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Short Report

Keywords:

Posted Date: August 3rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10482981/v1>

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Additional Declarations: Competing interest reported. J.P.E. is an employee of Arctoris Inc. All other authors were employees of Genentech, Inc., or its parent company, F. Hoffmann-La Roche Ltd, during the period in which this research was conducted. The authors declare no further competing interests.

TITLE

A Minimal Two-State Allosteric Dimer Model for SARM1 Regulation and Paradoxical Activation by Orthosteric Inhibitors

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ABSTRACT

Sterile alpha and Toll/interleukin-1 receptor motif-containing protein 1 (SARM1) is an oligomeric NAD⁺-consuming enzyme that drives programmed axonal degeneration. SARM1 activity is controlled by opposing metabolites: NAD⁺ stabilizes an inactive conformation, whereas nicotinamide mononucleotide (NMN) promotes activation. Orthosteric base-exchange inhibitors (BEIs) were developed to suppress SARM1 activity, but several studies have reported paradoxical activation at subinhibitory concentrations. Here, we integrate biochemical kinetics, binding measurements, structural analysis, and cellular assays to evaluate a two-state Monod-Wyman-Changeux framework for SARM1 regulation. The data support a model in which SARM1 favors an inactive T state without activating ligand, NMN stabilizes an active R state, and NAD⁺ both allosterically favors the inactive state and serves as substrate. The transition occurs concertedly through a minimal dimeric cooperative unit that generates two catalytic sites. This framework explains NAD⁺-dependent substrate inhibition, state-dependent cooperativity, and low-dose activation by orthosteric BEIs, with implications for targeting oligomeric enzymes with concerted transitions.

INTRODUCTION

Sterile alpha and Toll/interleukin-1 receptor motif-containing protein 1 (SARM1) is an octameric, multidomain enzyme and a central executioner of programmed axonal degeneration^{1–4}. Each SARM1 protomer contains an N-terminal mitochondrial targeting sequence (MTS), an armadillo repeat (ARM) domain that mediates allosteric regulation, two sterile alpha motif (SAM) domains that promote oligomerization, and a C-terminal Toll/interleukin-1 receptor (TIR) domain that carries catalytic activity (Figure 1A)^{5–7}. Structural studies indicate that the ARM allosteric site can adopt ligand-dependent conformations associated with inactive and active states of the enzyme (Figure 1A)^{7–9}.

SARM1 catalyzes base-exchange reactions in which the nicotinamide (NAM) moiety of nicotinamide adenine dinucleotide (NAD⁺) is replaced by water, its own adenine, or an exogenous heterocyclic base to yield ADPR, cADPR, or a base-exchange product, respectively^{3,8,10–13}. In neurons, SARM1 activity is regulated by the balance between NAD⁺ and its precursor nicotinamide mononucleotide (NMN). Under basal conditions, total cellular NAD⁺ levels are maintained in the 200–800 μ M range^{14,15}, whereas NMN remains in the low micromolar range¹³. In this NAD⁺-favored state, NAD⁺ binding at the ARM allosteric site stabilizes an autoinhibited conformation. Following axonal injury or disease, damage to the

1 microtubule network disrupts anterograde transport of NMNAT2, an axonal NAD⁺
2 biosynthetic enzyme that converts NMN to NAD⁺. Loss of NMNAT2 delivery
3 reduces local NAD⁺ synthesis while allowing NMN to accumulate, thereby shifting
4 the metabolite balance toward SARM1 activation¹³. As NMN accumulates, it
5 binds the ARM allosteric site and stabilizes the active conformation of SARM1.
6 Active SARM1 rapidly depletes NAD⁺, triggering bioenergetic failure and axon
7 fragmentation^{9,16}.

8 Because of its central role in axonal degeneration, SARM1 has emerged as an
9 attractive therapeutic target for the treatment of neurodegenerative conditions
10 such as peripheral neuropathies and amyotrophic lateral sclerosis. Much of the
11 published pharmacology has focused on orthosteric inhibitors that exploit the
12 SARM1 base-exchange mechanism^{8,12,17–23}. These base-exchange inhibitors
13 (BEIs) bind uncompetitively at the catalytic site and are converted by SARM1 into
14 base-exchange ADPR adducts that, as NAD⁺ analogs, compete with substrate
15 binding²². A prominent and unexpected recent finding is that low concentrations
16 of BEIs can paradoxically activate SARM1, leading to neurodegeneration and
17 serious adverse effects in vivo. This effect was first reported by Leahey et al.¹⁹
18 and subsequently supported by studies from other researchers^{17,18,21,23–25}.

19 Despite substantial progress in understanding SARM1 regulation by NAD⁺ and
20 NMN, a unifying kinetic framework for SARM1 regulation is still lacking. In
21 particular, current models do not fully account for three key observations:
22 substrate inhibition at high NAD⁺ concentrations, regulation by the NMN/NAD⁺
23 ratio, and paradoxical activation at low concentrations of orthosteric BEIs.
24 Existing frameworks, in which NAD⁺ and NMN compete for binding to the ARM
25 allosteric site to induce inactive or active conformations, explain activation and
26 inhibition qualitatively but do not readily explain substrate inhibition or low-dose
27 activation by orthosteric ligands.

28 An elegant and broadly applicable framework for explaining cooperative behavior
29 in oligomeric enzymes was proposed by Monod, Wyman, and Changeux (MWC)
30 in 1965^{26,27}. The MWC model describes oligomeric proteins made of identical
31 subunits (protomers) that can exist in two states, inactive (T), and active (R),
32 which are in equilibrium. Subunits transition between these states in a concerted
33 (all-or-none) manner. In its simplest form, ligands bind exclusively to either the T
34 or the R state and their affinities (dissociation constants) are identical for all
35 individual subunits and independent of occupancy of ligands at adjacent units.
36 Therefore, preferential binding of ligands to T or R states shifts the equilibrium
37 toward the corresponding conformation. This concerted transition explains the
38 basis of MWC allostery and cooperativity.

In this work, we integrate biochemical kinetics, surface plasmon resonance (SPR), cryogenic electron microscopy (cryo-EM), and cellular assays to show that a minimal two-state MWC framework with a dimeric cooperative unit provides an effective way to interpret SARM1 regulation by endogenous ligands and orthosteric inhibitors. This framework establishes quantitative criteria for assessing the risk of unintended activation when developing inhibitors for oligomeric enzymes that undergo concerted transitions between inactive and active states.

RESULTS

SARM1 preferentially adopts an inactive conformation

To assess the conformational state of ligand-free SARM1, we performed cryoEM classification of apo SARM1^{ΔMTS} particles (a SARM1 construct lacking a mitochondrial targeting sequence). Many individual ARM domains were poorly resolved, possibly due to partial denaturation at the air–water interface²⁸, but SARM1^{ΔMTS} protomers with well-defined ARM, SAM, and TIR domains exclusively displayed the ARM allosteric site in the inactive conformation (Figure 1B, Supplementary Table 1), structurally resembling the previously-described NAD⁺-bound state⁷.

To further investigate the resting state of SARM1, we characterized the regulatory effects of NAD⁺ and NMN on SARM1^{ΔMTS} activity. We used the PC6 fluorescence-based assay²⁹ to measure initial reaction velocities across a range of ligand concentrations (Supplementary Figure 1A). At all NMN concentrations tested, SARM1^{ΔMTS} showed substrate inhibition at high NAD⁺ concentrations (Figure 1D). In the absence of NMN, SARM1^{ΔMTS} activity was minimal. Together, minimal enzymatic activity in the absence of NMN and the apo structural data indicate that in the absence of ligands, SARM1^{ΔMTS} favors the inactive state.

SARM1 shows behavior consistent with a concerted allosteric transition

We next asked whether the transition between inactive and active SARM1 is consistent with a concerted allosteric mechanism. Cooperative behavior between ligand binding and enzymatic activity is a hallmark of such mechanisms. To probe cooperativity between activator (NMN) binding and SARM1^{ΔMTS} activity, we analyzed initial velocities across NMN titrations at varying NAD⁺ concentrations. Concentration-response curves were fit to a four-parameter logistic equation revealing weak but reproducible cooperativity, with a global Hill coefficient of approximately 1.4 (Figure 1C). To probe cooperativity with respect to substrate

binding, we analyzed initial velocities across NAD^+ titrations at varying NMN concentrations. After excluding data points affected by substrate inhibition, the rising phase of each curve was fit to the Hill equation (Figure 1D). At high NMN concentrations, where the enzyme population is expected to be biased toward an active state, Hill coefficients approached 1.0, consistent with near-Michaelis-Menten behavior. At low NMN concentrations, Hill coefficients rose to values greater than 1.5, indicating stronger cooperativity when $\text{SARM1}^{\Delta\text{MTS}}$ is biased toward an inactive state. Overall, these kinetic data indicate that the transition between inactive and active SARM1 is associated with detectable, albeit modest, state-dependent cooperativity.

Cryo-EM analysis revealed strong conformational coupling among ARM domains but provided more limited information about the organization of TIR-domain assemblies. Under apo and NAD^+ -only conditions (0.5 mM NAD^+), well-resolved $\text{SARM1}^{\Delta\text{MTS}}$ octamers showed all eight ARM domains in the inactive conformation, with TIR domains resolved and attached (Figure 1B, Supplementary Table 1). Under NMN-only conditions (0.5 mM NMN), all resolved ARM domains were in an active conformation and TIR density was not observed. In the presence of NAD^+ , NMN, and high concentrations of NB-3, an orthosteric uncompetitive binder, oligomeric TIR assemblies were observed above the ARM-SAM ring (Figure 1B, Supplementary Table 1), consistent with published findings which demonstrated stabilization of higher-order catalytic interfaces by orthosteric ligands⁸. An important finding was that in the presence of equimolar NAD^+ and NMN (0.5 mM), resolved $\text{SARM1}^{\Delta\text{MTS}}$ particles were found exclusively in either all-active or all-inactive ARM-domain conformations (Figure 1B, Supplementary Table 1), reflecting a highly cooperative transition to the active state. Under these conditions, consistent with early observations⁸, TIR assemblies were not observed, precluding reliable quantification of higher-order catalytic interfaces and suggesting that, for purified $\text{SARM1}^{\Delta\text{MTS}}$, such interfaces are dynamic or transient, and/or rare. While these structural findings indicate that a concerted transition of ARM domains liberates all TIR domains within an octamer, the stoichiometry of active TIR domains could not be directly determined. The observed cooperativity in enzyme kinetic experiments indicates that only a subset of liberated TIR domains form catalytically competent assemblies at physiological ligand concentrations.

State-selective preferential binding of NMN and NAD^+

To quantify ligand binding to the active and inactive states of SARM1 , we measured the affinities of NAD^+ and NMN using surface plasmon resonance (SPR). $\text{SARM1}^{\Delta\text{MTS}}$, SAM-TIR (with ARM domain truncated), and ARM-SAM constructs (with TIR domain truncated) were used to distinguish binding at the catalytic and allosteric sites. The catalytic site in the SAM-TIR construct and the

allosteric site in the ARM-SAM construct lack the structural regulation each domain poses on each other and are likely biased towards the active state. Immobilization of apo-SARM1^{ΔMTS} can potentially constrain SARM1 conformational dynamics in the hydrogel. To bias SARM1^{ΔMTS} toward the inactive state, we included Compound 1, an internally developed noncompetitive allosteric inhibitor that preferentially binds to the inactive conformation (Supplementary Table 2, Supplementary Fig 2). Conversely, to bias SARM1^{ΔMTS} toward the active state, NMN (250 μM) was included in the running buffer.

SPR measurements (Figure 1E, summarized in Table 1) showed that NAD⁺ bound to the catalytic site in the active-state-biased condition with a dissociation constant (K_D) of 55 μM. At the ARM allosteric site in the active state, NMN and NAD⁺ bound with K_D of 0.24 μM and 94 μM, respectively. In contrast, NAD⁺ bound to the allosteric site of Compound 1-biased SARM1^{ΔMTS} with substantially higher affinity (9 μM), representing an approximately 10-fold increase relative to the active state. The affinity of NAD⁺ at the monomeric TIR catalytic site in the inactive state was too weak to be measured. Overall, SPR data indicated that NAD⁺ as substrate and NMN preferentially bind to the active state while NAD⁺ as allosteric inhibitor preferentially binds to the inactive state.

A two-state MWC model accounts for key features of SARM1 regulation

The observed preference of SARM1 for an inactive state, detectable cooperativity, and state-preferential ligand binding, combined with SARM1's oligomeric architecture and paradoxical activation by orthosteric inhibitors, pointed us to an allosteric concerted transition MWC model as a unifying framework to explain SARM1 regulation by its endogenous and exogenous ligands. In this framework, NMN and NAD⁺ exhibit state preferences for binding at the ARM allosteric site: NMN binds preferentially to the active (R) state, while NAD⁺ binds preferentially to the inactive (T) state. NAD⁺ also binds as a substrate to both states at the TIR catalytic site, but only the R state is catalytically competent (Figure 1F).

In the absence of ligands, SARM1 predominantly adopts the T state. NMN binding shifts the equilibrium toward the R state without altering the intrinsic catalytic rate²⁷. Within this framework, substrate inhibition can be explained by asymmetric NAD⁺ binding stoichiometry between the T and R states: one at the ARM allosteric site and one at a nonproductive orthosteric site, whereas the R state binds only a single catalytic NAD⁺. As NAD⁺ concentration increases, the population of fully NAD⁺-loaded inactive enzyme increases quadratically, overwhelming the linearly increasing population of catalytically active R-state

enzyme and producing the characteristic bell-shaped velocity–substrate relationship.

To quantitatively capture SARM1 regulation within a concerted two-state framework, we formalized the MWC model describing ligand-dependent transitions between inactive (T) and active (R) states. Following the MWC model parameterizations²⁷, the allosteric constant L is defined as the ratio of ligand-free T and R states:

$$Eq. (1.1) \quad L = T_0/R_0$$

In the presence of ligands, the apparent allosteric constant becomes:

$$Eq. (1.2) \quad L' = L \left(\frac{(1 + \beta_1)^n (1 + \beta_2)^n}{(1 + \gamma)^n (1 + \alpha)^n} \right)$$

where n is the number of binding sites and α , β_1 , β_2 , and γ are normalized concentrations for each ligand at their respective binding sites, specifically:

$\alpha = [NAD^+]/K_S^R$ where K_S^R is a dissociation constant for NAD^+ at TIR orthosteric site in the R state

$\beta_1 = [NAD^+]/K_S^T$ where K_S^T is a dissociation constant for NAD^+ at TIR orthosteric site in the T state

$\beta_2 = [NAD^+]/K_I$ where K_I is a dissociation constant for NAD^+ at ARM allosteric site in the T state

$\gamma = [NMN]/K_A$ where K_A is a dissociation constant for NMN at ARM allosteric site in the R state

The fraction of SARM1 in the active state is:

$$Eq. (1.3) \quad \bar{R} = \frac{1}{L' + 1}$$

The normalized reaction velocity is then the product of the active-state fraction $\bar{R}$ and the Michaelis–Menten substrate binding saturation term $\frac{\alpha}{(1+\alpha)}$:

$$Eq. (1.4) \quad \frac{v}{V_{max}} = \bar{R} \frac{\alpha}{(1 + \alpha)} = \frac{1}{L' + 1} * \frac{\alpha}{(1 + \alpha)}$$

We next fitted the experimentally measured initial velocities (Figure 1G) to the MWC model using the SPR-derived binding affinities as initial values (Tables 1,2). Constraining all parameters to be shared across NMN conditions yielded best-fit values that closely matched SPR affinities (Figure 1G, Table 2, Supplementary Table 3). Notably, the allosteric NAD^+ K_D from SPR (9.0 μ M, measured with Compound 1) agrees within 1.4-fold with the kinetically fitted K_I (6.6 μ M, fitted without any tool compound). This convergence is the strongest evidence that Compound 1 does not create an artifactual T state. An optimal

fitted value for n was 1.27 (Table 2). In the MWC framework, n represents the physical number of binding sites per oligomer that contribute to concerted transition. For allosteric enzymes, fitted n values are typically rounded up to the nearest integer to estimate the theoretical upper limit of cooperativity²⁷. While both $n = 2$ and $n = 1$ explain NMN-induced activation and NAD⁺-mediated substrate inhibition (Supplementary Figure 4), the dimeric model is necessary to explain SARM1 activation at low concentrations of orthosteric inhibitors.

