

## A Multi-State Model of the CaMKII Dodecamer Suggests a Role for Calmodulin in Maintenance of Autophosphorylation

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## 1 **Abstract**

2  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) accounts for up to 2 percent of all brain  
3 protein and is essential to memory function. CaMKII activity is known to regulate dynamic shifts in the  
4 size and signaling strength of neuronal connections, a process known as synaptic plasticity. Increasingly,  
5 computational models are used to explore synaptic plasticity and the mechanisms regulating CaMKII  
6 activity. Conventional modeling approaches may exclude biophysical detail due to the impractical  
7 number of state combinations that arise when explicitly monitoring the conformational changes, ligand  
8 binding, and phosphorylation events that occur on each of the CaMKII holoenzyme's twelve subunits. To  
9 manage the combinatorial explosion without necessitating bias or loss in biological accuracy, we use a  
10 specialized syntax in the software MCell to create a rule-based model of the twelve-subunit CaMKII  
11 holoenzyme. Here we validate the rule-based model against previous measures of CaMKII activity and  
12 investigate molecular mechanisms of CaMKII regulation. Specifically, we explore how  $\text{Ca}^{2+}$ /CaM-  
13 binding may both stabilize CaMKII subunit activation and regulate maintenance of CaMKII  
14 autophosphorylation. Noting that  $\text{Ca}^{2+}$ /CaM and protein phosphatases bind CaMKII at nearby or  
15 overlapping sites, we compare model scenarios in which  $\text{Ca}^{2+}$ /CaM and protein phosphatase do or do not  
16 structurally exclude each other's binding to CaMKII. Our results suggest a functional mechanism for the  
17 so-called "CaM trapping" phenomenon, such that  $\text{Ca}^{2+}$ /CaM structurally excludes phosphatase binding  
18 and thereby prolongs CaMKII autophosphorylation. We conclude that structural protection of  
19 autophosphorylated CaMKII by  $\text{Ca}^{2+}$ /CaM may be an important mechanism for regulation of synaptic  
20 plasticity.

## 21 **Author summary**

22 In the hippocampus, the dynamic fluctuation in size and strength of neuronal connections is  
23 thought to underlie learning and memory processes. These fluctuations, called synaptic plasticity, are in-  
24 part regulated by the protein calcium/calmodulin-dependent kinase II (CaMKII). During synaptic  
25 plasticity, CaMKII becomes activated in the presence of calcium ions ( $\text{Ca}^{2+}$ ) and calmodulin (CaM),  
26 allowing it to interact enzymatically with downstream binding partners. Interestingly, activated CaMKII  
27 can phosphorylate itself, resulting in state changes that allow CaMKII to be functionally active  
28 independent of  $\text{Ca}^{2+}$ /CaM. Phosphorylation of CaMKII at Thr-286/287 has been shown to be a critical  
29 component of learning and memory. To explore the molecular mechanisms that regulate the activity of  
30 CaMKII holoenzymes, we use a rule-based approach that reduces computational complexity normally  
31 associated with representing the wide variety of functional states that a CaMKII holoenzyme can adopt.  
32 Using this approach we observe regulatory mechanisms that might be obscured by reductive approaches.  
33 Our results newly suggest that CaMKII phosphorylation at Thr-286/287 is stabilized by a mechanism in  
34 which CaM structurally excludes phosphatase binding at that site.

## 35 **Introduction**

36 CaMKII is a protein of interest because of its crucial role in synaptic plasticity [1-5]. In the  
37 hippocampus, synaptic plasticity in the post-synapse occurs within mushroom-shaped protrusions called  
38 dendritic spines [6]. Synaptic plasticity is dependent on calcium ion ( $\text{Ca}^{2+}$ ) flux through N-methyl-D-  
39 aspartate receptors (NMDARs) located on the dendritic spines of the post-synaptic neuron [7]. Depending  
40 on the magnitude, frequency, and location of  $\text{Ca}^{2+}$  flux, synaptic plasticity may produce increases or  
41 decreases (or neither) in synaptic strength [8, 9]. Large, higher-frequency  $\text{Ca}^{2+}$  spikes can induce an  
42 enduring up-regulation of synaptic strength, called long-term potentiation (LTP); while weak, lower-  
43 frequency  $\text{Ca}^{2+}$  spikes can induce an enduring down-regulation of synaptic strength, called long-term  
44 depression (LTD) [9, 10]. Whether  $\text{Ca}^{2+}$  spikes induce LTP or LTD depends on relative activation of  
45 intracellular protein signaling networks. When  $\text{Ca}^{2+}$  first enters the dendritic spine, it interacts with a

46 variety of buffer and sensor proteins, chiefly calmodulin (CaM), which has many protein targets in the  
47 spine, including CaMKII [5, 11, 12].

48 The CaMKII holoenzyme contains at least twelve subunits [13-16] arranged as two rings of six.  
49 As shown in Fig 1, each CaMKII subunit features an N-terminal kinase domain and C-terminal hub  
50 domain [17]. Between the kinase and hub domains is a flexible regulatory domain which lends to the  
51 subunit a wide range of movement away from the holoenzyme's central hub. A crystal structure of human  
52 alpha-CaMKII expressed in *E. coli* published by Chao *et al.* (2011) shows CaMKII subunits as able to  
53 rapidly and stochastically pivot between a “docked” and “undocked” conformation, seemingly mediated  
54 by residues on the kinase domain's activation loop and a spur structure on the hub domain (see Fig 3C in  
55 [17]), such that a docked subunit may be inaccessible to CaM binding. In contrast, a more recent work  
56 using electron microscopy with rat alpha-CaMKII expressed in Sf9 cells suggests that less than 3 percent  
57 of subunits exhibit a compact (or docked) conformation [18]. Given the uncertainty in the field, we  
58 include subunit docking and undocking in our model, allowing for future exploration of this possible  
59 subunit functionality. In addition to docking and undocking, each subunit can be in an “inactive”  
60 conformation when the regulatory domain is bound to the kinase domain (Fig 1B), or an “active”  
61 conformation when this binding is disrupted by the binding of Ca<sup>2+</sup>/CaM or phosphorylation at Thr-286  
62 [17, 19]. In the active conformation the catalytic domain of a subunit is able to bind and phosphorylate  
63 enzymatic substrates. A subunit may spontaneously return to an inactive conformation in the absence of  
64 Ca<sup>2+</sup>/CaM or phosphorylation at Thr-286 [19].

65 **Fig 1. Schematic of CaMKII Subunit Structure.** (A) Map of amino acid residues in a CaMKII subunit.  
66 The N-terminal kinase domain (blue) approximately spans residues 1-274. The regulatory domain  
67 (residues 275-314, yellow) binds to the kinase domain autoinhibiting the kinase activity of the each  
68 CaMKII subunit. The putative phosphatase binding site is also shown purple. The Ca<sup>2+</sup>/CaM binding site  
69 is shown in orange. Subunits self-associate via the hub domain (residues 315-475, green) to form  
70 multimeric complexes of 12-14 subunit holoenzymes. (B) The “inactive” CaMKII subunit (PDB: 3SOA)  
71 in which the regulatory domain (yellow) is closely associated with the kinase domain (blue). (C) A  
72 schematic of the “active” CaMKII subunit. The regulatory domain (yellow) is not bound to the kinase  
73 domain (blue). This schematic was generated by manually modifying PDB entry 3SOA to illustrate how  
74 the regulatory domain may be available for Ca<sup>2+</sup>/CaM binding and the kinase domain open for substrate

75 binding. (D) Cartoon depiction of all protein species in our model, in which Ca<sup>2+</sup>/CaM (orange) or  
76 phosphatase (purple) may bind to the regulatory domain (yellow) of a CaMKII subunit.

77  
78 CaMKII activity can become Ca<sup>2+</sup>/CaM-independent through phosphorylation at Thr-286, which  
79 is required for LTP [3, 20]. Importantly, this phenomenon is an autophosphorylation: it is thought to  
80 occur when an active subunit phosphorylates neighboring subunits within the same holoenzyme [21, 22].  
81 Autophosphorylation at Thr-286 (“pThr-286”) is thought to provide structural stability to a subunit’s  
82 active conformation (reviewed in [23]) [24]. Because CaMKII plays a key role in the induction of LTP,  
83 and ultimately learning and memory (reviewed in [4, 8]), we seek to better understand the biochemical  
84 regulation of CaMKII activation and autophosphorylation via computational modeling.

85 To characterize the spatiotemporal regulation of CaMKII, experimental studies are increasingly  
86 complemented by computational models [15, 17, 25, 26]. Computational models of Ca<sup>2+</sup>-dependent  
87 signaling implicate competition, binding kinetics, feedback loops, and spatial effects in regulating enzyme  
88 activation [7, 12, 24, 27, 28]. However, fully characterizing these and other mechanisms of CaMKII  
89 regulation is impeded by the challenge of accurately portraying the CaMKII holoenzyme. As described by  
90 previous work, combinatorial explosion applies to models of CaMKII (and similar biomolecules) because  
91 the protein exhibits a large number of functionally significant and not necessarily inter-dependent states  
92 [24, 26, 29-31]. The large number of possible states of CaMKII can neither be explicitly specified nor  
93 efficiently evaluated with conventional mass action-based methods. Indeed, for just one CaMKII hexamer  
94 ring, we estimate a state space of ~32 billion states, and for the full dodecamer approximately 10<sup>20</sup>  
95 possible states (See S1 Appendix). The numbers of possible CaMKII states far exceeds the number of  
96 CaMKII molecules in a dendritic spine, suggesting that some states never occur and are therefore not  
97 functionally important. Previous models leverage this observation to reduce the model state space and  
98 provide valuable insight to CaMKII binding and autophosphorylation dynamics [24, 31-34]. However, for  
99 CaMKII it remains unclear which states functionally participate in synaptic plasticity. Reduced models  
100 can inadvertently obscure key mechanisms regulating CaMKII activation and autophosphorylation. To

101 elucidate complex regulatory mechanisms, it may be necessary for models to provide for all possible  
102 states *ab initio*.

103 In this work, we use rule-based model specification and particle-based rule evaluation methods to  
104 overcome combinatorial explosion [26, 30, 35]. Rules are conditions, based primarily on experimental  
105 observations, that prescribe when an implicitly-defined reaction may occur. At a given iteration, only  
106 states that matter for the execution of a particular rule are explicitly declared. States that do not matter to a  
107 particular rule can be omitted, a principle that has been paraphrased as “don’t care, don’t write” [36]. We  
108 use rule- and particle-based methods within the spatial-stochastic software MCell 3.3 [28, 37] to present a  
109 comprehensive multi-state model of the CaMKII dodecamer. Other simulation platforms can also  
110 overcome combinatorial explosion through rule-based model specification (e.g. BioNetGen [38]) or  
111 network-free approaches (e.g. Nfsim [39]). Unlike other platforms, MCell 3.3 provides both spatial-  
112 stochastic and rule-based modeling, although multi-state molecules in MCell 3.3 cannot diffuse. We use  
113 MCell 3.3 in anticipation of future MCell versions accounting for multi-state molecule diffusion, and to  
114 eventually build on simulations with physiological dendritic spine geometries such as those by Bartol *et*  
115 *al.* (2015) [40].

116 Here, we validate this rule-based MCell model of CaMKII regulation against current descriptions  
117 of the Ca<sup>2+</sup> frequency-dependence of CaMKII activation. By varying the rules and model parameter  
118 values we can simulate different experimental manipulations of CaMKII interaction with Ca<sup>2+</sup>/CaM and  
119 phosphatase and thereby explore various mechanisms regulating CaMKII activity. In particular, we show  
120 that Ca<sup>2+</sup>/CaM is important not only for regulating activation of CaMKII but may also contribute to the  
121 maintenance of CaMKII phosphorylation at Thr-286. We hypothesize that by limiting access of  
122 phosphatases to CaMKII Thr-286 (perhaps by steric hindrance), Ca<sup>2+</sup>/CaM may prolong the lifetime of  
123 the auto-phosphorylated state.

## 124 **Results**

### 125 **Model Development**

126 **Molecular Species.** The model contains three protein species: CaM, protein phosphatase, and CaMKII.

