

The structural basis of human SAMD9 and the functional separation of its dual immune activities

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25 Abstract

26 Sterile alpha motif domain-containing protein 9 (SAMD9) is an interferon-inducible
 27 protein that has been implicated in both host antiviral defense and inherited human
 28 diseases. However, the structural basis governing full-length SAMD9 organization and
 29 innate immune signaling remains unclear. Here, we determined the atomic structure
 30 of human SAMD9 by cryo-electron microscopy and combined mutagenesis,
 31 biochemical, and cellular analyses to define structure-function relationship. SAMD9
 32 forms an integrated multidomain core stabilized by extensive intramolecular contacts,
 33 several of which coincide with disease-associated variants. The C-terminal
 34 oligonucleotide-binding fold domain adopts a self-inhibitory state through interactions
 35 with the NTPase and tetratricopeptide repeat domains. We also found that the majority
 36 of SAMD9 forms a homodimer mediated primarily by charged residues within the
 37 NTPase domain. Disruption of this interface shifts SAMD9 toward a monomeric
 38 conformation and significantly attenuates induction of interferon-stimulated genes. By
 39 contrast, although we identified a well-defined ATP-binding pocket, purified SAMD9
 40 shows minimal ATP hydrolysis activity. Mutations of ATP-contacting residues within
 41 SAMD9 reduced phenylalanine tRNA cleavage but did not affect interferon activation.
 42 Together, our results establish the oligonucleotide-binding fold as a structural
 43 determinant of SAMD9 auto-inhibition and provide a structural framework for the
 44 functional separation of interferon signaling from the previously established tRNA
 45 ribonuclease effector function.

46 Introduction

47 Sterile alpha motif domain-containing protein 9 (SAMD9) and its paralog SAMD9L
 48 are interferon-inducible proteins with potent antiviral and growth-restrictive activities^{1,2}.
 49 Interestingly, SAMD9 homologs in bacteria even precede the appearance of interferon
 50 signaling in zebrafish³ and seem to possess anti-bacteriophage activities⁴, reminiscent
 51 of cGAS, gasdermins, and other highly conserved innate immune defense systems⁵.
 52 In humans, dysregulation of SAMD9 or SAMD9L is associated with a broad spectrum
 53 of disorders. Mutations in SAMD9 are linked to normophosphatemic familial tumoral
 54 calcinosis^{6,7}, and multi-system MIRAGE syndrome characterized by Myelodysplasia,
 55 Infections, Restriction of growth, Adrenal hypoplasia, Genital phenotypes, and
 56 Enteropathy⁸⁻¹⁶. Pathogenic variants in SAMD9L similarly cause ataxia-pancytopenia,
 57 immunodeficiency, autoinflammatory disease, and predisposition to myeloid
 58 malignancies¹⁷⁻²⁰. A defining feature of SAMD9/9L syndromes is the strong selective
 59 pressure for somatic genetic rescue, including secondary loss-of-function variants,
 60 uniparental disomy, and loss of chromosome 7 carrying the mutant allele^{17,21-23}. These
 61 genetic observations underscore both the potent growth-restrictive activity of the so-
 62 called gain-of-function of SAMD9/9L and the need for tight control of their activation.

63 Sequence and structural analyses predict that SAMD9 contains a complex
 64 multidomain architecture comprising an N-terminal sterile alpha motif (SAM) domain,
 65 an AlbA2-related effector region, a SIR2-like domain, a central P-loop/NTPase domain,
 66 tandem tetratricopeptide repeat (TPR) regions, and a C-terminal
 67 oligonucleotide/oligosaccharide-binding (OB) fold^{3,4,24}. The overall organization of
 68 SAMD9 has been proposed to resemble that of STAND-family signaling proteins, in
 69 which a central P-loop NTPase module is embedded within a larger multidomain
 70 regulatory architecture³. The prokaryotic Avs, a primitive bacteria anti-phage defense
 71 system, has a very similar domain organization with AlbA2 followed by SIR2, P-loop
 72 NTPase, TPR, and OB-fold²⁵. Structural characterization of the N-terminal AlbA2-
 73 related region showed that it binds double-stranded nucleic acids and contributes to

the antiviral, antiproliferative, and translational-repressive activities of SAMD9/9L²⁴. By contrast, the structural organization and regulatory roles of the more C-terminal SIR2-like, P-loop/NTPase, TPR, and OB domains remain poorly defined. During the preparation of our manuscript, three preprints reporting human SAMD9 and SAMD9L structures became available²⁶⁻²⁸ and we discuss the similarities and differences between our findings and those reports throughout.

In addition to genetic disease relevance, SAMD9 and SAMD9L have emerged as components of cell-intrinsic antiviral defense. Human SAMD9 was subsequently shown to function as a poxvirus-activatable anticodon nuclease that selectively cleaves tRNA^{Phe}, leading to phenylalanine-codon-specific ribosomal pausing, translational arrest, and stress responses²⁹. Perhaps not surprisingly, SAMD9 is antagonized by poxvirus host-range proteins, including the myxoma virus M062 protein³⁰, while genetic screens subsequently established SAMD9 as a major barrier to replication of vaccinia viruses lacking the K1L and C7L host-range genes^{31,32}. SAMD9 also restricts rotavirus infection and is antagonized by the viral protein NSP1 via proteasomal degradation³³. In contrast, SAMD9L restricts HIV-1 and other primate lentiviruses through inhibition of viral and cellular translation, and has also been implicated in restriction of flaviviruses^{34,35}, showing distinct tropism in targeting different viral families. Here, we focus primarily on the structure and function of SAMD9.

We have recently shown that SAMD9, in addition to its tRNA cleavage and translational inhibition activities, directly binds to cytosolic double-stranded DNA and RNA, undergoes oligomerization, and promotes interferon responses through the MAVS-TBK1-IRF3 pathway³³. Importantly, disruption of tRNA binding (K350A) did not abolish IFNL3 induction, indicating that SAMD9-mediated nucleic-acid sensing and interferon signaling are functionally separable from its tRNA-directed effector activity³³. Additional lines of evidence seem to support that the N-terminal domains are critical for interferon induction but are dispensable for tRNA cleavage activity: adding epitopes to the SAM domain abrogates interferon expression³⁵ and deletion of the SAM domain does not impair tRNA cleavage but abolishes interferon activation³³. This distinction is

consistent with an emerging view that SAMD9/9L combine multiple antiviral outputs within a single multidomain protein, although the structural determinants that specify these activities remain largely unknown^{4,24,36}. In particular, the molecular basis of SAMD9 oligomerization, its relationship to signaling activation, and the function of the central P-loop/NTPase module have not been defined.

