Signal transduction systems enable organisms to monitor their external environments and accordingly adjust the cellular processes. In mast cells, the second messenger Ap₄A binds to the histidine triad nucleotide-binding protein 1 (HINT1), disrupts its interaction with the microphthalmia-associated transcription factor (MITF), and eventually activates the transcription of genes downstream of MITF in response to immunostimulation. How the HINT1 protein recognizes and is regulated by Ap₄A remain unclear. Here, using eight crystal structures, biochemical experiments, negative stain electron microscopy, and cellular experiments, we report that Ap₄A specifically polymerizes HINT1 in solution and in activated rat basophilic leukemia cells. The polymerization interface overlaps with the area on HINT1 for MITF interaction, suggesting a possible competitive mechanism to release MITF for transcriptional activation. The mechanism depends precisely on the length of the phosphodiester linkage of Ap₄A. These results highlight a direct polymerization signaling mechanism by the second messenger.
Second messengers propagate extracellular signals that are initiated by the binding of first messengers (e.g., hormones, allergens, and neurotransmitters) to cell membrane-bound receptors. By binding and activating target proteins including kinases, ion channels, or regulatory proteins, second messengers relay signaling cascades in the cytoplasm or nucleus. Diadenosine-5′,5′-P1,P4-tetraphosphate (Ap4A) is a ubiquitous second messenger from bacteria to higher eukaryotes, which responds to environmental stresses and cell cycle phases.

The lysyl-tRNA synthetase (LysRS) on the S207 residue through the mitogen-activated protein kinase (MAPK) pathway. The phosphorylated LysRS no longer functions in translation, but gains the ability to synthesize Ap4A molecules. The restoration from the stimulated signaling response is mediated by the Ap4A hydrolase. Knocking down the Ap4A hydrolase in rat basophilic leukemia (RBL) cells, which are widely used in allergy studies as a mast cell model, leads to the accumulation of cellular Ap4A, prolonged dissociation of MITF from HINT1, and increased transcription of MITF target genes.

MITF is a key transcription factor that regulates the expression of many genes critical for mast cell activation including proteases, cytokine receptors, and cell adhesion molecules. Mice lacking a functional Mitf gene (mit/mi) are essentially deficient in mast cells and are susceptible to death from parasite infections. Co-transfection of MITF with HINT1 inhibited up to 94% of the MITF-mediated transcriptional activation of mast cell protease-6, which indicates that HINT1 is an important regulator of MITF during mast cell activation. Besides the MAPK-LysRS-Ap4A-MITF signaling pathway, multiple pathways have been reported to regulate MITF activity and/or stability in mast cells, including the c-KIT and PI3K pathways.

HINT1 knockout mice (HINT1−/−) showed hyperalgesia caused by allergic or inflammatory responses, indicating aberrant mast cell activation. Both HINT1−/− and HINT1+/+ mice had high incidences of tumorigenesis after the exposure to the carcinogens 7,12-dimethylbenz(a)anthracene or dimethylbenz(a)anthracene and the released MITF eventually promotes the transcription of MITF target genes.

Of note, the sticking-out portion of Ap4A, containing the second adenine (A2) and the delta phosphate (Pδ), fit into an adjacent HINT1 dimer in the crystal structure (denoted as HINT1 Dimer II). The complex structures of HINT1H114A-Ap4A and Ap4A in the crystalline state closely resemble the HINT1 apo structure with overall RMSD about 0.238 Å. The complete Ap4A molecule was observed in one adenosine pocket of a HINT1 dimer (denoted as HINT1 Dimer I) in both co-crystallization and soaking structures, indicating that the ligands had been hydrolyzed by the HINT1WT protein during the crystallization, which took 3–7 days at 18 °C with a high protein concentration of ~2 mM.

