

1 **OLIGOMERIZATION OF THE HUMAN ADENOSINE A_{2A} RECEPTOR IS DRIVEN BY**
2 **THE INTRINSICALLY DISORDERED C-TERMINUS**

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17 **SIGNIFICANCE**

18 G protein-coupled receptors (GPCRs) are important drug targets in medicine. While it is widely
19 known that these receptors can form oligomers with unique functional consequences, the driving
20 factor of receptor oligomerization remains unclear. The intrinsically disordered C-terminus of
21 GPCRs is often thought to play no major role in receptor function and is thus usually removed to
22 simplify biophysical studies. Using the human adenosine A_{2A} receptor as a model GPCR, we find
23 instead that its C-terminus drives oligomer formation via an intricate network of interactions. This
24 finding suggests that the distinct properties associated with GPCR oligomerization may prevail
25 only when the C-terminus is present.

26 **ABSTRACT**

27 G protein-coupled receptors (GPCRs) have long been shown to exist as oligomers with functional
28 properties distinct from those of the monomeric counterparts, but the driving factors of GPCR
29 oligomerization remain relatively unexplored. In this study, we focus on the human adenosine A_{2A}
30 receptor (A_{2A}R), a model GPCR that forms oligomers both *in vitro* and *in vivo*. Combining
31 experimental and computational approaches, we discover that the intrinsically disordered C-
32 terminus of A_{2A}R drives the homo-oligomerization of the receptor. The formation of A_{2A}R
33 oligomers declines progressively and systematically with the shortening of the C-terminus.
34 Multiple interaction sites and types are responsible for A_{2A}R oligomerization, including disulfide
35 linkages, hydrogen bonds, electrostatic interactions, and hydrophobic interactions. These
36 interactions are enhanced by depletion interactions along the C-terminus, forming a tunable
37 network of bonds that allow A_{2A}R oligomers to adopt multiple interfaces. This study uncovers the
38 disordered C-terminus as a prominent driving factor for the oligomerization of a GPCR, offering
39 important guidance for structure-function studies of A_{2A}R and other GPCRs.

40 **INTRODUCTION**

41 G protein-coupled receptors (GPCRs) have long been studied as monomeric units, but
42 accumulating evidence demonstrates that these receptors can also form homo- and hetero-
43 oligomers with far-reaching functional implications. The properties emerging from these
44 oligomers can be distinct from those of the monomeric protomers in ligand binding(1–4), G protein

45 coupling(5–9), downstream signaling(10–13), and receptor internalization/desensitization(14–16).
46 With the vast number of genes identified in the human genome(17), GPCRs are able to form a
47 daunting number of combinations with unprecedented functional consequences. The existence of
48 this intricate network of interactions among GPCRs presents major challenges and opportunities
49 for the development of novel therapeutic approaches(18–23). Hence, it is crucial to identify the
50 driving factors that govern the oligomerization of GPCRs, such that the properties of GPCR
51 oligomers can be understood.

52 GPCR oligomers with multiple interfaces(24–28) can give rise to myriad ways by which these
53 complexes can be formed and their functions modulated. In the crystal structure of the turkey β_1 -
54 adrenergic receptor (β_1 AR), the receptor appears to dimerize via two different interfaces, one
55 formed via TM4/TM5 (transmembrane domains 4/5) and the other via TM1/TM2/H8 (helix 8)
56 contacts(29). Similarly, in the crystal structure of the antagonist-bound μ -opioid receptor (μ -OR),
57 the protomers also dimerize via two interfaces; however, only one of them is predicted to induce
58 a steric hindrance that prevents activation of both protomers(30), hinting at interface-specific
59 functional consequences. A recent computational study predicted that the adenosine A_{2A} receptor
60 (A_{2A} AR) forms homodimers via three different interfaces and that the resulting dimeric architectures
61 can modulate receptor function in different or even opposite ways(27). All of the above-mentioned
62 interfaces are symmetric, meaning that the two protomers are in face-to-face orientations, hence
63 forming strictly dimers. Asymmetric interfaces, reported in M_3 muscarinic receptor(31),
64 rhodopsin(32–34), and opsin(34), are in contrast formed with the protomers positioning face-to-
65 back, possibly enabling the association of higher-order oligomers.

66 Not only do GPCRs adopt multiple oligomeric interfaces, but various studies also suggest that
67 these interfaces may dynamically rearrange to activate receptor function(35). According to a recent
68 computational study, A_{2A} AR oligomers can adopt eight different interfaces that interconvert when
69 the receptor is activated or when there are changes in the local membrane environment(24).
70 Similarly, a recent study that combined experimental and computational data proposed that
71 neurotensin receptor 1 (NTS₁R) dimer is formed by “rolling” interfaces that co-exist and
72 interconvert when the receptor is activated(36). Clearly, meaningful functional studies of GPCRs
73 require exploring their dynamic, heterogeneous oligomeric interfaces.

74 The variable nature of GPCR oligomeric interfaces suggests that protomers of GPCR oligomers
75 may be connected by tunable interactions. In this study, we explore the role of an intrinsically
76 disordered region (IDR) of a model GPCR that could engage in diverse non-covalent interactions,
77 such as electrostatic interactions, hydrogen bonds, or hydrophobic interactions. These non-
78 covalent interactions are readily tunable by external factors, such as pH, salts, and solutes, and
79 further can be entropically stabilized by depletion interactions(37–39), leading to structure
80 formation and assembly(40–47). In a system where large protein molecules and small solute
81 particles typically coexist in solution, assembly of the protein molecules causes their excluded
82 volumes to overlap and the solvent volume accessible to the solutes to increase, raising the entropy
83 of the system. The type and concentration of solutes or ions can also remove water from the
84 hydration shell around the proteins, further enhancing entropy-driven protein-protein association
85 in what is known as the hydrophobic effect(48). This phenomenon is applied in the precipitation
86 of proteins upon addition of so-called salting-out ions according to the Hofmeister series(49). The
87 ability of IDRs to readily engage in these non-covalent interactions motivates our focus on the
88 potential role of IDRs in driving GPCR oligomerization.

89 The cytosolic carboxy (C-)terminus of GPCRs is usually an IDR(50, 51). Varying in length among
90 different GPCRs, the C-terminus is commonly removed in structural studies of GPCRs to enhance
91 receptor stability and conformational homogeneity. A striking example is A_{2A}R, a model GPCR
92 with a particularly long, 122-residue, C-terminus that is truncated in all published structural
93 biology studies(24, 27, 52–59). However, evidence is accumulating that such truncations—shown
94 to affect GPCR downstream signaling(60–62)—may abolish receptor oligomerization(63, 64). A
95 study using immunofluorescence has demonstrated that C-terminally truncated A_{2A}R does not
96 show protein aggregation or clustering on the cell surface, a process readily observed in the wild-
97 type form(65). Our recent study employing a tandem three-step chromatography approach
98 uncovered the impact of a single residue substitution of a C-terminal cysteine, C394S, in reducing
99 the receptor homo-oligomerization *in vitro*(63). In the context of heteromerization, mass
100 spectrometry and pull-down experiments have demonstrated that A_{2A}R-D₂R dimerization occurs
101 via direct electrostatic interactions between the C-terminus of A_{2A}R and the third intracellular loop
102 of D₂R(66). These results all suggest that the C-terminus may participate in A_{2A}R oligomer

103 formation. However, no studies to date have directly and systematically investigated the role of
104 the C-terminus, or any IDRs, in GPCR oligomerization.

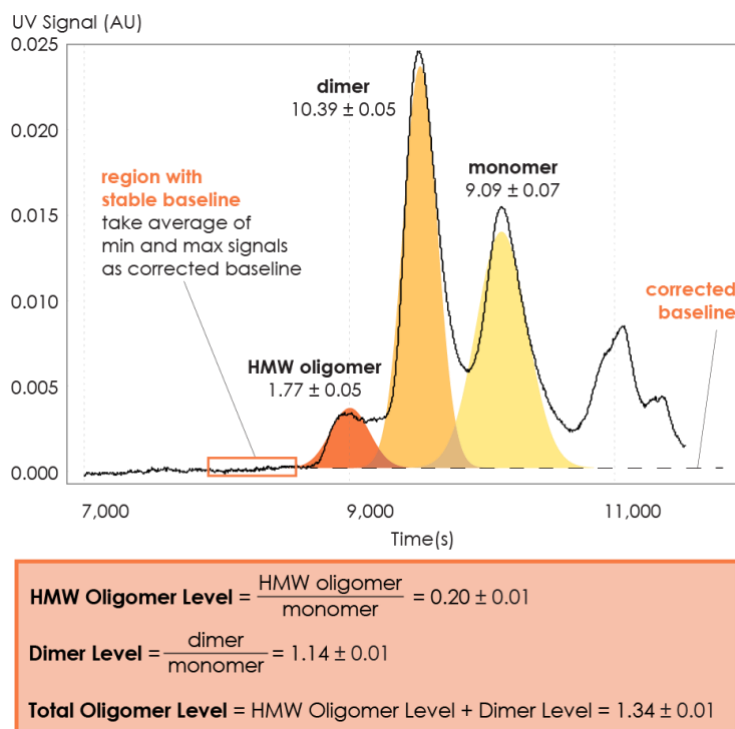
105 This study focuses on the homooligomerization of the human adenosine A_{2A}R, a model GPCR,
106 and seeks to address: (i) whether the C-terminus engages in A_{2A}R oligomerization, and if so, (ii)
107 whether the C-terminus forms multiple oligomeric interfaces. We use size-exclusion
108 chromatography (SEC) to assess the oligomerization levels of A_{2A}R variants with strategic C-
109 terminal modifications: mutations of a cysteine residue C394 and a cluster of charged residues
110 ³⁵⁵ERR³⁵⁷, as well as systematic truncations at eight different sites along its length. We
111 complemented our experimental study with an independent molecular dynamics study of A_{2A}R
112 dimers of five C-terminally truncated A_{2A}R variants designed to mirror the experimental constructs.
113 We furthermore examined the oligomerization level of select C-terminally modified A_{2A}R variants
114 under conditions of ionic strength ranging from 0.15 to 0.95 M. To test whether the C-termini
115 directly and independently promote A_{2A}R oligomerization, we recombinantly expressed the entire
116 A_{2A}R C-terminal segment sans the transmembrane portion of the receptor and investigated its
117 solubility and assembly properties with increasing ion concentration and temperature. This is the
118 first study designed to uncover the role of the intrinsically disordered C-terminus on the
119 oligomerization of a GPCR.

