of the native promoter (*cbu1752, cbu1387*) or under control of the *cbu1169* promoter (*cbu2013*), a weak constitutive promoter, and transformed into *C. burnetii* mutants and selected with kanamycin (375 µg/mL).

Cell Culture

HEK293 ISRE-Luciferase, HEK293T, HEK293T DDX58$^{+/+}$ and DDX58$^{-/-}$ cells (6) were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco™) supplemented with 10% heat inactivated fetal bovine serum (% v/v). THP-1 and J774A.1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (ATCC Modification, Gibco™) supplemented with 10% heat inactivated fetal bovine serum (% v/v). All cells were maintained in humidified air at 5% CO_2.

IFN-treated THP-1 GE analysis

THP-1 cells were seeded at a density of 5×10^4 cells per well in 24 well plate in RPMI 1640 (ATCC modification) with 10% FBS supplemented with 162 nM PMA overnight. The next day, the media was exchanged for media without PMA supplemented with 100 ng/mL IFN-β (R&D Systems) for 24 hours. Te next day, stationary phase *C. burnetii* cultures were quantified by qPCR as above. Bacteria were pelleted, resuspended in DMEM high glucose, and sonicated for 12 minutes (VWR Aquasonic 50T) prior to addition to cells. The media was aspirated, and bacteria were added to cells at an MOI of 20 in RPMI 1640 (ATCC modification) with 10% FBS. Four hours after infection, cells were washed with 1×PBS, and the media replaced with RPMI 1640 ATCC modification with 10% FBS. To enumerate intracellular *C. burnetii*, at indicated time points the media was aspirated, eukaryotic cells were washed once with 1×PBS and then lysed by resuspension in sterile, nuclease-free water. Cell lysates were centrifuged to collect the bacteria. The supernatant was aspirated, and the pellets were frozen until ready for further processing.

Genomic DNA was extracted using the Illustra Bacteria GenomicPrep Mini Prep Kit (Cytiva) according to the manufacturer's instructions. Genomic equivalents (GEs) were quantified by qPCR as described above and the fold replication was expressed as the number of GEs at indicated time point divided by the number of GEs at 4 hours post-infection (Day 0).

Cell-based ISRE and IFN-β-luciferase reporter assays

For assays involving the use of the HEK293 cells stably expressing an ISRE-firefly luciferase reporter, cells were seeded at $2x10^4$ cells per well of a 96 well plate in 100 µL DMEM high glucose with 10% FBS one day prior to infection. *C. burnetii* were grown to stationary phase in ACCM-2 and quantified by qPCR as described above. The bacteria were resuspended in DMEM with 10% FBS and added to cells at an MOI of 200. Twenty-four hours after infection, the cells were transfected using 1 ng/µl of poly(I:C) and X-tremegene 9 (Sigma-Aldrich) transfection reagent in DMEM per manufacturer's instructions. Twenty-four hours after transfection, the media was aspirated and 50 µl of 1× passive lysis buffer (Promega) was added. After incubation at RT for 15 minutes, 20 µl of lysate was removed from each well and transferred to a 96 well opaque white tissue culture plate (Falcon) and mixed 1:1 with luciferase substrate. Luminescence was measured using a Tecan Infinite M1000 plate reader (Tecan, Mannedorf, Switzerland). For assays involving ectopic expression of effector proteins or influenza A NS1 (7) , constructs were introduced to cells by transfection with TransIT-LT1 (Mirus) according to the manufacturers' guidelines at a ratio of 3:1 with indicated RIG-I agonist (polyI:C or pEBG-RIG-I 2CARD) and incubated for 48-60h prior to lysis and measurement of luciferase signal as previously described (8). For assays studying MDA5 signaling, HEK293T cells were transfected with pUNO1-MDA5 and an ISRE-luciferase reporter plasmid (Promega). To study the cGAS-STING pathway, HEK293T cells were reconstituted with plasmids encoding cGAS, STING and an ISRE-luciferase reporter (Promega). At 24hr after transfection, cells were lysed and analyzed as above.

For assays involving the human *IFNB* promoter luciferase plasmid, HEK293T cells were seeded in 96 well plates at a density of $2x10^4$ and reverse transfected with 20 ng of pGL3-*IFNB* (9) with TransIT-LT1 (Mirus) according to manufacturer's guidelines. The next day, cells were transfected with 100 ng of pcDNA4-3xF encoding either CBU1752 or EmcB. 24h later, cells were transfected with 100 ng of pEBG-RIG-I 2CARD or pEBG control. After 24h, cells were lysed and analyzed as above.