Here, we determined the cryo-EM structure of full-length human SAMD9 and combined structural, biochemical, and cellular analyses to define its regulatory architecture. The structure reveals an integrated multidomain core stabilized by extensive intramolecular contacts and identifies the P-loop/NTPase domain as the principal interface mediating SAMD9 homodimerization. Mapping of disease-associated variants highlights multiple interdomain regulatory sites, including a long-range NTPase-OB contact whose perturbation enhances basal interferon signaling. Disruption of the NTPase-mediated dimer interface shifts SAMD9 toward a monomeric state and markedly impairs innate immune gene expression, whereas mutations within the nucleotide-binding pocket retain substantial IFNL3-inducing activity. Together, these findings distinguish the assembly function of the P-loop/NTPase domain from its nucleotide-binding machinery and provide a structural framework for understanding how SAMD9 supports a direct translation-restricting effector function and an assembly-dependent innate immune signaling function through distinct structural determinants.

Results

Cryo-EM structure reveals an integrated multidomain architecture of SAMD9

To obtain a homogeneous sample suitable for structural characterization, we first evaluated four human SAMD9 constructs with distinct N- and C-terminal boundaries (**Fig. S1A-D**). These included full-length wild-type hSAMD9 (WT; residues 1–1589), an N-terminal truncation lacking the SAM and Alba2 domains [SAMD9(Δ SAM–Alba2), residues 387–1589], a C-terminal truncation lacking the OB domain [SAMD9(Δ OB), residues 1–1529], and a construct lacking both the N-terminal SAM–Alba2 region and

the C-terminal OB domain [SAMD9(Δ SAM–AlbA2/ Δ OB), residues 387–1529]. Before proceeding to cryo-EM analysis, we examined whether the purified proteins retained nucleic-acid-binding activity. Full-length hSAMD9 exhibited robust dsDNA binding *in vitro* (**Fig. S1E**), consistent with previous reports of nucleic-acid-binding activity in SAMD9^{24,37}. We next asked which domain(s) are responsible for dsDNA recognition. Notably, deletion of the C-terminal OB domain markedly reduced dsDNA binding (**Fig. S1E**), which is different from what we previously reported³³ and a recent preprint²⁸ showing OB is dispensable for dsDNA and dsRNA binding. This could be due to the His-MBP tag we had previously used and the lack of SAM domain in the new preprint, both of which could affect the N-terminal structural arrangement. Deletion of both the SAM-AlbA2 and OB domain led to the most pronounced binding defect (**Fig. S1E**). These observations extend previous findings by showing that, although the N-terminal AlbA2-containing region plays an important role in nucleic-acid recognition^{24,37}, efficient dsDNA binding by full-length SAMD9 also depends on the C-terminal OB-containing region. Together, these results support a multidomain mode of nucleic-acid recognition involving contributions from both the N- and C-terminal regions of SAMD9.

We next subjected purified full-length hSAMD9 to single-particle cryo-EM analysis (**Fig. S2 and Extended Data Table 1**). Two-dimensional class averages revealed well-defined particles with apparent two-fold symmetry, and subsequent three-dimensional classification identified a prominent homodimeric population. To improve the local density of individual protomers, we performed focused refinement using a mask encompassing a single protomer, yielding a reconstruction at an overall resolution of 2.9 Å. The resulting density enabled atomic modeling of residues 388–1589 (**Fig. 1A–C and Fig. S2**). The N-terminal SAM and AlbA2 regions were not resolved in the reconstruction (**Fig. S3**), indicating substantial conformational flexibility relative to the ordered C-terminal core, probably because of the lack of nucleic acid binding.

Based on sequence analysis (**Fig. S4**) together with the cryo-EM structure, hSAMD9 comprises an N-terminal sterile alpha motif (SAM) domain, an AlbA2-related tRNase effector region, a Silent Information Regulator 2 (SIR2)-like domain, a P-

loop/NTPase domain, two tetratricopeptide repeat domains (TPR1 and TPR2), and a C-terminal oligonucleotide/oligosaccharide-binding (OB) domain (**Fig. 1, S2, and S3**). The structural model begins at residue 388, corresponding to the N terminus of the SIR2-like domain, whereas the preceding SAM and AlbA2-related regions are not resolved in the cryo-EM reconstruction. This observation indicates substantial conformational flexibility between the N-terminal effector region and the ordered C-terminal core.

The resolved SAMD9 protomer adopts an overall L-shaped architecture, resembling a boot with dimensions of approximately 95 Å in length and 80 Å in height (**Fig. 1B–C**). In this orientation, the SIR2-like domain forms the upright portion, whereas the remaining domains extend counterclockwise to form the heel, instep, and toe of the boot-like structure.

The SIR2-like domain lies adjacent to the centrally positioned P-loop/NTPase domain and adopts a bilobed architecture comprising a large Rossmann-like $\alpha/\beta/\alpha$ fold and a smaller subdomain, with a prominent cleft at their interface (**Fig. 1B–C**). Although its overall fold resembles members of the SIR2/sirtuin family and there are known SIR2-containing proteins that can degrade nicotinamide adenine dinucleotide (NAD) and inhibit phage replication³⁸, comparison with canonical prokaryotic Sir2 proteins and eukaryotic sirtuins revealed marked differences. In particular, the SAMD9 SIR2-like domain lacks the NAD-binding cap and Zn^{2+} -binding subdomain characteristic of canonical sirtuins (**Fig. S5**), raising the possibility that its functional properties differ from those of conventional NAD-dependent deacylases. Previous studies of rabbit SAMD9 further showed that the minimal functional tRNase module extends into the SIR2-containing region³⁷, suggesting that the SIR2-like domain may contribute to the integrity or activity of the N-terminal tRNase effector module.

Adjacent to the SIR2-like domain, the P-loop/NTPase domain occupies a prominent central position and contains a conserved nucleotide-binding core, including the Walker A and Walker B motifs (**Fig. 1B–C and Fig. S6**). Its extensive contacts with

surrounding regions place it at a key structural junction within SAMD9. TPR1 and TPR2 extend from this region as elongated α -helical repeat arrays, forming a curved scaffold along one side of the molecule (**Fig. 1B–C and Fig. S2C–D**). At the distal end of TPR2, the C-terminal OB domain folds back toward the P-loop/NTPase region, bringing sequence-distant regions into close spatial proximity.

Disease-associated variants and C-terminal interdomain interactions reveal an autoinhibitory architecture of SAMD9

To date, approximately 95 pathogenic SAMD9 variants have been identified in patients with MIRAGE syndrome and other SAMD9-associated disorders¹¹. Mapping these variants onto our cryo-EM structure showed that they are broadly distributed across nearly all major structural domains, with more than half located within the SIR2-like and P-loop/NTPase domains (**Fig. 2A**). A subset lies at or near interdomain interfaces, suggesting that disruption of intramolecular contacts may contribute to SAMD9 dysregulation. We therefore examined these interfaces in greater detail.