We then made an H114A mutant to inactivate the hydrolysis activity of HINT1. The complex structures of HINT1H114A with Ap4A were then obtained through both co-crystallization (HINT1H114A-Ap4Aocrystallization, 0.95 Å) and soaking methods (HINT1H114A-Ap4Asoaking, 1.42 Å) (Supplementary Table 1). The complete Ap4A molecule was observed in one adenosine pocket of the HINT1 WT dimer (denoted as HINT1 Dimer I) in both co-crystallization and soaking structures. The two adenosine (A1) and the alpha-phosphate (Pa) of Ap4A bind inside the pocket, whereas the rest of the molecule adopts a unique Sticking-out conformation above the HINT1 WT surface (Fig. 1b). This conformation is significantly different from the flat conformation of Ap4A in the pocket of the homologous fragile histidine triad protein, due to the distinct C-terminal structures of the proteins. Interestingly, the Ap4A-complexed structure closely resembled the HINT1 apo structure with an overall RMSD about 0.238 Å (Fig. 1b and Supplementary Fig. 4b). Only three positions including the residues S107, I32, and D16 surrounding the adenosine pocket had minor shifts of 1.3, 3.8, and 1.9 Å upon Ap4A binding (Supplementary Fig. 4a–e). This was distinct from the large conformational change of target proteins induced by second messengers in the previous studies. These results suggested that Ap4A might regulate HINT1 through a distinct mechanism rather than inducing large, global conformational changes.

Of note, the sticking-out portion of Ap4A, containing the second adenosine (A2) and the delta phosphate (Pδ), fit into an adjacent HINT1 dimer in the crystal structure (denoted as HINT1 Dimer II; Fig. 1c and Supplementary Fig. 3a). The two HINT1 dimers formed a large cleft with their adjoining adenosine pockets that accommodated Ap4A (Fig. 1c, d). The HINT1 dimers I and II not only bound the same Ap4A molecule but also interacted with each other directly. The interacting residues include D16, I18, K21, H59, Q62, S107, and Y109, which is denoted as Ap4A-linked interface in Fig. 1c. Therefore, in the presence of Ap4A, two HINT1 dimers were integrated into a compact (HINT1)2-Ap4A-(HINT1)2 tetramer in the crystalline state.
The human HINT1 protein was previously co-crystallized with an Ap₄A analog JB419 \(^{47}\). In the JB419 molecule, the phosphate linkage was replaced by a non-hydrolysable bis-phosphorothioated glycerol structure (Supplementary Fig. 6a). Consistently, JB419 was shown to tether two HINT1 dimers into a tetramer in the crystal, supporting our observation that the two symmetric adenosine moieties could lead to HINT1 tetramerization (Supplementary Fig. 6b).

Interestingly, the relative orientation and detailed tetramer interface of HINT1 linked by JB419 and Ap₄A are quite different (rotated ~44°; Supplementary Fig. 6b). The resulting tetramer interface induced by JB419 is composed of I₁₈, I₄₄, S₄₅, S₁₀₂, S₁₀₇, and Q₁₂₀, which is significantly different from the tetramer interface in the HINT1-Ap₄A-HINT1 structure, which is composed by D₁₆, I₁₈, K₂₁, H₅₉, Q₆₂, S₁₀₇, and Y₁₀₉ (Supplementary Fig. 6d, e). These differences suggest that both the symmetric adenosines of Ap₄A and its natural tetraphosphate linkage are critical to modulate the orientation of HINT1 and subsequent polymerization.

Ap₄A induces HINT1 polymerization in solution. Crystal packing blocked the other two distal adenosine pockets of the (HINT1)₂-Ap₄A-(HINT1)₂ tetramer (Supplementary Fig. 3a–c). However, in solution, HINT1s with these available pockets should be able to bind additional Ap₄As to allow polymerization. To confirm the polymer in solution, the HINT1 \(_{H144A}\) protein was incubated with gradient concentrations of Ap₄A, from 0 to 700 μM; equivalent to the physiological concentration of Ap₄A in the activated BMMC cells \(^{14}\). HINT1 polymers were monitored by

