Supplementary Information

Structural insights into the agonist selectivity of the adenosine A_3 receptor

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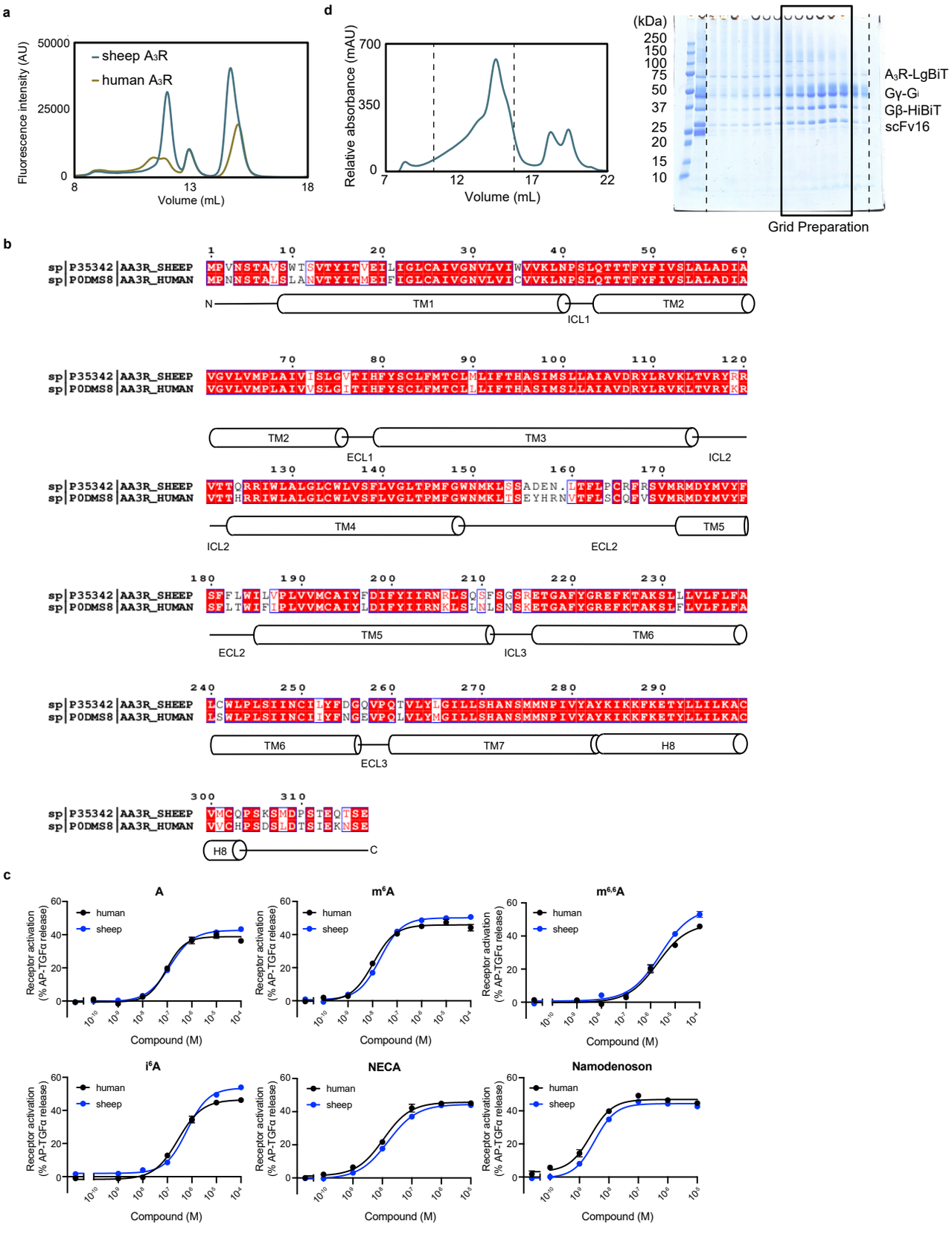
Tomoyoshi Kawakami, Aika Iwama, Hiroyuki H. Okamoto, Chisae Nagiri,