The SIR2-like domain forms extensive contacts with both the P-loop/NTPase and TPR1 domains (**Fig. 2B–C**). At the SIR2-like–NTPase interface, the first α -helix of the NTPase domain packs against helices and connecting loops on the outer surface of the SIR2-like domain through a combination of polar and hydrophobic interactions. Disease-associated variants including R459Q, N484S, and K643E lie within or adjacent to this interface (**Fig. 2B**). At the SIR2-like–TPR1 interface, D532 forms a salt bridge with K1024 at a distance of approximately 2.9 Å (**Fig. 2C**), providing a structural explanation for how the disease-associated K1024E substitution could weaken this interdomain contact. The P-loop/NTPase domain also contacts TPR1 through an extended interaction network involving T767 and residues surrounding the Walker B region (**Fig. 2D**). Together, these interfaces position the SIR2-like, P-loop/NTPase, and TPR1 domains within a tightly interconnected structural core.

Additional contacts organize the C-terminal half of SAMD9. TPR1 and TPR2 form an extensive helical interface containing several disease-associated variants, including

R1293Q/W, Y1274C, P1280L, and I1478T (**Fig. 2E**). TPR2 further contacts the C-terminal OB domain through a predominantly hydrophobic interface, with R1529 forming a salt bridge with D1585 near the C terminus of the OB fold (**Fig. 2F**). These interactions position the OB domain against the TPR scaffold and constrain its orientation within the compact C-terminal core. Strikingly, the OB domain also folds inward toward the P-loop/NTPase domain, generating a long-range contact between regions that are widely separated in the primary sequence (**Fig. 2G**). The recurrent disease-associated R824Q substitution maps to the NTPase side of this interface, whereas G1564S lies on the opposing OB surface, suggesting that this contact may represent a regulatory site susceptible to disease-associated perturbation.

To test whether this NTPase–OB interaction contributes to SAMD9 regulation, we reconstituted SAMD9-knockout HT-29 intestinal epithelial cells with wild-type SAMD9 or the R824Q variant in the presence or absence of the OB domain and measured basal levels of type I interferon (IFN β) expression without viral infection or poly(I:C) stimulation. The R824Q mutation markedly increased basal IFN β expression relative to wild-type SAMD9, and the effect was further enhanced by the additional OB deletion (**Fig. 2H**). Notably, SAMD9 R824Q mutant was expressed at a lower level compared to the other proteins (**Fig. 2I**), and yet it induced a much stronger innate immune response. These results indicate that the C-terminal OB-containing region contributes to maintaining SAMD9 in a restrained state under unstimulated conditions and that perturbation of the NTPase–OB interface can partially relieve this restraint. Together with the extensive contacts between the OB domain and the neighboring NTPase and TPR regions, these findings identify the C-terminal OB fold as a structural determinant of SAMD9 autoinhibition.

P-loop/NTPase-mediated dimerization is required for SAMD9-dependent innate immune signaling

Two-dimensional classification of the cryo-EM reconstruction revealed that hSAMD9 predominantly assembles as a homodimer (**Fig. 3A and S2A**). Although the

high-resolution structural details described above were obtained by focused refinement of a single protomer within the dimer, the overall reconstruction consistently showed a two-fold symmetric assembly. The hSAMD9 dimer adopts a butterfly-like architecture, with the two protomers forming opposing wing-like lobes connected through a central contact. The assembly measures approximately $135 \text{ \AA} \times 115 \text{ \AA} \times 90 \text{ \AA}$, and the dimer interface is formed primarily by the two P-loop/NTPase domains positioned at the center of the complex.

Closer inspection of this interface revealed a network of electrostatic and polar interactions between the two NTPase domains (**Fig. 3B**). K860, R863, and K870 from one protomer are positioned opposite acidic residues, including E699 and E874, from the neighboring protomer, with Y701 contributing additional contacts. The same dimer interface residues have been identified in Mou Z, bioRxiv, 2026 but not in Zou J, Langtaosha, 2026. Notably, the residues forming this central dimer interface are fully conserved in SAMD9L (**Fig. S4**), indicating strong conservation of the P-loop/NTPase contact surface between the two paralogs. To test the functionality of this interface, we introduced the charge-reversal mutations K860E, R863E, and K870E.

Biochemical analysis further supported the involvement of these residues in SAMD9 dimerization. Size-exclusion chromatography (SEC) showed that the K860E/R863E/K870E mutant eluted at a lower apparent molecular weight than wild-type SAMD9, suggesting that disruption of this interface weakens dimerization and shifts the protein toward a lower-order oligomeric state, potentially a monomeric species (**Fig. 3C**). Together with the structural observations, these results indicate that the NTPase-mediated interface provides an important structural basis for SAMD9 homodimerization in solution.

We next examined whether dimerization contributes to SAMD9-mediated innate immune signaling. Due to the low transfection efficiency in HT-29 cells, we switched to interferon-competent A549 lung epithelial cells. Overexpression of wild-type SAMD9 induced the transcript levels of type III interferon (IFNL3), chemokines CCL5, CXCL10, and interferon-stimulated gene MX1, whereas the K860E/R863E/K870E mutant showed substantially reduced responses (**Fig. 3D–G**). The reduction was most

pronounced for IFNL3, CCL5, and CXCL10, with MX1 expression also attenuated. Thus, disruption of the NTPase-mediated dimer interface compromises the ability of SAMD9 to activate multiple interferon-responsive genes.

Taken together, these findings identify the P-loop/NTPase domain as a major interface mediating SAMD9 homodimerization and show that disruption of this interface impairs SAMD9-dependent innate immune signaling. We next examined whether the nucleotide-binding properties of this domain are similarly required for signaling activity.

The ATP-binding module of SAMD9 is functionally separable from innate immune signaling

Given the role of the P-loop/NTPase domain in SAMD9 dimerization, we next asked whether its nucleotide-binding properties are similarly required for SAMD9 functions. The cryo-EM map revealed a well-defined nucleotide density within the P-loop/NTPase domain that could be modeled as ATP (**Fig. 4A–B, and S6A**). Because no nucleotide was added during cryo-EM sample preparation, the bound ATP was likely acquired during recombinant expression and retained throughout purification.

ATP is accommodated within a pocket formed by residues surrounding the Walker A and Walker B regions. The phosphate moieties are positioned adjacent to T744 and T745 and coordinated within a Mg^{2+} -containing environment, with D799 contributing to Mg^{2+} coordination. Additional interactions involving H737, R837, R982, and S986 further stabilize the nucleotide (**Fig. 4B**). The adenine moiety is accommodated within a predominantly hydrophobic cavity formed by F683, F705, F865, and F885 (**Fig. 4C**). Among these residues, R982 contributes indirectly to phosphate recognition through a water-mediated interaction. Notably, R982 is a recurrent disease-associated hotspot in SAMD9, with R982C and R982H variants reported in patients. The corresponding residue in SAMD9L, R986, is highly conserved and is likewise a recurrent pathogenic hotspot (**Fig. S4**), highlighting the potential regulatory importance of this region,

although whether these substitutions directly alter nucleotide binding remains to be determined.