C. burnetii GE analysis in THP-1 macrophages

THP-1 cells were seeded at a density of 4×10^4 cells per well in 96 well plate in RPMI 1640 (ATCC modification) with 10% FBS (media) supplemented with 162 nM PMA overnight. The next day, the media was exchanged for media without PMA for 24 hours. The day of the infection, *C. burnetii* cultures were pelleted and resuspended in media, enumerated by qPCR as above, and added to cells at an MOI of 30. Four hours after infection, the supernatant was aspirated, cells were washed twice and resuspended in fresh media. At Day 0 (4h post-infection), Day 3 and Day 5 of infection, the media was collected, the mammalian cells triturated in 200 µL of ultra-pure water (AmericanBio) and centrifuged at 17,000*g* for 15 minutes at 4ºC. The supernatant was aspirated, the pellets were frozen and stored at -20ºC until ready for further processing. Genomic DNA was extracted using the Illustra Bacteria GenomicPrep Mini Prep Kit (Cytiva) according to the manufacturer's instructions. Genomic equivalents (GEs) were quantified by qPCR as described above and the fold replication was expressed as the number of GEs at indicated time point divided by the number of GEs at 4-hours post-infection (Day 0).

Cytokine Expression Analysis in Macrophages and HEK293T cells

J774A.1 cells were seeded a density of $2x10^6$ in a 10 cm^2 dish in RPMI 1640 (ATCC modification) with 10% FBS and infected at an MOI of 500. After 4 days of infection, total RNA was extracted with the RNeasy mini kit (Qiagen) per manufacturer protocol. Fresh β-mercaptoethanol (1% v/v) was added to buffer RLT1 at the time of sample preparation, and on-column DNAse I digest was done to remove gDNA. cDNA was prepared with the iScript Advanced cDNA synthesis kit (Bio-Rad) and the abundance of cDNA for genes of interest was determined with quantitative PCR using iQ SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX thermocycler.

For analysis of *IFNB* expression in HEK293T, cells were seeded at a density of $3x10^5$ in 6 well plates in DMEM high glucose with 10% FBS and infected at an MOI of 1000. After 24h, cells were transfected with 1 ng/µL poly(I:C) using TransIT-LT1 (Mirus) according to manufacturer's instructions and incubated an additional 16h. Total RNA and qPCR analysis was done as above.

Protein Purification

Codon-optimized EmcB (Genscript) was cloned into a custom pET vector encoding an N-terminal 6×His-SUMO2-tagged fusion protein (10) and expressed in BL21 DE3 RIL *E. coli* (Agilent) containing the rare

tRNA plasmid pRARE2. *E. coli* was grown in 25 mL starter cultures of LB broth (miller) supplemented with 0.2% glucose, 100 µg/mL ampicillin and 12.5 µg/mL chloramphenicol. Cultures were back diluted 1:100 into LB broth (miller) with 100 µg/mL ampicillin and 12.5 µg/mL chloramphenicol and grown to an OD600 of 0.5-1.0 at 37ºC with 200 rpm. Upon reaching desired optical density, cultures were rapidly cooled in an ice water bath with vigorous intermittent shaking and then induced with 0.5 mM IPTG (GoldBio). Induced cultures were returned to the incubator overnight at 16ºC with 200 rpm. Bacteria were pelleted at 5000*g*, washed once with 1×PBS, flash frozen in liquid nitrogen, and stored at -80°C until ready for protein purification. Pellets were resuspended in a lysis buffer comprised of 20 mM HEPES-KOH pH 7.5, 250 mM NaCl, 20 mM imidazole, 10% glycerol and 1 mM DTT and lysed by sonication (10s, 20s off, 70% power, total time: 5 minutes). Lysates were clarified by centrifugation at 50000*g* for 20 minutes. Recombinant protein was purified by gravity chromatography and binding to Ni-NTA resin (QIAGEN). Resin was washed with lysis buffer supplemented to 1 M NaCl, washed with lysis buffer, and then eluted with lysis buffer supplemented to 300 mM imidazole. Purified protein was supplemented with recombinant human SENP2 protease (D364–L589, M497A) to remove the SUMO2 tag, and dialyzed overnight against 20 mM HEPES-KOH pH 7.5, 250 mM KCl, 1 mM DTT. Proteins were concentrated using a 10K or 30K-cutoff concentrator (Millipore) and further purified by size-exclusion chromatography on a Superdex 200 10/300 GL column (Cytiva) in 20 mM HEPES-KOH pH 7.5, 250 mM KCl, 1 mM DTT. Fractions containing protein were identified by absorbance at 280 nm, run on an SDS-PAGE, and those containing untagged EmcB were pooled and concentrated to 20−50 mg/mL in 20 mM HEPES-KOH pH 7.40, 250 mM KCl, 1 mM DTT or concentrated to 10 mg/mL and supplemented with 10% glycerol for working stocks. Protein was snap-frozen in liquid nitrogen and stored at −80°C in small aliquots until ready for use.