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**Fig. 1** Ap₄A integrated two HINT1 dimers into a HINT1-Ap₄A-HINT1 structure. a Schematics of adenosine polyphosphates (ApₙA). ApₙA is composed by two adenosine moieties linked through ribose 5′-carbons to a phosphate group chain of different length (n denotes the number of phosphate groups). b Superimposition of the HINT1-Ap₄A complex structure (pink) and HINT1_apo structure (PDB: 1KPB, gray). c Two HINT1 dimers (Dimer I and Dimer II) were integrated into a tetramer in the HINT1-Ap₄A complex structure. HINT1 proteins are shown as surface in black or white, and the Ap₄A molecule is shown as pink surface. d A new cleft built by two adenosine pockets with a long groove accommodated the Ap₄A molecule. The HINT1 proteins are shown as surface and the Ap₄A molecule is shown as sticks. e Zoom-in view of the Ap₄A-induced tetramer interface between HINT1 Dimer I (black) and Dimer II (white). The interacting residues are shown as sticks.
HAn formation breaks HINT1-MITF interactions in solution. Based on the HINT1-Ap4A tetramer crystal structure, a model of HAn polymers can be established. The HINT1 protein is a compact and symmetric dimer, with two adenosine pockets at opposite sides. The dimers I and II formed a tetramer with a shared Ap4A bound to their approximated adenosine pockets. The other adenosine pocket of dimer II could interact with an additional Ap4A and promote the interaction with a HINT1 dimer III, and so forth to polymerize into a filament (Fig. 3a and Supplementary Fig. 9a). The monomeric mutation V97D on the HINT1-Ap4A tetramer crystal structure model in activated RBL cells.

As HINT1 polymerized after stimulation and depolymerized after recovery in the RBL cells (Fig. 4a and Supplementary Fig. 10c), we expected that the cellular distribution of HINT1 would also change during this process. We observed the distribution pattern of the protein using laser confocal microscopy in the unstimulated RBL cells, HINT1 was present in both the nucleus and cytoplasm. And in the nucleus, it was mostly observed as bright dots (Fig. 4b). Interestingly, punctate localization of MITF in the nucleus has also been reported. We further found that HINT1 and MITF were co-localized on these dots in the unstimulated RBL cells (Supplementary Fig. 10a). The location of MITF cotransfection with Flag-HINT1 WT in activated RBL cells (Fig. 4a).

To further confirm our HAn structure model, we established HINT1 knockdown RBL cells (Supplementary Fig. 10a) and then stably expressed Flag_HINT1 WT and mutants (Y109D- at the Ap4A linked interface, could not form polymers in vitro; L53R, I22R at the adenosine binding pocket; V97D at the classic HINT1 dimer interface) in these cells (Supplementary Figs. 9 and 10b). However, only the Y109D mutant was expressed at a similar level as WT protein (Supplementary Fig. 10b) and therefore focused on the Y109D mutant for further analysis. Using anti-Flag antibody, we observed strong polymers of Flag_HINT1 WT after stimulation (Supplementary Fig. 10c). This phenotype was not seen with the Ap4A linked interface mutant Flag_HINT1_Y109D (Supplementary Fig. 10d), which is consistent with our in vitro results (Supplementary Fig. 9e) and supports our hypothesis that HINT1 polymerizes through the proposed HAn structure model in activated RBL cells.

HINT1 polymerizes in stimulated RBL cells. Cellular Ap4A levels are significantly increased upon mast cell activation with a peak concentration of >180 μM in the activated RBL cells and >700 μM in BMMCs. These concentration fluxes occur within 15–30 min and drop back to the basal level after 1 h17. These concentrations are sufficient to induce the formation of HAn in solution (Fig. 2a). Consistently, we observed the polymerization of endogenous HINT1 in activated RBL cells (Fig. 4a). We show that HINT1 forms polymers upon IgE-Antigen stimulation within 15–30 min and decreases after 2 h (Fig. 4a), which was synchronous with the changes in Ap4A concentration in the literature.

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These data were also consistent with the polymerization of HINT1 and the increased transcription of MITF downstream genes (c-Met, c-Kit), which reached peak levels at 30 min (Fig. 4h). After the peak, the levels of the c-Met and c-Kit transcripts declined slowly, and returned to basal level 4–8 h after the stimulation (Fig. 4h). We also determined the mRNA levels of c-Kit and c-Met in the HINT1 knockdown RBL cells expressing...
Flag\_HINT1\_WT and Flag\_HINT1\_Y109D (Supplementary Fig. 10d). The expression of Flag\_HINT1\_WT suppressed the mRNA levels of c-Kit and c-Met in the HINT1 knockdown RBL cells, and recovered the response to the stimulation (Supplementary Fig. 10d). On the contrary, the expression of Flag\_HINT1\_Y109D, which could not polymerize nor bind MITF, showed increased mRNA levels of c-Kit and c-Met throughout the stimulation process. In summary, the changes in Ap4A concentration, HAn formation, and gene transcription were synchronized in the mast cell activation process.