120 **RESULTS**

121 This study systematically investigates the role of the C-terminus on A_{2A}R oligomerization and the
122 nature of the interactions involved through strategic mutations and truncations at the C-terminus
123 as well as modulation of the ionic strength of solvent. The experimental assessment of A_{2A}R
124 oligomerization relies on size-exclusion chromatography (SEC) analysis.

125 **Size Exclusion Chromatography Quantifies A_{2A}R Oligomerization**

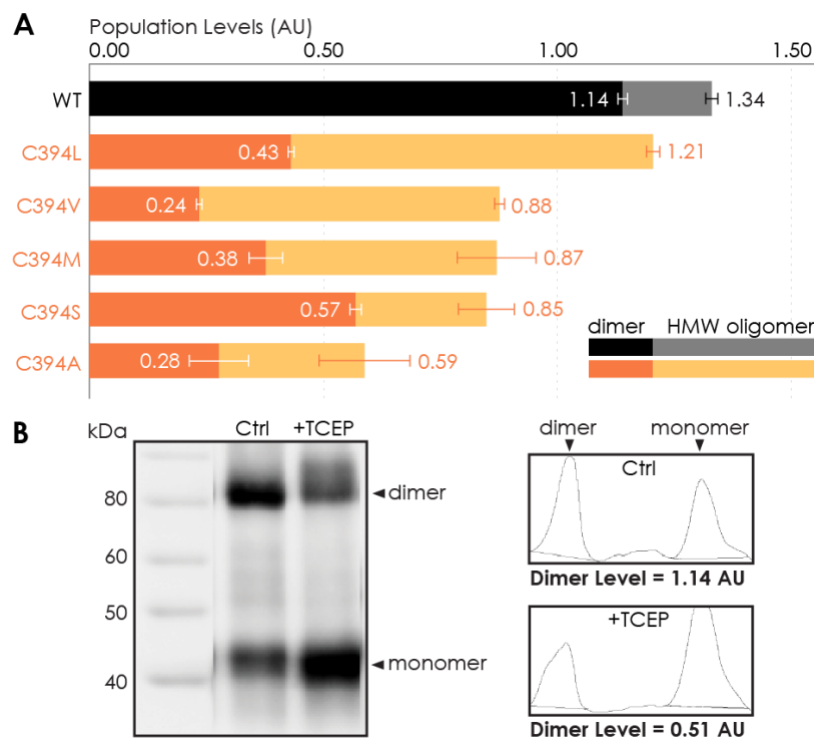
126 We performed SEC analysis on a mixture of ligand-active A_{2A}R purified from a custom
127 synthesized antagonist affinity column (**Fig. S1A**). Distinct oligomeric species were separated and
128 eluted in the following order: high-molecular-weight (HMW) oligomer, dimer, and monomer (**Fig.**
129 **1** and **Fig. S1B**). The population of each oligomeric species was quantified as the integral of each
130 Gaussian from a multiple-Gaussian curve fit of the SEC signal. The reported standard errors were
131 calculated from the variance of the fit that do not correspond to experimental errors (see **Table S1**
132 and **Fig. S2** for SEC data corresponding to all A_{2A}R variants in this study). As this study sought to
133 identify the factors that promote A_{2A}R oligomerization, the populations with oligomeric interfaces
134 (*i.e.*, dimer and HMW oligomer) were compared with those without such interfaces (*i.e.*,
135 monomer). Hence, the populations of the HMW oligomer and dimer were expressed relative to the
136 monomer population in arbitrary units as monomer-equivalent concentration ratios, henceforth
137 referred to as population levels (**Fig. 1**).



138 **Figure 1.** Method for collecting SEC data and assessing A_{2A}R oligomerization. The SEC data is recorded every second
139 as absorbance at 280 nm. The baseline is corrected to ensure uniform fitting and integration across the peaks. The
140 areas under the curve, resulting from a multiple-Gaussian curve fit, express the population of each oligomeric species.
141 The reported standard errors of integration are within a 95% confidence interval and are calculated from the variance
142 of the fit, not experimental errors. The levels of HMW oligomer and dimer are expressed relative to the monomeric
143 population in arbitrary units. A representative calculation defining the oligomer levels is given in the box.

144 C-Terminal Amino Acid Residue C394 Contributes to A₂AR Oligomerization

145 To investigate whether the C-terminus of A₂AR is involved in receptor oligomerization, we first
146 examined the role of residue C394, as a previous study demonstrated that the mutation C394S
147 dramatically reduced A₂AR oligomer levels(63). The C394S mutation was replicated in our
148 experiments, alongside other amino acid substitutions, namely alanine, leucine, methionine or
149 valine, generating five A₂AR-C394X variants. The HMW oligomer and dimer levels of A₂AR wild-
150 type (WT) were compared with those of the A₂AR-C394X variants. We found that the dimer level
151 of A₂AR-WT was significantly higher than that of the A₂AR-C394X variants (WT: 1.14; C394X:
152 0.24–0.57; **Fig. 2A**). A similar result, though less pronounced, was observed when the HMW
153 oligomer and dimer levels were considered together (WT: 1.34; C394X: 0.59–1.21; **Fig. 2A**). This
154 suggests that residue C394 plays a role in A₂AR oligomerization and more so in A₂AR dimers.



155

156 **Figure 2.** Residue C394 helps stabilize A₂AR oligomerization via disulfide bonds. (A) The effect of C394X substitutions
157 on A₂AR oligomerization. The levels of dimer (dark colors) and HMW oligomer (light colors) are expressed relative
158 to the monomeric population in arbitrary units, with reported errors calculated from the variance of the fit, not
159 experimental variation. (B) Line densitometry of Western Blot bands on SEC-separated dimeric population with and

160 *without 5 mM TCEP. The level of dimer is expressed relative to the monomeric population in arbitrary units similarly*
161 *to the SEC analysis. MagicMark protein ladder (LC5602) is used as the molecular weight standard.*

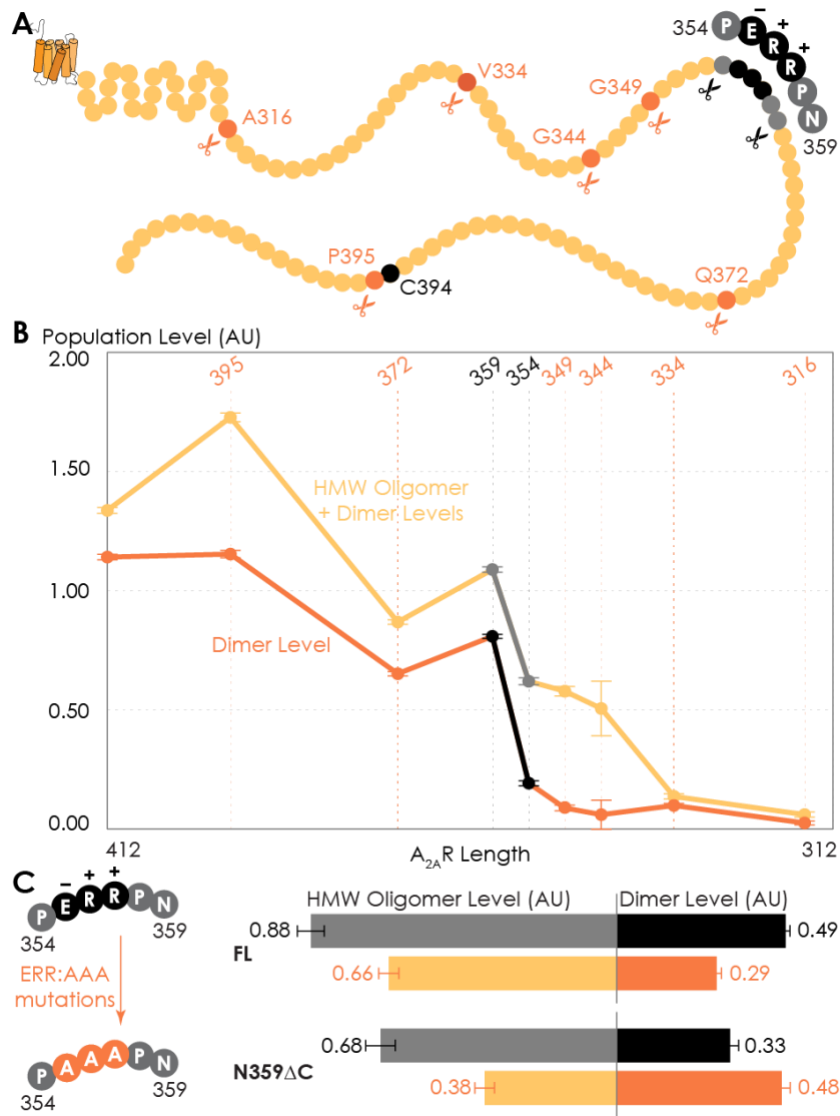
162 To test whether residue C394 stabilizes A_{2A}R dimerization by forming disulfide linkages, we
163 incubated SEC-separated A_{2A}R dimer with 5 mM of the reducing agent TCEP, followed by SDS-
164 PAGE and Western Blotting. The population of each species was determined as the area under the
165 densitometric trace. The dimer level was then expressed as monomer-equivalent concentration
166 ratios in a manner similar to that of the SEC experiment described above. Upon incubation with
167 TCEP, the dimer level of the sample decreased from 1.14 to 0.51 (**Fig. 2B**). This indicates that
168 disulfide bond formation via residue C394 is one possible mechanism for A_{2A}R dimerization.
169 However, a significant population of A_{2A}R dimer remained resistant to TCEP and C394X
170 mutations (**Fig. 2**), suggesting that disulfide linkages are not the only driving factor of A_{2A}R
171 oligomer formation. This finding agrees with a previous study showing that residue C394 in A_{2A}R
172 dimer is still available for nitroxide spin labeling,⁽⁶³⁾ suggesting that additional interfacial sites
173 help drive A_{2A}R dimer/oligomerization.