127  $\text{Ca}^{2+}/\text{CaM}$  facilitates CaMKII activation, which leads to autophosphorylation at Thr-286, and phosphatase  
128 activity facilitates de-phosphorylation at Thr-286. Both protein phosphatase 1 (PP1) and protein  
129 phosphatase 2A (PP2A) have been shown to dephosphorylate Thr-286, though in different subcellular  
130 fractions (reviewed by [21, 41-43]). Here we refer to them generally as protein phosphatase (PP).

131 CaM and PP are modeled in MCell as conventional cytosolic molecules. CaM is modeled as  
132 having one of two states: un-bound apo-CaM and fully-saturated  $\text{Ca}^{2+}/\text{CaM}$  (four  $\text{Ca}^{2+}$  bound to CaM).  
133 Although we and others have described the importance of sub-saturated  $\text{Ca}^{2+}/\text{CaM}$  states with fewer than  
134 4  $\text{Ca}^{2+}$  [12, 24, 31, 44-46], the dynamics of  $\text{Ca}^{2+}$ -CaM binding and the binding of sub-saturated  $\text{Ca}^{2+}/\text{CaM}$   
135 to CaMKII are beyond the scope of this current work. Indeed, accounting for sub-saturated  $\text{Ca}^{2+}/\text{CaM}$   
136 would here require a multi-state representation, and because multi-state molecules cannot diffuse in  
137 MCell 3.3, we simplify our  $\text{Ca}^{2+}/\text{CaM}$  model to allow CaM and CaMKII to interact. Thus, similarly to  
138 previous models [27, 47], we assume that apo-CaM has a negligible affinity for CaMKII; only fully-  
139 saturated  $\text{Ca}^{2+}/\text{CaM}$  binds CaMKII. In contrast to CaM, PP is modeled as single-state protein that is  
140 constitutively active and able to bind auto-phosphorylated CaMKII subunits. Our representation of  
141 constitutively active PP is consistent with previous models such as that by Lisman and Zhabotinsky  
142 (2001) [48].

143 CaMKII is modeled as a multi-subunit complex, defined using a specialized model syntax for  
144 complex molecules (COMPLEX\_MOLECULE) in MCell 3.3 [49]. This syntax allows for explicit  
145 representation of individual CaMKII dodecamers with distinguishable subunits. As shown in Fig 2, the  
146 holoenzyme is arranged as two directly-apposed, radially-symmetric rings each with six subunits. Each  
147 subunit features five “flags”, each standing for a particular state that each CaMKII subunit can adopt.  
148 Flags are used in rule evaluation, which occurs at each time step and for each individual subunit. That is,  
149 MCell repeatedly evaluates model rules against a given subunit’s flags (and those of the neighboring

150 subunits) to determine which state transitions a subunit undertakes at each time step. In the following sub-  
151 sections, we describe all CaMKII model flags, the state transitions that apply to each flag, the conditions  
152 and rate parameters for each state transition, and related model assumptions. In Fig 2, we visually convey  
153 how CaMKII subunits transition between states according to our model's rules. In S1 Appendix we  
154 summarize the state transition rules and rate parameter values.

155 **Fig 2. CaMKII holoenzyme state transitions.** (A) CaMKII has twelve subunits arranged in two radially  
156 symmetric, directly apposed rings. Subunits may spontaneously undock/extend from the central hub or  
157 dock/retract (if inactive). When undocked, subunits may spontaneously open/activate. (B) If two  
158 neighboring subunits are active, one may auto-phosphorylate the other at Thr-286. If auto-phosphorylated  
159 (pThr-286), a subunit may remain active even upon un-binding of CaM. A pThr-286 subunit un-bound to  
160 CaM may additionally phosphorylate at Thr-306, blocking subsequent re-binding of Ca<sup>2+</sup>/CaM. A pThr-  
161 286 subunit may also bind and become de-phosphorylated by PP (purple).  
162

163 **Flag 1: Subunit docking.** Docking is a binary flag that describes subunits as either “docked” or  
164 “undocked” to the CaMKII central hub. Subunits are instantiated in a docked state but may undergo  
165 numerous transitions between docked and undocked over the course of a simulation. At each time step,  
166 we assess a rule governing the subunit's transition from a docked to undocked state. If this rule is  
167 satisfied, meaning that the subunit's docking flag is verified as “docked”, then the transition is considered.  
168 Similarly, we assess a separate rule governing a transition from an undocked to docked state, which  
169 requires that the subunit not be bound to CaM and not phosphorylated at Thr-306 [17].

170 Subunit docking follows the structural model of Chao *et al.*, who showed that a subunit cannot  
171 bind CaM as long as the subunit is in a compact conformation, docked to its central hub [17]. Docking  
172 implies a two-step process in which the subunit must first un-dock before subsequent CaM-binding,  
173 which accounts for the reported difference in binding rate for CaM to CaMKII-derived peptide ( $1 \times 10^8$   
174  $M^{-1}s^{-1}$  [50]) and for CaM to full-length CaMKII-T286A ( $1.8 \times 10^6 M^{-1}s^{-1}$  [51]). Taking the ratio of these  
175 two rates gives an equilibrium constant for docking of 0.018, which is consistent with estimates by Chao  
176 *et al.*, who assumed  $K_{\text{docking}}$  to fall between 0.01 and 100 [17]. With this equilibrium constant, we estimate  
177 kinetic rates for docking and undocking. For this estimation, we first note that subunit docking involves a

178 structural conformation change on a relatively large scale. Referring to a separate, and notably smaller-  
179 scale, conformational change in our model, in which CaM quickly transitions from an initially- to fully-  
180 bound state (see Flag 3: CaM Binding), we assume the docked-to-undocked transition to proceed at an  
181 order of magnitude slower. We therefore arrive at an assumed rate for  $k_{\text{dock}}$  of  $35 \text{ s}^{-1}$ . In turn, this gives an  
182 undocking rate  $k_{\text{undock}} = k_{\text{dock}} \times K_{\text{docking}}$  of  $0.63 \text{ s}^{-1}$ , which lies within the range of  $0.01 \text{ s}^{-1}$  and  $100 \text{ s}^{-1}$  for  
183  $k_{\text{undock}}$  assumed by Chao *et al.*

184 **Flag 2: Subunit activation.** The activation flag describes subunits as either “active” or “inactive”. An  
185 inactive subunit has no catalytic activity because the regulatory domain is bound to the subunit’s catalytic  
186 site; others may refer to it as a closed subunit. Conversely, an active subunit has catalytic activity because  
187 the regulatory domain’s inhibition of the kinase domain is disrupted; in other words, an active subunit is  
188 an open subunit. When a subunit is active,  $\text{Ca}^{2+}/\text{CaM}$  and/or other proteins may access and bind CaMKII.  
189 In our model, the transition reaction from inactive to active (opening) involves no explicit rules (but  
190 rather occurs unconditionally and as governed by rates described below). In contrast, two rules inform the  
191 conditions for subunit inactivation: that the subunit is 1) not fully-bound to CaM, and 2) not  
192 phosphorylated at Thr-286.

193 To assign rate parameters for this flag, we first note that subunits can fluctuate between inactive  
194 and active states rapidly in the absence of  $\text{Ca}^{2+}/\text{CaM}$  (on the order of hundreds of nanoseconds) [19, 52].  
195 Noting this, we set the rate parameter for subunit inactivation at  $1 \times 10^7 \text{ s}^{-1}$ . Further, Stefan *et al.*  
196 determined that the activation probability (in the absence of CaM and phosphorylation) is 0.002, leading  
197 us to set our activation rate parameter to  $2 \times 10^4 \text{ s}^{-1}$  [29]. Thus, we arrive at a model in which CaMKII  
198 subunit activation is unstable until stabilized by CaM-binding or autophosphorylation.

199 **Flag 3: CaM binding.** CaM binding is a ternary flag meaning that each CaMKII subunit displays one  
200 of three states, where CaM may be “unbound”, “initially-bound”, or “fully bound”. Our model adapts  
201 previous work by Stefan *et al.* (2012) to describe CaM-binding to CaMKII as a two-step process [29].

202 First, CaM binds to the regulatory domain of a CaMKII subunit (residues 298-312), resulting in a low-  
203 affinity “initially bound” CaMKII state, which is compatible with both the closed and open subunit  
204 conformation. Second, if the initially bound CaMKII opens it may transition to a “fully bound” state that  
205 describes the complete, higher-affinity interaction between CaM and CaMKII along residues 291-312  
206 (see Figure 5 in [29]). We specify three rules to govern the transition from an unbound to initially bound  
207 state: the subunit must be 1) undocked, 2) not PP-bound, and 3) un-phosphorylated at Thr-306. The  
208 transition reaction from initially bound to a fully bound state is governed by a single rule that the subunit  
209 already be active/open. Dissociation of CaM from a fully bound CaM-CaMKII state proceeds through the  
210 initially bound state before becoming completely unbound from CaMKII.

211 In order to determine the parameters governing initial binding of CaM to CaMKII, we use data on  
212 CaM binding to CaMKII-derived peptides, rather than full-length CaMKII. This is done to separate the  
213 intrinsic binding constants from the parameters governing subunit activation/inactivation and  
214 docking/undocking. The microscopic  $k_{on}$  for CaM binding to CaMKII has been measured, using a  
215 CaMKII peptide and fluorescently labeled DA-CaM, as  $1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  [50]. For the  $K_D$  governing initial  
216 CaM binding, we use the  $K_D$  reported by Tse *et al.* for CaM binding to a low-affinity peptide (CaMKII  
217 residues 300-312), which is  $5.9 \times 10^{-6} \text{ M}$  [53]. From these two parameters, we can compute the  
218 dissociation rate of initially-bound CaM from CaMKII:  $k_{off\_CaM\_ini} = K_{d\_CaM\_ini} \times k_{on\_CaM} = 590 \text{ s}^{-1}$ .

219 In order to determine the parameters governing the transition from initially-bound to fully-bound  
220 CaM to CaMKII, we note that this transition involves a structural compaction of the CaM molecule,  
221 which has been measured using fluorescent labels [50, 51]. Using fluorescent labels to analyze the  
222 structural compaction of CaM is convenient in its exclusion of effects due to conformational changes  
223 within CaMKII subunits or the CaMKII holoenzyme. Thus, we use these measurements as a proxy for  
224 CaM binding to a CaMKII peptide and to estimate parameters governing the transition between initially-  
225 and fully-bound CaM-CaMKII. Based on experiments by Torok *et al.*, we identify a transition rate from  
226 initially- to fully-bound CaM-CaMKII of  $350 \text{ s}^{-1}$  and from fully- back to initially-bound CaM of  $4 \times 10^{-3}$

227  $s^{-1}$  [50]. This means that, in the absence of obstructions to binding, the likelihood of a bound CaM  
228 molecule being in the initial binding state (rather than the fully bound state) is  $4 \times 10^{-3} / 350 = \sim 1.1 \times 10^{-5}$ .  
229 This is consistent with a probability of CaM being bound to the high-affinity site of 0.99999 which was  
230 derived by Stefan *et al.* (2012) [29].

231 **Flag 4: Phosphorylation at Thr-286.** Phosphorylation at the residue Thr-286 is a ternary flag that  
232 describes this site as either “un-phosphorylated (uThr-286)”, “phosphorylated (pThr-286)”, or  
233 “phosphatase-bound”. We specify three rules to govern the reaction that transitions a subunit from uThr-  
234 286 to pThr-286: the subunit 1) be uThr-286, 2) be active, and 3) have an active neighbor subunit in the  
235 same holoenzyme ring. The neighboring subunit’s activation flag is considered because  
236 autophosphorylation is facilitated by its catalytic site. Our model only considers the counter-clockwise  
237 neighbor subunit because, in the absence of experimental observations to the contrary, we assume that  
238 steric effects cause autophosphorylation to occur in only one direction about a CaMKII ring, similar to  
239 previous work [54, 55]. The rate of autophosphorylation,  $1 s^{-1}$ , at Thr-286 is taken from an earlier study of  
240 CaMKII autophosphorylation in the presence of CaM [44].