For experiments in Fig 3A and 4H, MBP-EmcB was used. To purify, MBP-EmcB, EmcB was cloned into pMAL-C5X. MACH1 *E. coli* expressing pMAL-C5X-EmcB construct was grown in LB 0.2% glucose with 100 µg/mL ampicillin, diluted 1:100, grown to an OD600 of 0.5-1.0 at 37ºC with 200 rpm of shaking. Cultures at desired OD600 were rapidly chilled in an ice water bath for 15 minutes and induced with 0.5 mM IPTG (GoldBio). Cultures were returned to incubator at 16ºC with 225 rpm shaking overnight. Bacteria were pelleted at 5000*g* for 20 minutes, resuspended in 1×PBS, transferred to a 50 mL conical, pelleted at 3000*g* for 15 minutes, the supernatant aspirated, the pellets snap frozen in liquid nitrogen, and

stored at -80ºC until ready for purification. Pellets were resuspended in a lysis buffer composed of 20 mM HEPES-KOH pH 7.5, 250 mM NaCl, 10% glycerol and 2 mM DTT and lysed by sonication (10s, 20s off, 70% power, 5 minutes total). Lysates were clarified by centrifugation at 50000*g* for 20 minutes. Recombinant protein was purified by gravity chromatography and binding to Maltose resin (NEB). Resin was washed with lysis buffer supplemented to 1 M NaCl and then eluted with Lysis Buffer supplemented with 10 mM maltose. Protein was dialyzed overnight in 20 mM HEPES-KOH pH 7.40, 250 mM KCl, 1 mM DTT, 10% glycerol. Dialyzed protein was concentrated to 10 mg/mL, snap frozen in liquid nitrogen, and stored at −80°C in small aliquots until ready for use.

RIG-I ubiquitination assays

Purification of GST-RIG-I 2CARD from mammalian cells was done as previously described (8, 11). pEBG-RIG-I 2CARD (4 µg) and pcDNA4 carrying indicated gene or pCAGGS-NS1 (Influenza A virus, 10 µg) (7) and pGL4.45[luc2P/ISRE/Hygro] (Promega) (100 ng) were transfected with Mirus TransIT-LT1 into $5x10^6$ HEK293T cells in a 10 cm^2 dish in DMEM high glucose with 5% FBS per manufacturer guidelines. After 48h, the media was aspirated, cells were triturated from the dish in 2 mL of ice-cold 1×PBS with 2 mM EDTA and pelleted at 200*g* for 2 minute. The supernatant was aspirated, and the pellet was resuspended in 1 mL of ice-cold NP-40 lysis buffer (50 mM HEPES-KOH pH 7.40, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 50 mM NEM, 1 mM PMSF, 1% IGEPAL CA-630 (v/v) + Roche complete protease inhibitor tablet). Cells were incubated on ice for 15 minutes with occasional flicking and clarified by centrifugation for 10 minutes at 13000*g* at 4ºC. ISRE luciferase signal in clarified lysates was measured as above. For pull-downs, clarified lysates were transferred to 30 µL of glutathione beads (Pierce™) equilibrated in lysis buffer and incubated for two hours at 4˚C with constant rotation. Beads were washed five times with 20 volumes of lysis buffer for one minute each and then resuspend in one volume of 2×Laemmli sample buffer (65 mM Tris-HCl pH 6.80, 2% SDS, 10% glycerol, 0.004% Bromophenol blue, 5% 2-mercaptoethanol), snap frozen and stored at -20ºC until ready for use. Samples were thawed on the bench top and loaded onto to 4–20% Mini-PROTEAN TGX gels (BioRad). Resolved gels were transferred onto PVDF membranes and probed with a ubiquitin antibody (clone P4D1, Cell Signaling 1:1,000) and GST antibody (polyclonal, Cytiva 1:5,000). Blots were probed with cognate secondary antibodies conjugated to HRP and chemiluminescence was measured with an ImageQuant 800 biomolecular

imager. Ubiquitin conjugates were identified as specific bands of a higher than predicted molecular weight which reacted with both ubiquitin and GST antibodies. Densitometric analysis of ubiquitinated RIG-I was performed with ImageJ analysis (12). Briefly, images were inverted, and ubiquitin band intensity was measured for each condition and compared to that of the GST-RIG-I 2CARD control condition.