**Function of Ap₄A is determined by the phosphodiester linkage.** Early studies showed that only Ap₄A among the Ap₄A family could disrupt the HINT1-MITF interaction and activate MITF\(^{14}\). To understand the specificity of Ap₄A on HINT1 regulation, we investigated the ability of Ap₃A and Ap₅A to induce HINT1 polymerization through EMSA. Our results show that Ap₃A, Ap₅A, AMP, and ATP could not induce HINT1 polymerization (Fig. 5a). We designed an additional fluorescence resonance energy transfer (FRET) assay based on the monomeric CFP-HINT1\_V97D and YFP-HINT1\_V97D to monitor Ap₄A-induced HINT1 interactions (Fig. 5b). In this experiment, the V97D mutation was introduced to minimize the background FRET signal generated by the classic HINT1 dimerization\(^{48}\). The FRET signal between CFP-YFP tandems was measured and set to 100\% (Fig. 5b). Only Ap₄A, but not AMP, Ap₃A, or Ap₅A, increased the FRET signal (Fig. 5b), suggesting that ATP, Ap₃A, and Ap₅A were not able to induce HINT1 interactions through the Ap₄A linked interface. Estimated from the FRET signal, 16\% of HINT1\_V97D was recruited into polymer by 700 \(\mu\text{M}\) Ap₄A. Increasing Ap₄A concentration to 1400 \(\mu\text{M}\) further led to 23\% of HINT1\_V97D to polymer. The negative stain EM also showed little effect of Ap₃A, Ap₅A, or AMP to induce HINT1 filament compared with Ap₄A (Fig. 5c–e). Therefore, Ap₃A and Ap₅A are not able to polymerize HINT1.

Finally, to reveal the molecular specificity of Ap₄A to induce HAn, we further solved the co-crystal structures of...
HINT1H114A-ATP and HINT1H114A-Ap3A (Supplementary Table 1). Only the AMP portion of these molecules were resolved in the adenosine pockets of HINT1, with the remaining atoms of ATP or Ap3A untraceable (Fig. 5g, h and Supplementary Table 1). Neither ATP (with only one adenosine moiety) nor Ap3A (with a shorter phosphodiester linkage) could recruit HINT1 dimers to form polymer in solution. We also solved the HINT1H114A-Ap3A structure in a similar crystal packing state as HINT1H114A-Ap4A. The five-phosphate linkage in Ap3A adopted a bent and zigzag conformation compared with the straight conformation of Ap4A (Fig. 5i), suggesting that the increased length of Ap3A is too long to stabilize the HAn interface as Ap4A does. Together, these results indicate that the bivalent characteristic, the length of the phosphodiester linkage, and the interaction of phosphates of Ap4A with HINT1 were all critical for HAn formation.

**Discussion**

Together, this work shows that the second messenger Ap4A directly polymerizes its target protein HINT1. This polymerization interface is also a potential interface for MITF, and therefore oligomerization releases MITF from HINT in solution. In addition, we also observed synchronous changes in HINT1 cellular distribution patterns and the transcription of two MITF downstream genes (c-Met and c-Kit) during the RBL activation process. These results suggest a mechanism in which Ap4A polymerizes HINT1 to block the interaction with MITF, which subsequently releases MITF and activates downstream gene transcription during mast cell activation (Fig. 4i).

Mast cells play critical roles in asthma, allergy, and anaphylaxis, and generate inflammatory reactions by releasing mediators during the immune activation36. The aberrant, chronic, or systemic activation of mast cells leads to multiple pathological conditions and promotes harmful inflammation that damages host tissues30,31. Therefore, the activation of mast cells upon antigen stimulation must be tightly controlled. This stimulation-induced synthesis of Ap4A molecules leads to subsequent binding to HINT1 homodimers, thereby creating a HAn filament that is driven by a unique symmetric adenosine moieties in the second messenger Ap4A. This process requires a remarkable structural precision of Ap4A, in a length-dependent manner of its...
**Fig. 4** HINT1 polymerizes in stimulated RBL cells. 

**a** Western blotting of the endogenous HINT1 following by the IgE and antigen stimulation. 0 indicates the unstimulated state. Source data are provided as a Source Data file.

**b-g** Endogenous HINT1 in Rat basophilic leukemia (RBL) cell following the IgE and antigen stimulation (0–4 h) were immunostained and visualized by confocal laser scanning microscope or stimulated emission depletion microscope (STED). Scale bars, 10 μm. Nuclei were labeled with DAPI. One representative experiment out of three is shown.