241 De-phosphorylation of pThr-286 is facilitated by binding and enzymatic activity of protein  
242 phosphatases PP1 and PP2A, here referred to generally as PP [41, 42]. Two rules govern PP binding to a  
243 CaMKII subunit (the transition from pThr-286 to a phosphatase-bound state): that the subunit be 1) pThr-  
244 286 and 2) un-bound to CaM. It has been shown that a majority of autophosphorylated CaMKII in the  
245 PSD is dephosphorylated by PP1 [56, 57]; while in brain extracts autophosphorylated CaMKII is mostly  
246 dephosphorylated by PP2A [41]. The requirement that CaM be unbound from CaMKII in order for PP to  
247 bind to CaMKII is motivated by the observation that simultaneous binding of CaM and PP to the CaMKII  
248 regulatory domain may be mutually exclusive due to steric hindrance. CaM, having molecular weight 18  
249 kDa, binds to the CaMKII regulatory domain around residues 290–309 [54, 58, 59], which is at least 4  
250 residues, and at most 23 residues away from Thr-286 (again, see also Figure 5 in [29]). To the best of our  
251 knowledge, the peptide binding footprint of neither PP (PP1 nor PP2A) onto CaMKII is not yet fully

252 described. However, both PP1 and PP2A are widely known to target pThr-286 [56, 57, 60] and de-  
253 phosphorylate threonine residues nearby alpha helices in other substrates [61, 62]. Additionally, the  
254 catalytic subunit of PP1 has a molecular weight of 37 kDa, which is nearly twice that of CaM and more  
255 than half that of a CaMKII subunit. Taken together, we hypothesize that the PP binding footprint likely  
256 overlaps with the CaM binding site, such that the presence of bound PP likely structurally excludes or  
257 impedes upon a subsequent binding of CaM to CaMKII. Similarly, the presence of bound  $\text{Ca}^{2+}/\text{CaM}$   
258 structurally would exclude coincident binding of PP. In S1 Appendix, we further discuss the quantitative  
259 basis of this structural exclusion hypothesis in light of the crystal structure of the PP1-spinophilin  
260 interaction (PDB: 3EGG) [63]. In short, PP1 tends to bind substrates at a site  $>20\text{\AA}$  from the PP1 active  
261 site. Thus, if the PP1 binding footprint does not actually contain T286, then the furthest likely CaMKII  
262 residue of PP1 binding (at least on the hub domain side of T286) is G301, well within the CaM binding  
263 footprint (S1 Appendix). We examine the regulatory implications of this hypothesis by relaxing the rules  
264 of PP binding and requiring only that the subunit be pThr-286. The association, dissociation, and catalytic  
265 rates of PP for CaMKII are taken from Zhabotinsky (2000), using a Michaelis constant of  $6\ \mu\text{M}$  and a  
266 catalytic rate of  $2\ \text{s}^{-1}$  [47].

267 **Flag 5: Phosphorylation at Thr-306.** Phosphorylation at the residue Thr-306 is a binary flag that  
268 describes this site as either un-phosphorylated (“uThr-306”) or phosphorylated (“pThr-306”). We model  
269 the transition from uThr-306 to pThr-306 using three rules: that that the subunit be 1) uThr-306, 2) active,  
270 and 3) un-bound by CaM. Our model uses a forward rate parameter 50-fold slower than that of  
271 phosphorylation at Thr-286, based on past experimental measurements [33, 64]. Over the course of our  
272 simulation times, we observe very few pThr-306 transitions and therefore exclude the reverse transition  
273 reaction describing de-phosphorylation of pThr-306 into uThr-306.

#### 274 **Stimulation frequency correlates with subunit activity**

275 To validate our model, we assessed a variety of model outputs under various regimes of  
276  $\text{Ca}^{2+}/\text{CaM}$  stimulation. As a first assessment, we simulated a persistent  $\text{Ca}^{2+}/\text{CaM}$  bolus, similar to

277 experiments by Bradshaw *et al.* (2002), who monitored CaMKII autophosphorylation over time [55]. In  
278 Fig 3 we simultaneously monitored the time-course concentration of CaMKII subunit flags indicating:  
279 initially-bound Ca<sup>2+</sup>/CaM, fully-bound Ca<sup>2+</sup>/CaM, active CaMKII, and pThr-286. In the persistent,  
280 continuous presence of Ca<sup>2+</sup>/CaM, the concentration of subunits with initially-bound Ca<sup>2+</sup>/CaM (orange  
281 trace) is noisy and consistently low, implying that Ca<sup>2+</sup>/CaM transiently binds subunits in an initially-  
282 bound conformation. That is, initially-bound Ca<sup>2+</sup>/CaM seems rapidly to either dissociate or proceed to a  
283 fully-bound conformation. Fully-bound Ca<sup>2+</sup>/CaM (red trace) subunit concentrations closely follow those  
284 of active CaMKII subunits (dark blue trace) over time, providing evidence that Ca<sup>2+</sup>/CaM stabilizes  
285 CaMKII activation. Indeed, because the difference in concentrations of fully-bound Ca<sup>2+</sup>/CaM and active  
286 CaMKII is always small, we observe that although unbound CaMKII may spontaneously activate, these  
287 activated subunits rapidly return to an inactive state and are not likely to progress to a phosphorylated  
288 (pThr-286) state. We next observe that the increase of CaMKII autophosphorylation at Thr-286 (cyan  
289 trace) over time is strongly associated with increases in the number of subunits that are fully-bound to  
290 Ca<sup>2+</sup>/CaM and active subunits (dark blue and red traces, respectively). This is consistent with previous  
291 work showing that Ca<sup>2+</sup>/CaM must be bound to CaMKII for pThr-286 to occur [54] and CaMKII Ca<sup>2+</sup>-  
292 independent activity is strongly associated CaMKII autophosphorylation at Thr-286 [17, 51, 65, 66].  
293 Furthermore, we observe in Fig 3A that more than 80 percent of CaMKII subunits are autophosphorylated  
294 at t=20sec, which is of similar magnitude and timescale as observed by Bradshaw *et al.* (see Figure 2A in  
295 [55]).

296 **Fig 3. Validation of the Rule-based Model.** Bold traces (A-C) and solid circles (D) are the average of N  
297 = 50 executions. For each species (A-C), six representative traces are also shown (semi-transparent lines).  
298 (A) Model output resulting from stimulation with a large continuous bolus of Ca<sup>2+</sup>/CaM. Concentrations  
299 of active (red), initially CaM-bound (yellow), fully CaM-bound (blue), and pThr-286 (cyan) subunits. (B)  
300 Time-course average concentration (bold trace) of active subunits stimulated by 5 Hz or 50 Hz Ca<sup>2+</sup>/CaM.  
301 (C) Time-course concentration of pThr-286 subunits stimulated continuously by 5 Hz or 50 Hz  
302 Ca<sup>2+</sup>/CaM. (D) Frequency-dependent activation (red) and pThr-286 (cyan) of CaMKII subunits, with  
303 SEM error bars. Black dotted traces are linear fits.

304  
305 Next, we assessed model behavior under low- and high-frequency stimulating conditions.

306 CaMKII activation and autophosphorylation at Thr-286 in response to 5Hz and 50Hz Ca<sup>2+</sup>/CaM is plotted

307 in Figure 3B and 3C, respectively; 50 seeds were run for each condition, with 6 representative traces  
308 (transparent lines) and the average response (bold) plotted. As expected, the data showed significantly  
309 greater levels of CaMKII activation and autophosphorylation at 50Hz [12, 20]. Indeed, we compared our  
310 result in Fig 3C to work by Shifman *et al.* (2006), who observed much lower autophosphorylation at low  
311  $\text{Ca}^{2+}/\text{CaM}$  concentrations (less than 2  $\mu\text{M}$ ) than at high concentrations (see Figure 4D in [44]). Therefore,  
312 because our 50Hz model cumulatively exposes CaMKII to approximately ten times as much  $\text{Ca}^{2+}/\text{CaM}$   
313 per second as our 5Hz model, our results in Fig 3C are consistent with Shifman *et al.*, showing much  
314 higher autophosphorylation at 50Hz than 5Hz.

315 To further determine how stimulation frequency affects CaMKII activity, the model was  
316 stimulated at frequencies ranging from 1Hz to 50 Hz. At each frequency, models were sampled at 20  
317 seconds of simulation time. We observe a nearly linear correlation between both subunit activation ( $R^2 =$   
318 0.99) and pThr-286 ( $R^2 = 0.96$ ) and stimulation frequency (Fig 3D). This result is consistent with  
319 computational results from Chao *et al.*, who developed a stochastic model that also yielded a linear  
320 relationship between pThr-286 and stimulation frequency for frequencies greater than 1 Hz [15]. Taken  
321 together, these results (Fig 3) show that our model behaves as expected and is able to produce CaMKII  
322 activity and autophosphorylation behaviors similar to previous computational and experimental results.

### 323 **Exploring Switch-like Behavior in CaMKII**

324 CaMKII has long been theorized to exhibit switch-like or bistable behavior, which could underlie  
325 the importance of pThr-286 to learning and memory formation [4, 47, 48, 67, 68]. However, experimental  
326 efforts have struggled to identify a bistability between CaMKII and phosphatase activity. Though  
327 recently, Urakubo *et al.* used the chelator EGTA to control single pulses of  $\text{Ca}^{2+}$  in a mixture of CaM,  
328 CaMKII, PP1, and NMDAR peptides, leading to what seemed to be the first direct observation of  
329 CaMKII bistability [69]. Referring to Urakubo *et al.*, we explored whether a spatial stochastic model of  
330 the CaMKII dodecamer would exhibit near bistability or switch-like behavior for concentration  
331 parameters of  $\text{Ca}^{2+}$ , CaM, CaMKII, and PP known to exist in hippocampal spines. To explore this

332 bistability, we stimulated the model with a set of short  $\text{Ca}^{2+}/\text{CaM}$  input pulses (which could also be  
333 reproducible *in vitro*). Importantly, we did not aim to identify true bistability because exploring the many  
334 combinations of  $\text{Ca}^{2+}$ , kinase, and phosphatase concentrations was outside the scope of this paper. Instead  
335 we wondered if, by stimulating with brief pulses of  $\text{Ca}^{2+}/\text{CaM}$  of variable duration, our model would  
336 exhibit switch-reminiscent pThr-286 behavior. Specifically, we predicted a  $\text{Ca}^{2+}/\text{CaM}$  stimulation  
337 threshold below which pThr-286 was unachievable and above which pThr-286 was maintained.

338         In Fig 4 we exposed our model to single  $\text{Ca}^{2+}/\text{CaM}$  pulses of constant magnitude but of variable  
339 duration (similar to Figure 1B in [69]). The model was stimulated with single  $\text{Ca}^{2+}/\text{CaM}$  input pulses of  
340 magnitude 26  $\mu\text{M}$  and varying duration (0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 sec). Different pulse durations  
341 resulted in distinct levels of subunit activation, where longer pulse durations resulted in greater activation  
342 and autophosphorylation (p-Thr286) levels, (Fig 4A and B, respectively). Interestingly, subunits  
343 stimulated by even the shortest pulses of 0.05 or 0.1 sec, appeared to sustain their activation for the  
344 complete simulation period (120 sec). However, these short-pulse (0.05-0.1 sec) stimulations rarely  
345 resulted in autophosphorylation (pThr-286, Fig 4B). Longer (0.2-0.5 sec)  $\text{Ca}^{2+}/\text{CaM}$  pulses resulted in  
346 greater levels of subunit activation that started declining immediately after the  $\text{Ca}^{2+}/\text{CaM}$  pulse ended (Fig  
347 4A), but elicited pThr-286 levels that were generally sustained for the duration of a simulation (Fig.4B).  
348 Taken together, we found that CaMKII may be thresholded at a level of  $\text{Ca}^{2+}/\text{CaM}$  exposure below which  
349 pThr-286 is unobserved and above which pThr-286 is achieved and subsequently sustained across several  
350 minutes even in the presence of phosphatase.