Low concentrations of BEIs promote T→R transition and lead to SARM1 activation

A characteristic feature of concerted allosteric systems is the dual effect of orthosteric inhibitors: at low concentrations, they promote the T-to-R transition, thereby activating the enzyme, whereas at higher concentrations, they block the catalysis²⁷. This behavior is most prominent when SARM1 predominantly adopts an inactive (T) state. Previous reports have highlighted this “paradoxical” activation effect by BEIs^{17–19,21,23–25}.

To incorporate base-exchange inhibitors (BEIs) into the MWC framework, we extended the model to include an additional binding term (θ) describing BEI interaction with the catalytic site (Figure 2A):

Eq. (2.1) $\theta = [BEI]/K_{BEI}$ (normalized concentration) where K_{BEI} is a dissociation constant for a BEI at TIR catalytic site in the R state.

For competitive BEI-ADPR products, the inhibitor term is additive with substrate binding:

$$\text{Eq. (2.2)} \quad \frac{v}{v_{max}} = \frac{1}{L' + 1} * \frac{\alpha}{(1 + \alpha + \theta)} \quad \text{where } L' = L \left(\frac{(1 + \beta_1)^n (1 + \beta_2)^n}{(1 + \gamma)^n (1 + \alpha + \theta)^n} \right)$$

For uncompetitive BEI parent compounds, the inhibitor term enters as $\alpha\theta$ (see Supplementary Figure 3 for full polynomial factoring):

$$\text{Eq. (2.3)} \quad \frac{v}{v_{max}} = \frac{1}{L' + 1} * \frac{\alpha}{(1 + \alpha + \alpha\theta)} \quad \text{where } L' = L \left(\frac{(1 + \beta_1)^n (1 + \beta_2)^n}{(1 + \gamma)^n (1 + \alpha + \alpha\theta)^n} \right)$$

The extent of BEI-induced activation depends on the apparent allosteric constant L' , which defines the equilibrium between inactive (T) and active (R) states prior to inhibitor binding. When L' is large (conditions under which SARM1 predominantly adopts the T state), partial orthosteric occupancy promotes the T→R concerted transition and results in enzyme activation since for every n bound inhibitor there are $n - 1$ additionally transitioned catalytic sites. In contrast, when L' is small (biased toward the R state), BEIs no longer enhance T→R transition and instead act solely as inhibitors through active-site occupancy. This behavior is a defining feature of oligomeric systems with concerted transition and

provides a quantitative explanation for the paradoxical activation observed at subinhibitory BEI concentrations. Simulated data illustrate this predicted behavior (Figure 2B).

BEI-induced T→R transition is slow and kinetically distinct

To experimentally characterize this activation mechanism, we evaluated binding affinities, base-exchange catalytic rates, and reaction progress curves for three orthosteric BEIs: GNE-5152^{19,23}, NB-3²², and NB3-ADPR, the synthetic product of base-exchange reaction between NAD⁺ and NB-3.

SPR measurements demonstrated that NB-3 bound uncompetitively to SARM1^{ΔMTS} (Figure 2E, Suppl. Table 1), where NAD-bound TIR is a prerequisite for BEI parent binding (Figure 2E), while NB3-ADPR product bound competitively to the orthosteric site (Figure 2E). NMN affinity to the SARM1^{ΔMTS}-NB3-ADPR product complex was 10-fold more potent than for SARM1^{ΔMTS}-apo and showed similar affinity to the truncated ARM construct, consistent with preferred binding to the active state (Table 1). Neither the BE parent nor the BE-ADPR product showed binding to the ARM allosteric site in either the T or R states (Supplementary Table 2). An important finding was that both NB-3 and NB3-ADPR exhibited slow association and dissociation kinetics (Table 1, Supplementary Table 2).

In addition to their slow association and dissociation kinetics, BEIs are relatively poor substrates for SARM1-catalyzed base-exchange reactions (ADPR addition). To quantify the rate of conversion of BEI parent compounds to their corresponding ADPR adducts, we monitored formation of NB3-ADPR by LC-MS. Initial velocities of NB3-ADPR formation were fitted to the Michaelis-Menten model to estimate k_{cat} and K_M values for the second step of SARM1-catalyzed displacement reaction (Figure 2F; Table 3), in which the covalent ADPR-enzyme intermediate undergoes nucleophilic substitution. At low NB-3 concentrations, we observed higher rates of base exchange than predicted by the fitted model (Figure 2F open circles). We interpret this deviation as reflecting substoichiometric activation of SARM1, consistent with partial orthosteric occupancy promoting the T→R transition. Fitted k_{cat} and K_M values were reproducible across experiments performed at different enzyme concentrations (Table 3) and indicate that BEI turnover is orders of magnitude slower than reported NAD⁺ hydrolysis rates^{3,7,8,10,11,13}.

In biochemical PC6 assays, a prominent observation was a greater enzymatic activity in the presence of low concentration of inhibitors as compared to DMSO controls after 2 hours of reaction (red progress curves in Figure 2C). This persistent activity was less prominent at low NAD⁺ concentrations (SARM1 in R-

state, Figure 2C left panels). This slow onset of activation, which we speculate results from slow association kinetics, precluded rapid-equilibrium analysis of initial velocities for direct fitting to the MWC model.

We next examined the effects of BEIs on endogenous SARM1 activity in live SH-SY5Y human neuroblastoma cells using the 1a fluorescent probe, a PC6 analog with red-shifted excitation/emission spectra³⁰. We collected progress curves of the AD-1a formation (fluorescent product of SARM1-catalyzed conversion of the 1a parent to the AD-1a ADPR adduct) after SARM1 activation with a submaximal concentration of vacor (Figure 2D). Under these conditions, progress curves were sufficiently linear to permit estimation of initial velocities, and GNE-5152 caused activation at low, subinhibitory concentrations (0.39-5.8 nM, Figure 2D). Consistent with the model, this activation was not observed at high vacor concentrations, where the enzyme was expected to be predominantly R-biased.

DISCUSSION

Our kinetic, structural, and cellular data support a two-state MWC framework as a useful model for SARM1 regulation. In this framework, SARM1 has a strong preference toward an inactive basal state, NMN shifts the population toward an active state, and NAD⁺ allosterically stabilizes the inactive state while also serving as substrate at the catalytic site. This model provides a coherent explanation for three otherwise difficult to unify observations: regulation by the NMN/NAD⁺ balance, substrate inhibition at high NAD⁺ concentrations, and activation by low concentrations of orthosteric inhibitors, which is more pronounced under conditions that favor the inactive state.

A limitation of the quantitative MWC analysis presented here is that some fitted parameters are not independently identifiable. In particular, the allosteric constant L and the NMN affinity parameter K_A were strongly anticorrelated in the covariance matrix ($r = -0.98$), indicating that these parameters trade off against one another in describing ligand-dependent stabilization of the R state. Thus, the fitted values of L and K_A should be interpreted as a coupled parameter set rather than as independently precise estimates. Importantly, this limitation does not affect the central conclusion that SARM1 strongly favors the inactive T state in the absence of activating ligand. This conclusion is independently supported by cryo-EM analysis of apo SARM1^{ΔMTS}, near-zero enzymatic activity in the absence of NMN, and preferential NMN binding to the active state. Therefore, although the precise numerical value of L should be interpreted cautiously, the model-derived inference of a strong T-state bias is robust.

Although SARM1 forms an octameric assembly, our kinetic analyses indicate that a minimal catalytic dimer is sufficient to explain the observed kinetics if TIR

domains oligomerize as pairs (Figure 2G). The initial dimer is not expected to be catalytically active because the active site requires stabilization from a third TIR⁸. Once two pairs of dimers are formed, SARM1 has two active sites. Additional dimer TIR assemblies contribute additively rather than cooperatively. As a result, higher-order oligomerization increases catalytic capacity without introducing additional allosteric complexity. This kinetic inference should be viewed as model based rather than as a direct structural assignment. More broadly, the current data do not yet define whether the effective cooperative unit corresponds to a stable pair of protomers, a local TIR assembly, or another functionally coupled substructure within the octamer.

The BEI data also indicate that orthosteric activation is layered onto slower processes. In both biochemical and cellular assays, low BEI concentrations enhanced SARM1 activity under conditions that favored the inactive state, consistent with the MWC model. However, the delayed onset of activation in biochemical progress curves indicates that slow binding, slow conversion to BEI-ADPR products, progressive stabilization of TIR assemblies, or a combination of these processes contribute to the observed behavior. For that reason, the current equilibrium model is best viewed as a framework for interpreting the direction of the effect rather than as a full kinetic description of BEI action.

While direct measurement of enzymatic SARM1 activity in our biochemical and cellular experiments showed a modest BEI-induced increase, this effect led to near maximal SARM1 activation in previously reported assays that measured viability, axonal degeneration, and NfL release, readouts distal to SARM1 activation^{19,23,24}. This apparent potentiation of BEI-induced SARM1 activation in cells may be related to multiple factors. First, SARM1 activation in cells may have a threshold effect on viability, NfL release, or other distal readouts whereby cells resist SARM1-catalyzed depletion of NAD⁺ and accumulation of metabolites (e.g. cADPR) up to a certain “point of no return”. Once crossed, cells engage additional mechanisms to expedite the degradation. Second, some BEIs undergo NAMPT catalyzed reactions to form nicotinamide-substituted NMN analogs, which may bind to SARM1 and promote additional transition to the active state²⁰. A recent study proposed an aggregation mechanism, suggesting that activated SARM1 transitions to a super-stable active state via polymerization of its TIR domains¹⁷. Although recombinant TIR domains and a SARM1 octamer lacking the mitochondrial targeting sequence (MTS) were previously reported capable of forming stable polymers of active TIR domains¹⁷, we failed to observe polymerization of full-length SARM1 with an intact MTS in mouse cortical neurons (Supplementary Figure 5). Additionally, we observed inconsistent polymerization of SARM1^{ΔMTS} in various cell types: while we saw evidence of polymerization in primary mouse cortical neurons (Supplementary Figure 5), we failed to observe polymerization in HEK cells (Supplementary Figure 6). While

1 additional regulation by pathway non-linearities, BEI-substituted NMN analogs
2 and higher-order assemblies may modulate or amplify SARM1 activity in some
3 contexts, a minimal MWC framework is sufficient to explain both the regulation of
4 SARM1 by its native ligands and the BEI-induced SARM1 activation, especially if
5 the cellular conditions favor the T state.

6
7 More broadly, this work highlights the importance of considering concerted
8 allosteric regulation when developing orthosteric inhibitors for oligomeric
9 enzymes. The universality of the effect across 122 structurally diverse BEIs¹⁹
10 and other reported BEIs^{17,18,21,23–25} is consistent with the MWC prediction that
11 paradoxical activation is mechanism-based, not compound-specific. Similarly
12 “paradoxical” activation by substoichiometric inhibition has been observed for a
13 variety of other multimeric enzymes^{31,32}, underscoring that for enzymes
14 undergoing cooperative transitions between inactive and active states, partial
15 occupancy of the active site can unintentionally promote activation rather than
16 inhibition. These principles should therefore be incorporated early in drug
17 discovery to anticipate and mitigate the risk of unintended activation.

TABLES

Table 1. SPR-derived affinity and kinetic parameters for NMN, NAD⁺, and NB3-ADPR binding to immobilized SARM1 constructs.

Values are mean ± SE from n = 3 experiments. Abbreviations: K_D, equilibrium dissociation constant; k_a, association rate constant; k_d, dissociation rate constant; n.b., no binding observed; n.a., not applicable.

Immobilized Ligand	Analyte	Model	K _D (μM) [± SE]	k _a (M ⁻¹ s ⁻¹) [± SE]	k _d (s ⁻¹) [± SE]	% R _{max} [± SE]
SAM-TIR	NMN	Steady-state	n.b.			
	NAD ⁺	Steady-state	55.2 [± 8.5]	-	-	45 [± 0.4]
ARM-SAM	NMN	1:1 binding	0.24 [± 0.03]	1.8 [± 0.4] x 10 ⁵	4.1 [± 1.4] x 10 ⁻²	56 [± 7]
	NAD ⁺	Steady-state	94.4 [± 6.0]	-	-	44 [± 3]
SARM1 ^{ΔMTS} -apo	NMN	Steady-state	10.0 [± 1.6]	-	-	77 [± 18]
SARM1 ^{ΔMTS} -apo	NAD ⁺	Steady-state	96.3 [± 4.2]	-	-	58 [± 7]
SARM1 ^{ΔMTS} + 250 μM NMN	NAD ⁺	Steady-state	55.7 [± 4.0]	-	-	41 [± 3]
SARM1 ^{ΔMTS} + 100 μM Compound 1	NAD ⁺	Steady-state	9.0 [± 0.9]	-	-	56 [± 4.7]
SARM1 ^{ΔMTS} + 5 μM NB3-ADPR	NMN	Steady-state	0.8 [± 0.2]	-	-	43 [± 13]
SARM1 ^{ΔMTS} + 250 μM NMN	NB3-ADPR	Simple 2-site	0.034 [± 0.02]	8.0 [± 2.0] x 10 ³	3.0 [± 2.2] x 10 ⁻⁴	24 [± 5]
SARM1 ^{ΔMTS} + 1000 μM NAD ⁺	NB3-ADPR	n.a.	n.b.			

Table 2. Best-fit parameter values for the SARM1 MWC model and comparison with SPR- and cryoEM-based observations.

Best-fit model parameters are shown alongside experimentally derived SPR values or qualitative cryoEM comparison where available. See Supplementary Table 3 for the parameter covariance matrix and goodness-of-fit metrics.

Parameters	MWC model best fit [$\pm$ SE]	SPR measurements average [$\pm$ SE]	CryoEM results
K_S^R	14.2 [$\pm$ 0.2]	55.2 [$\pm$ 8.5]	NAD ⁺ resolved
K_S^T	250 [$\pm$ 15]	N/A (> 100 μ M)	NAD ⁺ at TIR in T state not resolved but partial binding site in TIR is solvent exposed
K_I	6.6 [$\pm$ 1.1]	9.0 [$\pm$ 0.9]	NAD ⁺ resolved
K_A	0.51 [$\pm$ 0.42]	0.24 [$\pm$ 0.03]	NMN resolved
L	73 [$\pm$ 71]	(K_D of NMN ^T / NMN ^R) = 10 / 0.2 = 50	Most resolved particles are in T state
n	1.27 [$\pm$ 0.02]	N/A	N/A

Table 3. Kinetic parameters for NB-3 conversion to NB3-ADPR at two SARM1 concentrations.

K_M , k_{cat} , and k_{cat}/K_M were estimated from Michaelis-Menten fits to the initial velocities shown in Figure 2F. Reported values for the hydrolysis of NAD^+ are 0.2-0.8 (s^{-1}) for the k_{cat} and 6,000-30,000 ($M^{-1}s^{-1}$) for the k_{cat}/K_M values^{7,33}.