174 **C-Terminus Truncation Systematically Reduces A_{2A}R Oligomerization**

175 To determine which interfacial sites in the C-terminus other than C394 drive A_{2A}R
176 dimer/oligomerization, we carried out systematic truncations at eight sites along the C-terminus
177 (A316, V334, G344, G349, P354, N359, Q372, and P395), generating eight A_{2A}R-ΔC variants
178 (**Fig. 3A**). The A_{2A}R-A316ΔC variant corresponds to the removal of the entire disordered C-
179 terminal region as previously performed in all published structural studies^(24, 27, 52–59). Using
180 the SEC analysis described earlier (**Fig. 1**) we evaluated the HMW oligomer and dimer levels of
181 the A_{2A}R-ΔC variants relative to that of the A_{2A}R full-length-wild-type (FL-WT) control. Both the
182 dimer and the total oligomer levels of A_{2A}R decreased progressively with the shortening of the C-
183 terminus, with almost no oligomerization detected upon complete truncation of the C-terminus at
184 site A316 (**Fig. 3B**). This result shows that the C-terminus drives A_{2A}R oligomerization, with
185 multiple potential interaction sites positioned along much of its length.

186 Interestingly, there occurred a dramatic decrease in the dimer level between the N359 and P354
187 truncation sites, from a value of 0.81 to 0.19, respectively (**Fig. 3B**). A similar result, though less
188 pronounced, was observed on the total oligomer level, with a decrease from 1.09 to 0.62 for the

189 N359 and P354 truncation sites, respectively (**Fig. 3B**). Clearly, the C-terminal segment
 190 encompassing residues 354–359 (highlighted in black in **Fig. 3A**) is a key constituent of the A_{2A}R
 191 oligomeric interface.



192

193 **Figure 3. Truncating the C-terminus systematically affects A_{2A}R oligomerization.** (A) Depiction of where the
 194 truncation points are located on the C-terminus, with region 354–359 highlighted (in black) showing critical residues.
 195 (B) The levels of dimer and HMW oligomer are expressed relative to the monomeric population as an arbitrary unit
 196 and plotted against the residue number of the truncation sites, with reported errors calculated from the variance of
 197 the fit, not experimental variation. Region 354–359 is emphasized (in black and gray) due to a drastic change in the
 198 dimer and HMW oligomer levels. (C) The dependence of A_{2A}R oligomerization on three consecutive charged residues
 199 ³⁵⁵ERR³⁵⁷. The substitution of residues ³⁵⁵ERR³⁵⁷ to ³⁵⁵AAA³⁵⁷ is referred to as the ERR:AAA mutations. The levels of

200 *dimer and HMW oligomer are expressed relative to the monomeric population as an arbitrary unit, with reported*
201 *errors calculated from the variance of the fit, not experimental variation.*

202 Since segment 354–359 contains three consecutive charged residues (³⁵⁵ERR³⁵⁷; **Fig. 3A**), which
203 could be involved in electrostatic interactions, we hypothesized that this ³⁵⁵ERR³⁵⁷ cluster could
204 strengthen inter-protomer A_{2A}R-A_{2A}R association. To test this hypothesis, residues ³⁵⁵ERR³⁵⁷ were
205 substituted by ³⁵⁵AAA³⁵⁷ on A_{2A}R-FL-WT and A_{2A}R-N359ΔC to generate A_{2A}R-ERR:AAA
206 variants (**Fig. 3C**). We then compared the HMW oligomer and dimer levels of the resulting
207 variants with controls (same A_{2A}R variants but without the ERR:AAA mutations). We found that
208 the ERR:AAA mutations had varied effects on the dimer level: decreasing for A_{2A}R-FL-WT (ctrl:
209 0.49; ERR:AAA: 0.29) but increasing for A_{2A}R-N359ΔC (ctrl: 0.33; ERR:AAA: 0.48) (**Fig. 3C**).
210 In contrast, the ERR:AAA mutations reduced the HMW oligomer level of both A_{2A}R-FL-WT (ctrl:
211 0.88; ERR:AAA: 0.66) and A_{2A}R-N359ΔC (ctrl: 0.68; ERR:AAA: 0.38) (**Fig. 3C**). Consistently,
212 the ERR:AAA mutation lowered the total oligomer level of both A_{2A}R-FL-WT (ctrl: 1.37;
213 ERR:AAA: 0.94) and A_{2A}R-N359ΔC (ctrl: 1.01; ERR:AAA: 0.85) (**Fig. 3C**). These results
214 suggest that the charged residues ³⁵⁵ERR³⁵⁷ participate in A_{2A}R oligomerization, with a greater
215 effect in the context of a longer C-terminus and for higher-order oligomer formation. The question
216 then arises as to what types of interactions are formed along the C-terminus that help stabilize
217 A_{2A}R oligomerization.

218 **C-Terminus Truncation Disrupts Complex Network of Non-Bonded Interactions Necessary** 219 **for A_{2A}R Dimerization.**

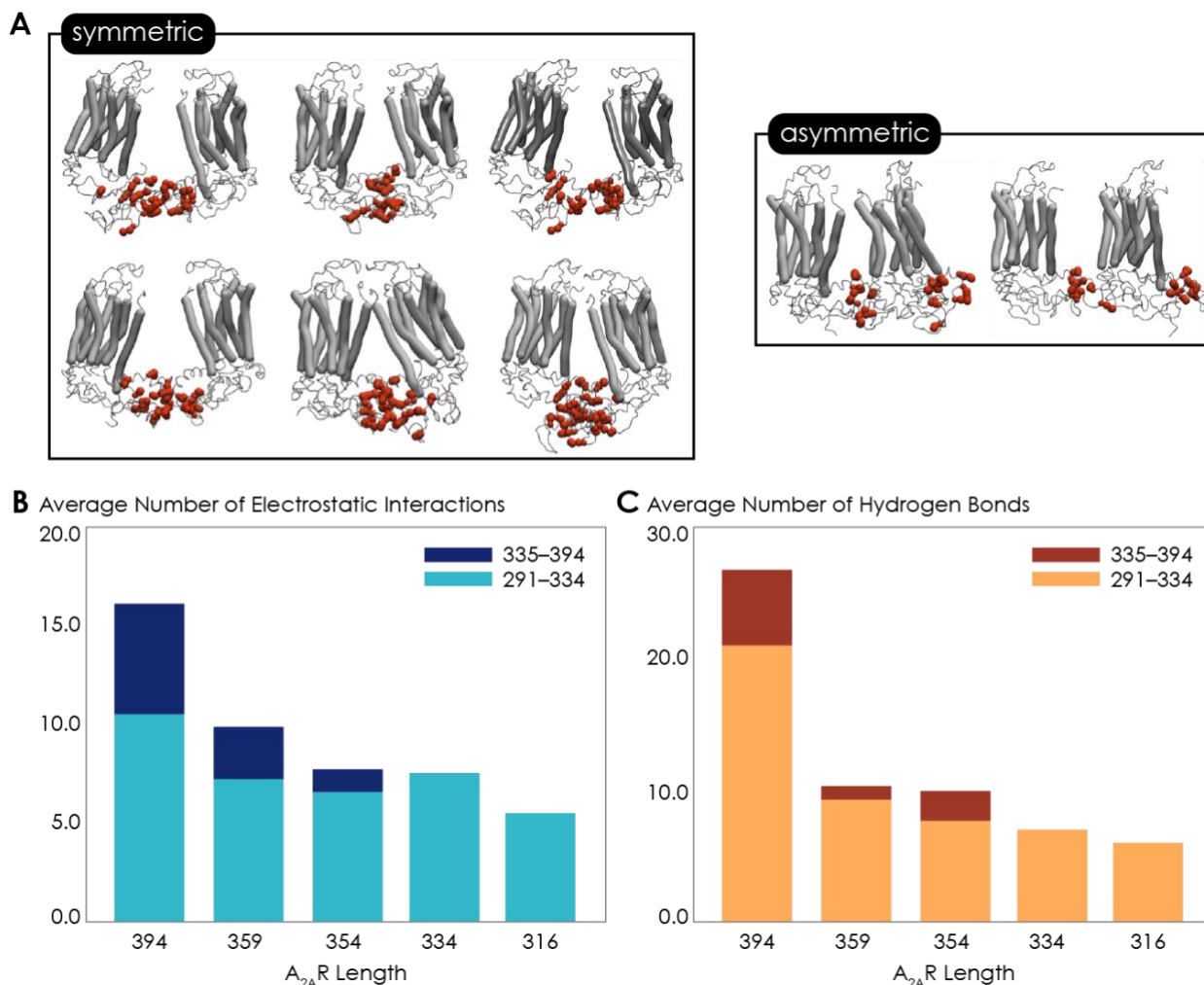
220 Given that the structure of A_{2A}R dimers or oligomers are unknown, we next used molecular
221 dynamics (MD) simulations to seek molecular-level insights into the role of the C-terminus in
222 driving A_{2A}R dimerization and to determine the specific interaction types and sites involved in this
223 process. First, to explore A_{2A}R dimeric interface, we performed coarse-grained (CG) MD
224 simulations, which can access the length and time scales relevant to membrane protein
225 oligomerization, albeit at the expense of atomic-level details. We carried out a series of CGMD
226 simulations on five A_{2A}R-ΔC variants designed to mirror the experiments by systematic truncation
227 at five sites along the C-terminus (A316, V334, P354, N359, and C394). Our results revealed that
228 A_{2A}R dimers were formed with multiple interfaces, all involving the C-terminus (**Fig. 4A and S3A**).