351 **Fig 4. Response to short  $\text{Ca}^{2+}/\text{CaM}$  pulse stimulation.** Average concentration of (A) active and (B)  
352 pThr-286 CaMKII subunits over time, following  $\text{Ca}^{2+}/\text{CaM}$  stimulating pulses of length 0.05  
353 (red), 0.1 (blue), 0.2 (green), 0.3 (purple), 0.4 (yellow), and 0.5 (orange) seconds. Each trace  
354 represents the average of N=50 executions. See Appendix S1 for identical data shown with SEM  
355 error bars and over the first two seconds of simulated time.  
356

357         We briefly explored how this  $\text{Ca}^{2+}/\text{CaM}$  threshold may depend on the number of directions by  
358 which subunits can autophosphorylate their neighbors. Note that in the results up to this point,

359 autophosphorylation was limited to occurring in a single direction, or degree of freedom. That is, subunits  
360 could only autophosphorylate their adjacent neighbors [17]. We therefore created alternative versions of  
361 our model in which autophosphorylation could occur with multiple degrees of freedom, both intra- and/or  
362 trans- holoenzyme ring. We used these higher-degree of freedom models to monitor the rates of pThr-286  
363 formation both in bulk and on an individual subunit basis. As expected, pThr-286 formation and intra-  
364 holoenzyme propagation rates increased with increasing degrees of freedom (see S1 Appendix, Figure  
365 S2.2), though the differences would likely not be distinguishable by bench-top experimentation. In  
366 addition, the length of time in which consecutive subunits remained autophosphorylated also increased  
367 with increasing degrees of freedom. This implied that subunits may be more frequently  
368 autophosphorylated on time-average with increasing degrees of freedom (See Appendix S1, figures S2.3  
369 and S2.4). Future experimental and computational studies could perhaps explore autophosphorylation  
370 with higher degrees of freedom.

371 Figure 4 suggested a threshold of  $\text{Ca}^{2+}/\text{CaM}$  activation beyond which CaMKII remains  
372 autophosphorylated, implying a balance between kinase and phosphatase activity. We wondered how a  
373 putative balance between CaMKII autophosphorylation and phosphatase activity might be regulated. In  
374 the previous experimental work by Urakubo *et al.*, maximally-phosphorylated CaMKII was maintained in  
375 the presence of PP1 and GluN2B peptide for as long as 8 hours (at 4°C). In that work, addition of the  
376 kinase inhibitor K252a to phosphorylated CaMKII resulted in a steady decline in pThr-286 towards basal  
377 levels, suggesting that maintenance of pThr-286 over time was not due to low phosphatase activity, but  
378 rather a recovery of de-phosphorylated subunits back a phosphorylated state. To recreate inhibition of  
379 kinase activity in our model, at time  $t=30$  sec we introduced a high concentration (21  $\mu\text{M}$ ) of K252a,  
380 enough to bind all CaMKII subunits in the model. K252a binding results in a blocked CaMKII state that  
381 cannot be autophosphorylated (see Flag 2 in S1 Appendix). Importantly, the blocked CaMKII subunit can  
382 still be de-phosphorylated at pThr-286. In separate simulations we explored the effects of a phosphatase  
383 inhibitor, which was also introduced at  $t=30$ sec. To simulate the introduction of a phosphatase inhibitor,

384 we defined the catalytic rate of de-phosphorylation by PP1 ( $k_{\text{cat}}^{\text{PP1}}$ ) as a time-dependent variable that  
385 assumed a value of zero at  $t = 30\text{sec}$ . This implementation of kinase and phosphatase inhibition preserved  
386 normal CaM and PP1 binding dynamics.

387 We compared non-inhibited, control versions of our model to versions in which kinase activity or  
388 phosphatase activity was inhibited after stimulating with  $\text{Ca}^{2+}/\text{CaM}$  for 2 sec (Fig 5). As expected,  
389 inhibiting phosphatase activity (green trace) caused kinase activity to dominate, resulting in a steady  
390 increase in pThr-286 compared to the control (black trace). Surprisingly, the kinase-inhibited model (blue  
391 trace) showed little difference compared to the control. Instead of causing phosphatase activity to  
392 dominate, simulated kinase inhibition caused pThr-286 activity to persist as if no kinase inhibitor were  
393 present. Because pThr-286 persisted even in the presence of kinase inhibitor, we hypothesized that some  
394 other, non-enzymatic mechanism in our model was contributing to the maintenance of pThr-286.

395 **Fig 5. Blocking kinase or phosphatase activity.** Average concentration of pThr-286 CaMKII subunits  
396 over time. For all traces, the model is stimulated by a 2 sec pulse of  $\text{Ca}^{2+}/\text{CaM}$ . At time  $t=30$  sec  
397 (arrowhead), either a kinase inhibitor (blue trace) or phosphatase inhibitor (green trace) is introduced. No  
398 inhibitor is introduced in the control (black trace). Each trace represents the average of  $N=50$  executions.  
399

#### 400 **CaM-dependent exclusion of PP1 binding stabilizes autophosphorylation**

401 To understand why our model as-presented in Fig 5 showed no significant response to kinase  
402 inhibition, we wondered if another mechanism was regulating the putative balance between kinase and  
403 phosphatase activity. In every simulation presented thus far, we assumed that CaM binding to the  
404 CaMKII regulatory domain sterically hinders PP binding to the regulatory domain, and vice-versa. This  
405 was implemented in the model via a rule that requires a subunit be unbound by CaM in order for PP to  
406 bind.

407 To test the role of PP exclusion by CaM, we created a second version of our model in which PP  
408 binding would become allowable regardless of the presence of CaM. In contrast to our original  
409 “exclusive” model, the “non-exclusive” model required only that a subunit be pThr-286 in order for PP  
410 binding to be allowable. In other words, the non-exclusive model allowed  $\text{Ca}^{2+}/\text{CaM}$  and PP to bind

411 CaMKII agnostically of each other. Aside from this rule adjustment, our exclusive and non-exclusive  
412 models utilized identical parameters. As in Fig 5, we selected a  $\text{Ca}^{2+}/\text{CaM}$  bolus time of 2 sec. Again, we  
413 monitored both CaMKII activation (Figs 6A and 6B) and pThr-286 (Figs 6C and 6D) over 120 seconds of  
414 simulated time. Critically, both the exclusive and non-exclusive models were examined with high (purple  
415 trace) and low (orange trace) association rate parameter values for PP binding to CaMKII. Increasing and  
416 decreasing the association rate of PP ( $k_{\text{on}}^{\text{PP}}$  is normally set to  $3 \mu\text{M}^{-1}\text{sec}^{-1}$ ) to CaMKII by one order of  
417 magnitude accounted for parameter uncertainty and provided a magnified view of the signaling effects of  
418 CaM-mediated exclusion of PP binding.

419 **Fig 6. Comparison of Exclusive and Non-exclusive Models.** For all traces, models are stimulated by a  
420 2sec pulse of  $\text{Ca}^{2+}/\text{CaM}$ . (A) Active CaMKII subunits over time in our exclusive model. (B) Active  
421 CaMKII subunits over time in our non-exclusive model. (C) pThr-286 subunits over time in our exclusive  
422 model. (D) pThr-286 subunits over time in our non-exclusive model. (A-D) The parameter value for the  
423 rate of PP association ( $k_{\text{on}}^{\text{PP1}}$ ) with CaMKII is either increased (purple traces) or decreased (orange traces)  
424 by one order of magnitude. (E) Extension of Fig 5 to include non-exclusive model results. At time  
425  $t=30\text{sec}$  (arrows), either a kinase inhibitor (light blue trace) or phosphatase inhibitor (light green trace) is  
426 introduced. No inhibitor is introduced in the control (grey trace). All traces are the average of  $N=50$   
427 executions.

428  
429 Our results suggested that CaM-dependent exclusion of PP is an important regulatory mechanism  
430 for maintaining CaMKII autophosphorylation levels. While the PP exclusion rule had little to no effect on  
431 CaMKII subunit activation (Fig 6A and Fig 6B), pThr-286 (Fig 6C and Fig 6D) was highly influenced by  
432 the PP exclusion rule. In the exclusive model (Fig 6C), pThr-286 levels were steady and stable despite  
433 varying the PP association rate parameter by two orders of magnitude. In contrast, the non-exclusive  
434 model (Fig 6D) showed that for a high PP association rate, significant pThr-286 levels were never  
435 achieved. Moreover, for a low PP association rate, the non-exclusive model briefly attained pThr-286  
436 levels similar to those achieved in the exclusive model, but the pThr-286 levels then declined while also  
437 displaying a high level of noise. It seemed that in order to maintain pThr-286 over longer time periods,  
438 CaMKII required a mechanism regulating phosphatase access, and a regulator of phosphatase access  
439 could be CaM itself.

440 To reinforce our assertion that CaM-dependent structural exclusion of PP binding stabilizes pThr-  
441 286, we repeated simulations shown in Fig 5, but with our non-exclusive model. In Fig 6E, we stimulated  
442 our non-exclusive model with a 2sec pulse of Ca<sup>2+</sup>/CaM and then monitored pThr-286 over time. For  
443 these simulations,  $k_{on}^{PP1}$  was restored to its standard value of 3  $\mu\text{M}^{-1}\text{sec}^{-1}$ . As in Fig 5, in separate  
444 simulations we inhibited at  $t=30\text{sec}$  either phosphatase activity, kinase activity, or neither (control). The  
445 control (grey trace) was reminiscent of results in Fig 6D, in which pThr-286 was achieved but then slowly  
446 declined on a steady yet noisy basis. Notably, all non-exclusive model variants were much noisier than  
447 their exclusive model counterparts in Fig 6E. Inhibiting phosphatase activity (light green trace) in the  
448 non-exclusive model again caused kinase activity to dominate and pThr-286 levels to generally increase  
449 over time, similarly to the exclusive model. In contrast to the exclusive model, inhibiting kinase activity  
450 (light blue trace) in the non-exclusive model rapidly and totally abolished pThr-286. It seemed that for the  
451 non-exclusive model, in which CaM and PP could bind simultaneously, inhibiting kinase activity caused  
452 phosphatase activity to dominate. Taken together, these results suggested that in addition to supporting  
453 CaMKII subunit activation, CaM also has a role in maintaining CaMKII activity by blocking phosphatase  
454 access and thereby slowing down dephosphorylation.

## 455 Discussion

456 In this work, we use rule- and particle-based methods with the software MCell to model the  
457 complete CaMKII holoenzyme. Rule-based modeling allows us to account for and monitor multiple  
458 CaMKII states simultaneously without eliciting combinatorial explosion. By explicitly accounting for  
459 multiple CaMKII states, we can use this model to explore regulatory mechanisms such as the CaM-  
460 dependent maintenance of pThr-286 by structural exclusion of phosphatase binding to CaMKII.

461 Previous multi-state models of CaMKII exist but are different in focus and in scope from the  
462 present model. For example, our model is based on an earlier multi-state model by Stefan *et al.* (2012)  
463 [29] implemented in the particle-based stochastic simulator StochSim [70]. StochSim accounts for subunit  
464 topology (i.e. the user can specify whether a subunit is adjacent to another, and reactions can be neighbor-

465 sensitive), but StochSim does not explicitly account for spatial information. MCell, as a spatial simulator,  
466 offers more possibilities to precisely account for spatial effects and to situate models in spatially realistic  
467 representations of cellular compartments. In addition, the model by Stefan *et al.* provides only for  
468 interactions between adjacent CaMKII molecules on the same hexamer ring and therefore models  
469 CaMKII as a hexamer, not a dodecamer. Similarly, another previous model of CaMKII by Michalski and  
470 Loew (2012) uses the softwares BioNetGen and VCell to offer an infinite subunit holoenzyme  
471 approximation (ISHA) of the CaMKII hexamer [71-73]. The ISHA asserts that under certain enzymatic  
472 assumptions, the output of a multi-state CaMKII model is independent of holoenzyme size when the  
473 number of subunits exceeds six. However, Michalski's ISHA model is most suitable for systems  
474 containing only one holoenzyme structure-dependent reaction such as the autophosphorylation at Thr-  
475 286. Additional reactions to describe actin binding [74] or subunit exchange [14, 15] may invalidate  
476 Michalski's ISHA, whereas our model can in the future readily accommodate additional, holoenzyme  
477 structure-dependent phenomena. Finally, a more recent rule-based model of the CaMKII holoenzyme by  
478 Li and Holmes [26] offers a detailed representation of how CaM binds to Ca<sup>2+</sup> and subsequently activates  
479 CaMKII subunits, based on earlier results of CaM regulation [75]. Li and Holmes offer valuable and  
480 detailed insight into how CaM binding to CaMKII depends on Ca<sup>2+</sup> dynamics. While our model is less  
481 detailed in representing the regulation of CaM itself, our model is much more detailed in representing  
482 other aspects of CaMKII regulation, including multiple modes of CaM binding, conformational change,  
483 detailed holoenzyme structure, multiple phosphorylation sites, and dephosphorylation. We can in the  
484 future expand our MCell model to account for multiple holoenzyme structure-dependent phenomena and  
485 simultaneously incorporate this model into the broader Ca<sup>2+</sup>-dependent signaling network.