Despite the presence of a well-defined ATP-binding pocket, SAMD9 contains a noncanonical Walker A motif. Structural comparison with the nucleotide-binding domain of Apaf-1 revealed an overall similar P-loop/NTPase fold but substantial differences around the Walker A region (**Fig. 4D and Fig. S6B–D**). In Apaf-1, the conserved Walker A lysine is represented by K160, whereas the equivalent position in SAMD9 is occupied by G743. This noncanonical configuration is also conserved in SAMD9L (**Fig. S4 and Fig. S6D**). By contrast, the Walker B residue D799 occupies a position similar to that of D243 in Apaf-1. Thus, SAMD9 retains a recognizable nucleotide-binding architecture despite divergence of a key feature of the canonical Walker A motif.

We next examined whether purified SAMD9 exhibits detectable ATP hydrolysis activity. No measurable ATP hydrolysis was observed under the conditions tested (**Fig. 4E**), indicating that purified SAMD9 has minimal detectable hydrolytic activity under these conditions. Whether the noncanonical Walker A configuration contributes directly to this behavior remains to be established.

To determine whether the ATP-binding pocket is required for SAMD9-mediated signaling, we introduced mutations at several ATP-contacting residues, including F705A, T744A, D799A, R837A, and F865A. None of these substitutions substantially reduced IFNL3 induction relative to wild-type SAMD9 (**Fig. 4F**), and immunoblotting confirmed comparable expression of the corresponding mutant proteins (**Fig. 4G**). Thus, in contrast to the recently reported R837A mutant that loses anti-poxvirus activity²⁶, perturbation of individual ATP-contacting residues was largely tolerated in the IFNL3 response.

Together, these findings functionally separate the nucleotide-binding properties of the P-loop/NTPase domain from SAMD9-mediated interferon activation. Although SAMD9 contains a well-defined ATP-binding pocket, its purified form shows minimal detectable hydrolytic activity, and mutations of individual ATP-contacting residues do not substantially impair IFNL3 induction. These results indicate that the contribution of

the P-loop/NTPase domain to SAMD9-dependent innate immune signaling is more closely associated with protein assembly than with the integrity of its nucleotide-binding or hydrolytic machinery.

Discussion

SAMD9 and its paralog SAMD9L are interferon-inducible antiviral proteins with potent growth-restrictive activities and broad relevance to human disease. Previous studies established a tRNA-directed effector function for SAMD9, in which cleavage of tRNA^{Phe} leads to translational restriction^{24,29}, whereas our recent work showed that SAMD9 also promotes interferon signaling following cytosolic double-stranded nucleic-acid recognition³³. The present study provides a structural framework for understanding how these activities are organized within full-length SAMD9 and identifies distinct structural determinants underlying its regulatory and signaling functions.

Our cryo-EM structure reveals that SAMD9 forms an integrated multidomain core stabilized by extensive intramolecular interactions among the SIR2-like, P-loop/NTPase, TPR, and OB domains (**Fig. 1**). Disease-associated variants are distributed throughout this structural network, with several mapping directly to interdomain interfaces. These observations suggest that the integrity of the intramolecular interaction network is important for maintaining the regulated state of SAMD9 and provide a structural context for understanding how disease-associated substitutions may perturb its conformational organization.

A particularly important feature of this architecture is the C-terminal OB fold, which is positioned through contacts with both the P-loop/NTPase and TPR regions (**Fig. 2**). Functional analysis of the NTPase–OB interface showed that the disease-associated R824Q substitution increased basal IFNB expression, while deletion of the OB domain further enhanced this response. Together, these findings support a model in which the C-terminal OB-containing region contributes to a self-inhibitory state that restrains SAMD9 under basal conditions. Thus, beyond its contribution to nucleic-acid

recognition, the OB fold also serves as a structural determinant of SAMD9 auto-inhibition.

The cryo-EM reconstruction further revealed that the majority of SAMD9 adopts a homodimeric organization mediated primarily by charged residues within the P-loop/NTPase domain (**Fig. 3**). Our previous work showed that nucleic-acid engagement promotes SAMD9 oligomerization³³, but the structural basis and functional significance of this process were previously unclear. Disruption of the dimer interface shifted SAMD9 toward a species of smaller apparent molecular size, consistent with a transition from a dimeric toward a monomeric state, and markedly reduced induction of multiple interferon-stimulated genes. These results establish a direct link between NTPase-mediated assembly and SAMD9-dependent innate immune signaling, indicating that formation or stabilization of the dimeric state is an important component of the signaling-competent configuration of SAMD9.

By contrast, the nucleotide-binding properties of the P-loop/NTPase domain appear to make a more limited contribution to interferon activation (**Fig. 4**). Although SAMD9 contains a well-defined ATP-binding pocket, the domain also harbors a noncanonical Walker A configuration, and purified SAMD9 exhibits minimal detectable hydrolytic activity under the conditions tested. More importantly, mutations of several ATP-contacting residues did not substantially impair IFNL3 induction, in clear contrast to mutations that disrupt the dimer interface. These findings indicate that the contribution of the P-loop/NTPase domain to innate immune signaling is more closely associated with protein assembly than with the integrity of its nucleotide-binding or hydrolytic machinery.

The recurrent disease-associated R982 position nevertheless suggests that the nucleotide-binding region may have additional regulatory significance. R982 contributes to the nucleotide-binding environment, and the homologous R986 residue in SAMD9L is likewise a recurrent pathogenic hotspot^{17,18}. This conservation points to a shared regulatory vulnerability in the two paralogs. The consequences of these substitutions remain unclear, however, and may involve nucleotide recognition, structural stability, or conformational coupling rather than nucleotide hydrolysis itself.

Together with previous functional studies, our results support a mechanistic separation between SAMD9-mediated interferon signaling and its established tRNA ribonuclease activity. The N-terminal region mediates tRNA^{Phe}-directed translational restriction^{24,29}, whereas interferon signaling remains largely intact when tRNA binding is disrupted but is strongly impaired when P-loop/NTPase-mediated assembly is perturbed. In this framework, the N-terminal region functions primarily as a direct antiviral effector module, while the C-terminal core provides regulatory and assembly determinants required for innate immune signaling. The OB-containing region contributes to maintaining an autoinhibited state, whereas P-loop/NTPase-mediated dimerization promotes a signaling-competent configuration.

Several questions remain unresolved. The N-terminal effector region is conformationally flexible and was not visualized in the present reconstruction, preventing direct observation of how it communicates with the ordered C-terminal core. The physiological significance of ATP binding also remains unclear, as does the relationship between the homodimer observed here and the higher-order assemblies formed following nucleic-acid engagement. Defining how nucleic-acid recognition, relief of auto-inhibition, dimerization, and tRNase activation are coordinated will be important for understanding how SAMD9 transitions between its distinct immune functions.

In summary, our study defines a structural basis for SAMD9 regulation and functional specialization. An integrated multidomain core stabilized by disease-relevant intramolecular interactions maintains the regulated architecture of the protein, the C-terminal OB fold contributes to auto-inhibition, and P-loop/NTPase-mediated homodimerization is required for efficient interferon signaling. By contrast, the nucleotide-binding pocket is largely dispensable for interferon activation. These findings provide a structural framework for understanding the functional separation of SAMD9-dependent interferon signaling from its previously established tRNA ribonuclease effector function.