Fan-Yan Wei, Wataru Shihoya, Osamu Nureki



Supplementary Figure 1 | Comparison of A_3Rs and sample preparation

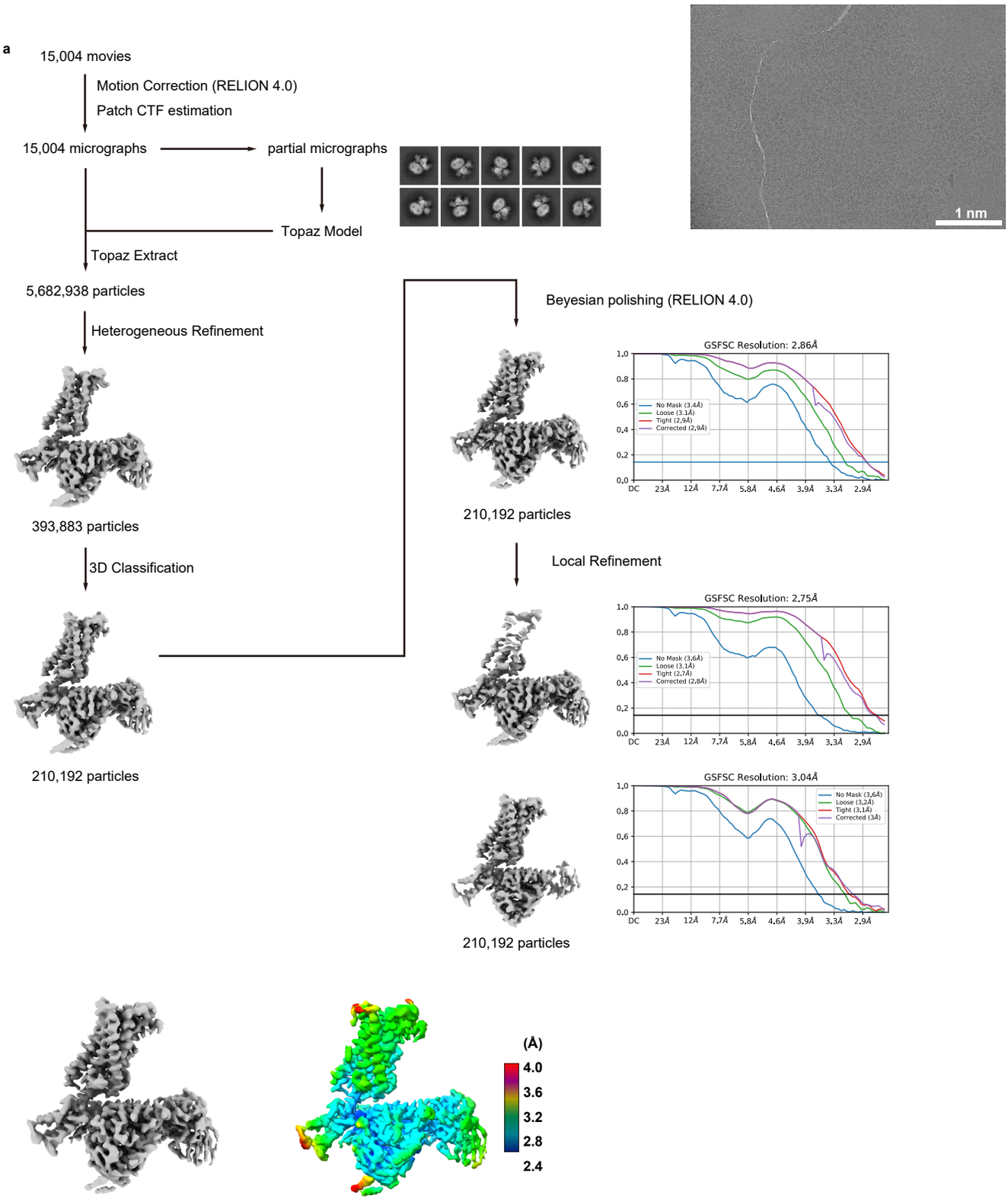
(a) Fluorescence-detection size-exclusion chromatography (FSEC) analysis of the complex formations by sheep and human A_3R.

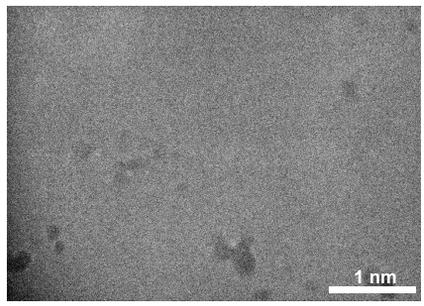
(b) Alignment of the sheep and human A_3R amino acid sequences. Secondary structures are represented by schematic models (based on GPCRdb).

(c) TGF-α shedding assays of sheep and human A_3R. Both A_3Rs exhibited similar ligand binding profiles. Symbols and error bars are means ± SEM of representative data (n=3-6) from three independent experiments. The values for human A, m6A, m66A, and i6A used as a reference are the same as those in Figure 1b-d.

(d) Representive size-exclusion chromatography elution profiles of A_3R and SDS-PAGE analysis of the gel filtration fractions. Corresponding fractions are represented by black dashed lines.