For *in vitro* RIG-I deubiquitination assays, GST-RIG-I 2CARD was purified from cells as above with the following modifications. After five washes with 20 volumes of lysis buffer, beads were washed five times with 20 volumes of wash buffer (50 mM HEPES-KOH pH 7.40, 150 mM NaCl, 5 mM DTT). EmcB was preincubated in wash buffer and added to 15 µL of bead slurry in wash buffer to a final concentration of 40 µM (estimated ~200 µM GST-RIG-I 2CARD). Reactions were incubated for one hour at 37ºC and then quenched with 10 µL of 4×Laemmli sample buffer (130 mM Tris-HCl pH 6.80, 4% SDS, 20% glycerol, 0.008% Bromophenol blue), and processed as above. Where indicated, EmcB was pre-incubated in wash buffer supplemented with 50 mM NEM or 20 mM EDTA for 15 minutes prior to co-incubation with GST-RIG-I 2CARD.

For measuring the ubiquitination of full length RIG-I, $5x10^6$ HEK293T cells were seeded in a 10 cm^2 dish in DMEM high glucose with 5% FBS and reverse transfected with pcDNA4-3xF-EmcB or empty vector, pEBG-RIG-I Full-length and pRK5-HA-Ubiquitin K63 only (K6R, K11R, K27R, K29R, K33R, K48R) (13) with Trans-IT LT1 transfection reagent (Mirus) per manufacturer's guidelines. After 48h, cells were lysed, and GST-RIG-I (FL) was isolated and analyzed as was done for GST-RIG-I 2CARD. Ubiquitin conjugates associated with RIG-I were expressed as the HA to GST signal in the immunoprecipitated samples. HA antibody (Millipore-Sigma, Clone 3F10) was used for HA western blots at a dilution of 1:3333 in 5% milk in TBS.

Di-ubiquitin cleavage assay with MBP EmcB

DUB activity assays were performed similar to as previously described (14, 15). In brief, purified MBP or MBP-EmcB (1 µM) was incubated with 0.5 µM di-ubiquitin in 10 µL reactions of 50 mM Tris-HCl pH 7.50, 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT for two hours before being quenched with SDS-PAGE sample buffer and resolved on a 4–20% Mini-PROTEAN TGX gel (Bio-Rad) and stained with Sypro Ruby (Sigma-Aldrich) per the manufacturer's guidelines. Stained gels were imaged on an Amersham ImageQuant 800 biomolecular imager in the UV channel.

Cy5-Ubiquitin-VME gel shift assay

Ubiquitin-VME gel shifts were performed as previously described (16). Purified EmcB or indicated point mutant was diluted to 0.6 µM in 10 µL reactions of 25 mM Tris-HCl pH 7.40, 150 mM KCl, 2 mM DTT with or without of 2.5 µM Cy5-Ubiquitin-VME (UBPBio) for 1.5 hours wrapped in foil at room temperature. Reactions were quenched with addition of 4 µL of 4× Laemmli sample buffer without reducing agent and resolved on a 4–20% Mini-PROTEAN TGX gel (Bio-Rad). Resolved gels were immediately imaged on an Amersham ImageQuant 800 biomolecular imager in the Cy5 channel, and then fixed and silver stained(Pierce™ Silver Stain Kit). Silver-stained gels were imaged with the Amersham ImageQuant 800 biomolecular imager using the optical density measurement feature.

Ubiquitin-propargylamine gel shift assay

Ubiquitin-PA gel shifts were performed as previously described (17, 18). EmcB or EmcB C156A was diluted to 0.6 µM in 10 µL reactions of 25 mM Tris-HCl pH 7.40, 150 mM KCl, 10 mM DTT with or without addition of 2.5 µM N-terminally HA-tagged ubiquitin-propargylamine (HA-Ub-PA) (South Bay Bio) for 1 hour. Reactions were quenched with 4 µL of 4× Laemmli sample buffer without reducing agent and resolved on a 10% SDS-PAGE. Gels were stained with Sypro Ruby (Sigma-Aldrich) or silver stained (Pierce™ Silver Stain Kit) per manufacturers guidelines and imaged on an Amersham ImageQuant 800 biomolecular imager.