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Contributions

N.Y. and S.D. conceived and designed the study. J.Z. and X.C. prepared samples for cryo-EM analysis and generated all constructs. J.Z. and X.C. collected the cryo-EM data. J.Z. processed the cryo-EM data and built and refined the atomic models. G.H. and D.C. performed the cellular experiments and analyzed interferon-responsive gene expression in HT-29 and A549 cells. X.C. performed the EMSA experiments. J.Z., X.C., G.H., and D.C. prepared the figures. J.Z. and S.D. analyzed and interpreted the results. J.Z. and S.D. wrote the manuscript, and all authors reviewed and revised the manuscript.

Materials and Methods

Protein expression and purification

The gene encoding full-length human SAMD9 (hSAMD9; UniProt accession Q5K651) was amplified from a cDNA library and cloned into a modified pFastBac vector (Thermo Fisher Scientific). A Twin-Strep tag was fused to the N terminus to facilitate protein expression and purification. Truncated variants, including SAMD9(Δ SAM–AlbA2) (residues 387–1589), SAMD9(Δ OB) (residues 1–1529), and SAMD9(Δ SAM–AlbA2/ Δ OB) (residues 387–1529), were generated and purified using

the same procedure. All point mutations were introduced by standard site-directed mutagenesis, and all recombinant plasmids were verified by DNA sequencing. SAMD9 proteins were expressed in *Spodoptera frugiperda* Sf9 cells using the Bac-to-Bac system (Invitrogen). When the cell density reached approximately 2.5×10^6 cells/ml, cells were infected with P2 recombinant baculovirus at a ratio of 1:100 (v/v). Cells were harvested after 60 h after infection and stored at -80°C until purification.

Cell pellets were resuspended in buffer A containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM β -mercaptoethanol (β -ME), and a protease inhibitor cocktail containing 1 mM PMSF, 0.8 μM pepstatin, 2 μM leupeptin, and 2 μM aprotinin, followed by disruption using a Dounce homogenizer. Insoluble material was removed by ultracentrifugation at $100,000 \times g$ for 35 min. The supernatant was passed through a 0.22- μm filter and incubated with Strep-Tactin resin. The resin was washed with 10 column volumes of buffer A, and bound protein was eluted with buffer A supplemented with 5 mM desthiobiotin. The eluate was concentrated and further purified by size-exclusion chromatography using a Superose 6 Increase 10/300 GL column equilibrated with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM β -ME. Peak fractions were pooled and concentrated to approximately 5 mg/mL for cryo-EM sample preparation.

Cryo-EM sample preparation and data collection

Aliquots of 3.5 μL freshly prepared hSAMD9 samples were applied to glow-discharged holey gold grids (Quantifoil Au R1.2/1.3, 300 mesh, Germany). The grids were blotted for 3.0–4.5 s and plunge-frozen in liquid ethane cooled by liquid nitrogen using an FEI Vitrobot Mark IV (Thermo Fisher Scientific, USA) at 8°C and 100% humidity. Cryo-EM data were collected on a 300 kV Titan Krios transmission electron microscope (Thermo Fisher Scientific, USA) equipped with a K2 Summit direct electron detector (Gatan, USA) and a Quantum GIF energy filter (Gatan, USA) with a slit width of 20 eV. Movie stacks were automatically acquired using SerialEM at a calibrated magnification of $\times 130,000$, corresponding to a physical pixel size of 1.0825 Å (0.54125

Å in super-resolution mode). The defocus range was set from -1.2 to -2.0 μm . The dose rate was adjusted to 10 counts/pixel/s, with an exposure time of 6.4 s, resulting in a total dose of approximately $50 \text{ e}^-/\text{\AA}^2$. A total of 1,910 movie stacks were collected for hSAMD9, with each movie fractionated into 32 frames.

Data processing

Movie stacks were first subjected to beam-induced motion correction, two-fold binning, and dose weighting using MotionCor2. The resulting summed micrographs were manually inspected to remove poor-quality images. Contrast transfer function parameters were estimated for each micrograph, and only micrographs with CTF fits better than 5 Å were retained for particle picking. A total of 2,388,417 particles were automatically picked for hSAMD9. Subsequent two-dimensional classification, three-dimensional classification, particle refinement, and CTF refinement were performed in cryoSPARC³⁹. After iterative classification and refinement, the final hSAMD9 reconstruction reached an overall resolution of 2.9 Å. The detailed cryo-EM data-processing workflow is shown in Extended Data Fig. 2.

Model building

To build the atomic model of hSAMD9, the AlphaFold3-predicted model was initially fitted into the corresponding cryo-EM density map using UCSF Chimera⁴⁰. The model was subsequently subjected to multiple rounds of manual inspection and adjustment in Coot⁴¹, followed by real-space refinement in PHENIX⁴². Model-to-map Fourier shell correlation (FSC) curves were calculated using phenix.real_space_refine. Statistics for cryo-EM data collection and model refinement are summarized in Supplementary Table 1. Structural analyses and figure preparation were performed using PyMOL (Schrödinger, LLC), GraphPad Prism 8.0.1 (GraphPad Software), and UCSF ChimeraX⁴³.

EMSA assay

To generate 512-bp dsDNA fragments, we designed a pair of primers based on a randomly selected sequence from pUC19. The dsDNA fragments were amplified by KOD DNA polymerase (Sigma-Aldrich # KMM-101NVS) and purified from agarose gel using Universal DNA Purification Kit (TIANGEN #DP214-03).

Each SAMD9 protein variant (1 μ M), including SAMD9(WT), SAMD9 Δ SAM-AlbA2, SAMD9 Δ OB, SAMD9 Δ SAM-AlbA2/ Δ OB, and SAMD9(K860E/R863E/K870E), was incubated with 512-bp dsDNA (50 nM) in EMSA buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 5 mM MgCl₂, and 4% glycerol. The mixtures were incubated at 30 °C for 1.5 h. The mixtures were then loaded onto 1% agarose gel prepared in 1x tris-acetate-EDTA (TAE) buffer and run at 180V for 30 min at room temperature. The gels were stained with Goldview Nucleic Acid Gel Stain (Yeasen, #41003) and imaged using a ChemiDoc MP Imaging System (Bio-Rad).

RT-qPCR analysis of interferon-responsive gene expression

RNA samples were extracted as described⁴⁴. RNA was reverse-transcribed by High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific #4368814) and the quantitative PCR was performed by SYBR Green qPCR ReadyMix (Sigma-Aldrich #KCQS00). The primers used in this study were: GAPDH forward primer: 5'-GGAGCGAGATCCCTC CAAAAT-3', reverse primer: 5'-GGCTGTTGTCATACTTCTCATGG-3'; IFNB forward primer: 5'-ATGACCAACAAGT GTCTCCTCC-3', reverse primer: 5'-GGAATCCAAGCAA GTTGTAGCTC-3'; IFNL3 forward primer: 5'-TAAGAGGGCCAAAGATGCCTT-3', reverse primer: 5'-CTGGTCCAA GACATCCCCC-3'; CCL5 forward primer: 5'-CCAGCAGTCGTC TTTGTAC-3', reverse primer: 5'-CTCTGGGTTGGCACACACTT-3'; CXCL10 forward primer: 5'-GTGGCATT C AAGGAGTACCTC-3', reverse primer: 5'-TGATGGCCTTCGAT TCTGGATT-3'; MX1 forward primer: 5'-GTGGCTGAGAACAACCTGTG-3', reverse primer: 5'-GGCATCTGG TCACGATCCC-3'.