486 This work in-part demonstrates the value of MCell as a rule-based modeling framework. Rule-  
487 based modeling accommodates much larger state spaces than is possible using conventional systems of  
488 differential equations. Admittedly, not all models (including models of CaMKII) require extensive state  
489 spaces, but rule-based modeling results can help justify the assumptions typically used to reduce a state

490 space. For example, our model conditions yield, as shown in Fig 3A, negligible levels of initially-bound  
491 CaM compared to other states such as fully-bound CaM or pThr-286. Therefore, it might sometimes be  
492 appropriate to exclude an initially-bound CaM state from future implementations in frameworks for  
493 which combinatorial explosion is a concern. Aside from addressing combinatorial explosion, rule-based  
494 models are especially well-suited to discern otherwise concealed mechanisms, as exemplified by Di  
495 Camillo *et al.* who used rule-based models to identify a robustness-lending negative feedback mechanism  
496 in the insulin signaling pathway [49]. Furthermore, MCell describes CaMKII holoenzymes as discrete  
497 particles in space, which will lend realism to future spatial-stochastic models of Ca<sup>2+</sup>-dependent signaling  
498 networks in the dendritic spine, a compartment in which the Law of Mass Action is invalid [24]. This  
499 particle-based framework also allows for individual subunit monitoring, which works in conjunction with  
500 the Blender software plugin, CellBlender (see S1 Movie).

501         One of the results of this work is the identification of distinct levels of CaMKII activation and  
502 pThr-286 in response to distinct pulses of Ca<sup>2+</sup>/CaM stimulation. Distinct levels of CaMKII activation  
503 could tune the selectivity of CaMKII for certain downstream binding targets such as AMPA receptors or  
504 the structural protein PSD-95. If stimulation-dependent tuning of CaMKII activation were observed, it  
505 would be reminiscent of other studies that have implicated feedback loops [27] and binding dynamics  
506 [24] as regulators of Ca<sup>2+</sup>-dependent enzyme activation. For example, a recent study suggests that  
507 competition is an emergent property that tunes the Ca<sup>2+</sup> frequency dependence of CaM binding to  
508 downstream targets, leading Ca<sup>2+</sup>/CaM to set distinct levels of calcineurin- and CaMKII-binding [12].  
509 Similarly, CaMKII itself could preferentially select downstream binding partners as a function of its level  
510 of activation by Ca<sup>2+</sup>/CaM, possibly providing a mechanism by which CaMKII facilitates certain LTP-  
511 related molecular events. Additionally, our observation of distinct levels of CaMKII activation and  
512 thresholded pThr-286 could be an indication of long-hypothesized switch-like behavior in synaptic  
513 plasticity [4, 67]. If switch-like behavior in fact occurs, then pThr-286 is likely maintained by a balance in  
514 kinase and phosphatase activity.

515           While investigating a putative interplay in CaMKII kinase and PP phosphatase activity in  
516 maintaining pThr-286 levels, we may have identified a CaM-dependent mechanism that blocks PP  
517 binding to CaMKII. In a model that excludes simultaneous binding of CaM and PP to CaMKII, pThr-286  
518 significantly increases upon phosphatase inhibition, yet in the same model kinase inhibition causes little  
519 change in pThr-286 over time (Fig 5). In contrast, a non-exclusive model that allows simultaneous  
520 binding of CaM and PP shows that introduction of a kinase inhibitor rapidly abolishes pThr-286. These  
521 results suggest that CaM-dependent exclusion of PP may provide a stabilizing mechanism. Additionally,  
522 we use our MCell-based implementation of the model to monitor transitions between multiple states of  
523 distinct subunits within holoenzymes (Fig 7 and S1 Movie).

524 **Fig 7. Visualizing Individual Subunits with MCell and CellBlender.** In the exclusive model, PP does  
525 not bind a pThr-286 subunit until Ca<sup>2+</sup>/CaM dissociation (see t = 85 sec, comparing rows B and C). Each  
526 frame depicts the same CaMKII holoenzyme, from the same perspective, at identical time points under  
527 50Hz Ca<sup>2+</sup>/CaM stimulation. Each dodecahedron is a single CaMKII subunit. (A) Inactive CaMKII  
528 subunits (white) spontaneously become active (black) and remain active while bound to Ca<sup>2+</sup>/CaM. (B)  
529 Un-bound CaMKII subunits (yellow) will not bind Ca<sup>2+</sup>/CaM (red) and become Ca<sup>2+</sup>/CaM-bound (purple)  
530 unless the subunit had previously activated. (C) uThr-286 subunits (green) become pThr-286 (blue). If  
531 Ca<sup>2+</sup>/CaM dissociates from a pThr-286 subunit, then PP can bind and form a PP-CaMKII complex (cyan).  
532

533           The major outcome of this work is a proposed mechanism in which bound Ca<sup>2+</sup>/CaM could  
534 exclude PP from accessing CaMKII subunits, thereby protecting pThr-286. We assert that CaM-  
535 dependent exclusion of PP could provide a functional role for so-called “CaM trapping” [54] and possibly  
536 contribute to CaMKII bistability. Indeed, a model by Zhabotinsky (2000) explored CaMKII bistability,  
537 indicating that two stable states of pThr-286 would in-part require very high CaMKII concentrations,  
538 seemingly to bolster kinase activity in the system [47]. However, the Zhabotinsky model assumes that  
539 CaM and PP1 could bind CaMKII simultaneously, possibly exaggerating the ability of PP1 to de-  
540 phosphorylate at Thr-286. If PP1 binding were to be encumbered in the Zhabotinsky model, perhaps  
541 through CaM-dependent exclusion, then bistability might be achievable at lower CaMKII concentrations.

542           Previous studies have sought to explore the dependence of CaMKII de-phosphorylation on the  
543 presence of Ca<sup>2+</sup>/CaM. An experiment by Bradshaw *et al.* (2003) quantifies PP1-mediated de-

544 phosphorylation rates of pThr-286 *in vitro*, in the presence or absence of the Ca<sup>2+</sup> chelator EGTA (see  
545 Figure 4B in [76]). The Bradshaw results suggest that PP1 activity at 0°C is unaffected by the presence of  
546 bound-CaM to CaMKII, seemingly at odds with the results of our model. However, EGTA can only act  
547 on free Ca<sup>2+</sup>, Ca<sup>2+</sup>/CaM has a low dissociation rate for Ca<sup>2+</sup>, and phospho-CaMKII has a low dissociation  
548 rate for Ca<sup>2+</sup>/CaM. Also, other studies [12, 24, 44] indicate that sub-saturated Ca<sup>2+</sup>/CaM may in fact  
549 significantly bind CaMKII. Thus, because the Bradshaw experiment does not remove CaM from the  
550 reaction mixture, the Bradshaw results may be confounded by a slow unbinding of CaM from CaMKII.  
551 Our results motivate a revised phosphatase assay, which a) uses physiological temperatures, b) ensures  
552 detection of de-phosphorylation solely of pThr-286, and c) totally removes CaM from the reaction  
553 mixture.

554 Future work may need to explore the ability of PP1 to bind CaMKII in the presence of sub-  
555 saturated Ca<sup>2+</sup>/CaM. Referring to Fig 6, although some CaM may remain bound to CaMKII when Ca<sup>2+</sup>  
556 concentration decreases, the resulting sub-saturated (lower affinity) Ca<sup>2+</sup>/CaM state might be out-  
557 competed by PP1. In other words, if only the CaM C-terminus were bound to CaMKII, would PP1 have  
558 sufficient structural access and/or affinity to bind also? Thus, in addition to the revised phosphatase assay  
559 mentioned above, further structural studies of the CaM-CaMKII and PP1-CaMKII interaction are needed.  
560 Also, because this work could only model multi-state CaMKII (but not also multi-state CaM) due to  
561 limitations in MCell 3.3, perhaps a future version of MCell should provide for the diffusion of multiple  
562 multi-state proteins. With a platform that can handle multiple multi-state proteins, a model could much  
563 more explicitly handle Ca<sup>2+</sup>/CaM-binding and further explore our results.

564 CaM-dependent PP exclusion could provide an added layer of robustness to similar mechanisms  
565 that may protect pThr-286 from de-phosphorylation. For example, Mullasseril *et al.* (2007) observe that  
566 endogenous, PSD-resident PP1 cannot de-phosphorylate CaMKII at pThr-286, whereas adding exogenous  
567 PP1 does cause de-phosphorylation [68]. The results by Mullasseril *et al.* suggest that endogenous PP1 is  
568 somehow sequestered by the PSD scaffold, and only upon saturation of this scaffold by exogenous PP1

569 does pThr-286 become de-phosphorylated. Our results indicate that perhaps in addition to saturating the  
570 PSD scaffold, the added exogenous PP1 could be out-competing CaM for binding to CaMKII, thereby  
571 terminating protection of pThr-286 by CaM. As another example, Urakubo *et al.* suggest that pThr-286  
572 could be protected from PP activity by GluN2B binding, showing that GluN2B peptides are necessary for  
573 an apparent CaMKII bistability *in vitro* [69]. Notably, Urakubo *et al.* observe a decline in pThr-286 upon  
574 kinase inhibition, in contrast with our exclusive model, though this is likely due to differences in the  
575 conditions and timescales between Urakubo's experiments and our model. Overall, it seems scaffold-  
576 dependent sequestration of PP1 [68], GluN2B-dependent PP exclusion [69], and CaM-dependent PP  
577 exclusion could together provide considerable robustness of pThr-286 to phosphatase activity.

## 578 **Methods**

### 579 **Simulation Methods**

580 In each MCell execution, proteins are instantiated at time zero having random positions within a  
581  $0.32 \mu\text{m}^3$  (0.32 fL) cube. All proteins are described as three-dimensional volume molecules having the  
582 following concentrations:  $1.52 \mu\text{M}$  CaMKII (30 holoenzymes, 360 subunits),  $22.8 \mu\text{M}$  CaM (450 discrete  
583 proteins), and  $0.86 \mu\text{M}$  PP1 (17 discrete proteins). Because CaMKII particles are modeled using the  
584 specialized COMPLEX\_MOLECULE syntax and MCell 3.3 does not accommodate diffusion for such  
585 particles, CaMKII is given no diffusion constant. In contrast, CaM and PP1 are simple volume-type  
586 molecules that move about the model space with a diffusion constant  $6 \times 10^{-6} \mu\text{m}^2/\text{sec}$ . All models are run  
587 at a time step of  $0.1 \mu\text{s}$  for a total of either 20 or 120 seconds of simulation time, depending on the model  
588 variant. For statistical significance, all model variants are repeated 50 times each.

589 CaM activation/inactivation is modeled by a pair of forcing functions which serve as a proxy for  
590  $\text{Ca}^{2+}$  flux. Both forcing functions are time-dependent square waves and inform the rates at which free  
591 CaM transitions between states. Equation 1 rapidly transitions all free CaM towards an active ( $\text{Ca}^{2+}/\text{CaM}$ )  
592 state, and Equation 2 rapidly transitions all free CaM towards an inactive (apo-CaM) state.