SARM1 ^{ΔMTS} concentration (nM)	NB-3 K_M (M)	NB-3 k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)
20	6.3×10^{-7}	9.3×10^{-5}	1.4×10^2
1	5.8×10^{-7}	1.1×10^{-4}	1.8×10^2

FIGURES AND FIGURE LEGENDS

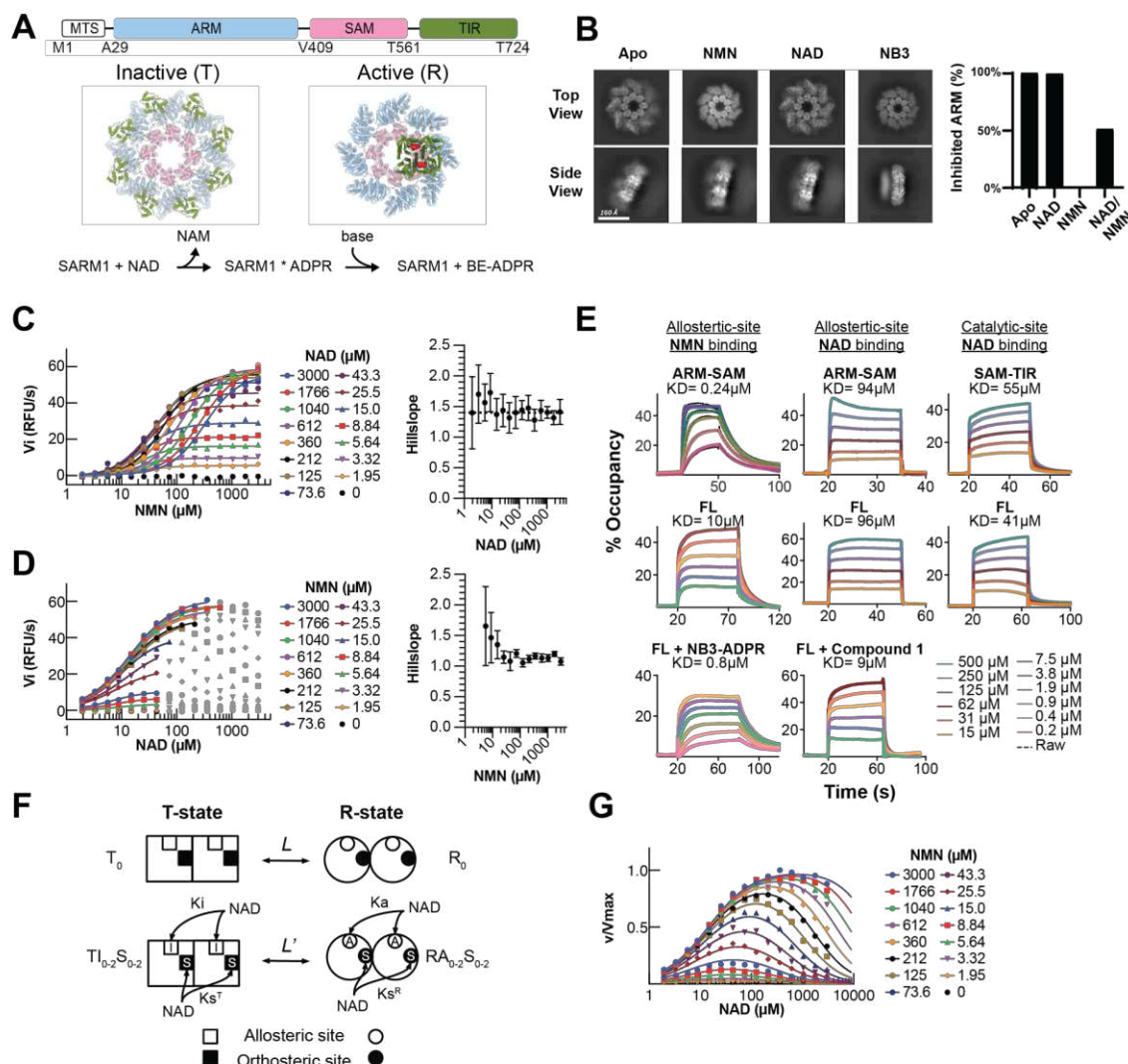


Figure 1. SARM1 structure, kinetics, and a proposed concerted two-state dimer model.

(A) Top: Domain architecture of human SARM1 and boundaries of the constructs used in this study. Middle: Modeled structures of SARM1 in inactive (T) and active (R) conformations based on PDB 7ANW, PDB 7NAL (ARM-SAM), and PDB 7NAK (TIR), with simulated SAM-TIR linkers. In the inactive state, all ARM and TIR domains adopt the autoinhibited arrangement. In the active state, in the presence of endogenous ligands, all ARM domains adopt the activated conformation and all TIR domains are released; the illustrated catalytic dimer assembly contains four TIR domains. Bottom: SARM1 catalyzes base-exchange reaction via a double-displacement catalytic mechanism by first cleaving nicotinamide (NAM) from NAD⁺ and forming a covalent

intermediate with ADPR, followed by addition of water, cis-adenine, or a suitable base to form base-exchange-ADPR product (ADPR, cADPR, or a base-exchange product, respectively).

(B) Left: CryoEM class averages of SARM1^{ΔMTS} particles under apo, 0.5 mM NMN, 0.5 mM NAD⁺, and 47 μM NB-3 conditions. Under apo and NAD⁺-only conditions, resolved particles show all ARM domains in the inactive conformation with all TIR domains attached (top and bottom panels). Under NMN conditions, all ARM domains adopt the active conformation and TIR density is not resolved. In the presence of NMN, NAD⁺, and NB-3, additional TIR assemblies are observed above the ARM-SAM ring (side view panel). Right: Percentage of SARM1^{ΔMTS} particles classified with ARM domains in the inactive conformation under the indicated conditions.

(C) Left: Effect of NMN on initial velocity at varying NAD⁺ concentrations, fitted locally to a four-parameter logistic equation. Right: Hill slopes from individual fits plotted as a function of NAD⁺ concentration. The dotted line indicates the globally fitted Hill slope. Error bars represent standard error of the fitted parameters.

(D) Left: Initial velocity as a function of NAD⁺ at varying NMN concentrations. The rising phase of each curve was fitted locally to the Hill equation. The descending phase of the substrate inhibition curves was excluded. Right: Hill coefficients from individual fits plotted as a function of NMN concentration. Error bars represent standard error of the fitted parameters.

(E) Representative SPR sensorgrams for NAD⁺ and NMN binding to immobilized SARM1 constructs. FL denotes SARM1^{ΔMTS}. Steady-state affinities were obtained from multi-cycle kinetic (MCK) analysis. Experimental data is displayed in colors corresponding to concentrations shown in the legend. X-axis is time (s) and the Y-axis is the % target occupancy.

(F) Proposed two-state MWC model of SARM1. In the absence of ligands, SARM1 exists in equilibrium between inactive (T) and active (R) states, defined by $L = T_0 / R_0$. NAD⁺ and NMN, as effector ligands, bind preferentially to T and R states respectively. NAD⁺, as a substrate, binds non-exclusively to both R and T states. Only R state is catalytically active. L' defines T/R equilibrium in the presence of ligands.

(G) Global fit of the data in panel D to the MWC model (Eq. 1.4).

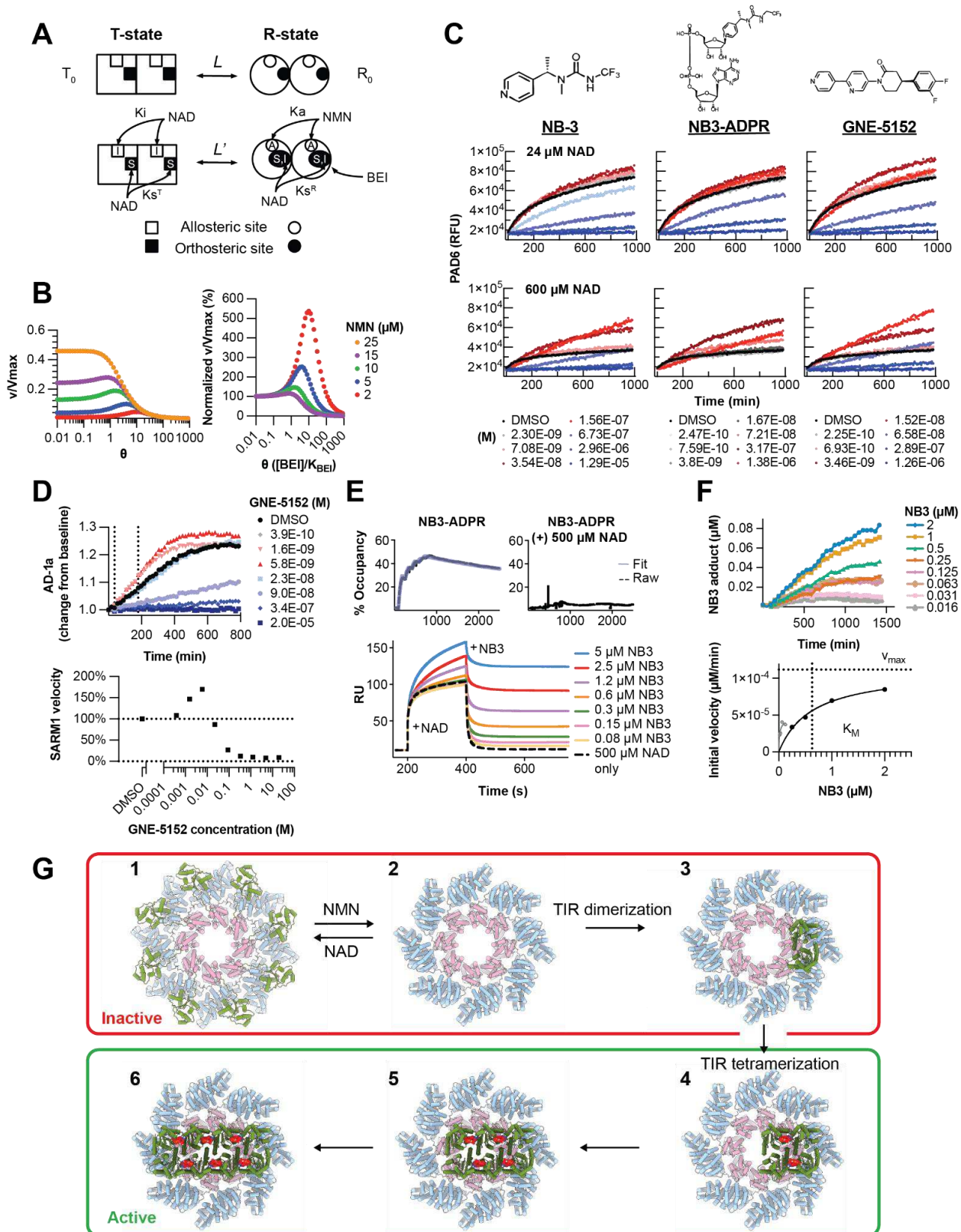


Figure 2. BEIs show slow binding kinetics and catalytic conversion.

(A) MWC scheme incorporating orthosteric BEIs, which bind to the orthosteric site.

(B) Modeled effect of $\theta = [BEI]/K_{BEI}$ on v/V_{max} using Eq. 1.4, the parameters in Table 2, and $n = 2$. Left: predicted activation effect is observed when SARM1 is strongly biased toward the T state. Right: the same curves replotted after normalization to the $\theta=0$ condition.

(C) SARM1^{ΔMTS} progress curves at 24 μM NMN with 24 μM NAD⁺ (top) or 600 μM NAD⁺ (bottom) and varying concentrations of NB-3 (left), NB3-ADPR (middle), or GNE-5152 (right). Blue traces indicate inhibition; red traces indicate delayed activation when compared to DMSO controls (black lines).

(D) Top: SH-SY5Y 1a progress curves, normalized to $t = 0$, across a concentration series of GNE-5152. Bottom: quantified SARM1 velocities showing activation at subinhibitory concentrations. Velocities were calculated from the linear range of the progress curves between 18 and 179 min.

(E) Representative SPR sensorgrams showing NB3-ADPR binding to immobilized SARM1^{ΔMTS} with or without 500 μM NAD⁺ (top) and concentration-dependent binding of NB3 to immobilized TIR in the presence of constant 500 μM NAD⁺ (bottom). Colors correspond to analyte concentrations indicated in the legend. The X-axis of all sensorgrams is time (s) and the Y-axis is target occupancy or response units (RU).

(F) LC-MS/MS quantification of the second step in SARM1-catalyzed conversion of NB-3 to NB3-ADPR. Top: product-formation progress curves across NB-3 concentrations. Bottom: initial velocities were fitted to the Michaelis-Menten equation to estimate k_{cat} and K_M . Filled circles were included in the fit; open circles were excluded.

(G) Sequential SARM1 dimer activation scheme. Proposed sequence of events during activation. (1) In the inactive state, all ARM domains are in the NAD⁺-bound conformation and all TIR domains are bound to ARM domains. (2) In the presence of NMN, all ARM domains are in the NMN-bound conformation and most or all TIR domains are released and disordered (tethered by 20-residue-long flexible linker); no TIR tetramers are assembled at this stage. (3) TIR domains can dimerize across an approximately 643 Å² contact surface (the alternative dimerization interface, across the catalytic site, is only approximately 384 Å²). No catalytic sites have formed yet. (4) Two TIR dimers associate to form two catalytic sites. Note: a single TIR domain may also bind a preformed dimer to form one catalytic site, but the kinetic model presented here is most consistent with cooperative formation of paired catalytic sites. (5) Additional TIR dimers may associate with the TIR oligomer. (6) Each SARM1 octamer may form up to 6 catalytic sites. It is also possible that multiple SARM1 octamers may contribute to higher-order assemblies of TIR domains¹⁷.

METHODS

Preparation of recombinant human SARM1^{AMTS} protein. A pRK5 plasmid containing human SARM1 (E26-T724, EC 3.2.2.6) with N-terminal His and C-terminal Avi and Flag tags was transfected into Expi293F™ cells (ThermoFisher Scientific #A14527). Cells were cultured in Expi293 expression medium at +37°C, 8% CO₂. Cells were seeded at 2.5–3 × 10⁶ viable cells per mL and transfected with 0.8 mg/mL DNA construct using 1:3 PEI Max transfection reagent (PolySciences #24765). Post-transfection, cells were fed and 4 mM valproic acid was added. The cells were harvested after 48 hours by centrifuging at 500×g for 15 minutes at +4°C. For purification, cell paste was resuspended in ice-cold lysis buffer (100 mL per liter of cell paste) consisting of 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5% Glycerol, 1.0 mM TCEP (Tris-HCl(2-carboxyethyl)phosphine), and Roche Complete EDTA-Free Protease Inhibitor Cocktail (Millipore Sigma #4693132001, 2 tablets per 100 mL lysis buffer). Cells were homogenized and then disrupted by double passage using a Microfluidizer Processor (Microfluidics Model M-110Y). Insoluble matter was separated by ultracentrifugation at 40,000 RPM for 1 hour. The soluble supernatant was decanted and passed through a monoclonal ANTI-FLAG® M2 antibody resin column. The resin column was equilibrated with Lysis Buffer, and the bound protein was eluted with 100 µg/mL of 3X FLAG® Peptide (Sigma-Aldrich #F4799). The eluted protein was then injected onto a Superdex 200 16/60 column (Amersham Biosciences # GE28-9893-35) equilibrated in 25 mM Tris pH 8.0, 1 mM TCEP, 150 mM NaCl, 5% Glycerol.