ATPase activity assay

ATPase activity of recombinant human SAMD9 was measured using the ATPase-Glo Assay (Promega) according to the manufacturer's instructions with modifications. Purified His-tagged SAMD9 was prepared in protein buffer containing 20 mM phosphate buffer (PB, pH 7.0), 300 mM NaCl, and 10% glycerol. ATPase reactions were performed in a final volume of 10 μ l containing 0.138 μ M His-SAMD9, 1.5 mM $MgCl_2$, 1 mM DTT, and 100 μ M ATP. Control reactions contained an equivalent volume of protein buffer in place of His-SAMD9. Reaction mixtures were incubated for 1 h at 30°C.

Following the ATP hydrolysis reaction, 10 μ l of ATPase-Glo reagent was added to each 10 μ l reaction mixture, and incubated for 40 min at room temperature. Subsequently, 20 μ l of Detection Reagent was added, followed by an additional 40 min incubation at room temperature. Luminescence was measured in a white 96-well plate using a microplate reader with an integration time of 1 s per well. The ATPase-Glo assay quantifies residual ATP, in which lower luminescence values indicate greater ATP hydrolysis and thus higher ATPase activity.

Data Availability

The UniProt accession code for the sequence of human SAMD9 is Q5K651. The three-dimensional cryo-EM density map of hSAMD9 has been deposited in the Electron Microscopy Data Bank under accession code EMD-83565. The corresponding atomic coordinates have been deposited in the Protein Data Bank under accession code 45VL.

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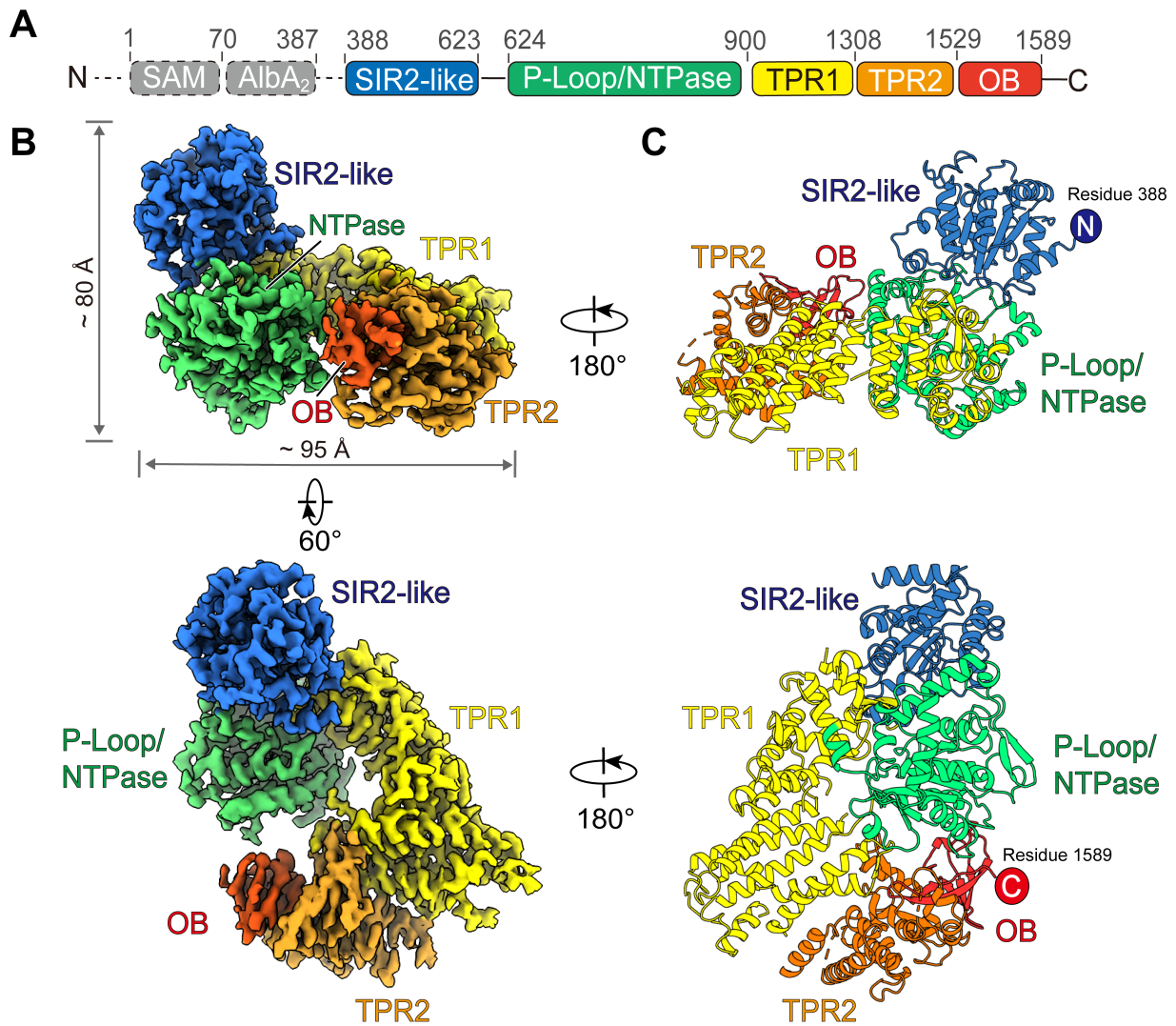


Figure 1. Cryo-EM structure of human SAMD9

A, Domain organization of human SAMD9. **B**, Cryo-EM density map of SAMD9 shown in two views. **C**, Cartoon representation of the SAMD9 structure. The SIR2-like, P-loop/NTPase, TPR1, TPR2, and OB domains are colored blue, green, yellow, orange, and red, respectively. The N- and C-terminal residues resolved in the structure are indicated.