593 
$$(1)F_{activation}(t) = \begin{cases} 100000000, & t = n_i \\ 0, & t \neq n_i \end{cases}$$

594 
$$(2)F_{inactivation}(t) = \begin{cases} 0, t = n_i \\ 100000000, t \neq n_i \end{cases}$$

595 For both Equations 1 and 2,  $n = i/f$  where  $i$  is the number of time step iterations and  $f$  is frequency. Time  $t$   
596 iterates at 0.01sec intervals for the complete duration of a simulation. Equations 1 and 2 therefore yield a  
597 peak width of 0.01sec regardless of frequency, which allows us to directly compare the effect of different  
598  $Ca^{2+}/CaM$  frequencies on CaMKII activity, without having to account for variable amounts of  $Ca^{2+}/CaM$   
599 exposure per pulse. In separate simulations without frequency dependence (i.e.  $Ca^{2+}/CaM$  is continuously  
600 available to CaMKII), Equation 1 is adjusted to always fulfill the  $t=n_i$  condition. Similarly, for pulse  
601 simulations in which  $Ca^{2+}/CaM$  becomes withdrawn or blocked, Equations 1 and 2 are given abbreviated  
602 time domains.

603 All MCell code and associated files are available online at Github, the Purdue University  
604 Research Repository (DOI: 10.4231/MBPK-D277), and the University of Edinburgh Repository.

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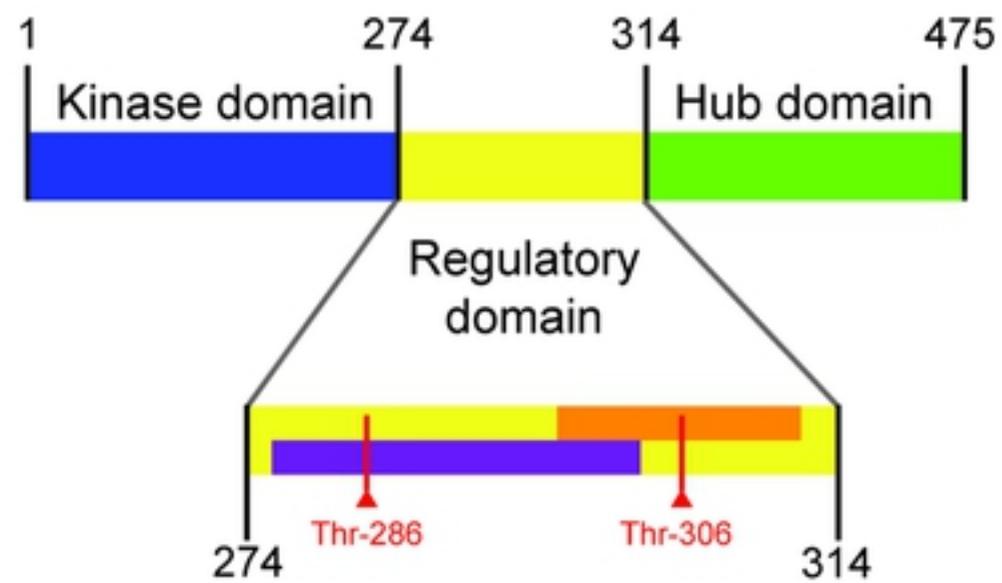
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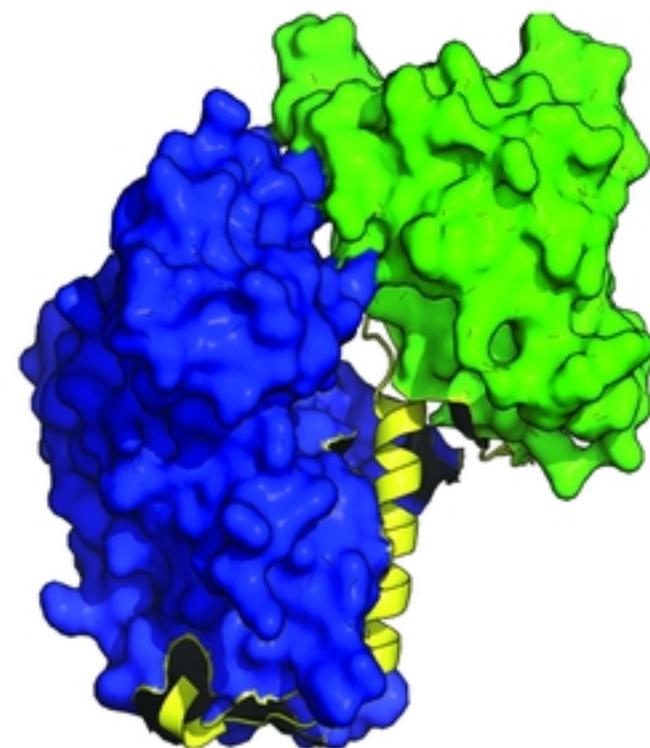
## 838 **Supporting Information**

839 **S1 Appendix. Appendix.** This document enumerates the model parameters, discusses  
840 combinatorial explosion, shows alternative visualizations of select data, and discusses the  
841 quantitative basis for PP1/CaM mutual exclusion from CaMKII binding.