Source data are provided as a Source Data file.


a
15,004 movies
Motion Correction (RELION 4.0)
Patch CTF estimation
15,004 micrographs
partial micrographs
Topaz Model
Topaz Extract
5,682,938 particles
Heterogeneous Refinement
393,883 particles
3D Classification
210,192 particles
Beyesian polishing (RELION 4.0)
210,192 particles
GSFSC Resolution: 2.86Å
No Mask (3.4Å)
Loose (3.1Å)
Tight (2.9Å)
Corrected (2.9Å)
Local Refinement
210,192 particles
GSFSC Resolution: 2.75Å
No Mask (3.6Å)
Loose (3.1Å)
Tight (2.7Å)
Corrected (2.8Å)
210,192 particles
GSFSC Resolution: 3.04Å
No Mask (3.6Å)
Loose (3.2Å)
Tight (3.1Å)
Corrected (3Å)
DC
23Å
12Å
7.7Å
5.8Å
4.6Å
3.9Å
3.3Å
2.9Å
1 nm
(Å)
4.0
3.6
3.2
2.8
2.4



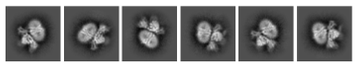
b

21,810 movies

Motion Correction (RELION 4.0)

Patch CTF estimation

21,810 micrographs → partial micrographs

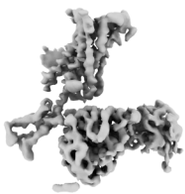


Topaz Model

Topaz Extract

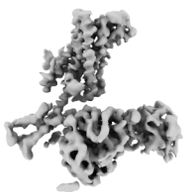
6,981,460 particles

Heterogeneous Refinement



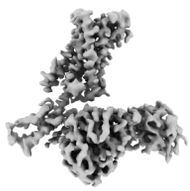
342,540 particles

3D Classification

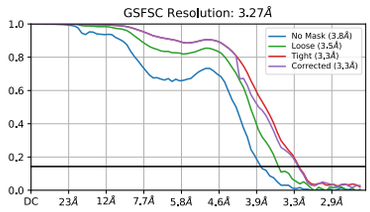


160,357 particles

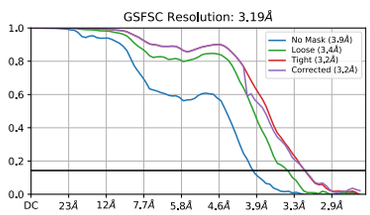
Beyesian polishing (RELION 4.0)



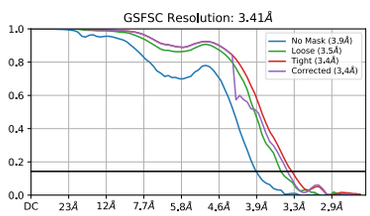
160,357 particles

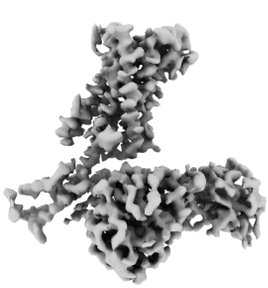


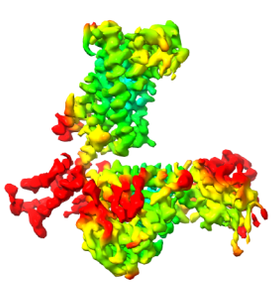
Local Refinement



160,357 particles







(Å)

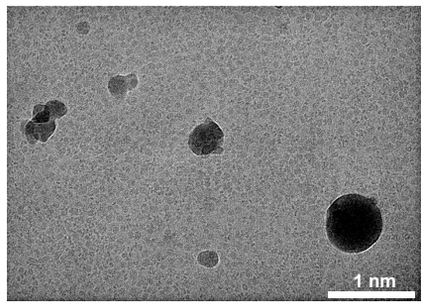
4.0

3.6

3.2

2.8

2.4



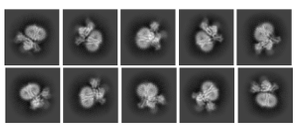
c

12,663 movies

Motion Correction (RELION 4.0)