229 The vast majority of A_{2A}R dimers were symmetric, with the C-termini of the protomers directly
230 interacting with each other. A smaller fraction of the dimers had asymmetric orientations, with the
231 C-terminus of one protomer interacting with other parts of the other protomer, such as ICL2 (the
232 second intracellular loop), ICL3, and ECL2 (the second extracellular loop) (**Fig. 4A**).

233 Our observation of multiple A_{2A}R oligomeric interfaces, consistent with previous studies(24, 27),
234 suggests that tunable, non-covalent intermolecular interactions are involved in receptor
235 dimerization. We dissected two key non-covalent interaction types: electrostatic and hydrogen
236 bonding interactions. (The criteria for designating inter-A_{2A}R contacts as electrostatic interactions
237 or hydrogen bonds are described in detail in **Materials and Methods**.) Electrostatic interactions
238 were calculated from CGMD simulations. Hydrogen bonds were quantified from atomistic MD
239 simulation, given that the CG model merges all hydrogens into a coarse-grained bead and hence
240 cannot report on hydrogen bonds. This analysis was performed on the symmetric dimers as they
241 constituted the majority of the assemblies. With the least truncated A_{2A}R variant containing the
242 longest C-terminus, A_{2A}R-C394ΔC, we observed an average of 15.9 electrostatic contacts (**Fig.**
243 **4B**) and 26.7 hydrogen bonds (**Fig. 4C**) between the C-termini of the protomers. This result shows
244 that both electrostatic interactions and hydrogen bonds play important roles in A_{2A}R dimer
245 formation.

246 Upon further C-terminus truncation, the average number of both electrostatic contacts and
247 hydrogen bonds involving C-terminal residues progressively declined, respectively reaching 5.4
248 and 6.0 for A_{2A}R-A316ΔC (in which the disordered region of the C-terminus is removed) (**Fig. 4B**
249 and **4C**). This result is consistent with the experimental result, which demonstrated a progressive
250 decrease of A_{2A}R oligomerization with the shortening of the C-terminus (**Fig. 3B**). Interestingly,
251 upon systematic truncation of the C-terminal segment 335–394, we observed in segment 291–334
252 a steady decrease in the average number of electrostatic contacts, from 10.4 to 7.4 (**Fig. 4B**). This
253 trend was even more pronounced with hydrogen bonding contacts involving segment 291–334
254 decreasing drastically from 21.0 to 7.0 as segment 335–394 was gradually removed (**Fig. 4C**).
255 This observation, namely that truncation of a C-terminal segment reduces inter-A_{2A}R contacts
256 elsewhere along the C-terminus, indicates that a cooperative mechanism of dimerization exists, in
257 which an extended C-terminus of A_{2A}R stabilizes inter-A_{2A}R interactions near the heptahelical
258 bundles of the dimeric complex. Besides the intermolecular interactions, we also identified a

259 network of intramolecular salt bridges involving residues on the C-termini, including cluster
260 ³⁵⁵ERR³⁵⁷ (Fig. 7A). These results demonstrate that A_{2A}R dimers can be formed via multiple
261 interfaces predominantly in symmetric orientations, facilitated a cooperative network of
262 electrostatic interactions and hydrogen bonds along much of its C-terminus.



263 **Figure 4.** Non-bonded interactions of the extended C-terminus of A_{2A}R play a critical role in stabilization of the
264 dimeric interface. (A) Dimer configurations from cluster analysis in GROMACS of the 394-residue variant identify
265 two major clusters involving either 1) the C-terminus of one protomer and the C-terminus, ICL2, and ICL3 of the
266 second protomer or 2) the C-terminus of one protomer and ICL2, ICL3, and ECL2 of the second protomer. Spheres:
267 residues forming intermolecular electrostatic contacts. (B) Average number of residues that form electrostatic
268 contacts as a function of sequence length of A_{2A}R. (C) Average number of residues that form hydrogen bonds as a
269 function of sequence length of A_{2A}R.

270 Ionic Strength Modulates Oligomerization of C-Terminally Truncated A_{2A}R Variants

271 So far, we have demonstrated that the C-terminus clearly plays a role in forming A_{2A}R oligomeric
272 interfaces. However, the driving factors of A_{2A}R oligomerization remain unknown. The variable
273 nature of A_{2A}R oligomeric interfaces suggests that the main driving forces must be non-covalent
274 interactions, such as electrostatic interactions and hydrogen bonds as identified by the above MD
275 simulations. Modulating the solvent ionic strength is an effective method to identify the types of
276 non-covalent interaction(s) at play. Specifically, with increasing ionic strength, electrostatic
277 interactions can be weakened (based on Debye-Hückel theory, most electrostatic bonds at a
278 distance greater than 5 Å are screened out at an ionic strength of 0.34 M at 4°C), depletion
279 interactions are enhanced with salting-out salts, and hydrogen bonds remain relatively impervious.
280 For this reason, we subjected various A_{2A}R variants (FL-WT, FL-ERR:AAA, N359ΔC, and
281 V334ΔC) to ionic strength ranging from 0.15 to 0.95 M by adding NaCl (buffer composition
282 shown in **Table 1**). The HMW oligomer and dimer levels of the four A_{2A}R variants were
283 determined and plotted as a function of ionic strengths.

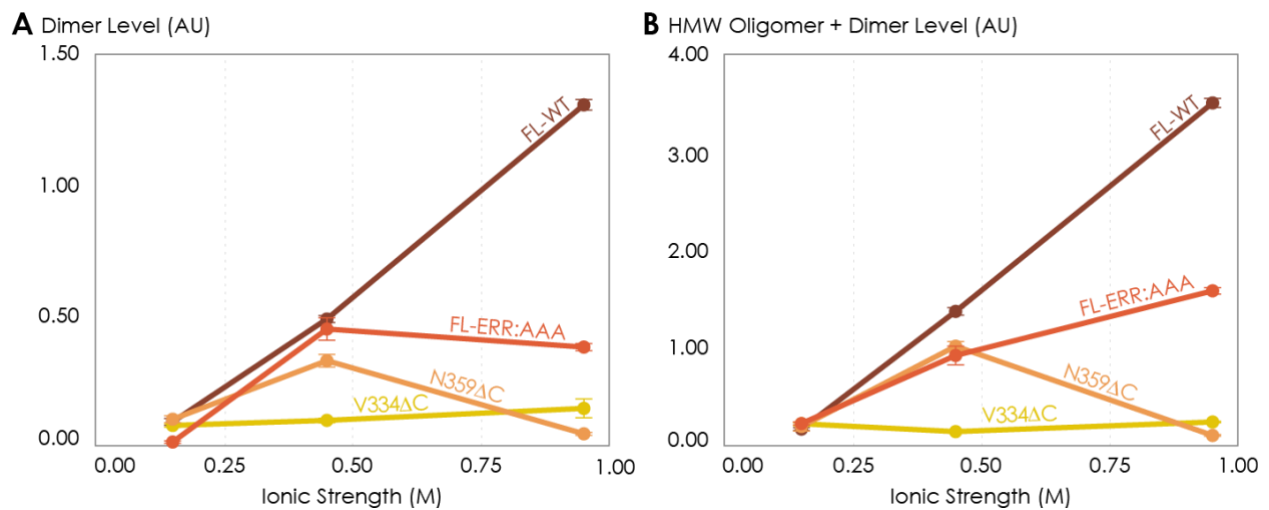
284 The low ionic strength of 0.15 M should not affect hydrogen bonds or electrostatic interactions, if
285 present. We found that the dimer and total oligomer levels of all four variants were near zero (**Fig.**
286 **5**). This is a striking observation, as it already excludes electrostatic and hydrogen-bonding
287 interactions as the dominant force for A_{2A}R association. The question remains whether depletion
288 interactions could be involved.

289 At higher ionic strengths of 0.45 M and 0.95 M, the dimer and total oligomer levels of A_{2A}R-
290 V334ΔC still remained near zero (**Fig. 5**). In contrast, we observed a progressive and significant
291 increase in the dimer and total oligomer levels of A_{2A}R-FL-WT with increasing ionic strength (**Fig.**
292 **5**). This result indicates A_{2A}R oligomerization must be driven by depletion interactions, which are
293 enhanced with increasing ionic strength, and that these interactions involve the C-terminal segment
294 after residue V334.