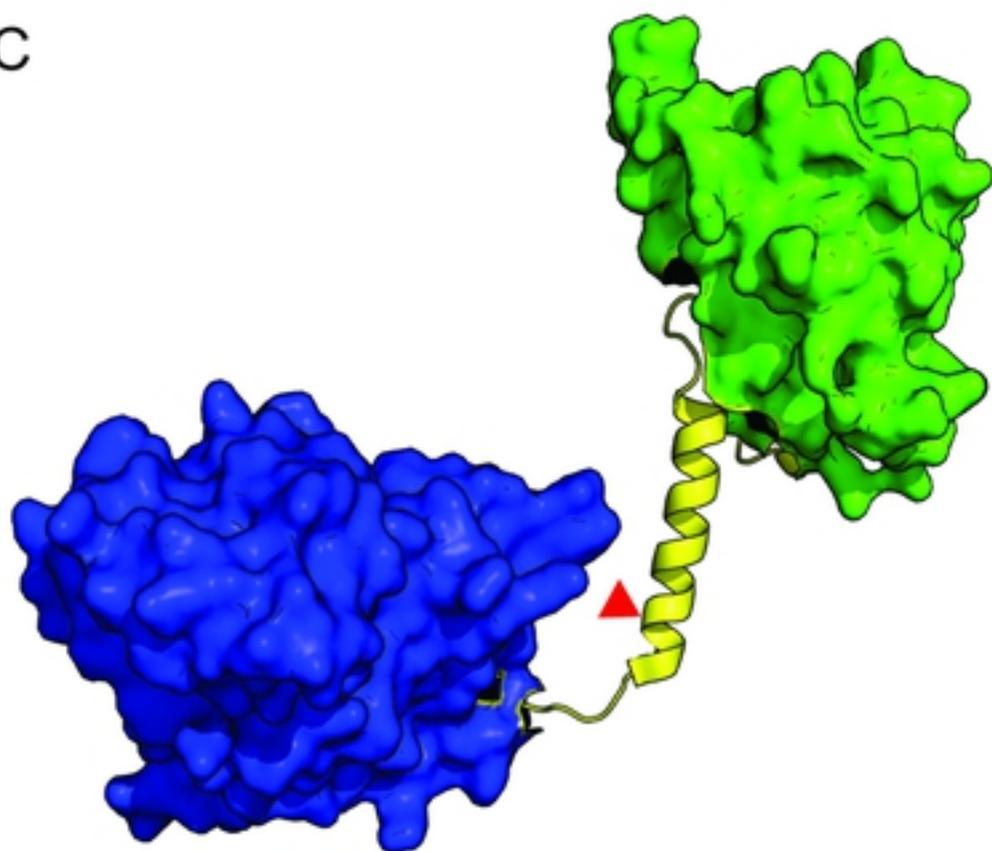
A



B



C



D

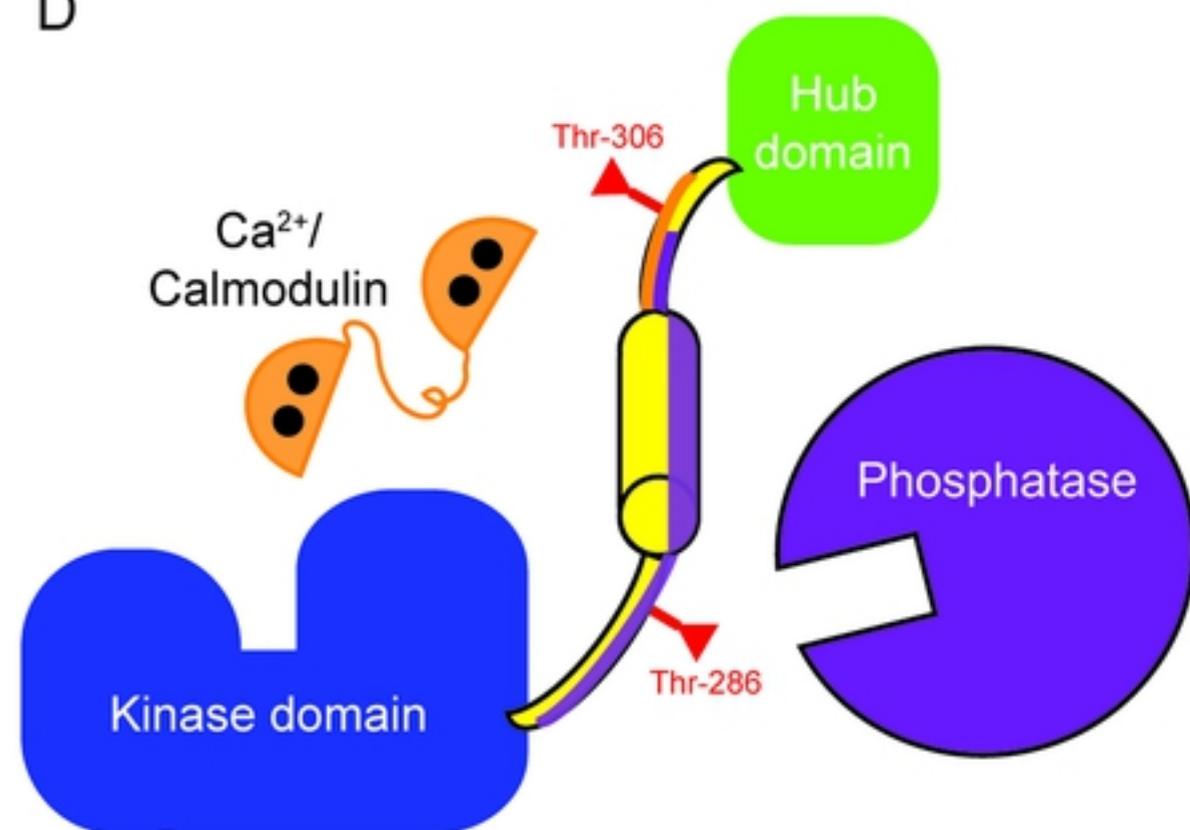


Figure 1

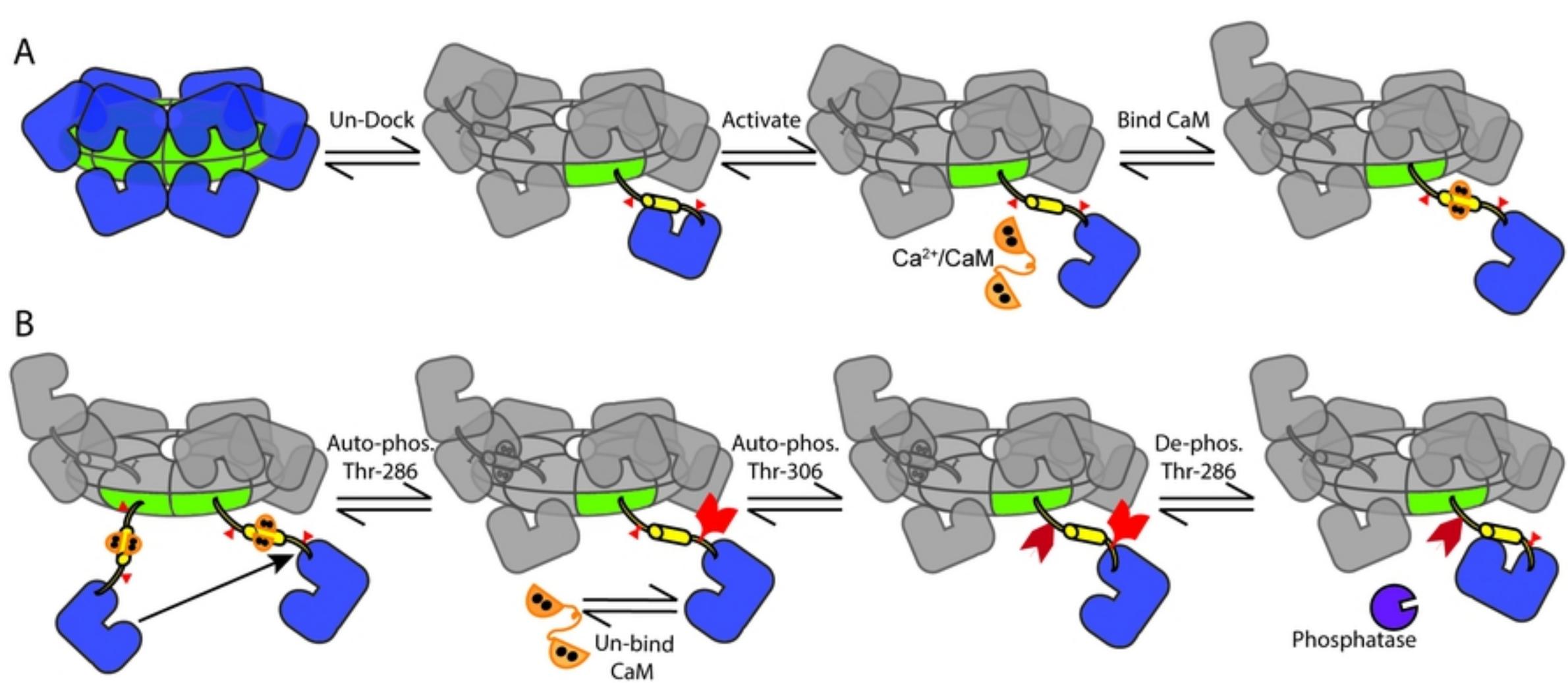


Figure 2

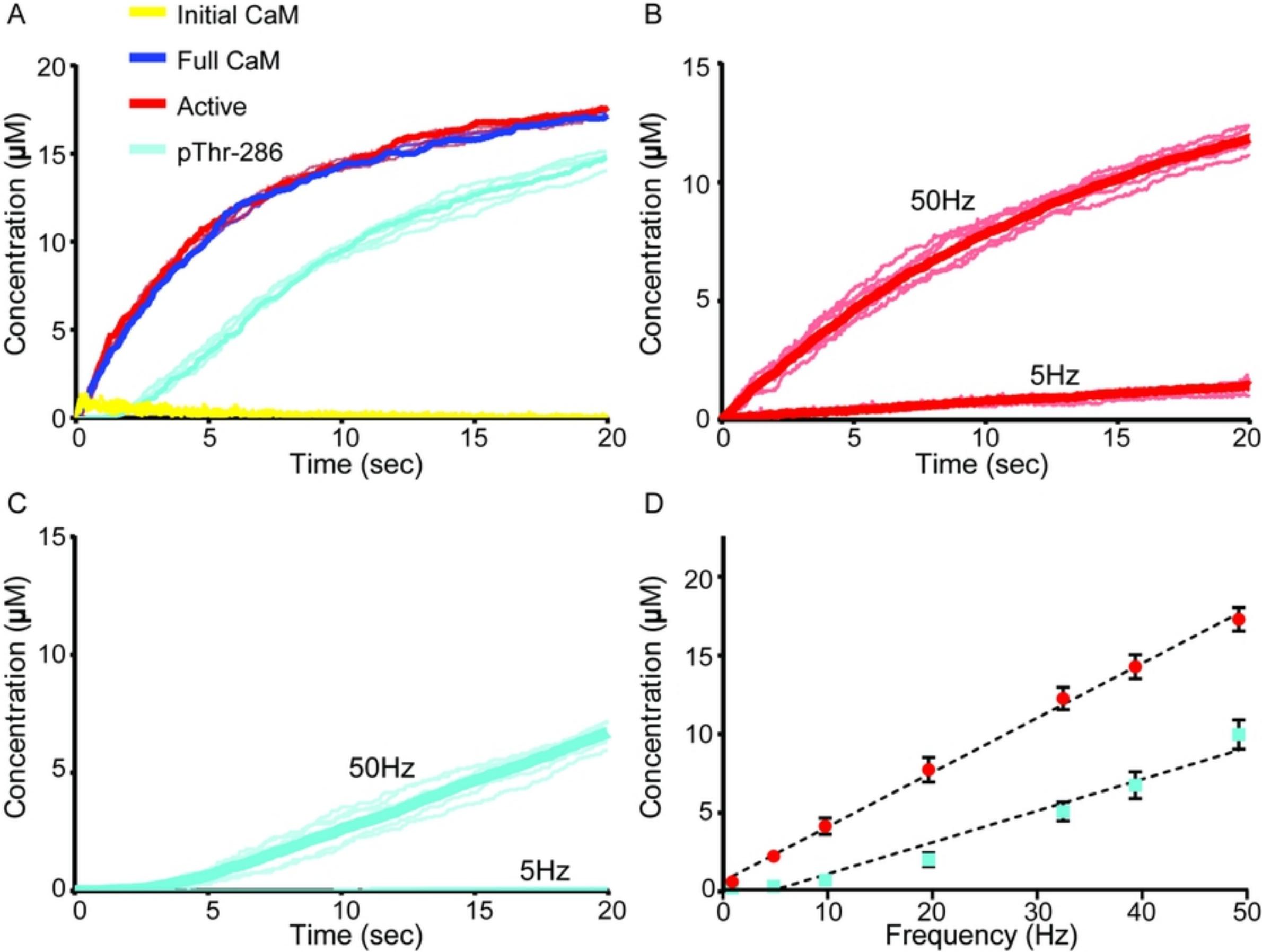


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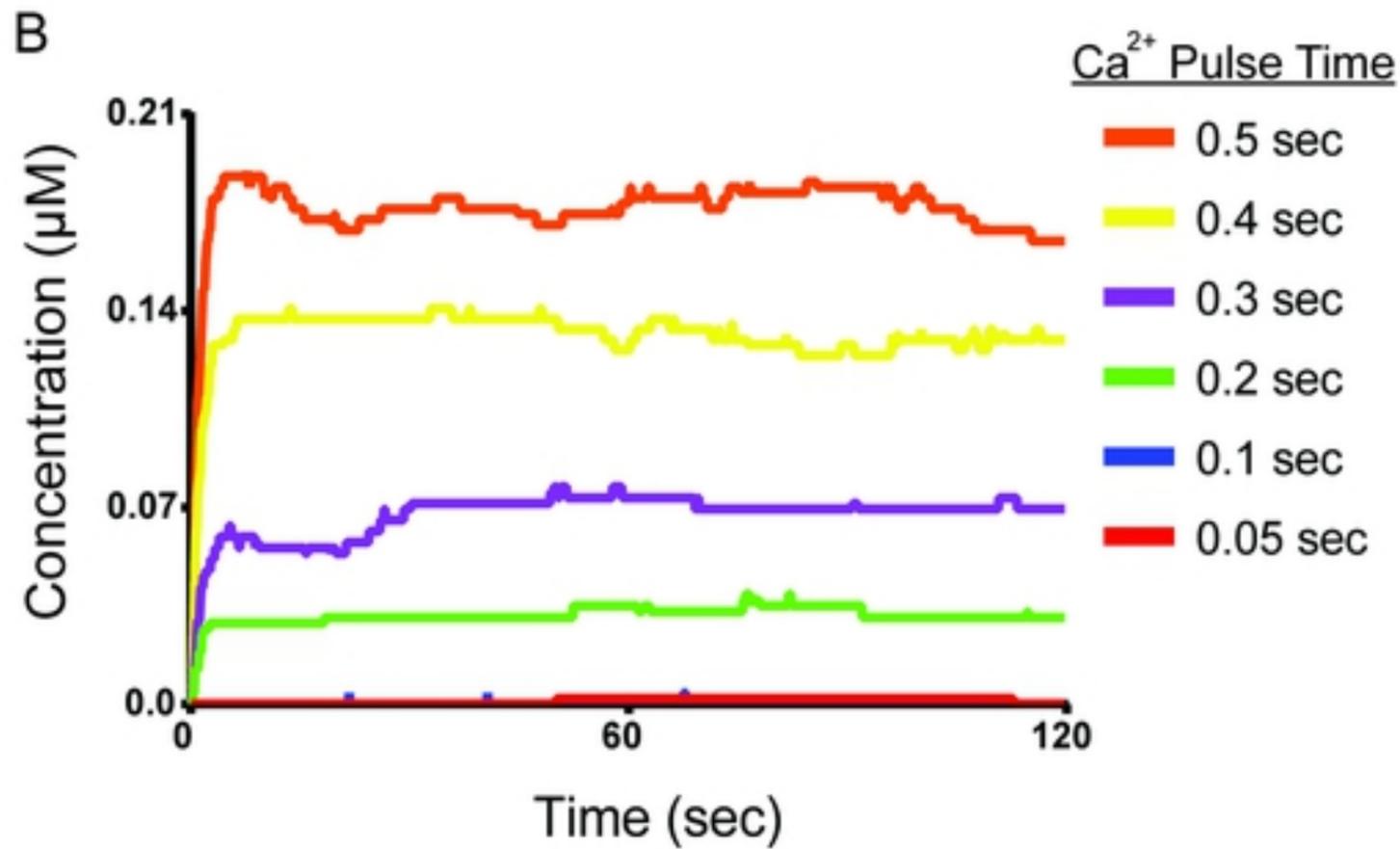
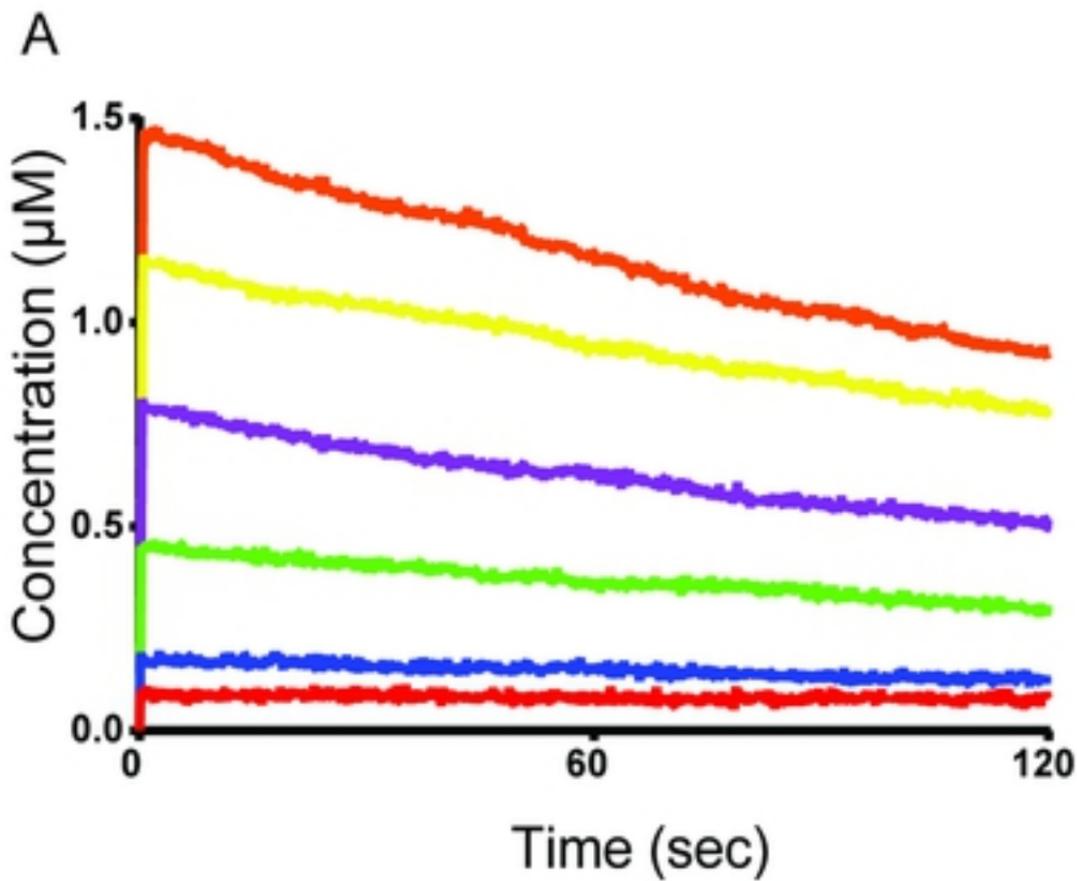