Preparation of recombinant human SAM-TIR protein. An *E. coli* pet52b plasmid containing human SAM-TIR SARM1 (V409-T724) with N-terminal tags His6-MBP-20XGS-TEV-Avi was transformed into BL21-Gold(DE3) competent cells (Agilent #230132) and cultured in TB autoinduction media supplemented with 2 mM nicotinamide riboside at +16°C. Cells were harvested after 16 hours by centrifugation at 5000 rpm for 15 minutes at +4°C. For purification, cell paste was resuspended in lysis buffer consisting of 50mM HEPES, 400mM NaCl, 10% glycerol, 1mM TCEP, 5mM imidazole, EDTA-free PI tablets, 200mM arginine, and benzonase; final pH 7.8. Cells were homogenized, lysed by sonication, clarified by ultracentrifugation at 40,000rpm for 1h, and passed over a Nalgene 0.8 µm filter. The sample was loaded onto a 5 mL HisTRAP FF column (Cytiva #17525501) and eluted with a 30CV gradient from zero to 250 mM imidazole. The His6-MBP-20XGS tag was removed by TEV digestion and passed over a 1:1 Ni-NTA Superflow: Amylose resin (Cytiva #30410 and NEB #E802ALVIAL) gravity flow column to remove the TEV protease and cleaved tag. Avi-SAM-TIR protein was biotinylated with a BirA biotinylation kit (Avidity #BirA500) followed by binding and elution from a STREP-XT column (Cytiva #2901322) to isolate only biotinylated protein. Eluted protein was injected onto a Superose 6 Increase HiScale 16/40 SEC column (Cytiva 29-3219-03). Biotinylated Avi-SAM-TIR was buffer exchanged using Zeba Desalting Columns (ThermoFisher #89892) into a final buffer of 25 mM HEPES, 200 mM NaCl, 5% glycerol, 0.5 mM TCEP; final pH 7.8.

Preparation of recombinant human ARM-SAM protein. An intracellular baculovirus pAcGP67 plasmid containing human ARM-SAM SARM1 (A29-R561) with N-terminal tags His6-MBP-3XGS-TEV-Avi was transfected at an MOI of 2.0 into *Tni* insect cells, grown in shake flasks with ESF921 media. Cells were harvested after 48 hours by centrifugation at 5000 rpm for 15 minutes at +4°C. For purification, cell paste was resuspended in lysis buffer consisting of 50 mM HEPES, 400 mM NaCl, 10% glycerol, 200 mM arginine, 5 mM imidazole, 1 mM TCEP, EDTA-free PI tablets, benzonase; final pH 7.8. Cells were homogenized, lysed by sonication, clarified by ultracentrifugation at 40,000 rpm for 1 hour, and passed over a Nalgene 0.8 µm filter. The cleared lysate was loaded onto a 5 mL HisTRAP FF column (Cytiva #17525501) and the flowthrough fraction was saved. The flowthrough was added to 10 mL of amylose resin (NEB

#E802ALVIAL), batch bound overnight at +4°C, and eluted with a lysis buffer containing 50 mM maltose. The His6-MBP-3XGS tag was removed by TEV digestion and passed over a 1:1 Ni-NTA Superflow:Amylose resin (Cytiva #30410 and NEB #E802ALVIAL) gravity flow column to remove the TEV protease and cleaved tag. Avi-ARM-SAM protein was biotinylated with a BirA biotinylation kit (Avidity #BirA500). Biotinylated ARM-SAM protein was dialysed overnight at +4°C into the final SEC buffer containing 25 mM HEPES, 200 mM NaCl, 5% glycerol, 25 mM arginine, 0.5 mM TCEP; final pH 7.8 and injected onto a Superose 6 Increase HiScale 16/40 SEC column (Cytiva 29-3219-03).

SH-SY5Y 1a assay. SH-SY5Y cells were maintained at 37°C with 5% CO₂ in RPMI-1640 medium supplemented with 10% FBS and 2 mM L-glutamine. On the day of the experiment, cells were transferred into Opti-MEM medium (Gibco, 11058021) supplemented with 1% FBS and 25 μM 1a fluorogenic probe³⁰, and dispensed into 384-well plates (Greiner, 781091) at 60,000 cells per well, followed by brief centrifugation at 300 × g to distribute cells evenly across the well bottom. Compound dilution series were prepared from 10 mM DMSO stock solutions and dispensed into assay plates using an Echo 555 (Labcyte/Beckman Coulter) before cell addition (0.2% final DMSO). After a 30 min preincubation at 37°C, vacor was added to a final concentration of 4.8 μM to activate SARM1. After a further 1-hour incubation at 37°C, plates were sealed and placed in a plate reader (EnVision, PerkinElmer). SARM1 activity was monitored by quantifying AD-1a fluorescence intensity (excitation/emission 450/595 nm) during a continuous 16-hour kinetic read at room temperature using bottom optics, with 9 reads per well.

Biochemical assays. Biochemical investigations were conducted at Arctaris (Abingdon, United Kingdom). The activity of recombinant human SARM1^{ΔMTS} was monitored using the PC6 fluorogenic probe²⁹. Reaction mixtures contained 1 nM SARM1^{ΔMTS}, variable concentrations of NAD⁺ (Roche, 10127973001) and NMN (USBiological, 018099), 10 μM PC6. Assay buffer contained 50 mM Tris HCl pH 7.5 (Teknova, T1074), 0.5 mM DTT (Roche, 03117014007), 0.0036% Tween-20 (Sigma, P1379), and 0.05% bovine gamma globulin (BGG, Sigma, P1379). Enzyme and substrate solutions were dispensed using a DragonFly Discovery instrument (SPT Labtech). Compound dilution series were prepared from 10 mM DMSO stock solutions and dispensed using a Tecan D300e digital dispenser to a final DMSO concentration of 1%. PAD6 fluorescence (excitation/emission 390/520 nm) was measured at room temperature for 16.5 hours at 5-min intervals using a PHERAstar plate reader (BMG). Initial velocities were determined from the initial linear phase of progress curves. All experiments were repeated twice, and a representative set was chosen for analysis and graphing. The SPR-measured k_{on} ($1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) and k_{off} ($4.1 \times 10^{-2} \text{ s}^{-1}$) for NMN at the ARM site give a relaxation time of ~1–2 seconds at the lowest NMN concentrations tested, which is >100× faster than the 5-minute measurement intervals.

Curve fitting was performed in GraphPad Prism (version 11) using least squares regression (no weighting). For Figure 1C, the data were fitted to a four-parameter logistic equation: $Y = \text{Bottom} + ((\text{Top} - \text{Bottom}) \times X^{\text{HillSlope}}) / (\text{EC50}^{\text{HillSlope}} + X^{\text{HillSlope}})$ where Y is the observed SARM1 initial velocity (RFU/s), X is NMN concentration (μM), Top and Bottom are plateaus in the same units as Y, EC50 has the same concentration units as X, and HillSlope is the empirical slope factor (unitless). For Figure 1D, the data were fitted to a Hill equation: $Y = (V_{\text{max}} \times X^{\text{HillSlope}}) / (K_{\text{half}}^{\text{HillSlope}} + X^{\text{HillSlope}})$ where Y is the observed SARM1 initial velocity (RFU/s), X is NAD⁺ concentration (μM), V_{max} is the maximal velocity in the same units as Y, K_{half} is the substrate concentration that gives half-maximal velocity, and HillSlope is the empirical Hill coefficient (unitless). For Figure 1G, the data were fitted to equation 1.4 with all parameters shared across NMN conditions. Initial affinity values were set to the SPR measurements, initial n value was set to the kinetically estimated 1.4

1 and initial L value was set to the SPR suggested 50 (summarized in Table 2). For Figure 2F, the
2 data were fitted to the Michaelis-Menten equation: $Y = (V_{\max} \times X) / (K_m + X)$ where Y is the
3 observed SARM1 initial velocity ($\mu\text{M}/\text{min}$), X is substrate concentration, $V_{\max}$ is the maximal
4 velocity, and K_m is the Michaelis constant. Since $V_{\max} = E_t \times k_{\text{cat}}$, k_{cat} was calculated from the
5 fitted $V_{\max}$ values.

6 **SPR analysis of NMN and NAD⁺ interactions with SARM1 constructs.** The following
7 huSARM1 constructs were used for immobilization on the SPR surface: biotin-TIR(D560-Q700),
8 biotin-ARM-SAM(A29-T561), SAM-TIR(V409-T724) or FL(E26-T724)(minimal chemical
9 biotinylation, Thermofisher #A35389). The constructs were immobilized onto the SA sensor chip
10 (Cytiva) between 3,000 - 5,000 RU using capture buffer (50 mM HEPES pH 7.5, 150 mM NaCl,
11 2% glycerol, 0.5 mM TCEP, +/- 20 mM arginine, +/- 500 μM - 1000 μM NAD⁺ or +/- 250 μM
12 NMN) at 5 $\mu\text{L}/\text{min}$ flow rate at 20 °C. The reference flow cell was left empty. All channels were
13 capped with biotin. The system was primed with running buffer (50 mM HEPES pH 7.5, 150 mM
14 NaCl, 1% glycerol, 0.5 mM TCEP, +/- 20 mM arginine, +/- 500 μM - 1000 μM NAD⁺ or +/- 250
15 μM NMN or +/- 10 μM NB3 adduct, +/- 1% DMSO) at 20 °C. Compounds titrated via 2-fold
16 dilution, 6-point dose response, were injected at a flow rate of 50 $\mu\text{L}/\text{min}$ for 45 – 200 s with
17 dissociation times between 30 – 2000 s via multi-cycle (MCK) or single-cycle kinetic (SCK)
18 mode. Model fitting of binding sensorgrams was performed using Biacore S200 Evaluation v1
19 software. A simple steady state or 1:1 binding model was applied to fit the binding profile. The
20 following statistical parameters were evaluated to assess the quality of curve fitting: the
21 standard error of the fit (SEF) should be 10% of the estimated parameter value and chi squared
22 (χ^2) should generally be 5% of the calculated $R_{\max}$. Graphpad Prism 10 was used to
23 generate final binding sensorgrams.

24 **CryoEM**

25 For cryoEM grid preparation, 2.5 microliters of sample were applied to glow-dishcharged gold
26 R1.1/1.2 UltrAuFoil grids (Quantifoil), and plunge-frozen using a Vitrobot (Thermo-Fisher
27 Scientific). Data collection was performed using a Titan Krios equipped with a Falcon F4i direct
28 detector, Selectris energy filter, and EPU automation software (Thermo-Fisher Scientific).
29 Instrument parameters were as follows: acceleration voltage 300 kV, calibrated pixel size 0.731
30 Å (165,000x magnification), energy filter slit width 10 eV, and a total fluence of 40 electrons per
31 square Ångstrom.

32
33
34 All data processing was performed using cryoSPARC³⁴. Briefly, following preprocessing (motion
35 correction, CTF estimation, template-based particle picking, and extraction), extracted particles
36 were subjected to 2D classification. To quantify particle distributions across different
37 conformations, particles were selected from 2D classification and subjected to *ab initio*
38 reconstruction and heterogenous refinement to separate selections into conformational bins to
39 be quantified and compared across different ligand states.

40 **LC-MS/MS measurements of NB-3-ADPR**

41 Enzymatic reactions were carried out at 20 °C in the MS assay buffer (50 mM Tris, pH 7.5, 0.5
42 mM DTT, 0.01% (w/v) BGG). Each reaction contained 500 μM NAD⁺ and 100 μM NMN. NB3
43 was titrated in serial dilutions from 2 μM to 16 nM. Reactions were initiated by addition of FL
44 SARM1 (final concentration of 1 nM or 20 nM) to reaction mixtures. Kinetic measurements were
45 obtained by direct sampling of each reaction mixture at multiple time points.

46
47
48 Samples were injected onto a Waters ACQUITY UPLC BEH C8 1.7 μm 2.1x50 mm column
49 maintained at 55 °C on a Waters Acquity I-Class UPLC operating at a flow rate of 0.8 mL/min.
50 An optimized 2.5-min gradient was employed using solvent A (water + 0.1% formic acid) and

solvent B (acetonitrile + 0.1% formic acid): 1% B for 0.3 min, ramping linearly to 20% B at 1.9 min, 100% B at 2.0 min, followed by re-equilibration at 1% B to 2.5 min.

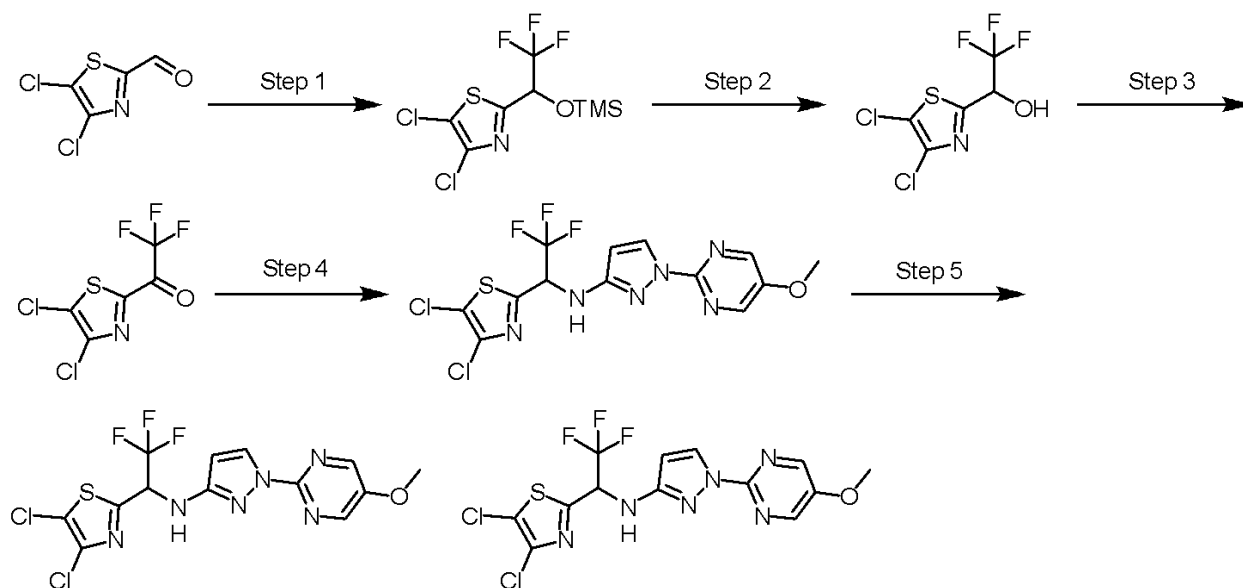
MRM-MS data were acquired on a Sciex QTRAP 6500 triple quadrupole mass spectrometer in positive ion mode using the following parameters: IonSpray voltage = 5500 V, source temperature = 650 °C, curtain gas = 25 psi, CAD = medium, ion source gases 1 and 2 = 60 psi, declustering potential = 30 V, entrance potential = 10 V, and collision cell exit potential = 13 V. Q1 and Q3 resolutions were set to "Unit" and "Low," respectively. The NB3-ADPR transition (402/262 m/z) was monitored for quantification.

Absolute quantification was performed using a standard curve prepared from serial dilutions of NB3-ADPR standards (1 μ M to 150 pM) prepared in the MS assay buffer. Integrated peak areas were determined using Sciex MultiQuant software (v3.03) and used for downstream kinetic analysis. Initial slopes were calculated using the linear portion of the reaction progress and these values were fitted to determine kinetic parameters ($v_{\max}$ and K_M) by nonlinear regression fitting to the Michaelis-Menten equation. Enzyme concentrations were used to calculate k_{cat} . Data was analyzed using GraphPad Prism.

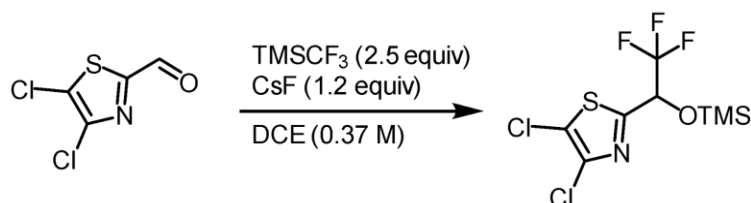
Compounds

Commercially available reagents were purchased. Vacor (pyrinuron), PC6 and 1a fluorogenic probes were synthesized as previously described^{29,30,35}.