295 Upon closer examination, we recognize that at the very high ionic strength of 0.95 M, the increase
296 in the dimer and total oligomer levels was robust for A_{2A}R-FL-WT, but less pronounced for A_{2A}R-
297 FL-ERR:AAA (**Fig. 5**). Furthermore, this high ionic strength even had an opposite effect on A_{2A}R-
298 N359ΔC, with both its dimer and total oligomer levels abolished (**Fig. 5**). These results indicate

299 that the charged cluster ³⁵⁵ERR³⁵⁷ and the C-terminal segment after residue N359 are required for
300 depletion interactions to promote A₂AR oligomerization to the full extent.

301 Taken together, we demonstrated that A₂AR oligomerization is more robust when the C-terminus
302 is fully present and the ionic strength is higher, suggesting that depletion interactions via the C-
303 terminus are a strong driving factor of A₂AR oligomerization. The question then arises whether
304 such depletion interactions are the result of the C-termini directly interacting with one another,
305 necessitating an experiment that investigates the behavior of A₂AR C-terminus sans the
306 transmembrane domains.



307

308 **Figure 5.** The effects of ionic strength on the oligomerization of various A₂AR variants reveal the involvement of
309 depletion interactions. The levels of dimer and HMW oligomer are expressed relative to the monomeric population as
310 an arbitrary unit and plotted against ionic strength, with reported errors calculated from the variance of the fit, not
311 experimental variation. NaCl concentration is varied to achieve ionic strengths of 0.15, 0.45, and 0.95 M.

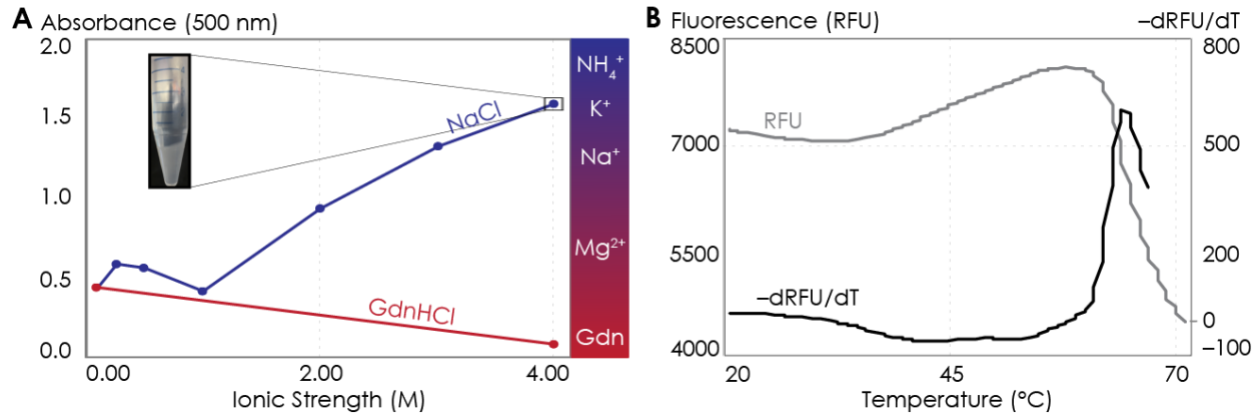
312 The Isolated A₂AR C-Terminus Is Prone to Aggregation

313 To test whether A₂AR oligomerization is driven by direct depletion interactions among the C-
314 termini of the protomers, we assayed the solubility and assembly properties of the stand-alone
315 A₂AR C-terminus—an intrinsically disordered peptide—sans the upstream transmembrane regions.
316 Since depletion interactions can be manifested via the hydrophobic effect(48), we examined
317 whether this effect can cause A₂AR C-terminal peptides to associate.

318 It is an active debate(67) whether the hydrophobic effect can be promoted or suppressed by ions
319 with salting-out or salting-in tendency, respectively(68–70). We increased the solvent ionic
320 strength using either sodium (salting-out) or guanidinium (salting-in) ions and assessed the
321 aggregation propensity of the C-terminal peptides using UV-Vis absorption at 450 nm. We first
322 observed the behavior of the C-terminus with increasing salting-out NaCl concentrations. At NaCl
323 concentrations below 1 M, the peptide was dominantly monomeric, despite showing slight
324 aggregation at NaCl concentrations between 250–500 mM (**Fig. 6A**). At NaCl concentrations
325 above 1 M, A_{2A}R C-terminal peptides strongly associated into insoluble aggregates (**Fig. 6A**).
326 Consistent with the observations made with the intact receptor (**Fig. 5**), A_{2A}R C-terminus showed
327 the tendency to progressively precipitate with increasing ionic strengths, suggesting that depletion
328 interactions drive the association and precipitation of the peptides. We next observed the behavior
329 of the C-terminus with increasing concentrations of guanidine hydrochloride (GdnHCl), which
330 contains salting-in cations that do not cause proteins to precipitate and instead facilitate the
331 solubilization of proteins(71, 72). Our results demonstrated that the A_{2A}R C-terminus incubated in
332 4 M GdnHCl showed no aggregation propensity (**Fig. 6A**), validating our expectation that
333 depletion interactions are not enhanced by salting-out salts. These observations demonstrate that
334 the C-terminal peptide in and of itself can directly interact with other C-terminal peptides to form
335 self-aggregates in the presence of ions, and presumably solutes, that have salting-out effects.

336 Attractive hydrophobic interactions among the hydrophobic residues are further enhanced by water
337 solvating the protein having more favorable interactions with other water molecules, ions or
338 solutes than with the protein, here the truncated C-terminus(73–75). We explored the possible
339 contribution of hydrophobic interactions to the aggregation of the C-terminal peptides using
340 differential scanning fluorimetry (DSF). In particular, we gradually increased the temperature to
341 melt the C-terminal peptides, exposing any previously buried hydrophobic residues (**Fig. S4A**)
342 which then bound to the SYPRO orange fluorophore, resulting in an increase in fluorescence signal.
343 Our results showed that as the temperature increased, a steady rise in fluorescence was observed
344 (**Fig. 6B**), indicating that multiple hydrophobic residues were gradually exposed to the SYPRO
345 dye. However, at approximately 65°C, the melt peak signal was abruptly quenched (**Fig. 6B**),
346 indicating that the hydrophobic residues were no longer exposed to the dye. This observation
347 suggests that, at 65°C, enough hydrophobic residues in the C-terminal peptides were exposed such

348 that they collapsed on one another (thus expelling the bound dye molecules), resulting in
349 aggregation. Clearly, the hydrophobic effect can cause A_{2A}R C-terminal peptides to directly
350 associate. These results demonstrate that A_{2A}R oligomer formation can be driven by depletion
351 interactions among the C-termini of the protomers.



352

353 **Figure 6.** The A_{2A}R C-terminus is prone to aggregation. (A) Absorbance at 500 nm of the A_{2A}R C-terminus in solution,
354 with NaCl and GdnHCl concentrations varied to achieve ionic strengths 0–4 M. Inset: the solution at ionic strength
355 4 M achieved with NaCl. The Hofmeister series is provided to show the ability of cations to salt out (blue) or salt in
356 (red) proteins. (B) SYPRO orange fluorescence of solutions containing the A_{2A}R C-terminus as the temperature was
357 varied from 20 to 70°C (grey). The change in fluorescence, measured in relative fluorescence unit (RFU), was
358 calculated by taking the first derivative of the fluorescence curve (black).

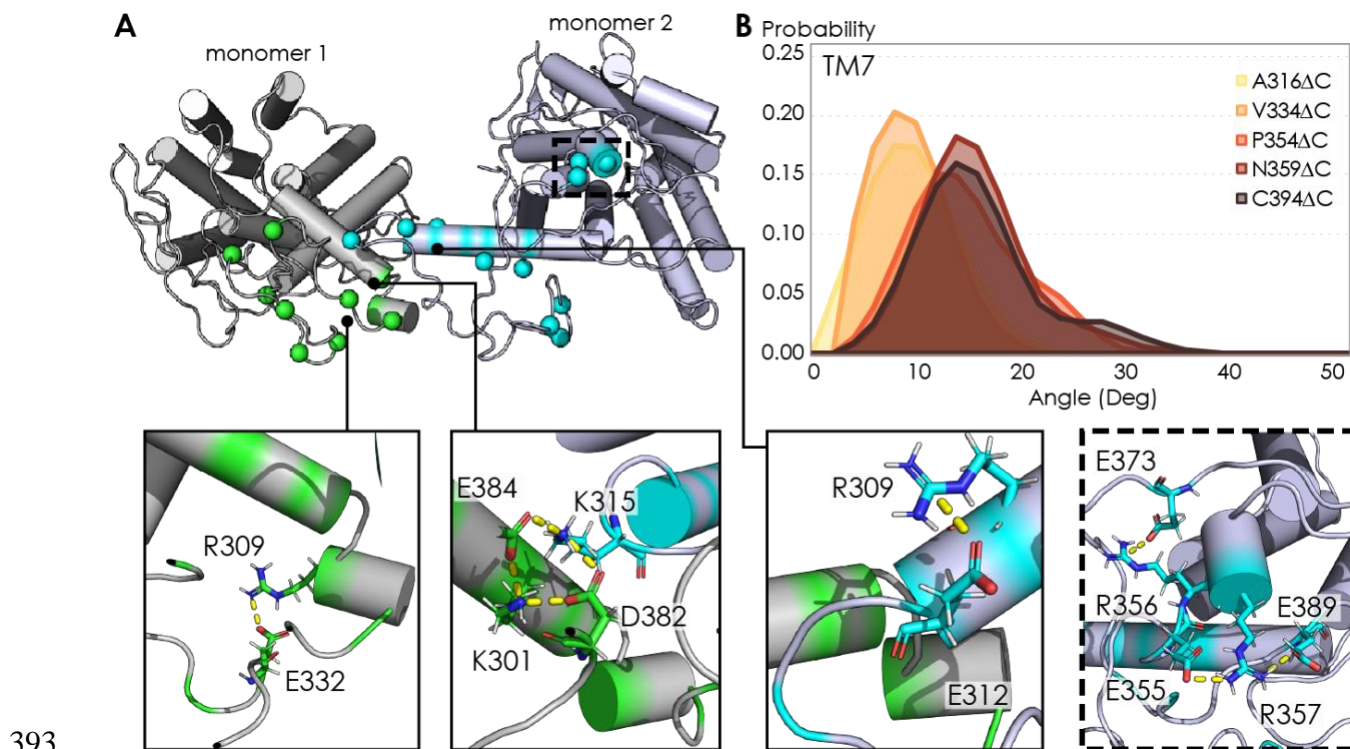
359 DISCUSSION

360 The key finding of this study is that the C-terminus of A_{2A}R, removed in all previously published
361 structural studies of this receptor, is directly responsible for receptor oligomerization. Using a
362 combination of experimental and computational approaches, we demonstrate that the C-terminus
363 drives A_{2A}R oligomerization via a combination of disulfide linkages, hydrogen bonds, electrostatic
364 interactions, and hydrophobic interactions. This diverse combination of interactions is greatly
365 enhanced by depletion interactions, forming a network of malleable bonds that give rise to the
366 existence of multiple A_{2A}R oligomeric interfaces.