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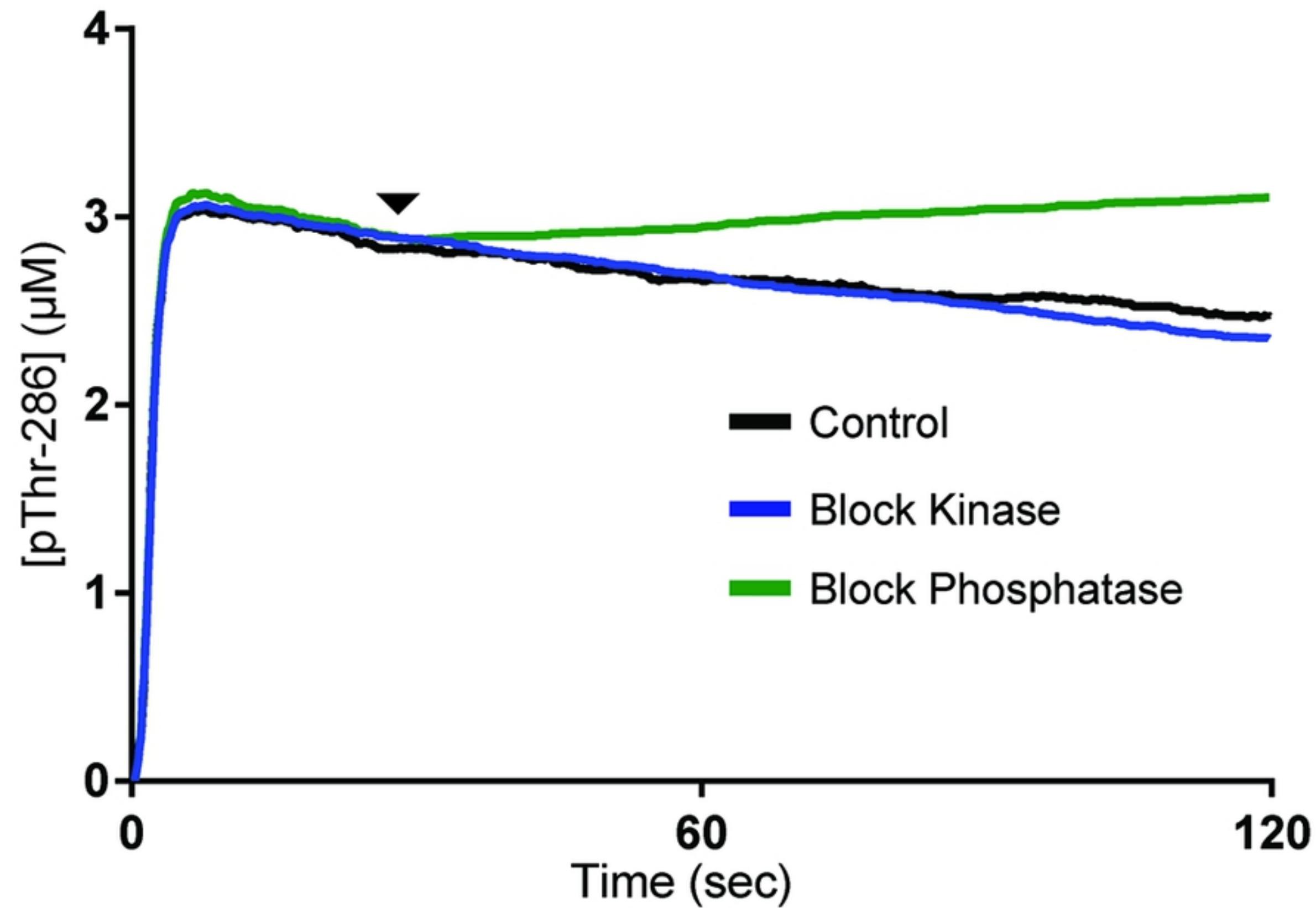


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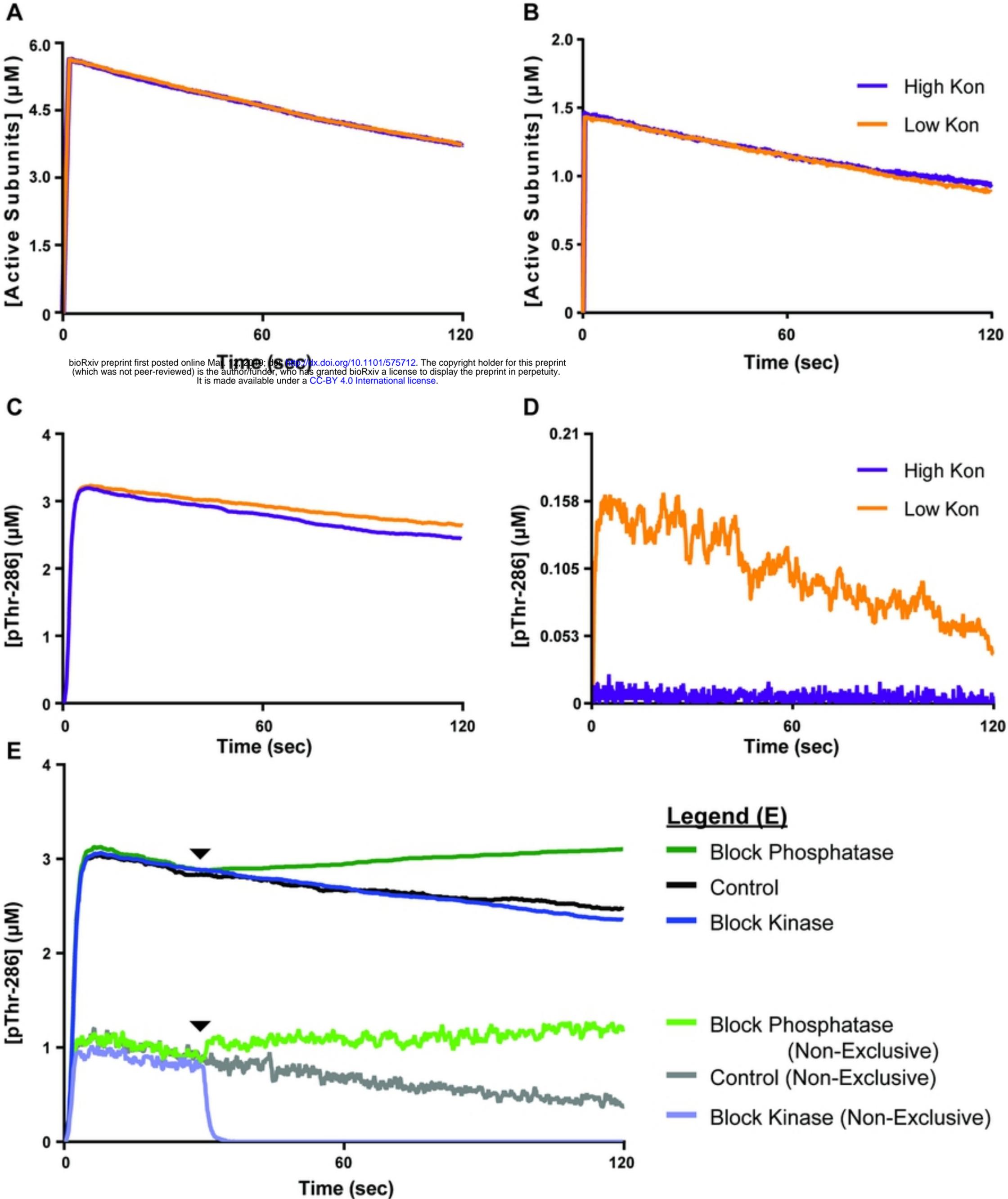


Figure 6

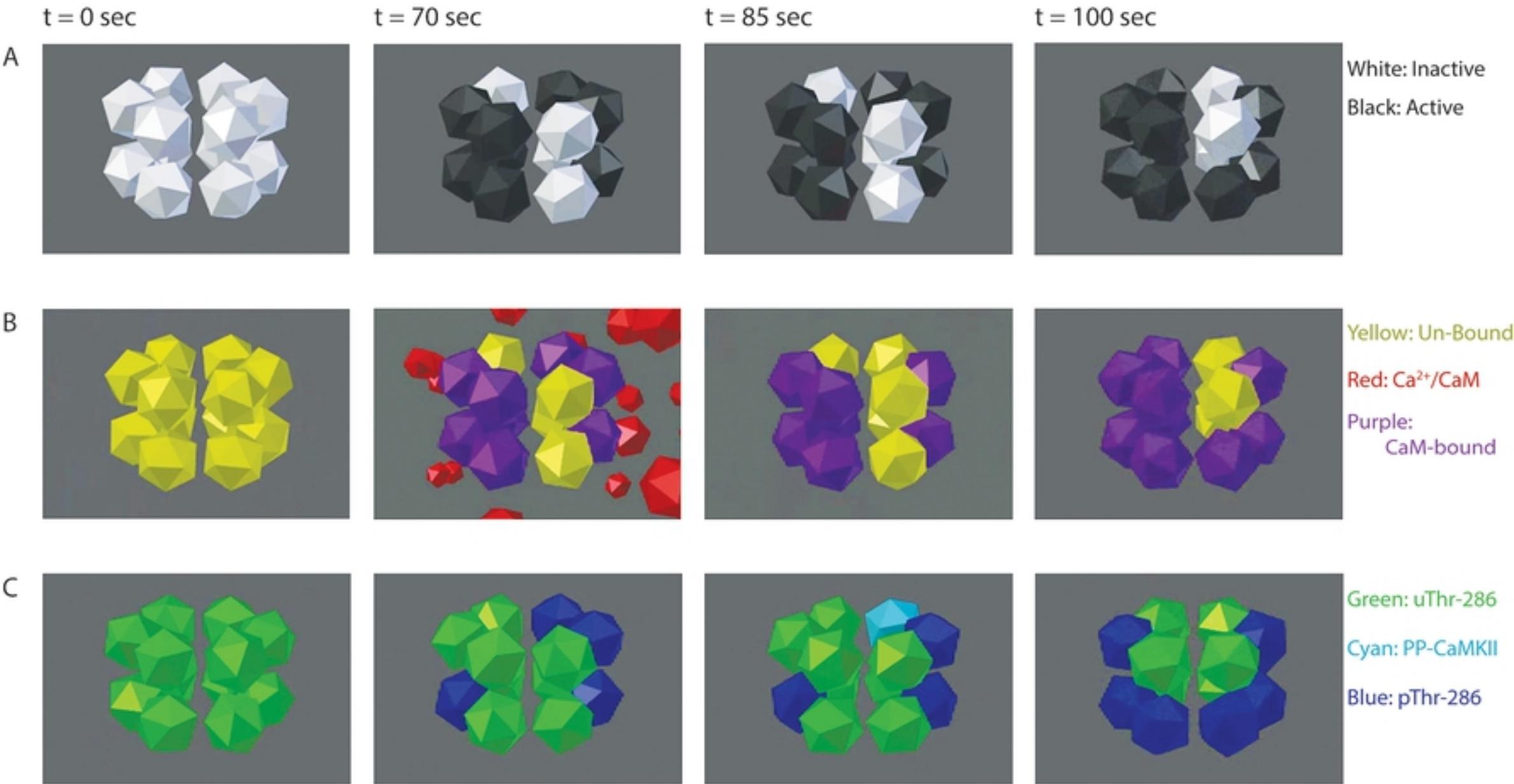


Figure 7