Synthesis of Compound 1



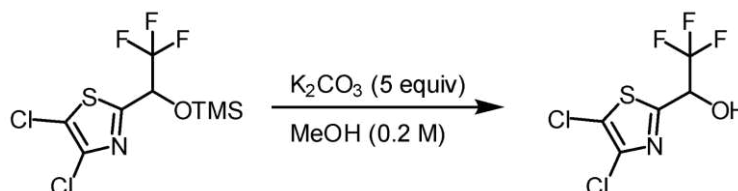
Step 1: Synthesis of 4,5-Dichloro-2-(2,2,2-trifluoro-1-((trimethylsilyloxy)oxy)ethyl)thiazol



To a solution of 4,5-dichlorothiazole-2-carbaldehyde (1 g, 5.49 mmol, 1 equiv) in DCE (15 mL, 0.37 M) was added CsF (1 g, 6.59 mmol, 1.2 equiv) at 0 °C. After stirring at 0 °C for 5 minutes, TMSCF₃ (2.03 mL, 13.73 mmol, 2.5 equiv) was added slowly at 0 °C. The mixture was stirred at room temperature for 6 hours. After completion, the mixture was poured into water (10 mL) and extracted with EtOAc (3 x 30 mL). The combined organics were washed with brine, dried over sodium sulfate, filtered, and concentrated. The material was purified by column chromatography (7% ethyl acetate in *n*-hexane) to yield 4,5-dichloro-2-(2,2,2-trifluoro-1-((trimethylsilyl)oxy)ethyl)thiazole (1.1 g, 62% yield) as a white solid.

¹H NMR (400 MHz, DMSO): δ 5.97 (q, *J* = 6.4 Hz, 1H), 0.00 (s, 9H).

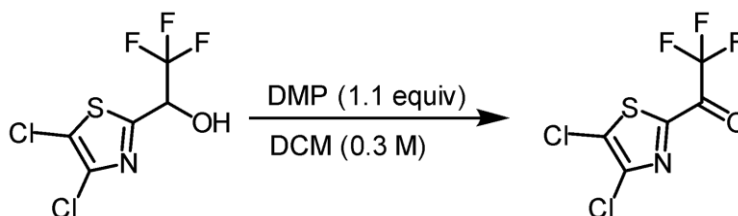
Step 2: Synthesis of 1-(4,5-Dichlorothiazol-2-yl)-2,2,2-trifluoroethan-1-ol



To a solution of 4,5-dichloro-2-(2,2,2-trifluoro-1-((trimethylsilyl)oxy)ethyl)thiazole (1 g, 3.08 mmol, 1 equiv) in MeOH (15 mL, 0.2 M) was added K₂CO₃ (2.1g, 15.42 mmol, 5 equiv). The reaction was stirred at room temperature for 5 hours. After completion, the mixture was poured into water (100 mL) and extracted with EtOAc (3 x 30 mL). The combined organics were washed with brine, dried over sodium sulfate, filtered, and concentrated to yield 1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethan-1-ol (770 mg, 99% yield) as a white solid.

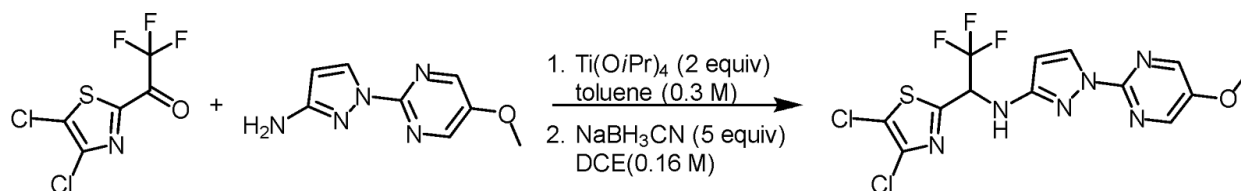
¹H NMR (400 MHz, DMSO): δ 8.32 - 8.23 (m, 1H), 5.63 - 5.61 (m, 1H).

Step 3: Synthesis of 1-(4,5-Dichlorothiazol-2-yl)-2,2,2-trifluoroethan-1-one



To a solution of 1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethan-1-ol (770 mg, 3.06 mmol, 1 equiv) in DCM (10 mL, 0.3 M) was added DMP (1.4 g, 3.36 mmol, 1.1 equiv). The mixture was stirred at room temperature for 1 h. After completion, the mixture was poured into water (100 mL) and extracted with EtOAc (3 x 30 mL). The combined organics were washed with brine, dried over sodium sulfate, filtered, and concentrated. The material was purified by column chromatography (10% ethyl acetate in *n*-hexane) to yield 1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethan-1-one (560 mg, 76% yield) as a white solid.

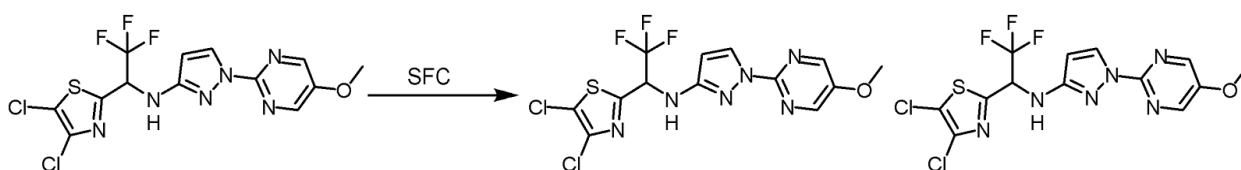
Step 4: Synthesis of N-(1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethyl)-1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine



To a solution of 1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethan-1-one (320 mg, 1.28 mmol, 1 equiv) in toluene (4 mL) were added 1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine (245 mg, 1.28 mmol, 1 equiv) and $\text{Ti}(\text{OiPr})_4$ (0.76 mL, 2.56 mmol, 2 equiv). The reaction was stirred at 110 °C for 2 h. After cooling to room temperature, DCE (4 mL) was added to the mixture and then NaBH_3CN (371 mg, 5.91 mmol, 5 equiv) was added. The mixture was stirred for another 1 hour. After completion, the reaction was diluted with water (10 mL) and extracted with DCM (3 x 20 mL). The combined organics were washed with brine, dried over sodium sulfate, filtered, and concentrated. The material was purified by column chromatography (0-10% MeOH in DCM) to yield *N*-(1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethyl)-1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine (120 mg, 24% yield) as a white solid.

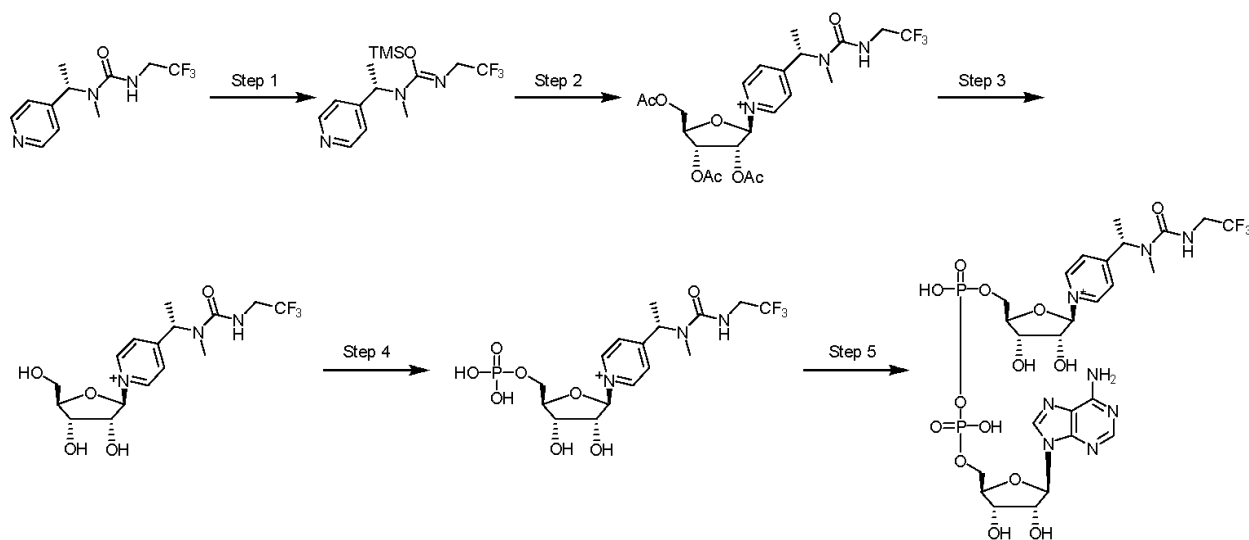
LCMS: (ESI, m/z) $[\text{M}+\text{H}]^+ = 424.9$

Step 5: Synthesis of *N*-(1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethyl)-1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine & *N*-(1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethyl)-1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine



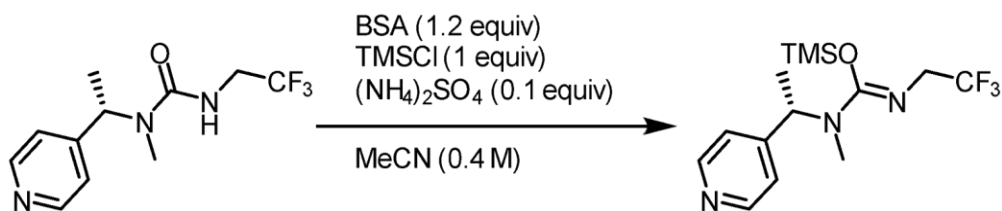
N-(1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethyl)-1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine (120 mg, 0.28 mmol) was separated by chiral SFC (Daicel Chiralpak IC (250 mm x 30 mm, 10 μm), Supercritical CO_2 / EtOH + 0.1% $\text{NH}_3\text{H}_2\text{O}$ = 70/30; 150 mL/min) to yield *N*-(1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethyl)-1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine (peak 2, R_t = 2.881 min, 38.1 mg, 31% yield) and *N*-(1-(4,5-dichlorothiazol-2-yl)-2,2,2-trifluoroethyl)-1-(5-methoxypyrimidin-2-yl)-1H-pyrazol-3-amine (**Compound 1**, peak 1, R_t = 2.252 min, 23.1 mg, 19% yield) both as white solid. Peak 2 $^1\text{H NMR}$ (400 MHz, DMSO): δ 8.51 (s, 2H), 8.34 (d, J = 2.8 Hz, 1H), 7.72 (d, J = 10.0 Hz, 1H), 6.12 - 6.03 (m, 2H), 3.91 (s, 3H). **LCMS** (ESI, m/z) $[\text{M}+\text{H}]^+ = 424.9$. **Compound 1:** $^1\text{H NMR}$ (400 MHz, DMSO): δ 8.51 (s, 2H), 8.34 (d, J = 2.8 Hz, 1H), 7.72 (d, J = 9.6 Hz, 1H), 6.12 - 6.03 (m, 2H), 3.91 (s, 3H). **LCMS** (ESI, m/z) $[\text{M}+\text{H}]^+ = 424.9$

Synthesis of NB3-ADPR adduct

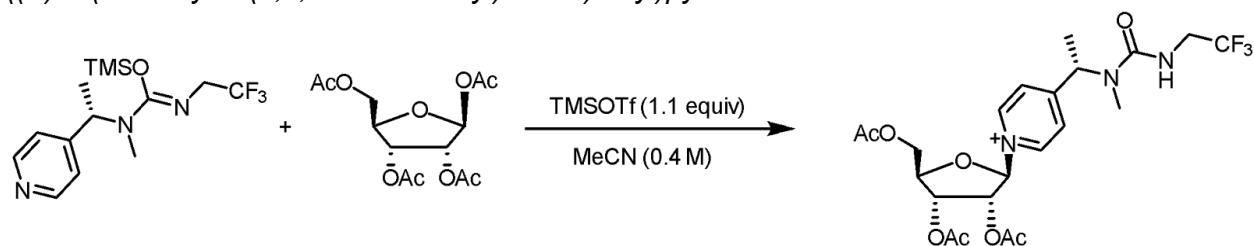


Step 1: Synthesis of trimethylsilyl (S,Z)-N-methyl-N-(1-(pyridin-4-yl)ethyl)-N'-(2,2,2-trifluoroethyl)carbamimidate

To a solution of (S)-1-methyl-1-(1-(pyridin-4-yl)ethyl)-3-(2,2,2-trifluoroethyl)urea (2.4 g, 9.19 mmol), *N,O*-bis(trimethylsilyl)acetamide (2.7 mL, 11.02 mmol) and (NH₄)₂SO₄ (121 mg, 0.92 mmol, 0.1 equiv) in anhydrous acetonitrile (24 mL, 0.4 M) was added TMSCl (1.17 mL, 9.19 mmol, 1 equiv). The reaction was stirred at room temperature for 4 hours under nitrogen condition. After completion, the reaction mixture was used directly in the next step without purification.



Step 2: Synthesis of 1-((2R,3R,4R,5R)-3,4-diacetoxy-5-(acetoxymethyl)tetrahydrofuran-2-yl)-4-((S)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium

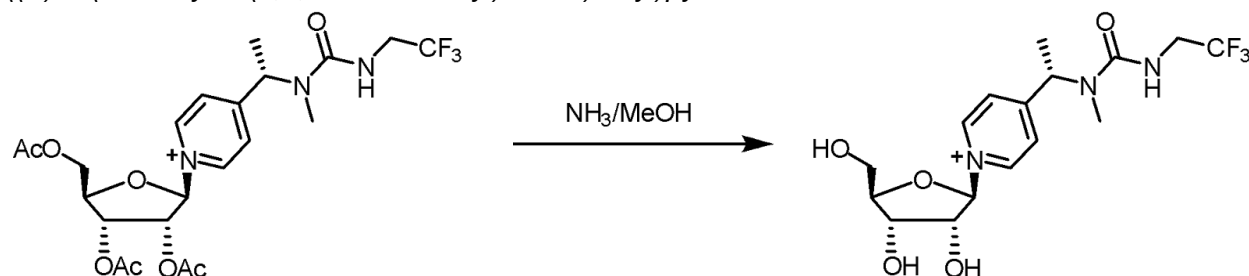


To a solution of trimethylsilyl (S,Z)-N-methyl-N-(1-(pyridin-4-yl)ethyl)-N'-(2,2,2-trifluoroethyl)carbamimidate in anhydrous acetonitrile (24 mL) from the last step was added (2S,3R,4R,5R)-5-(acetoxymethyl)tetrahydrofuran-2,3,4-triyl triacetate (2.86 g, 9 mmol, 1 equiv). Then, TMSOTf (2.04 g, 9.9 mmol, 1.1 equiv) was added to the mixture dropwise over 20 min at room temperature under nitrogen condition. The reaction was stirred at room temperature for 12 hours. The reaction was filtered and concentrated. The resulting residue was purified by prep-HPLC (Xtimate C18 column 150 x 40 mm, 10 μm) using 0.225% FA in water with a gradient of 16–46% MeCN at 25 mL/min to yield 1-((2R,3R,4R,5R)-3,4-diacetoxy-5-

1 (acetoxymethyl)tetrahydrofuran-2-yl)-4-((S)-1-(1-methyl-3-(2,2,2-
2 trifluoroethyl)ureido)ethyl)pyridin-1-ium (1.2 g, 25% yield) as a yellow solid.