367 The intermolecular disulfide linkages associated with residue C394 play a role in A_{2A}R
368 oligomerization. However, it is unclear which cysteine on the second protomer is linked to this
369 cysteine. A previous study showed that residue C394 in A_{2A}R dimer is available for nitroxide spin

370 labeling(63), suggesting that some of these disulfide bonds may be between residue C394 and
371 another cysteine in the hydrophobic core of A_{2A}R that do not form intramolecular disulfide
372 bonds(76–78). Many examples exist where disulfide linkages help drive GPCR oligomerization,
373 including the CaR-mGluR₁ heterodimer(79), homodimers of mGluR₅(80), M₃R(81), V₂R(82), 5-
374 HT₄R(83) and 5-HT_{1D}R(84), and even higher-order oligomers of D₂R(85). However, although
375 unconventional cytoplasmic disulfide bonds have been reported(86, 87), no study has shown how
376 such linkages would be formed *in vivo*, as the cytoplasm lacks the conditions and machinery
377 required for disulfide bond formation(88–91). Nevertheless, residue C394 is highly conserved and
378 a C-terminal cysteine is almost always present among A_{2A}R homologs(92), suggesting that this
379 cysteine cannot be excluded for serving an important role *in vivo*.

380 The electrostatic interactions that stabilize A_{2A}R oligomer formation come from multiple sites
381 along the C-terminus. From a representative snapshot of a A_{2A}R-C394ΔC dimer from our MD
382 simulations (**Fig. 7A**), we could visualize not only the intermolecular interactions calculated from
383 the CGMD simulations (**Fig. 4B**), but also intramolecular salt bridges. In particular, the ³⁵⁵ERR³⁵⁷
384 cluster of charged residues lies distal from the dimeric interface, yet still forms several salt bridges
385 (**Fig. 7A**, inset). This observation is supported by our experimental results showing that
386 substituting this charged cluster with alanines reduces the total A_{2A}R oligomer levels (**Fig. 3C**).
387 However, it is unclear how such salt bridges involving this ³⁵⁵ERR³⁵⁷ cluster are enhanced by
388 depletion interactions (**Fig. 5**), as electrostatic interactions are usually screened out at high ionic
389 strengths. In our MD simulations, we also observed networks of salt bridges along the dimeric
390 interface, for example between K315 of one monomer and D382 and E384 of the other monomer
391 (**Fig. 7A**, inset). The innate flexibility of the C-terminus could facilitate the formation of such salt
392 bridges, which then acts as a potential scaffold to stabilize A_{2A}R dimers.



393
 394 **Figure 7.** (A) Representative snapshot of $A_{2A}R$ -C394AC dimers shows salt bridge formation between a sample
 395 trajectory. The insets are close-ups of the salt bridges, which can be both intra- and intermolecular. The last inset
 396 shows a network of salt bridges with the charged cluster $^{355}ERR^{357}$ involved. (B) Helical tilt angles for TM7 helix in
 397 $A_{2A}R$ as a function of protein length. Systematic truncations of the C-terminus lead to rearrangement of the
 398 heptahelical bundle. The participation of the C-terminus in $A_{2A}R$ dimerization increases the tilting of the TM7 domain,
 399 which is in closest proximity to the C-terminus.

400 We also found that depletion interactions can enhance the diversity of interactions that stabilize
 401 $A_{2A}R$ oligomer formation (Fig. 5 and 6). Depletion interactions could be the key factor to the
 402 cooperative mechanism by which $A_{2A}R$ oligomerization occurs. As revealed by our MD
 403 simulations, an increasing number of contacts are formed along segment 291–334 when the rest
 404 of C-terminus is present (Fig. 4B and 4C). As more of the C-terminus is preserved, the greater
 405 extent of depletion interactions limits the available dimer arrangements, forcing segment 291–334
 406 into an orientation that optimizes intermolecular interactions.

407 Our finding that $A_{2A}R$ forms homo-oligomers via multiple interfaces (Fig. 4A) agrees with the
 408 increasing number of studies reporting multiple and interconverting oligomeric interfaces in $A_{2A}R$
 409 and other GPCRs(24–36). When translated to *in vivo* situations, GPCR oligomers can also

410 transiently associate and dissociate(93–96). Such fast conformational changes require that the
411 oligomeric interfaces be formed by interactions that can easily be modulated. This is consistent
412 with our study, which demonstrates that depletion interactions via the intrinsically disordered,
413 malleable C-terminus drive A_{2A}R oligomerization. Because depletion interactions can be readily
414 tuned by environmental factors, such as ionic strength, molecular crowding, and temperature, the
415 formation of GPCR oligomeric complexes could be dynamically modulated in response to
416 environmental cues to regulate receptor function.

417 Not only did we find multiple A_{2A}R oligomeric interfaces, we also found that these interfaces can
418 be either symmetric or asymmetric. This finding is supported by a growing body of evidence that
419 there exists both symmetric and asymmetric oligomeric interfaces for A_{2A}R(24) and many other
420 GPCRs. Studies using various biochemical and biophysical techniques have shown that
421 heterotetrameric GPCR complexes can be formed by dimers of dimers, including μ OR- δ OR(97),
422 CXC₄R-CC₂R(98), CB₁R/D₂R(99) as well as those involving A_{2A}R, such as A₁R-A_{2A}R(61, 100)
423 and A_{2A}R-D₂R(101). The quaternary structures identified in these studies required specific
424 orientations of each protomer, with the most viable model involving a stagger of homodimers with
425 symmetric interfaces(102). On the other hand, since symmetric interfaces limit the degree of
426 receptor association to dimers, the HMW oligomer of A_{2A}R observed in this(24) and other
427 studies(63, 103) can only be formed via asymmetric interfaces. It is indeed tempting to suggest
428 that the formation of the HMW oligomer of A_{2A}R may even arise from combinations of different
429 interfaces. In any case, the wide variation of GPCR oligomerization requires the existence of both
430 symmetric and asymmetric oligomeric interfaces.

431 In the case of A_{2A}R, displacement of the transmembrane domains have been demonstrated to be
432 the hallmark of receptor activation(104–107). However, no studies have linked receptor
433 oligomerization with the arrangement of the TM bundles in A_{2A}R. Our MD simulations revealed
434 that C-terminus truncation resulted in structural changes in the heptahelical bundles of A_{2A}R
435 dimers. Specifically, as more of the C-terminus was preserved, we observed a progressive increase
436 in the helical tilt of TM7 (**Fig. 7B**). This change in helical tilt occurred for the entire heptahelical
437 bundle, with an increase in tilt for TM1, TM2, TM3, TM5, and TM7, and a decrease in tilt for
438 TM4 and TM6 (**Fig. S3**). The longer C-terminus in the full-length A_{2A}R permits greater
439 rearrangements in the transmembrane regions, leading to the observed change in helical tilt. This

440 result hints at potential conformational changes of A_{2A}R upon oligomerization, necessitating future
441 investigation on functional consequences.

442 C-terminal truncations prior to crystallization and structural studies may be the main reason for
443 the scarcity of GPCR structures featuring oligomers. In that context, this study offers valuable
444 insights and approaches to tune the oligomerization of A_{2A}R and potentially of other GPCRs using
445 its intrinsically disordered C-terminus. The presence of A_{2A}R oligomeric populations with partial
446 C-terminal truncations means that one can now study its oligomerization with less perturbation
447 from the C-terminus. We also present evidence that the multiple C-terminal interactions that drive
448 A_{2A}R oligomerization can be easily modulated by ionic strength and specific salts (**Fig. 5** and **6**).
449 Given that ~75% and ~15% of all class-A GPCRs possess a C-terminus of > 50 and > 100 amino
450 acid residues(108), respectively, it will be worthwhile to explore the prospect of tuning GPCR
451 oligomerization not only by shortening the C-terminus but also with simpler approaches such as
452 modulating ionic strength and the surrounding salt environment.

453 **CONCLUSION**

454 This study emphasizes for the first time the definite impact of the C-terminus on A_{2A}R
455 oligomerization, which can be extended to include the oligomers formed by other GPCRs with a
456 protracted C-terminus. We have shown that the oligomerization of A_{2A}R is strongly driven by
457 depletion interactions along the C-terminus, further modulating and enhancing the multiple
458 interfaces formed via a combination of hydrogen, electrostatic, hydrophobic, and covalent
459 disulfide interactions. The task remains to link A_{2A}R oligomerization to functional roles of the
460 receptor(109). From a structural biology standpoint, visualizing the multiple oligomeric interfaces
461 of A_{2A}R in the presence of the full-length C-terminus is key to investigating whether these
462 interfaces give rise to different oligomer functions.