**h** The transcript level of c-Met and c-Kit following the IgE and antigen stimulation. Error bars represent the SEM of three experimental repeats. Source data are provided as a Source Data file.

**i** Schematic cartoon showing Ap4A induces the formation of HAn polymer to release MITF for transcriptional activity in allergic response.
tetra-phosphate linkage. HINT1 polymerization may block the MITF-HINT1 interface on HINT1 that releases MITF for the transcriptional activation. Our results on the Ap4A-HINT1-MITF pathway at the molecular level provides a basis for developing potential approaches to treating asthma and anaphylaxis.

HINT1 is involved in a broad spectrum of deficiencies in mammals. It is an ancient and conserved haplo-insufficient tumor suppressor. The deficiency of HINT1 in mice results in increased susceptibility to both spontaneous and carcinogen-induced tumor formation. The regulation of HINT1-MITF is also found in melanoma cells. MITF is the master regulator of melanocyte development and melanoma formation, as well as proliferation and relapse. K21 and Y109 in HINT1, which are located on the HAn interface, are two of the most prevalent post-translationally modified residues in HINT1 in multiple cancers including colorectal, liver, gastric cancer, and leukemia. We show that the mutants K21D and Y109D lost the ability to polymerize and are also unable to...
regulate MITF. Further, loss-of-function mutations in HINT1 were found in inherited peripheral neuropathies. Importantly, the tumor suppressor functions of HINT1 appear to be independent of its enzymatic activity, as a mutant HINT1 (H112N) defective in AMP-NH2-hydrolyzing activity was not impaired for induction of apoptosis. Here, the finding that ApA regulates HINT1 through induction of polymerization hints that this non-enzymatic regulation may serve as a broad-spectrum mechanism for HINT1 functions.

It has been widely reported that second messengers regulate target proteins by modifying their structural conformations. For example, 3',5'-cyclic AMP binds to the regulatory subunit of protein kinase A (PKA) causing a dramatic conformational change that uncouples the large lobe of the catalytic subunit of PKA. InsP3 binds to the ligand-binding core of InsP3 receptors and evokes conformational changes that open the channel gate to release Ca2+.

**Methods**

**Protein preparation** Human full-length WT or mutant HINT1 protein (residue 1–126) was constructed with an N-terminal 6×His-tag with a TEV protease cleavage site in a pHisTEV vector. HisTEV-HINT1 expression was induced by isopropyl β-D-1-thiogalactopyranoside in the bacterial strain BL21 (DE3) at 16°C for 20 h and the His-tagged protein was purified by nickel affinity column. The purified protein was further polished by a size-exclusion column (Superdex 75, GE Healthcare, Piscataway, NJ) for biochemical assays. For crystallization, the 6×His-tag was cleaved by TEV protease before gel filtration.

**Crystallization, data collection, and structure refinement** Crystallization was done by the sitting drop method. To co-crystallize HINT1 with ATP/ApA/ApA/ApA, ApA, 30 mg ml⁻¹ HINT1 protein (WT, H114A) was mixed with 10 mM different ligands (ATP, ApA, ApA, or ApA) separately and incubated on ice for 0.5 h. A drop then contained the above 0.15 µl of HINT1-ligand solution with 0.15 µl of precipitant solution, containing 100 mM HEPES pH 7.5, 35–40% PEG3350. Crystals were obtained after incubation at 18°C for 3–7 days. To obtain the HINT1 ApA soaked crystals, the HINT111111A-co-crystals were further soaked in solution containing 100 mM HEPES pH 7.5, 35–40% PEG3350, 20 mM ApA for 20 h.

The crystals were flash-frozen in liquid nitrogen with cryo-solution containing 0.75 mM HEPES pH 7.5, 26–30% PEG3350, and 25% glycerol for data collection.