3 **¹H NMR** (400 MHz, DMSO): δ 9.04 (d, J = 6.8 Hz, 2H), 8.04 (d, J = 6.4 Hz, 2H), 7.34 -
4 7.23 (m, 1H), 6.55 (d, J = 4.0 Hz, 1H), 5.65 - 5.53 (m, 2H), 5.45 - 5.39 (m, 1H), 4.72 - 4.62 (m,
5 1H), 4.49 - 4.37 (m, 2H), 3.94 - 3.78 (m, 2H), 2.80 (s, 3H), 2.12 (d, J = 6.0 Hz, 6H), 2.06 (s, 3H),
6 1.57 (d, J = 7.2 Hz, 3H). **LCMS** (ESI, m/z) $[M]^+$ = 520.2

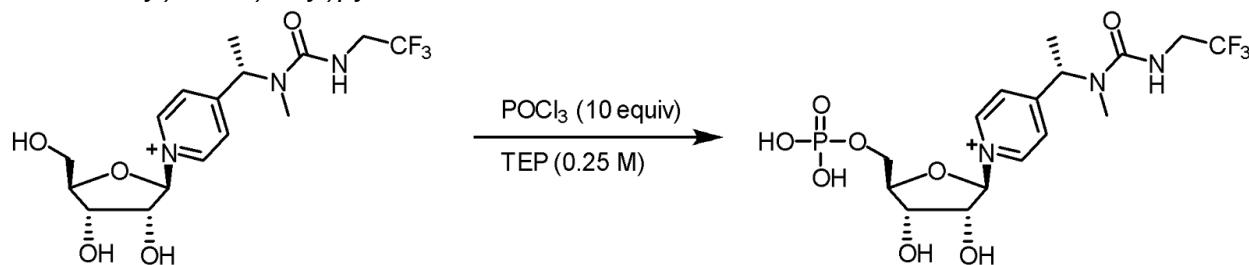
7
8 **Step 3: Synthesis of 1-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-4-**
9 **((S)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium**



10
11
12 A solution of 1-((2R,3R,4R,5R)-3,4-diacetoxy-5-(acetoxymethyl)tetrahydrofuran-2-yl)-4-
13 ((S)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium (1.2 g, 2.31 mmol, 1 equiv) in
14 NH₃/methanol (7 M, 20.34 mL, 142.37 mmol) was stirred at 0 °C for 3 hours. Then the reaction
15 was stirred at room temperature for 12 hours. After completion, the reaction mixture was
16 concentrated to yield crude 1-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-
17 2-yl)-4-((S)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium (900 mg, 99% yield) as
18 a brown oil.

19 **LCMS** (ESI, m/z) $[M]^+$ = 394.2

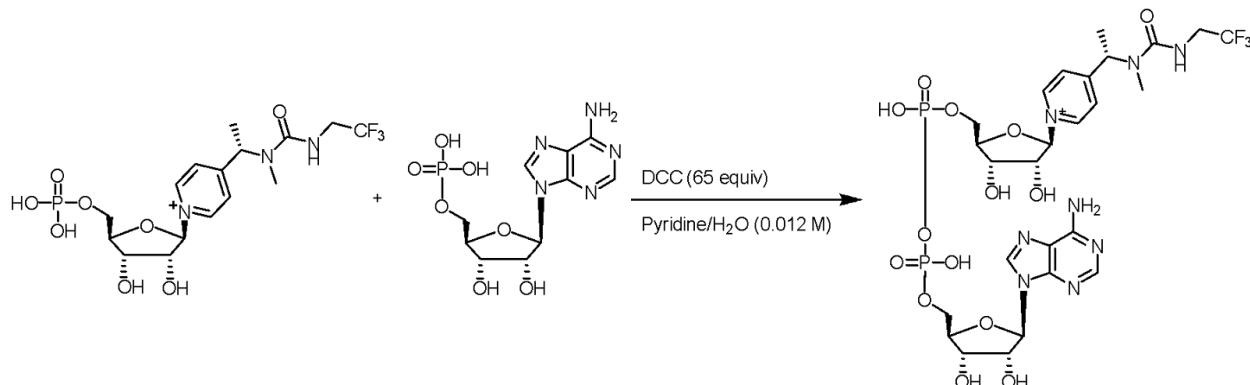
20
21
22 **Step 4: Synthesis of 1-((2R,3R,4S,5R)-3,4-dihydroxy-5-**
23 **((phosphonooxy)methyl)tetrahydrofuran-2-yl)-4-((S)-1-(1-methyl-3-(2,2,2-**
24 **trifluoroethyl)ureido)ethyl)pyridin-1-ium**



25
26
27 To a solution of 1-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-
28 4-((S)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium (500 mg, 1.27 mmol, 1 equiv)
29 in triethyl phosphate (5 mL, 29.43 mmol) at 0 °C was added phosphorus oxychloride (1.18 mL,
30 12.68 mmol, 10 equiv). The reaction mixture was stirred at 4 °C for 4 hours. After completion,
31 the reaction was quenched with water (0.5 mL), filtered, and then MeCN (1 mL) was added to
32 the filtrate. The resulting solution was purified directly via prep-HPLC (Xtimate C18 column 100
33 x 30 mm, 3 μ m) using 10 mM NH₄HCO₃ in water with a gradient of 0-20% MeCN at 25 mL/min)
34 to yield 1-((2R,3R,4S,5R)-3,4-dihydroxy-5-((phosphonooxy)methyl)tetrahydrofuran-2-yl)-4-((S)-
35 1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium (200 mg, 33% yield) as a yellow
36 solid.

37 **LCMS** (ESI, m/z) $[M]^+$ = 474.2

Step 5: Synthesis of 1-((2*R*,3*R*,4*S*,5*R*)-5-((((((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)oxy)(hydroxy)phosphoryl)oxy)methyl)-3,4-dihydroxytetrahydrofuran-2-yl)-4-((*S*)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium



To a solution of 1-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-((phosphonooxy)methyl)tetrahydrofuran-2-yl)-4-((*S*)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium (173 mg, 0.36 mmol), ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl dihydrogen phosphate (203 mg, 0.58 mmol, 1 equiv) in pyridine (24 mL) and water (6 mL) was added DCC (4.86 g, 23.57 mmol, 65 equiv). The reaction was stirred at room temperature for 7 days. After completion, the resulting mixture was poured into 100 mL of cold distilled water and left at 4 °C for 2 hours, allowing the DCU to precipitate. Then, DCU was filtered off and the filtrate was extracted with DCM (3 x 50 mL) to remove other water insoluble organic impurities. The aqueous layer was collected and dried in vacuo to yield 290 mg of crude product. The crude product was further purified via prep-HPLC (Waters Xbridge BEH C18 column 19 x 150 mm) using 100 mM HFIP + 5 mM TEA in water with a gradient of 0-10% MeCN at 25 mL/min) to yield 1-((2*R*,3*R*,4*S*,5*R*)-5-((((((((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)oxy)(hydroxy)phosphoryl)oxy)methyl)-3,4-dihydroxytetrahydrofuran-2-yl)-4-((*S*)-1-(1-methyl-3-(2,2,2-trifluoroethyl)ureido)ethyl)pyridin-1-ium (TEA salt, 21.7 mg, 5% yield) as a yellow solid. ¹H NMR (400 MHz, DMSO): δ 8.78 (d, *J* = 6.8 Hz, 2H), 8.40 (d, *J* = 9.6 Hz, 1H), 8.11 (s, 1H), 7.87 - 7.77 (m, 2H), 6.02 - 5.96 (m, 1H), 5.92 (d, *J* = 5.2 Hz, 1H), 5.49 - 5.39 (m, 1H), 4.48 (br s, 2H), 4.38 - 4.31 (m, 4H), 4.25 - 4.15 (m, 4H), 3.93 - 3.81 (m, 2H), 3.16 (q, *J* = 7.2 Hz, 6H), 2.78 - 2.72 (m, 3H), 1.54 (d, *J* = 6.0 Hz, 3H), 1.54 (d, *J* = 6.0 Hz, 9H). LCMS (ESI, *m/z*) [*M*]⁺ = 803.5

ACKNOWLEDGMENTS

We thank Bing-Yan Zhu, Jessica Grandner, Jun Liang, Matthew Del Bel, Mingshuo Zeng, Marie- Gabrielle Braun and Russell Smith for their compound design contributions and Adam Johnson for first suggesting the MWC framework for our SARM1 data. We thank the chemistry team members at Wuxi.

AUTHOR CONTRIBUTIONS

S.R., B.B., J.N., K.M., M.B., A.R., M.J., F.I.H., M.M., J.P.E., and A.D. designed, performed or analyzed experiments. S.G., G.B., and H.A.W. synthesized reagents. B.C. and Y.X. designed experiments. S.R., B.B., Y.X., and A.D. wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

J.P.E. is an employee of Arctoris Inc. All other authors were employees of Genentech, Inc., or its parent company, F. Hoffmann-La Roche Ltd, during the period in which this research was conducted. The authors declare no further competing interests.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. CryoEM particle statistics

Total number of 2D SARM1 octamer particles and classification of protomeric ARM allosteric site conformations in the resolved SARM1 octamer particles.

Condition	Number of 2D particles	ARM unresolved	ARM inhibited (%)	ARM uninhibited
apo	777,216	5,921,107	296,621 (5%)	0
NAD ⁺ (0.5 M)	1,053,441	8,229,231	198,297	0
NMN (0.5 M)	1,456,765	11,654,120	0	161,290
NAD ⁺ + NMN (each 0.5 M)	1,230,736	9,169,656	322,179	354,053

Supplementary Table 2. SPR-derived affinity and kinetic parameters for Compound 1 and NB3 binding.

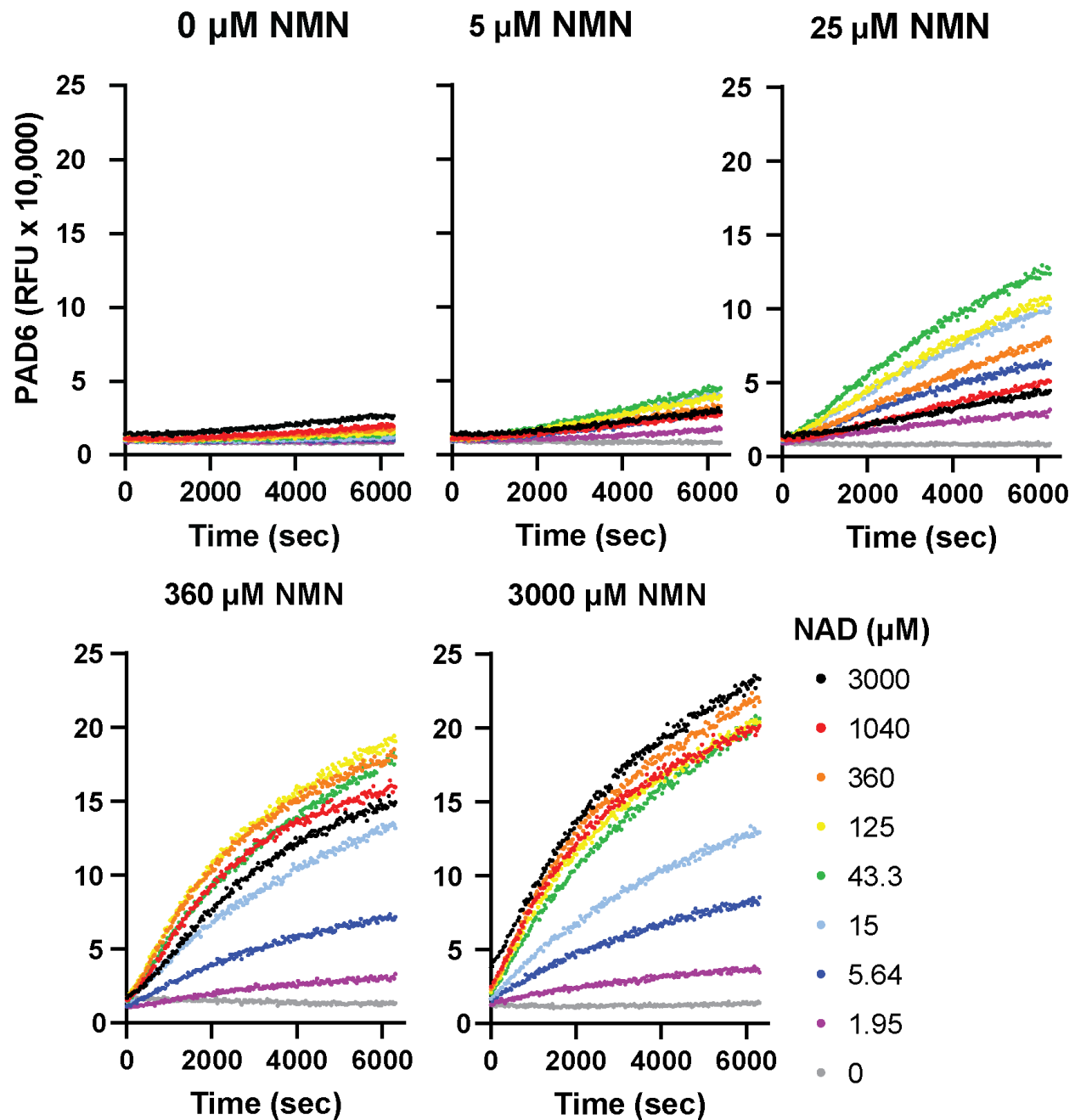
Values are mean $\pm$ SE from n = 3 experiments. Abbreviations: K_D , equilibrium dissociation constant; k_a , association rate constant; k_d , dissociation rate constant; n.a., not applicable.

Immobilized Ligand	Analyte	Model	K_D (μ M) [$\pm$ SE]	k_a ($M^{-1}s^{-1}$) [$\pm$ SE]	k_d (s^{-1}) [$\pm$ SE]	% R_{max} [$\pm$ SE]
SARM1 Δ MTS apo	Compound 1	Steady-state	7.3 [$\pm$ 0.9]	-	-	61 [$\pm$ 6.4]
SARM1 Δ MTS + 250 μ M NMN	Compound 1	n.a.	> 20	-	-	-
TIR + apo	NB3 parent	n.a.	> 10			
TIR + 500 μ M NAD ⁺	NB3 parent	1:1 binding	0.016 [$\pm$ 0.01]	6.6 [$\pm$ 0.5] x 10 ³	1.1 [$\pm$ 0.8] x 10 ⁻⁴	68 [$\pm$ 28]
ARM-SAM	NB3 parent		> 100			
ARM-SAM	NB3-ADPR		> 100			

Supplementary Table 3. Goodness-of-fit metrics and covariance matrix for MWC model parameters.