463 **MATERIALS AND METHODS**

464 *Cloning, Gene Expression, and Protein Purification*

465 The multi-integrating pITy plasmid(110), previously used for overexpression of A_{2A}R in
466 *Saccharomyces cerevisiae*(111), was employed in this study. pITy contains a Gal1–10 promoter

467 for galactose-induced expression, a synthetic pre-pro leader sequence which directs protein
468 trafficking(112, 113), and the yeast alpha terminator. The genes encoding A_{2A}R variants with 10-
469 His C-terminal tag were cloned into pITy downstream of the pre-pro leader sequence, using either
470 splice overlapping extension(114) or USER cloning using X7 polymerase(115, 116), with primers
471 provided in **Table S3**. The plasmids were then transformed into *S. cerevisiae* strain BJ5464
472 (MAT α ura3-52 trp1 leu2 Δ 1 his3 Δ 200 pep4::HIS3 prb1 Δ 1.6R can1 GAL) (provided by the lab of
473 Anne Robinson at Carnegie Mellon University) using the lithium-acetate/PEG method(117).
474 Transformants were selected on YPD G-418 plates (1% yeast extract, 2% peptone, 2% dextrose,
475 2.0 mg/mL G-418).

476 Receptor was expressed and purified following the previously described protocol(118). In brief,
477 from freshly streaked YPD plates (1% yeast extract, 2% peptone, 2% dextrose), single colonies
478 were grown in 5-mL YPD cultures over night at 30°C. From these 5-mL cultures, 50-mL cultures
479 were grown with a starting OD of 0.5 over night at 30°C. To induce expression, yeast cells from
480 these 50-mL cultures were centrifuged at 3,000 x g to remove YPD before resuspended in YPG
481 medium (1% yeast, 2% peptone, 2% D-galactose) at a starting OD of 0.5. The receptor was
482 expressed for 24 hours over night at 30°C with 250 r.p.m shaking. Cells were pelleted by
483 centrifugation at 3,000 x g, washed in sterile PBS buffer, and pelleted again before storage at –
484 80°C until purification.

485 Mechanical bead lysis of cells was done, per 250 mL of cell culture, by performing 12 pulses of
486 60 s intense vortexing (with at least 60 s of rest in between pulses) in 10 mL 0.5-mm zirconia silica
487 beads (BioSpec, Bartlesville, OK, USA; #11079105z), 25 mL of lysis buffer (50 mM sodium
488 phosphate, 300 mM sodium chloride, 10% (v/v) glycerol, pH = 8.0, 2% (w/v) n-Dodecyl- β -D-
489 maltopyranoside (DDM; Anatrace, Maumee, OH, USA; #D310), 1% (w/v) 3-[(3-
490 Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Anatrace; #C216), and 0.2%
491 (w/v) cholesteryl hemisuccinate (CHS; Anatrace; #CH210) and an appropriate amount of 100x
492 Pierce Halt EDTA-free protease inhibitor (Pierce, Rockford, IL, USA #78439)). Beads were
493 separated using a Kontex column. Unlysed cells were removed by centrifugation at 3,220 x g for
494 10 min. Receptor was let solubilized on rotary mixer for 3 hours before cell debris was removed
495 by centrifugation at 10,000 x g for 30 min. Solubilized protein was incubated with Ni-NTA resin
496 (Pierce; #88221) over night. Protein-resin mixture was then washed extensively in purification

497 buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10% (v/v) glycerol, 0.1% (w/v) DDM,
498 0.1% (w/v) CHAPS and 0.2% (w/v) CHS, pH = 8.0) containing low imidazole concentrations (20–
499 50 mM). A_{2A}R was eluted into purification buffer containing 500 mM imidazole. Prior to further
500 chromatographic purification, imidazole was removed using a PD-10 desalting column (GE
501 Healthcare, Pittsburgh, PA, USA; # 17085101).

502 Ligand affinity resin was prepared as previously described for purification of active A_{2A}R.(119)
503 (120) In brief, 8 mL of isopropanol-washed Affigel 10 resin (BioRad; # 1536099) was mixed
504 gently in an Erlenmeyer flask for 20 h at room temperature with 48 mL of DMSO containing 24
505 mg of xanthine amine congener (XAC, high-affinity A_{2A}R antagonist, K_D = 32 nM; Sigma, St.
506 Louis, MO, USA; #X103). The absorbance at 310 nm of the XAC-DMSO solution before and after
507 the coupling reaction was measured in 10 mM HCl and compared to a standard curve. The amount
508 of resin bound to ligand was estimated to be 5.6 μM. The coupling reaction was quenched by
509 washing the resin with DMSO, then with Tris-HCl 50 mM (pH = 7.4), then with 20% (v/v) ethanol.
510 The resin was packed into a Tricorn 10/50 column (GE Healthcare) under pressure via a BioRad
511 Duoflow FPLC (BioRad).

512 For purification of active A_{2A}R, the column was equilibrated with 4 CV of purification buffer. The
513 IMAC-purified A_{2A}R was desalted and diluted to 5.5 mL before applied to a 5-mL sample loop on
514 the BioRad Duoflow FPLC, from which the sample was loaded onto the column at a rate of 0.1
515 mL/min. Inactive A_{2A}R was washed from the column by flowing 10 mL of purification buffer at
516 0.2 mL/min, followed by 16 mL at 0.4 mL/min. Active A_{2A}R was eluted from the column by
517 flowing purification buffer containing 20 mM theophylline (low-affinity A_{2A}R antagonist, K_D =
518 1.6 μM; Sigma, St. Louis, MO, USA; #T1633). Western blot analysis was performed to determine
519 4-mL fractions with active A_{2A}R collected with a BioFrac fraction collector (BioRad; Hercules,
520 CA, USA), which were then concentrated through a 30-kDa MWCO centrifugal filter (Millipore,
521 Billerica, MA, USA; # UFC803096) and desalted to remove excess theophylline. For the
522 experiments where the salt concentrations were varied, the buffer exchange was done also by this
523 last desalting step.

524 *Size-Exclusion Chromatography*

525 To separate oligomeric species of active A_{2A}R, a prepacked Tricorn Superdex 200 10/300 GL
526 column (GE Healthcare) connected to a BioRad Duoflow FPLC was equilibrated with 60 mL of
527 running buffer (150 mM sodium chloride, 50 mM sodium phosphate, 10% (v/v) glycerol, 0.1%
528 (w/v) DDM, 0.1% (w/v) CHAPS, 0.02% (w/v) CHS, pH = 8.0) at a flow rate of 0.2 mL/min. 0.5-
529 mL fractions were collected with a BioFrac fraction collector in 30 mL of running buffer at the
530 same flow rate. Analysis of SDS/PAGE and western blot was done to determine oligomeric states
531 of the eluted A_{2A}R.

532 *SEC Peak Analysis*

533 SEC chromatograms were analyzed using OriginLab using the nonlinear curve fit (Gaussian)
534 function. The area under the curve and the peak width were manually defined in cases where the
535 SNR of the SEC trace were too low. The R² values reached > 0.96 for most cases. The population
536 of each oligomeric species was expressed as the integral of each Gaussian this curve fit of the SEC
537 signal. The HMW oligomer peak in some cases could not be fitted with one curve and thus was
538 fitted with two curves instead. The reported standard errors were calculated from the variance of
539 the fit and did not correspond to experimental errors. The results are detailed in **Fig. S2** and **Table**
540 **S1**.

541 *SDS-PAGE and Western Blotting*

542 10% SDS-PAGE gels were hand-casted in BioRad Criterion empty cassettes (BioRad; #3459902,
543 3459903). Lysate controls were prepared by lysis of 5 OD cell pellets with 35 μ L of YPER (Fisher
544 Scientific, Waltham, MA, USA # 8990) at RT for 20 min, incubation with 2x Laemmli buffer (4%
545 (w/v) SDS, 16% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 167 M Tris, pH 6.8) at 37°C for
546 1 h, and centrifugation at 3,000 \times g for 1 min to pellet cell debris. Protein samples were prepared
547 by incubation with 2x Laemmli buffer at 37°C for 30 min. For all samples, 14 μ L (for 26-well gel)
548 or 20 μ L (for 18-well gel) was loaded per lane, except for 7 μ L of Magic Mark XP Western protein
549 ladder (Thermo Scientific, Waltham, MA, USA; # LC5602) as a standard. Electrophoresis was
550 carried out at 120 V for 100 min. Proteins were transferred to 0.2- μ m nitrocellulose membranes
551 (BioRad; # 170-4159) via electroblotting using a BioRad Transblot Turbo, mixed MW protocol.
552 Membranes were blocked in Tris-buffered saline with Tween (TBST; 150 mM sodium chloride,

553 15.2 mM Tris-HCl, 4.6 mM Tris base, pH = 7.4, 0.1% (v/v) Tween 20 (BioRad; # 1706531))
554 containing 5% (w/v) dry milk, then probed with anti-A_{2A}R antibody, clone 7F6-G5-A2 (Millipore,
555 Burlington, MA, USA; # 05-717) at 1:500 in TBST with 0.5% (w/v) dry milk. Probing with
556 secondary antibody was done with a fluorescent DyLight 550 antibody (Abcam, Cambridge, MA,
557 USA; ab96880) at 1:600 in TBST containing 0.5% (w/v) milk.