The datasets were obtained from beam line LS-CAT 21-ID-F at Advanced Photon Source (Argonne, IL) or beam line 7-1 at the Stanford Synchrotron Radiation Lightsource (LBNL, Menlo Park, CA). All datasets were processed with HKL2000. The structures were solved by molecular replacement using the HINT1 structure (PDB: 4EQQ) with the program Molrep. Iterative model building and refinement were performed using Coot and Phenix. Data collection and refinement statistics are given in Supplementary Table 1. Representative views of the electron density maps are shown in Supplementary Fig. 12. In the HINT111111A-co-crystal structures, the ApA and ApA bind two HINT1 dimers in two alternative modes, which are related by a twofold symmetry. For both structures, one of the two equivalent modes are selected for analysis in the manuscript.

**Electrophoretic mobility shift assay and immunoblotting** HINT1WT (25 µM) or HINT111111A were mixed with either 700 µM ApA, ApA, ApA, AMP, ATP, or blank buffer and incubated at 4°C rotating gently for 2 h. The working buffer includes 25 mM HEPES pH 8.0, 400 mM NaCl, and 2 mM EDTA. Samples were separated by 4–20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) without pre-heating. RealBand 3-color High Range Protein Marker (BBI Life Science, Rockville, MD) was used to identify molecular weight of target bands. Gel under 25 kDa marker was cut for Coomassie brilliant blue staining. Gel above 25 kDa was transferred onto nitrocellulose membranes. Blottings were blocked in 5% non-fat milk for 30 min at room temperature, then incubated in Anti-His antibody (TransGen Biotech, catalog number H1001-2, dilution 1:3000) overnight at 4°C followed by 1 h incubation in horseradish peroxidase (HRP)-conjugated Goat Anti-Mouse IgG (BBI Life Science, catalog number D110087, 1:5000 dilution) at room temperature. Blottings were then treated with Pierce ECL Western Blotting Substrate and visualized with Amersham TM Imager 600 (GE Healthcare, Piscataway, NJ). The molecular weights of protein bands were calculated with three independent experiments by Amersham TM Imager 600 Analysis Software Version 1.0 (GE Healthcare, Piscataway, NJ).

**Negative stain electron microscopy** HINT1WT proteins were mixed with either ApA, ApA, ApA, AMP, or blank buffer. The final system contained 2 mM EDTA, 25 mM HEPES pH 8.0, 400 mM NaCl, 10 µM HINT1WT protein, and substrates with indicated concentration (e.g., 700 µM for AMP/ApA/ApA/ApA/ApA, 400 µM for ApA) Samples were well mixed and incubated at 4°C for 2 h. Five microliters of each sample was then transferred directly to a freshly glow-discharged transmission electron microscope (TEM) grid (Beijing Zhongjingkeyi Technology Co., Ltd) for 45 s. The grid was rinsed with 5 µl 3% w/v uranyl acetate pre-cooled at 4°C, followed by staining with 5 µl 3% w/v uranyl acetate for 45 s. The excess buffer was removed. The grid was dried in air before TEM imaging. Images of the specimens were obtained at 120 kV on a FEI Tecnai T12.

**Fluorescence anisotropy assay** Fluorescence anisotropy measurements were carried out using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). The interaction between 5IAF-labeled MITF (5IAF-MITF) and HINT1WT through fluorescence anisotropy assay was first established based on a previous study. HINT1WT (final concentration 500 nM) was mixed with gradient ApA and ATP (0–1400 µM) in buffer containing 20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM MgCl2, 1% (v/v) Glycerol. After a 2 h incubation at 4°C, 5IAF-MITF was added to the mixtures to initiate the reaction with final concentration of 150 nM. The measurement was performed after 2 h of incubation. Excitation beam at 494 nm and emission at 518 nm were used to measure the fluorescence anisotropy.

**Fluorescence resonance energy transfer assay** FRET measurements were carried out using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Purified 500 nM CFP-HINT111111A and 500 nM YFP-HINT111111A were incubated with gradient ApA/ApA/ApA/ApA/ApA up to 20 µM reaction volume. The reaction buffer contained 20 mM HEPES pH 7.5, 250 mM NaCl. The mixtures were incubated at 4°C for 3 h before the measurement. Excitation beam at 436 nm and emission at 480 nm, 525 nm were used to detect the FRET efficiency.

**Cell culture and activation.** RBL-2H3 cells were obtained from the cell bank of the Chinese Academy of Science (catalog number 10177), cultured and maintained at 37°C in growth medium containing RPMI, 2 mM l-glutamine, 2 mM nonessential amino acids, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 10% fetal bovine serum. For IgE and antigen activation, RBL-2H3 cells were treated with mouse IgE antibody (Sigma-Aldrich, catalog number D8460) at 400 ng ml⁻¹ for 2 h at 37°C. Followed by 3x wash with warm phosphate-buffered saline (PBS) buffer, cells were stimulated with 400 ng ml⁻¹ DNP-albumin (Sigma-Aldrich).