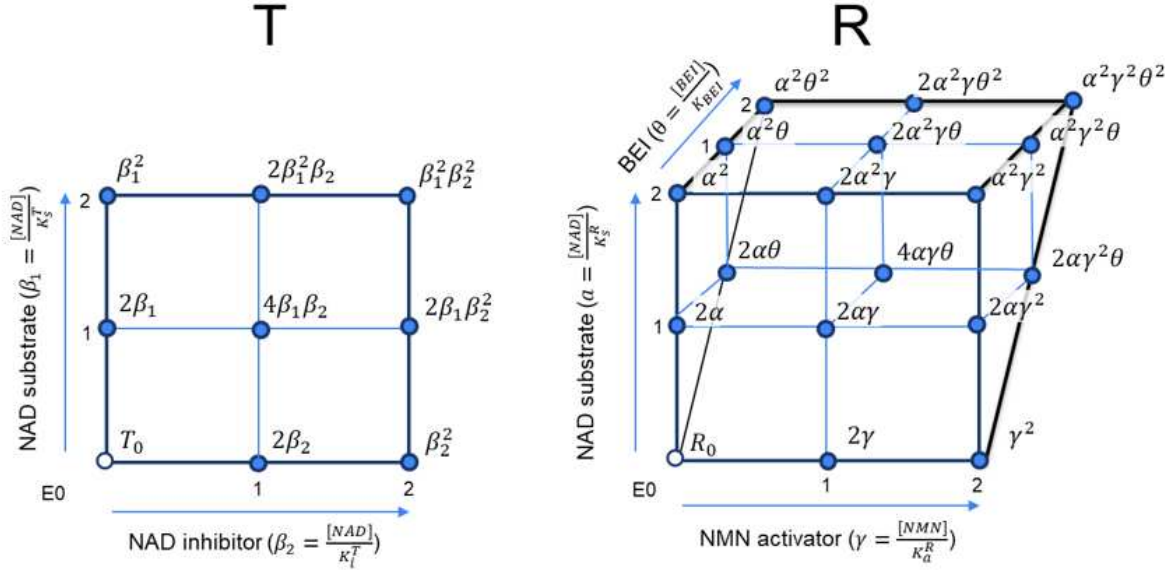
	Goodness-of-fit	
NMN (μM)	R squared	AICc
3000	0.994	
1766	0.996	
1040	0.997	
612	0.991	
360	0.996	
212	0.995	
125	0.986	
73.6	0.984	
43.3	0.972	
25.5	0.964	
15.0	0.779	
8.84	0.814	
5.64	0.057	
3.32	-3.974	
1.95	-4.966	
0	-17.060	
Global (shared)	0.995	-1948

Model parameters	Covariance
Ksr & n	0.22
Ksr & L	-0.02
Ksr & Kit	-0.17
Ksr & Kst	-0.05
Ksr & Ka	-0.02
n & L	-0.57
n & Kit	0.04
n & Kst	0.19
n & Ka	0.62
L & Kit	0.02
L & Kst	-0.41
L & Ka	-0.98
Kit & Kst	-0.62
Kit & Ka	0.14
Kst & Ka	0.32



Supplementary Figure 1. Representative SARM1^{ΔMTS} progress curves from NAD⁺:NMN titrations.

Representative SARM1^{ΔMTS} progress curves acquired across a full 16 × 16 combinatorial matrix of NAD⁺ and NMN concentrations in 1536-well format. NAD⁺ and NMN concentrations ranged from 0 to 3000 μM.



9 T states:

$$T_0(1 + 2\beta_1 + \beta_1^2 + 2\beta_2 + 4\beta_1\beta_2 + 2\beta_1^2\beta_2 + \beta_2^2 + 2\beta_1\beta_2^2 + \beta_1^2\beta_2^2) = T_0(1 + \beta_1)^2(1 + \beta_2)^2$$

18 R states:

$$\begin{aligned} R_0(1 + 2\alpha + \alpha^2 + 2\alpha\theta + 2\alpha^2\theta + \alpha^2\theta^2 + 2\gamma + 4\alpha\gamma + 2\alpha^2\gamma + 4\alpha\theta\gamma + 4\alpha^2\theta\gamma + 2\alpha^2\theta^2\gamma + \gamma^2 + 2\alpha\gamma^2 + \alpha^2\gamma^2 \\ + 2\alpha\theta\gamma^2 + 2\alpha^2\theta\gamma^2 + \alpha^2\theta^2\gamma^2) = R_0(1 + 2\gamma + \gamma^2)(1 + \alpha^2 + \alpha^2\theta^2 + 2\alpha + 2\alpha\theta + 2\alpha^2\theta) \\ = R_0(1 + \gamma)^2(1 + \alpha + \alpha\theta)^2 \end{aligned}$$

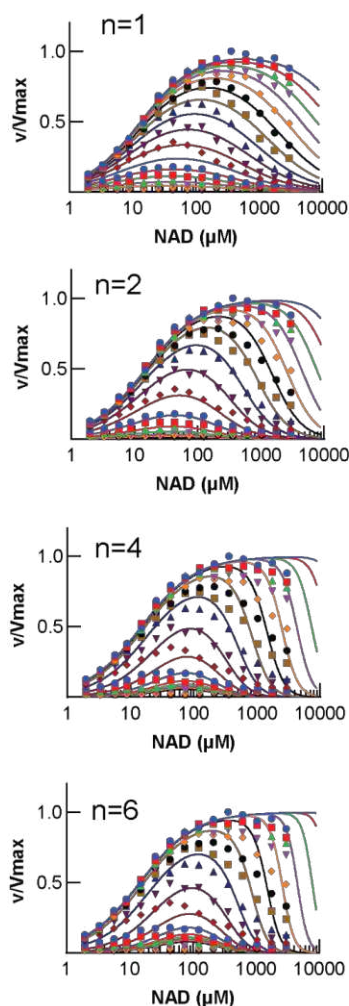
Fraction of SARM1 in the R state:

$$\begin{aligned} \bar{R} &= \frac{18 \text{ R states}}{9 \text{ T states} + 18 \text{ R states}} = \frac{R_0(1 + \alpha)^2(1 + \alpha + \alpha\theta)^2}{T_0(1 + \beta_1)^2(1 + \beta_2)^2 + R_0(1 + \alpha)^2(1 + \alpha + \alpha\theta)^2} \\ &= \frac{1}{L \left(\frac{(1 + \beta_1)^2(1 + \beta_2)^2}{(1 + \alpha)^2(1 + \alpha + \alpha\theta)^2} \right) + 1} = \frac{1}{L' + 1} \end{aligned}$$

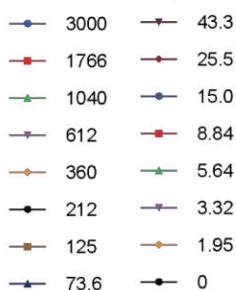
where $L = T_0/R_0$

Supplementary Figure 3: Mass balance schematic and polynomial factoring for the uncompetitive SARM1 MWC dimer model.

Full enumeration and polynomial factoring of 9 T-state species (left) and 18 R-state species (right) in the presence of NAD⁺, NMN, and uncompetitive BEIs.



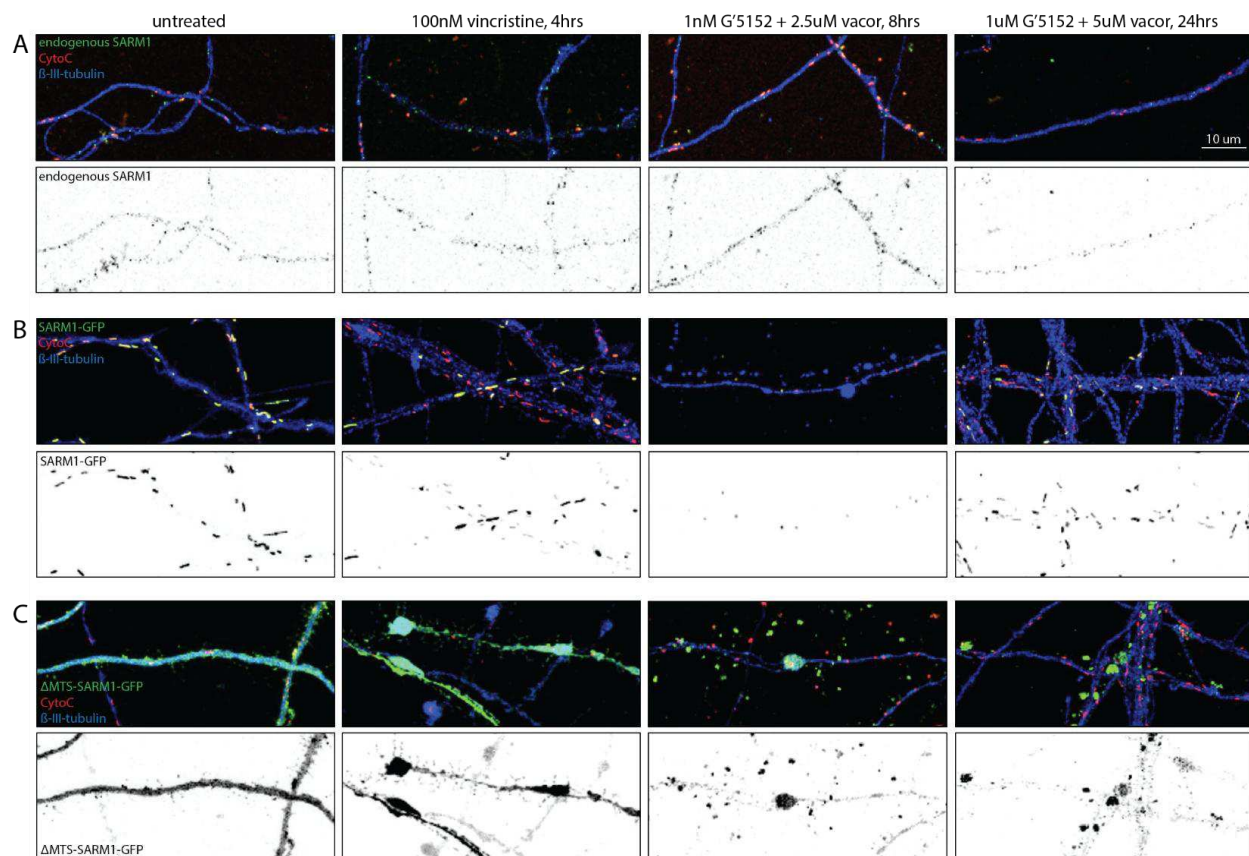
NMN (μM)



	n=1	n=2	n=4	n=6
Best-fit values				
Ksr	13.26	15.4	14.87	14.57
L	~ 50.00	~ 50.00	~ 50.00	~ 50.00
Kit	3.747	15.47	27.65	22.92
Kst	286.2	217.9	409.5	804
Ka	~ 0.2000	4.142	39.51	86.72
Std. Error				
Ksr	0.3481	0.4501	0.7303	0.8095
L				
Kit	1.259	2.993	4.091	2.398
Kst	25.47	25.5	58.93	98.04
Ka		1.144	4.57	8.516
Goodness of Fit				
Degrees of Freedom	250	250	250	250
R squared	0.9895	0.9824	0.9504	0.937
Adjusted R squared	0.9894	0.9821	0.9496	0.936
AICc	-1744	-1611	-1347	-1286
Covariance Matrix				
Ksr & L	0.1217	0.02034	-0.2038	-0.2497
Ksr & Kit	-0.2557	-0.06125	0.1731	0.3472
Ksr & Kst	-0.1155	-0.08752	-0.1407	-0.1542
Ksr & Ka	-0.1918	-0.2042	-0.1853	-0.1792
L & Kit	0.0375	0.4592	0.8335	0.7692
L & Kst	-0.2897	-0.653	-0.7285	-0.6416
L & Ka	-0.9821	-0.8527	-0.5486	-0.4676
Kit & Kst	-0.5768	-0.7471	-0.7889	-0.7004
Kit & Ka	0.1482	0.02975	-0.3358	-0.3752
Kst & Ka	0.2008	0.4154	0.699	0.7745
Constraints				
Ksr	Ksr is shared			
L	L > 50 and shared			
Kit	Kit is shared			
Kst	Kst is shared			
Ka	Ka > 0.2 and shared			
Number of points				
# of X values	16	16	16	16
# Y values analyzed	16	16	16	16
Note: (~) Hit constraint				

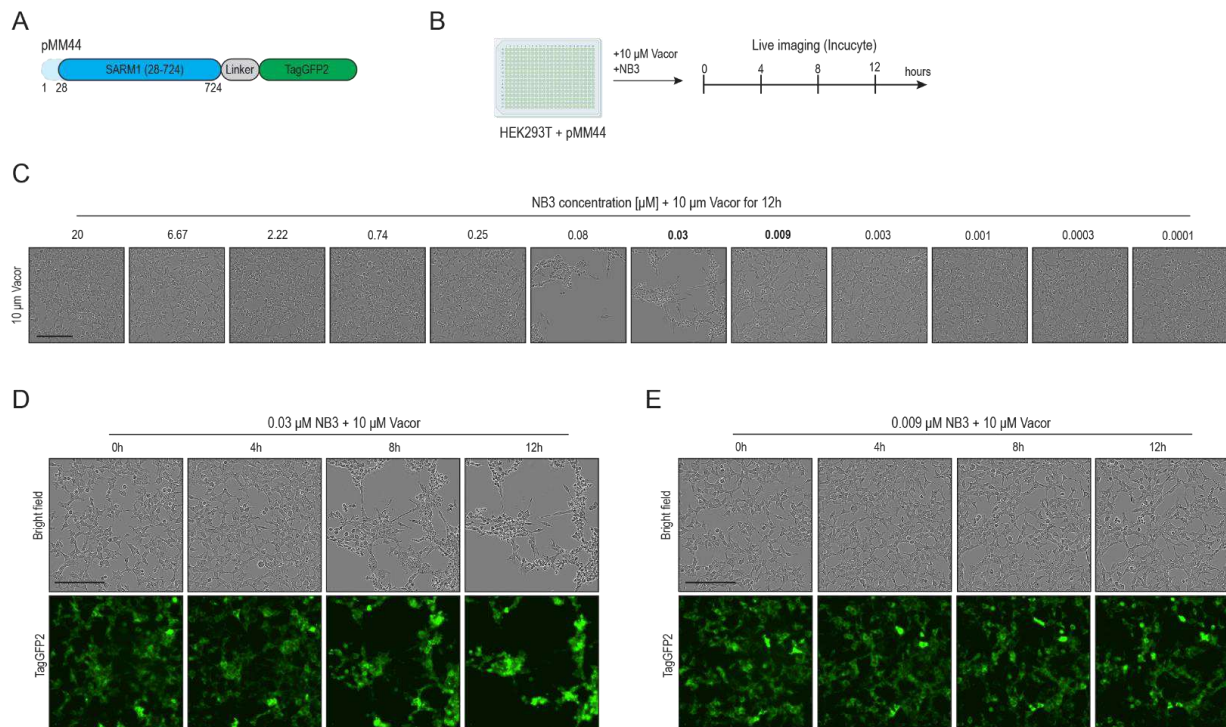
Supplementary Figure 4: Comparison of fitted parameters and goodness-of-fit metrics for fitting the MWC model with fixed values of n.

MWC model (Eq. 1.4) was fitted to the 2D titration data in Figure 1D with n fixed to 1, 2, 4, and 6. Given the anticorrelated nature and K_a and L parameters, these values were constrained (see the inserted table).



Supplementary Figure 5. Heterologous SARM1^{ΔMTS} but not full-length endogenous or heterologous SARM1 form large aggregates in mouse cortical neuron axons after BEI treatment.

(A) DIV7 mouse primary neurons treated as indicated and stained for endogenous SARM1 (green), mitochondrial marker Cytochrome C (red) and axonal marker β -III-tubulin (blue).
 (B) DIV7 mouse primary neurons overexpressing full length SARM1-GFP treated as indicated and stained for Cytochrome C (red) and β -III-tubulin (blue).
 (C) DIV7 mouse primary neurons overexpressing Δ MTS-SARM1-GFP treated as indicated and stained for Cytochrome C (red) and β -III-tubulin (blue). Upper panel shows merged image, lower panel shows only SARM1 signal. SARM1 aggregates are seen only in stress conditions or with addition of BEI when cells are overexpressing Δ MTS-SARM1-GFP.



Supplementary Figure 6. No visible SARM1 aggregation was observed in HEK293T cells expressing Δ MTS-SARM1-GFP.

(A) Schematic of the Δ MTS-SARM1-GFP construct (pMM44) used for live imaging experiments. The construct consists of human SARM1 lacking the N-terminal mitochondrial targeting sequence (amino acids 28-724), fused via a linker (2xSGGGG) to a C-terminal TagGFP2 fluorescent reporter.

(B) Experimental workflow for live-cell imaging. HEK293T cells stably expressing Δ MTS-SARM1-GFP were simultaneously treated with 10 μ M vacor to induce SARM1 activation and varying concentrations of the orthosteric inhibitor NB-3. Live imaging was performed using an Incucyte system over a 12-hour period.