558 Western blot was analyzed with Fiji. The Gels analysis plugin was used to define each sample lane,
559 and to generate an intensity profile. Peaks were manually selected and integrated with the measure
560 tool to determine the amount of protein present.

561 *Coarse-Grained MD Simulations*

562 Initial configuration of A_{2A}R was based on the crystal structure of the receptor in the active state
563 (PDB 5G53). All non-receptor components were removed, and missing residues added using
564 MODELLER 9.23(121). Default protonation states of ionizable residues were used. The resulting
565 structure was converted to MARTINI coarse-grained topology using the martinize.py script(122).
566 The ELNeDyn elastic network(123) was used to constrain protein secondary and tertiary structures
567 with a force constant of 500 kJ/mol/nm² and a cutoff of 1.5 nm. To optimize loop refinement of
568 the model, a single copy was embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
569 (POPC) bilayer using the insane.py script, solvated with MARTINI polarizable water, neutralized
570 with 0.15 M NaCl, and a short MD (1.5 μs) run to equilibrate the loop regions. Subsequently, two
571 monomers of the equilibrated A_{2A}R were randomly rotated and placed at the center of a 13 nm ×
572 13 nm × 11 nm (xyz) box, 3.5 nm apart, with their principal transmembrane axis aligned parallel
573 to the z axis. The proteins were then embedded in a POPC bilayer using the insane.py script.
574 Sodium and chloride ions were added to neutralize the system and obtain a concentration of 0.15
575 M NaCl. Total system size was typically in the range of 34,000 CG particles, with a 280:1
576 lipid:protein ratio. Ten independent copies were generated for each A_{2A}R truncated variant.

577 v2.2 of the MARTINI coarse-grained force field(124) was used for the protein and water, and v2.0
578 was used for POPC. All coarse-grained simulations were carried out in GROMACS 2016(125) in
579 the NPT ensemble (P = 1 atm, T = 310 K). The Bussi velocity rescaling thermostat was used for
580 temperature control with a coupling constant of $\tau_t = 1.0$ ps(126), while the Parrinello-

581 Rahman barostat(127) was used to control the pressure semi-isotropically with a coupling constant
582 of $\tau_t = 12.0$ ps and compressibility of 3×10^{-4} bar⁻¹. Reaction field electrostatics was used with
583 Coulomb cut-off of 1.1 nm. Non-bonded Lennard-Jones interactions were treated with a cut-off of
584 1.1 nm. All simulations were run with a 15 fs timestep, updating neighbor lists every 10 steps.
585 Cubic periodic boundary conditions along the x, y and z axes were used. Each simulation was run
586 for 8 μ s.

587 *Atomistic MD Simulations*

588 Three snapshots of symmetric dimers of A₂A_R for each respective truncated variant were randomly
589 selected from the CG simulations as starting structures for backmapping. Coarse-grained systems
590 were converted to atomistic resolution using the backward.py script(128). All simulations were
591 run in Gromacs2019 in the *NPT* ensemble ($P = 1$ bar, $T = 310$ K) with all bonds restrained using
592 the LINCS method(129). The Parrinello-Rahman barostat was used to control the pressure semi-
593 isotropically with a coupling constant of $\tau_t = 1.0$ ps and a compressibility of 4.5×10^{-5} bar⁻¹, while
594 the Bussi velocity rescaling thermostat was used for temperature control with a coupling constant
595 of $\tau_t = 0.1$ ps. Proteins, lipids, and solvents were separately coupled to the thermostat. The
596 CHARMM36 and TIP3P force fields(130, 131) were used to model all molecular interactions.
597 Periodic boundary conditions were set in the x, y, and z directions. Particle mesh Ewald (PME)
598 electrostatics was used with a cut-off of 1.0 nm. A 2-fs time step was used for all atomistic runs,
599 and each simulation was run for 50 ns.

600 *Analysis of Computational Results*

601 All trajectories were post-processed using gromacs tools and in-house scripts. We ran a clustering
602 analysis of all dimer frames from the CG simulations using Daura et. al.'s clustering algorithm(132)
603 implemented in GROMACS, with an RMSD cutoff of 1.5 Å. (An interface was considered dimeric
604 if the minimum center of mass distance between the protomers was less than 5 Å.) This method
605 uses an RMSD cutoff to group all conformations with the largest number of neighbors into a cluster
606 and eliminates these from the pool, then repeats the process until the pool is empty. We focused
607 our analysis on the most populated cluster from each truncated variant. Electrostatic interactions
608 in the dimer were calculated from CG systems with LOOS(133) using a distance cutoff of 5.0 Å.

609 Transmembrane helical tilt angles were also calculated in LOOS from CG simulations. Hydrogen
610 bonds were calculated from AA simulations using the hydrogen bonds plugin in VMD(134), with
611 a distance cutoff of 3.5 Å and an angle cutoff of 20°. Only C-terminal residues were included in
612 hydrogen bond analysis. PyMOL(135) was used for molecular visualizations.

613 *Assessing A_{2A}R Oligomerization with Increasing Ionic Strength*

614 Na₂HPO₄ and NaH₂PO₄ in the buffer make up an ionic strength of 0.15 M, to which NaCl was
615 added to increase the ionic strength to 0.45 M and furthermore to 0.95 M. The A_{2A}R variants were
616 purified at 0.45 M ionic strength and then exchanged into buffers of different ionic strengths using
617 a PD-10 desalting column prior to subjecting the samples to SEC. The buffer composition is
618 detailed below.

<i>Buffers</i>	<i>Components</i>	<i>Conc.</i> <i>(mM)</i>	<i>Ionic Strength</i> <i>(mM)</i>
<i>0.15 M Ionic Strength</i>	NaCl	0	0
	NaH ₂ PO ₄	4	4
	Na ₂ HPO ₄	49	146
<i>0.45 M Ionic Strength</i>	NaCl	300	300
	NaH ₂ PO ₄	4	4
	Na ₂ HPO ₄	49	146
<i>0.95 M Ionic Strength</i>	NaCl	800	800
	NaH ₂ PO ₄	4	4
	Na ₂ HPO ₄	49	146

619 **Table 1.** Calculations regarding composition of the buffers used in the experiments where salt concentrations are
620 varied. Only NaCl concentration (in bold) is varied to achieve the different ionic strengths.

621 *Isolated C-Terminus Purification*

622 *Escherichia coli* BL21 (DE3) cells were transfected with pET28a DNA plasmids containing the
623 desired A_{2A}R sequence with a 6x His tag attached for purification. Cells from glycerol stock were
624 grown in 10 mL luria broth (LB, Sigma Aldrich, L3022) overnight at 37°C and then used to
625 inoculate 1 L of fresh LB and 10 µg/mL kanamycin (Fisher Scientific, BP906). Growth of cells
626 were performed at 37°C, 200 rpm until optical density at λ = 600 nm reached 0.6–0.8. Expression
627 was induced by incubation with 1 mM isopropyl-β-D-thiogalactoside (Fisher Bioreagents,
628 BP175510) for 3 hrs.

629 Cells were harvested with centrifugation at 5000 rpm for 30 min. Harvested cells were resuspended
630 in 25 mL Tris-HCl, pH = 7.4, 100 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA with 1 Pierce protease
631 inhibitor tablet (Thermo Scientific, A32965), 1 mM PMSF, 2 mg/mL lysozyme, 20 μ g/mL DNase
632 (Sigma, DN25) and 10 mM MgCl₂, and incubated on ice for 30 min. Samples were then incubated
633 at 30°C for 20 minutes, then flash frozen and thawed 3 times in LN₂. Samples were then centrifuged
634 at 10,000 rpm for 10 min to remove cell debris. 1 mM PMSF was added again and the resulting
635 supernatant was incubated while rotating for at least 4 hrs with Ni-NTA resin. The resin was loaded
636 to a column and washed with 25 mL 20 mM sodium phosphate, pH = 7.0, 1 M NaCl, 20 mM
637 imidazole, 0.5 mM DTT, 100 μ M EDTA. Purified protein was eluted with 15 mL of 20 mM sodium
638 phosphate, pH = 7.0, 0.5 mM DTT, 100 mM NaCl, 300 mM imidazole. The protein was
639 concentrated to a volume of 2.5mL and was buffer exchanged into 20 mM ammonium acetate
640 buffer, pH = 7.4, 100 mM NaCl using a GE PD-10 desalting column. Purity of sample was
641 confirmed with SDS-PAGE and western blot.

642 *Aggregation Assay to Assess A_{2A}R C-Terminus Assembly*

643 Absorbance was measured at 450 nm using a Shimadzu UV-1601 spectrophotometer with 120 μ L
644 sample size. Prior to reading, samples were incubated at 40°C for 5 minutes. Samples were
645 vigorously pipetted to homogenize any precipitate before absorbance was measured. Protein
646 concentration was 50 μ M in a 20 mM ammonium acetate buffer (pH = 7.4).

647 *Differential Scanning Fluorimetry (DSF)*

648 DSF was conducted with a Bio-rad CFX90 real-time PCR machine. A starting temperature 20°C
649 was increased at a rate of 0.5°C per 30 seconds to a final temperature of 85°C. All samples
650 contained 40 μ L of 40 μ M A_{2A}R C-terminus, 9x SYPRO orange (ThermoFisher S6650), 200 mM
651 NaCl, and 20 mM MES. Fluorescence was detected in real-time at 570 nm. All samples were
652 conducted in triplicate.

653 *Hydrophobicity and Charge Profile of C-Terminus*

654 The hydrophobicity profile reported in **Fig. S4** was determined with ProtScale using method
655 described by Kyte & Doolittle(136), window size of 3.

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