Ubl-AMC assay

Ubiquitin-like modifier AMC-cleavage experiments were done as previously described (15, 19). Indicated conjugate, Ub-AMC, NEDD8-AMC, ISG15-AMC (South Bay Bio), was diluted to 666 nM to a total volume of 60 µL AMC-cleavage buffer (50 mM Tris-HCl, pH 7.50, 0.5 mM EDTA, 5 mM DTT, 0.1 % (w/v) bovine serum albumin) in a 96-well black polystyrene plate (Costar). AMC conjugates were equilibrated by shaking at 30°C for 5 min in a fluorescent plate reader (Tecan). EmcB was equilibrated at room temperature for 10 minutes in AMC buffer, and then 40 µL was added to the AMC conjugates to a final concentration of 3.5 nM (total volume 100 µL), mixed for 10s by shaking, and then fluorescence was measured every 30 seconds for 60 min by 340 nm/441 nm (20 nM bandwidth) excitation/emission.

Enzyme kinetics were determined using Graphpad Prism analysis of the linear velocity of DUB activity as previously described (20). Enzyme kinetic assays were done in AMC buffer with EmcB at a concentration of 150 nM which was determined from linear range of enzyme kinetic assays with 1 µM of Ub-AMC.

Ubiquitin-propargylamine inhibition of DUB activity assay

MBP-EmcB was diluted to 8.2 nM in 39 µL AMC buffer. HA-Ub-PA was prepared at 40× desired concentration in AMC buffer and 1 µL at indicated concentrate was added to MBP-EmcB. Mixtures were incubated for 15 minutes at room temperature before adding 10 µL MBP-EmcB and HA-UB-PA mixture to 30 µL of Ub-AMC in AMC buffer at a concentration of 333.3 nM (Total volume, 40 µL, final concentrations: 250 nM Ub-AMC, 2 nM EmcB). The samples were mixed by 15 s of shaking and Ub-AMC cleavage was measured as above on a Tecan plate reader every 30s for 60 minutes.

Ubiquitin cleavage assay by varied chain length

To measure kinetic cleavage of ubiquitin chains, EmcB was diluted to 15 µM in DUB buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM DTT) and then 1 µL was added to 30 µL of 1 µM K63-linked hexa-ubiquitin or diubiquitin (Boston Biochem) in DUB buffer. At indicated time, 4 µL of reaction was sampled, quenched with 2 µL 4×Laemmli sample buffer (130 mM Tris-HCl pH 6.80, 4% SDS, 20% glycerol, 0.008% Bromophenol blue), and stored on ice until all samples were collected. Samples were resolved on a 4–20% Mini-PROTEAN TGX gel (BioRad) and stained with SYPRO Ruby per manufacturer's instructions. Stained gels were imaged in the UV channel with an ImageQuant 800.

Thermal Denaturation Assay

Thermal denaturation assays to assess protein folding were done as previously described (10, 21). Indicated protein was diluted to a final concentration of 5 µM in 20 µL of 20 mM HEPES-KOH pH 7.40, 150 mM KCl and 3× SYPRO Orange dye in technical duplicate. Using a Bio-Rad CFX thermocycler, samples were brought from 25ºC to 95ºC at a rate of 1ºC per minute while measuring SYBR fluorescence. The derivative of SYBR fluorescence over time was calculated using Bio-Rad CFX Manager (dRFU/dT), then expressed as a percent of the maximum change in fluorescence for each sample. The melting temperature was determined automatically by the Bio-Rad CFX Manager as the inflection of dRFU/dT.

Phylogenetic tree ubiquitin-like modifiers

Ubiquitin-like modifiers were identified from published work (22). Sequences were extracted from Uniprot for indicated human ubiquitin-like modifier for the specified ubiquitin-like domain. In the case of ISG15, which contains tandem ubiquitin like domains, the C-terminal ubiquitin-like domain was selected for alignment. Sequences were aligned with Clustal Omega (23), exported to Jalview (24) and formatted into a tree by average distance by PID. Trees were visualized with Interactive tree of life (iTOL) v5 (25). Tree was rooted on ATG12.

Measurement of FLAG-tagged effector production

HEK293T cells were seeded at a density of $3x10^5$ in 6 well plates in DMEM high glucose with 10% FBS and transfected with pcDNA4/TO-3xF containing indicated effector and incubated for 48h. Cells were harvested in 1×PBS and resuspended in NP-40 Lysis buffer (50 mM HEPES-KOH pH 7.40, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630 (v/v) + Roche complete protease inhibitor tablet). Samples were incubated on ice for 15 minutes, clarified by centrifugation at 13000*g* at 4ºC for 15 minutes, and the supernatant harvested and 4x Lammeli buffer added to 1x. Samples were boiled for 10 minutes, loaded onto to 4–20% Mini-PROTEAN TGX gels (BioRad). Resolved gels were transferred onto PVDF membranes and probed with M2 FLAG antibody (Sigma, 1:5000) and α-actinin antibody (BD Biosciences, 1:1000).

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