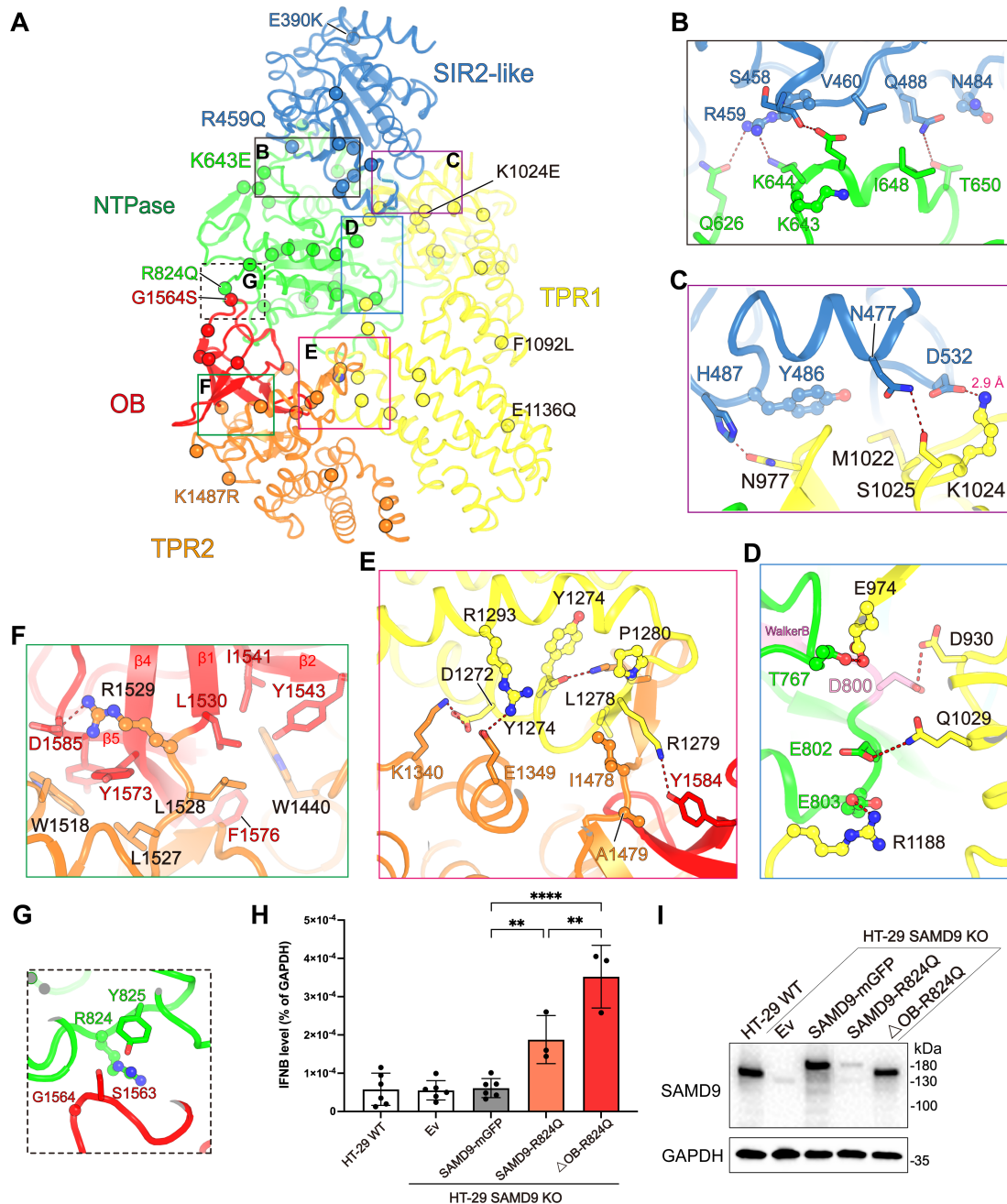


Figure 2. Disease-associated interdomain interactions reveal an autoinhibitory role of the C-terminal OB domain

A, Distribution of disease-associated variants on the SAMD9 structure. Dashed boxes indicate the interdomain interfaces shown in **B–G**. **B**, Interface between the SIR2-like and P-loop/NTPase domains. **C**, Interface between the SIR2-like and TPR1 domains. **D**, Interface between the P-loop/NTPase and TPR1 domains. **E**, Interface between the TPR1 and TPR2 domains. **F**, Interface between the TPR2 and OB domains. **G**, Interface between the P-loop/NTPase and OB domains. Key interacting residues are shown as sticks, and dashed lines indicate polar or electrostatic interactions. **H**, Basal IFN β expression in wild-type (WT) HT-29 cells and SAMD9-knockout HT-29 cells expressing empty vector (Ev), SAMD9 WT or the indicated variants. Note that the reconstituted SAMD9-mGFP migrates more slowly than endogenous SAMD9 protein. **** $P < 0.0001$ and ** $P < 0.01$ for one-way ANOVA test. **I**, Western blot analysis of SAMD9 WT and the indicated variants expressed in SAMD9-knockout HT-29 cells.