**Analyses of the endogenous HINT1 oligomerization by EMSA.** To analyze endogenous HINT1 oligomers during RBL activation, the cells with or without activation were collected with ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 350 mM NaCl, 1% Triton X-100, 0.1% SDS, 1× protease inhibitor cocktail B14002 (Bimake, Bimake, USA) and 1× phosphatase inhibitor cocktail B15002 (Bimake, Bimake, USA). The samples were homogenized by passing through a...
21-gauge syringe needle and then separated by 8–16% gradient SDS-PAGE without preheating. Immunoblots were blocked in 5% non-fat milk for 30 min at room temperature and then incubated in the Anti-HINT1 antibody (Abcam, catalog number ab124912, 1:3000 dilution) for 48 h at 4 °C and followed by 1 h incubation in the HRP-conjugated Goat Anti-rabbit IgG (BBI Life Science, catalog number D110058, 1:5000 dilution) at room temperature. Blotting was then treated with Cyanine 3 dye from TSA-Plus Fluorescence systemsNEL744 (PerkinElmer, Waltham, MA) for 10 min at room temperature shielded from light. Visualization was performed with Amersham TM Image 600 (GE Healthcare, Piscataway, NJ) under fluorescence detection mode with stimulation light 520 nm and Cy3 filter. To analyze the polymerization of Flag_HINT1 WT, Flag_HINT1_Y109D, Flag_HINT1_V97D, and HINT1_L53R during the RBL activation, an Anti-FLAG antibody (Proteintech, catalog number 20543-1-AP, 1:3000 dilution) was used and followed by 1 h incubation in HRP-conjugated Goat Anti-Rabbit IgG (BBI Life Science, catalog number D110058, 1:5000 dilution) at room temperature. Anti-β tubulin antibody (Proteintech, catalog number 10068-1-AP, 1:3000 dilution), and HRP-conjugated Goat Anti-mouse IgG (BBI Life Science, catalog number D110087, 1:5000 dilution) were used as primary and secondary antibodies for the Tubulin control in the western blotting. Blotting were then treated with Pierce ECL. Western Blotting Substrate and visualized with Amersham TM Image 600 (GE Healthcare, Piscataway, NJ).

**Cell imaging**. Cells were collected at different time points after DNP-albumin treatment by fixing the cells with 100% methanol/acetone at −20 °C. Cells were then incubated in HINT1 polyclonal antibody (Proteintech, catalog number 10717-1-AP, 1:500 dilution) for 2 h at room temperature. After washing with PBS, the samples were incubated with the secondary antibody (Abcam, catalog number ab6939, 1:1000 dilution) for 1 h at room temperature and shielded from light. DAPI (4’,6-diamidino-2-phenylindole) staining was used to detect the nucleus. Protein visualization was visualized with a laser scanning microscope (Leica-microsystems) both in the confocal mode and the stimulated emission depletion mode (STED). Negative control experiments were done without primary antibodies.

**Hydrolysis activity of HINT1 toward ApA measurement**. HINT1 WT protein (25 μM) was incubated with 1 mM ApA in the buffer containing 25 mM HEPES pH 8.0, 400 mM NaCl at 37 °C for multiple time courses (0 min, 15 min, 30 min, 1 h, 2 h, 4 h), with total volume of 50 μl. At the end of each incubation, the sample was heated at 95 °C for 10 min and centrifuged at 13,500 g for 10 min. Supernatant was then diluted into 200 μl buffer containing 25 mM HEPES pH 8.0 and analyzed through source 150 Column by high-performance liquid chromatography. A continuous sedimentation coefficient distribution model using program SEDFIT®.

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J.W., M.G., P.F., G.Z., C.L., J.K. and E.R. designed all experiments. J.W., P.F. and M.G. wrote the manuscript. J.Y., F.L., J.Z., Y.L., Z.L., C.T., M.Z. and A.M. performed the experiments. All authors analyzed the data and contributed to manuscript preparation.

Competing interests

The authors declare no competing interests.

Additional information

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