(C) Representative bright-field images of HEK293T cells treated with a constant concentration of 10 μ M vacor and a titration of NB-3 ranging from 20 μ M to 0.0001 μ M. Images show cell morphology after 12 hours of treatment.

(D) Time-lapse imaging of HEK293T cells treated with 10 μ M vacor and 0.03 μ M NB-3. Bright-field (top) and TagGFP2 fluorescence (bottom) images are shown at 0, 4, 8, and 12 hours post-treatment. The TagGFP2 signal remains diffused within the cytoplasm, indicating no formation of visible SARM1 aggregates or condensates during the activation time course.

(E) Time-lapse imaging of HEK293T cells treated with 10 μ M vacor and 0.009 μ M NB-3. Bright-field (top) and TagGFP2 fluorescence (bottom) images are shown at 0, 4, 8, and 12 hours. Similar to the 0.03 μ M NB-3 condition, SARM1-TagGFP2 fluorescence remains diffuse, further supporting the absence of large-scale SARM1 polymerization or phase transition in HEK293T cells under conditions where SARM1 is activated by vacor and modulated by sub-saturating concentrations of orthosteric inhibitors.

Supplementary Methods

Animals

To prepare primary embryonic cortical cultures, embryos were obtained from timed pregnant C57BL/6N mice from Charles River Laboratories (MA, USA). Mice were housed on a regular light/dark cycle (14:10 hour (hr)) with ad libitum access to food (LabDiet 5010) and water. All animal care/handling procedures were reviewed and approved by Genentech's Institutional Animal Care and Use Committee, and were conducted in full compliance with regulatory statutes, Institutional Animal Care and Use Committee policies, and National Institutes of Health guidelines.

Dissociated primary mouse cortical neuron cultures & AAV transduction

Cortices from day 15 embryos (E15) were dissected, stripped of meninges, washed 3x with cold HBSS (Invitrogen, 14170-112), and incubated for 10 min at 37°C in HBSS supplemented with 0.25% trypsin (Invitrogen, 15090-046) and DNase I (Roche, 104159). Tissue was washed 3x with HBSS and triturated in plating media containing DNase I (Neurobasal Medium (Thermo Fisher, 21103-049), 20% heat-inactivated horse serum (Thermo Fisher, 26050-088), 25 mM sucrose, and 0.25% Gibco GlutaMAX (Thermo Fisher, 35050-061)). Dissociated cells were centrifuged at 125 g for 5 min at 4°C, resuspended in plating medium, and plated in poly-L-lysine-coated (Millipore Sigma, P1274) plates. After 24 hrs, the plating medium was replaced with NActv4 (Brain Bits, NB4500). Cells were maintained at 37°C with 5% CO₂ and the medium was renewed using 50% exchange every 3-4 days. At DIV2, neuronal cultures were transduced with 10k MOI of AAV.DJ, hSyn hSARM1-GFP or AAV.DJ, hSyn ΔMTS-hSARM1-GFP (missing first 25 N-terminal amino acids) for approx. 10hrs.

Immunocytochemistry

At DIV7, neurons were fixed with 4% PFA in PBS for 20 minutes at 37°C, washed 3x with PBS and permeabilized with a blocking buffer (0.1% triton, 10% FBS) for 20 minutes. Cells were then incubated with respective antibodies in the blocking buffer overnight at 4°C, washed 3x with PBS and incubated with AlexaFluor-conjugated secondary antibodies (1:500) for two hours at room temperature. Cells were washed 3x with PBS and imaged using Zeiss LSM980 confocal microscope. Data acquisition was done through Zen Microscopy software (Blue edition) (Zeiss). Primary antibodies: Cytochrome C (Abcam, ab110325), SARM1 (Biolegend, 696602), β-III-tubulin (Aves).

Cloning and Vector Design

The pMM44 expression construct was designed in Geneious Prime (Dotmatics) and synthesized by GenScript. The expression cassette is driven by a human PGK promoter. The construct encodes a truncated human SARM1 (residues 28–724, mitochondrial localization signal removed) fused at the C-terminus to TagGFP2 via a flexible 2xSGGGG linker. For stable cell line selection, the vector utilizes an internal ribosome entry site (IRES) immediately downstream of the TagGFP2 fusion, allowing for co-expression of the puromycin resistance gene (PuroR). The detailed sequence of the construct is described in [To be added].

Cell Culture and Cell Lines

Human embryonic kidney 293T (HEK293T) cells (Cat# CRL-3216, ATCC) were maintained in DMEM High-Glucose (Cat# 11965092, Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Cat# A3160402, Gibco) and 1% penicillin-streptomycin (10,000 U/mL stock; Cat#

15140122, Gibco), hereafter referred to as DMEM CM (complete medium). Cells were passaged every 2–3 days to maintain exponential growth using 0.05% Trypsin-EDTA (Cat# 25300054, Gibco) for detachment. Puromycin (1 µg/mL; Cat# A1113803, Gibco) was included in the medium to select for stably transfected cells. Cells were grown in a 5% CO₂, 95% air-humidified incubator at 37°C.

Stable Cell Line Generation using PiggyBac

Stable cell lines were generated using the PiggyBac transposon system to achieve stable genomic integration of the expression cassettes. pMM44 plasmid was transfected with a helper plasmid encoding the PiggyBac (PBO) transposase at a 3:1 ratio using Lipofectamine 2000 (Cat# 11668019, Invitrogen). Two days after transfection, cells expressing pMM44 were selected for 7 days using DMEM CM supplemented with 1 µg/mL puromycin (Cat# A1113803, Gibco).

Incucyte imaging assay

HEK293T cells stably expressing pMM44 were seeded into 384-well black tissue-culture treated plates (Cat#353962, Falcon) at a density of 5,000 cells per well in 20 µL of DMEM CM. After 24 h, NB-3 was dispensed into the assay plates (100 nL per well) using an Echo liquid handler. NB-3 was prepared as a 12-point, 3-fold serial dilution series with a top final assay concentration of 20 µM. Following compound addition, Vacor (5 µL/well), a SARM1 activator, was added to achieve a final concentration of 10 µM. Plates were centrifuged at 100 × g for 1 min and placed into the Incucyte S3 Live-Cell Analysis System (Sartorius). Bright-field and GFP images (300 ms exposure) were acquired using a 20x objective lens every 4 h for a total duration of 12 h.

pMM44 sequence

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TTGCGCCTTTTCCAAGGCAGCCCTGGGTTTGCGCAGGGACGCGGCTGCTCTGGGCGTGG
TTCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTCGCACATTCTTCACGTCCGTTTCGCAG
CGTCACCCGGATCTTCGCCGCTACCCTTGTTGGGCCCCCGGCGACGCTTCCTGCTCCGC
CCCTAAGTCGGGAAGGTTCTTGCGGTTTCGCGGCGTGCCGGACGTGACAAACGGAAGCC
GCACGTCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCG
ACCGCGATGGGCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCCGAGAGCAGCGGC
CGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCTCTGC
CCGCGCGGTGTTCCGCATTCTGCAAGCCTCCGGAGCGCACGTGCGGACGTCGGCTCCCTC
GTTGACCGAATCACCGACCTCTCTCCCCAGCGTTTTAGTGAACCGTCAGATCGCCTGGAGA
CGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGACTCTAGAGCCACCGGATCCAG
GTAGCCTTGCAAGATTGGTCGTGAGGCACTGGGCAGGTAAGTATCAAGGTTACAAGACA
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TGGGCTGCGGGTGGCCGCGGGCCCCGCGAAGTGTGCGCGGGGGCAGGCACCGAGGTG
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GACGCGTGTGCAGGCCGCGCGCCTGCTGGAGCAGATCCTGGTGGCTGAGAACCGAGACC
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ATGCCAGAGGCTGGTGGCGGCCGCGGCCCTGGACGCGGTGCTGTATTGGTGCCGCCGC
ACGGACCCCGCGCTGCTGCGCCACTGCGCGCTGGCGCTGGGCAACTGCGCGCTGCACG
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1 GGGGCCAGGCGGTGCAGCGACGCATGGTAGAGAAGCGCGCAGCCGAGTGGCTCTTCCC
2 GCTCGCCTTCTCCAAGGAGGACGAGCTGCTTCGGCTGCACGCCTGCCTCGCAGTAGCGG
3 TGTTGGCGACTAACAAAGGAGGTGGAGCGCGAGGTGGAGCGCTCGGGCAGCTGGCGCTC
4 GTGGAGCCGCTTGTGGCCTCGCTGGACCCTGGCCGCTTCGCCCGCTGTCTGGTGGACGC
5 CAGCGACACAAGCCAGGGCCGCGGGCCCGACGACCTGCAGCGCCTCGTGCCGTTGCTC
6 GACTCTAACCGCTTGGAGGCGCAGTGCATCGGGGCTTTCTACCTCTGCGCCGAGGCTGCC
7 ATCAAGAGCCTGCAAGGCAAGACCAAGGTGTTTCAGCGACATCGGCGCCATCCAGAGCCTG
8 AAACGCCTGGTTTCCTACTCTACCAATGGCACTAAGTCGGCGCTGGCCAAGCGCGCGCTG
9 CGCCTGCTGGGCGAGGAGGTGCCACGGCCCATCCTGCCCTCCGTGCCCAGCTGGAAGGA
10 GGCCGAGGTTTCAGACGTGGCTGCAGCAGATCGGTTTCTCCAAGTACTGCGAGAGCTTCCG
11 GGAGCAGCAGGTGGATGGCGACCTGCTTCTGCGGCTCACGGAGGAGGAACCTCCAGACCG
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13 AGACCTTCGCCAACTATTCTACGTGCGACCGCAGCAACCTGGCGGACTGGCTGGGCAGCC
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15 CTGCTGCACCGCGTGTCTGAGCAGCAGCTGCTGGAAGACTGCGGCATCCACCTGGGCGT
16 GCACCGCGCCCGCATCCTCACGGCGGCCAGAGAAATGCTACACTCCCCGCTGCCCTGTA
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18 GTTCCAGCTGGCCAGTCTCCTGAAGGTGCACCTGCAGCTGCATGGCTTCAGTGTCTTCA
19 TTGATGTGGAGAAGCTGGAAGCAGGCAAGTTCGAGGACAAACTCATCCAGAGTGTATGG
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21 ATGACTGCAAGGATTGGGTGCATAAGGAGATTGTGACTGCTTTAAGCTGCGGCAAGAACAT
22 TGTGCCCATCATTGATGGCTTCGAGTGGCCTGAGCCCCAGGTCCTGCCTGAGGACATGCA
23 GGCTGTGCTTACTTTCAACGGTATCAAGTGGTCCCACGAATACCAGGAGGCCACCATTGA
24 GAAGATCATCCGCTTCTGTCAGGGCCGCTCCTCCCGGGACTCATCTGCAGGCTCTGACAC
25 CAGTTTGGAGGGTGCTGCACCCATGGGTCCAACCTCTGGTGGCGGAGGCTCGGGCGGAG
26 GTGGGTGAGCGGGGGCGAGGAGCTGTTCCCGGCATCGTGCCCGTGCTGATCGAGCT
27 GGACGGCGACGTGCACGGCCACAAGTTCAGCGTGCGCGGCGAGGGCGAGGGCGACGCC
28 GACTACGGCAAGCTGGAGATCAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGG
29 CCCACCCTGGTGACCACCCTCTGCTACGGCATCCAGTGCTTCGCCCGCTACCCCGAGCAC
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31 ATCCAGTTCAGGACGACGGCAAGTACAAGACCCGCGGCGAGGTGAAGTTCAGGGGCGA
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34 CAACAACGGCCTGGAGGCTAACTTCAAGACCCGCCACAACATCGAGGGCGGGCGGCGTGC
35 AGCTGGCCGACCACTACCAGACCAACGTGCCCTGGGCGACGGCCCCGTGCTGATCCCC
36 ATCAACCACTACCTGAGCACTCAGACCAAGATCAGCAAGGACCGCAACGAGGCCCCGCGAC
37 CACATGGTGTCTCCTGGAGTCCTTCAGCGCCTGCTGCCACACCCACGGCATGGACGAGCTG
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39 CCCCACGACCCGCGAGCGCCCGACCGAAAGGAGCGCACGACCCCATCATCCAATTCCGCC
40 CCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTA
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43 TGAATGTCGTGAAGGAAGCAGTTCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGC
44 GACCCTTTGCAGGCAGCGGAACCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGC
45 CACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCAGTGCCACGTTGTGAGTTGGA
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51 CCCGCCACGCGCCACACCGTCGATCCGGACCGCCACATCGAGCGGGTCACCGAGCTGCA

1 AGAACTCTTCCTCACGCGCGTCGGGCTCGACATCGGCAAGGTGTGGGTGCGGGACGACG
2 GCGCCGCGGTGGCGGTCTGGACCACGCCGGAGAGCGTCGAAGCGGGGGCGGTGTTTCGC
3 CGAGATCGGCCCGCGCATGGCCGAGTTGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGA
4 TGGAAGGCCTCCTGGCGCCGCACCGGCCCAAGGAGCCCGCGTGGTTCCTGGCCACCGTC
5 GGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGGCAGCGCCGTCGTGCTCCCCGGAG
6 TGGAGGCGGCCGAGCGCGCCGGGGTGGCCGCCTTCCTGGAGACCTCCGCGCCCCGCAA
7 CCTCCCCTTCTACGAGCGGCTCGGCTTCACCGTCACCGCCGACGTCGAGGTGCCCGAAG
8 GACCGCGCACCTGGTGCATGACCCGCAAGCCCGGTGCCTAAGCTAGCAACTTGTATTG
9 CAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTT
10 CACTGCATTCTAGTTGTGGTTTGTCCAACTCATCAATGTATCTTA

11 PGK promoter Intron KOZAK SARM1 28 - 724 2xSGGGG linker TagGFP2 IRES element PuroR
12 SV40 polyA

13
14 **SPR analysis of Compound 1, NB3, and NB3-ADPR injected over immobilized SARM1**
15 **constructs.** The following huSARM1 constructs were used for immobilization on the SPR
16 surface: biotin-TIR(D560-Q700), biotin-ARM-SAM(A29-T561), or FL(E26-T724)(minimal
17 chemical biotinylation, Thermofisher #A35389). The constructs were immobilized onto the SA
18 sensor chip (Cytiva) between 3,000 - 5,000 RU using capture buffer (50 mM HEPES pH 7.5,
19 150 mM NaCl, 2% glycerol, 0.5 mM TCEP, +/- 20 mM arginine, +/- 500 μ M - 1000 μ M NAD⁺ or
20 +/- 250 μ M NMN) at 5 μ L/min flow rate at 20 °C. The reference flow cell was left empty. All
21 channels were capped with biotin. The system was primed with running buffer (50 mM HEPES
22 pH 7.5, 150 mM NaCl, 1% glycerol, 0.5 mM TCEP, +/- 20 mM arginine, +/- 500 μ M - 1000 μ M
23 NAD⁺ or +/- 250 μ M NMN, 1% DMSO) at 20 °C. Compounds titrated via 2-fold dilution, 6-point
24 dose response, were injected at a flow rate of 50 μ L/min for 45 – 200 s with dissociation times
25 between 30 – 2000 s via multi-cycle (MCK) or single-cycle kinetic (SCK) mode. Model fitting of
26 binding sensorgrams was performed using Biacore S200 Evaluation v1 software. A simple
27 steady state or 1:1 binding model was applied to fit the binding profile. The following statistical
28 parameters were evaluated to assess the quality of curve fitting: the standard error of the fit
29 (SEF) should be 10% of the estimated parameter value and chi squared should generally be
30 5% of the calculated Rmax. Graphpad Prism 10 was used to generate final binding
31 sensorgrams.