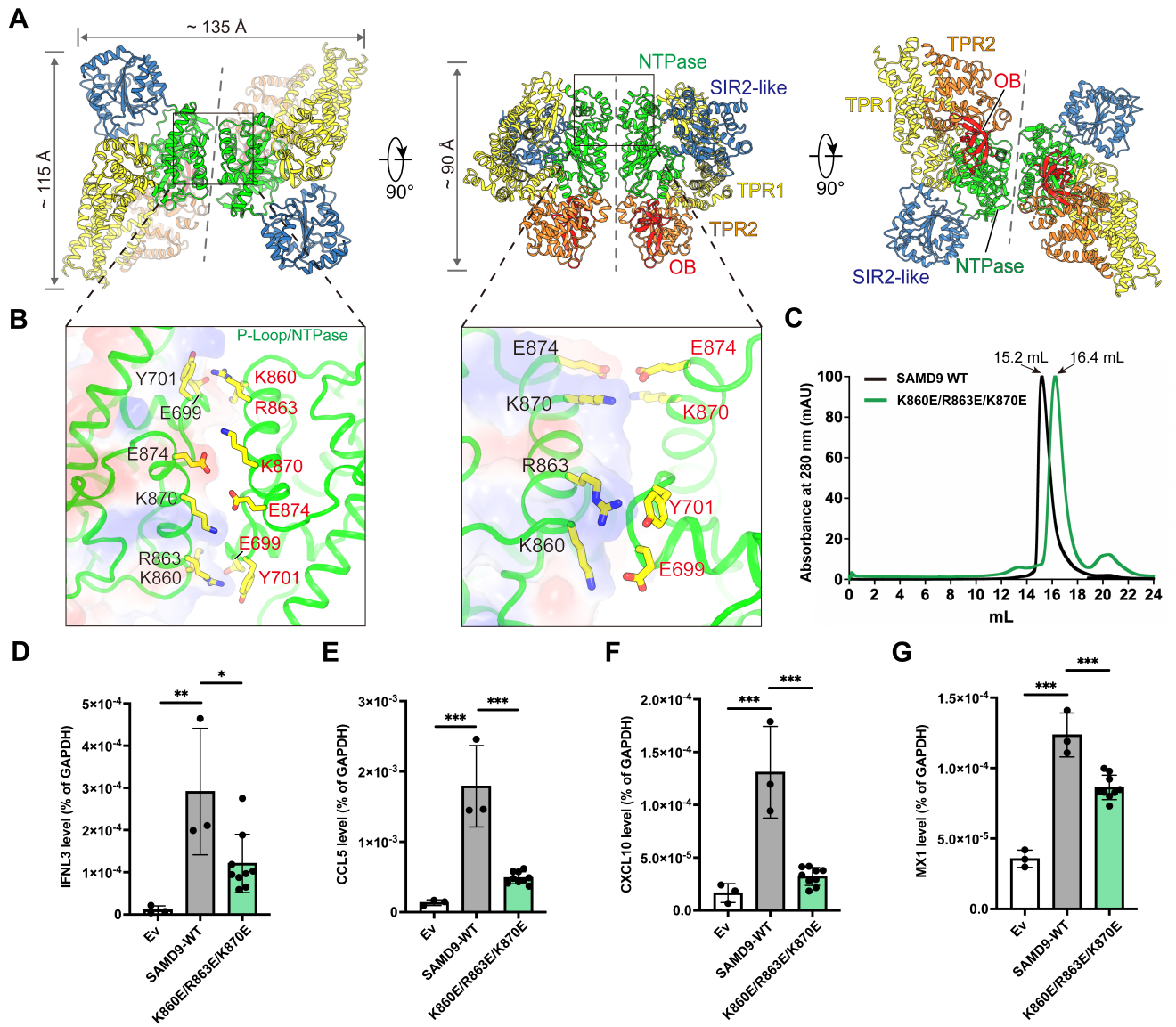


Figure 3. NTPase-mediated homodimerization is required for SAMD9-dependent interferon signaling

A, Cartoon representation of the SAMD9 homodimer shown in different views. **B**, Detailed interactions at the P-loop/NTPase-mediated dimer interface. Key interacting residues are shown as sticks. **C**, Representative gel filtration profiles of purified SAMD9 WT and the K860E/R863E/K870E mutant. **D–G**, Expression levels of IFNL3 (**D**), CCL5 (**E**), CXCL10 (**F**), and MX1 (**G**) in A549 cells expressing empty vector (Ev), SAMD9 WT or the K860E/R863E/K870E mutant. **H**, Western blot analysis of SAMD9 WT and the K860E/R863E/K870E mutant in A549 cells. Data are shown as means $\pm$ SD. Statistical significance is indicated in the figure.

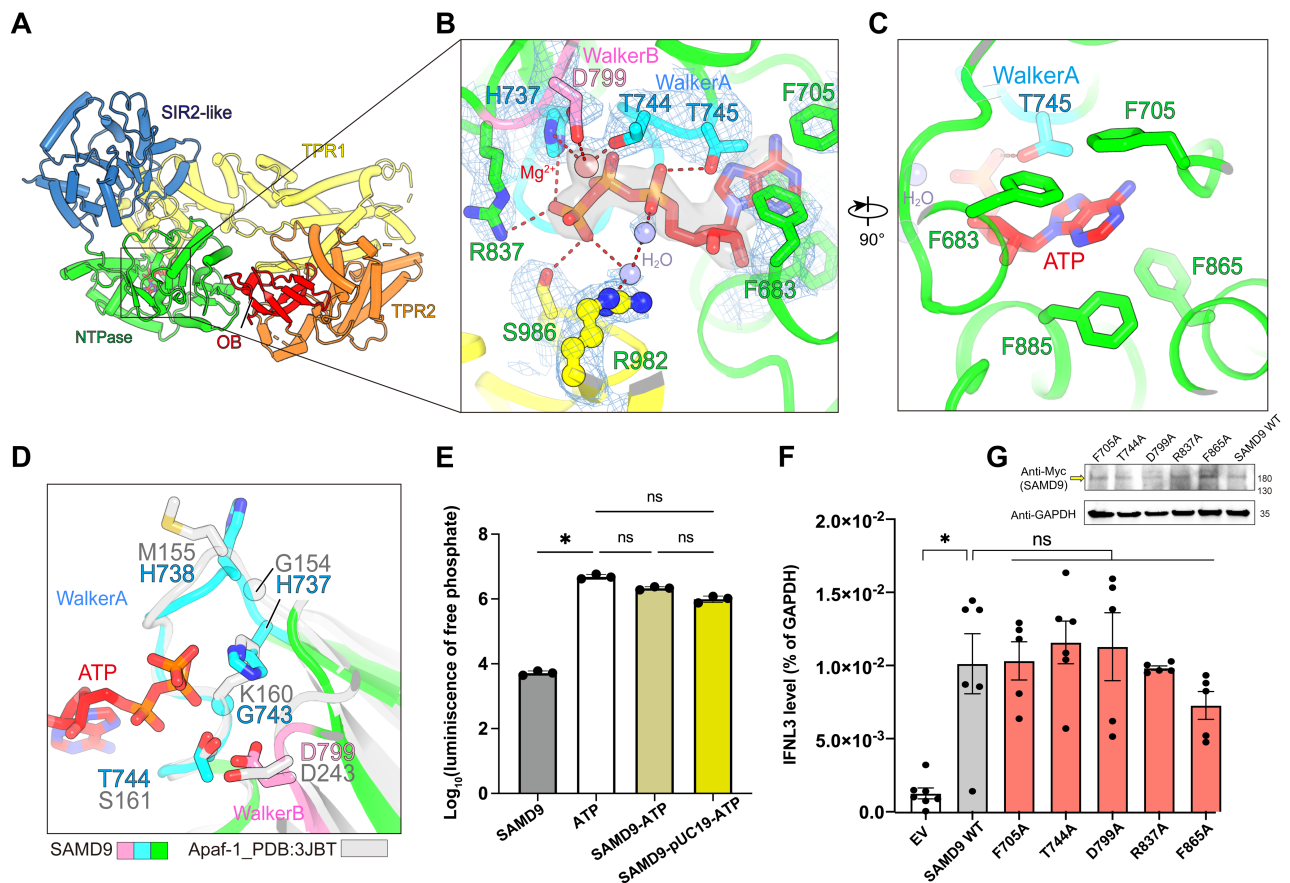


Figure 4. ATP binding is functionally separable from SAMD9-mediated interferon activation

A, Overall structure of SAMD9 showing the location of the ATP-binding pocket. **B**, Detailed interactions of ATP with residues surrounding the Walker A and Walker B motifs. Cryo-EM densities for ATP and the Mg^{2+} ion are shown as surfaces, whereas densities for the surrounding residues are shown as mesh; all densities are contoured at 8σ . Ordered water molecules involved in ATP coordination are indicated. **C**, Hydrophobic interactions surrounding the adenine moiety of ATP. **D**, Structural comparison of the Walker A and Walker B regions of SAMD9 and Apaf-1 (PDB: 3JBT). **E**, ATP hydrolysis assay of purified SAMD9 with and without dsDNA binding. **F**, IFNL3 expression in A549 cells expressing empty vector, SAMD9 WT or the indicated ATP-contacting mutants. **G**, Western blot analysis of SAMD9 WT and the indicated mutants in A549 cells. Data are shown as means $\pm$ SD.