Patch CTF estimation

12,207 micrographs → partial micrographs

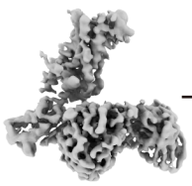


Topaz Model

Topaz Extract

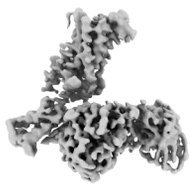
3,575,189 particles

Heterogeneous Refinement

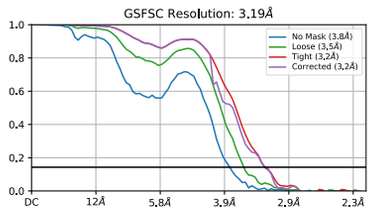


198,745 particles

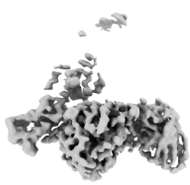
Reference Based Motion Correction

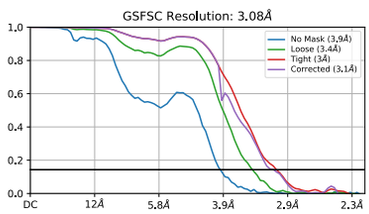


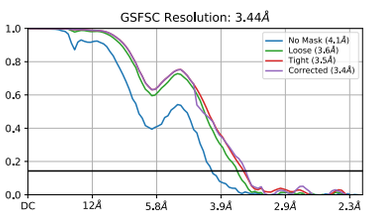
156,891 particles



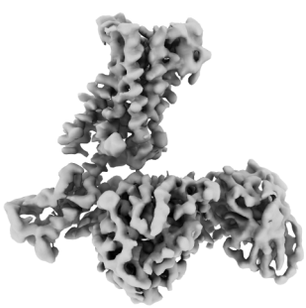
Local Refinement

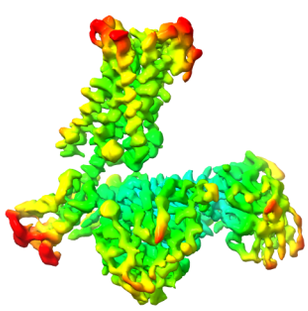


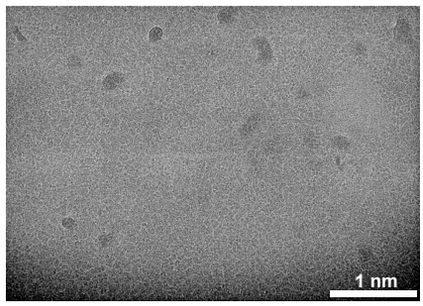




160,357 particles







d

21,300 movies

Motion Correction (RELION 4.0)

Patch CTF estimation

20,787 micrographs → partial micrographs

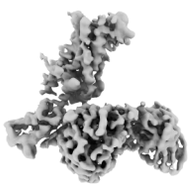
2D templates

Template Picker

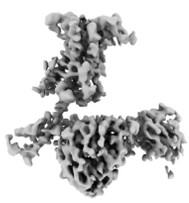
17,004,284 particles

2D classification

Heterogeneous Refinement

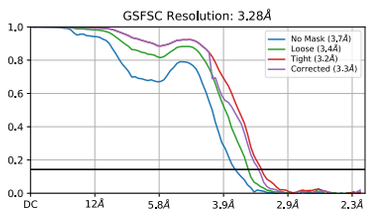


Reference Based Motion Correction

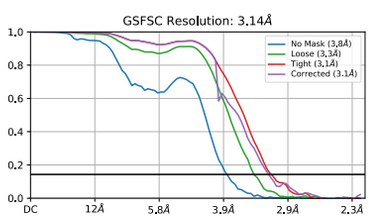


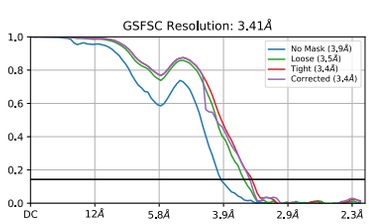
184,579 particles

183,625 particles

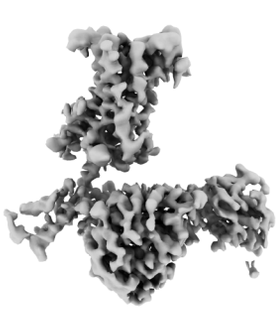


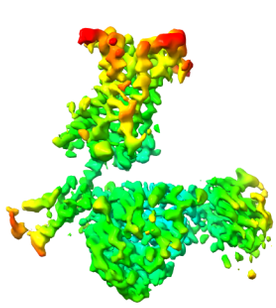
Local Refinement

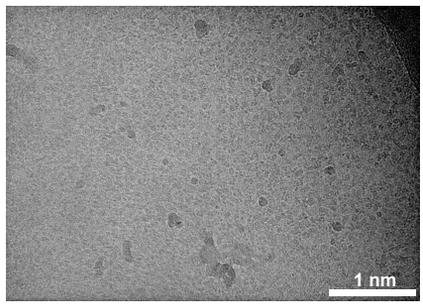




183,625 particles

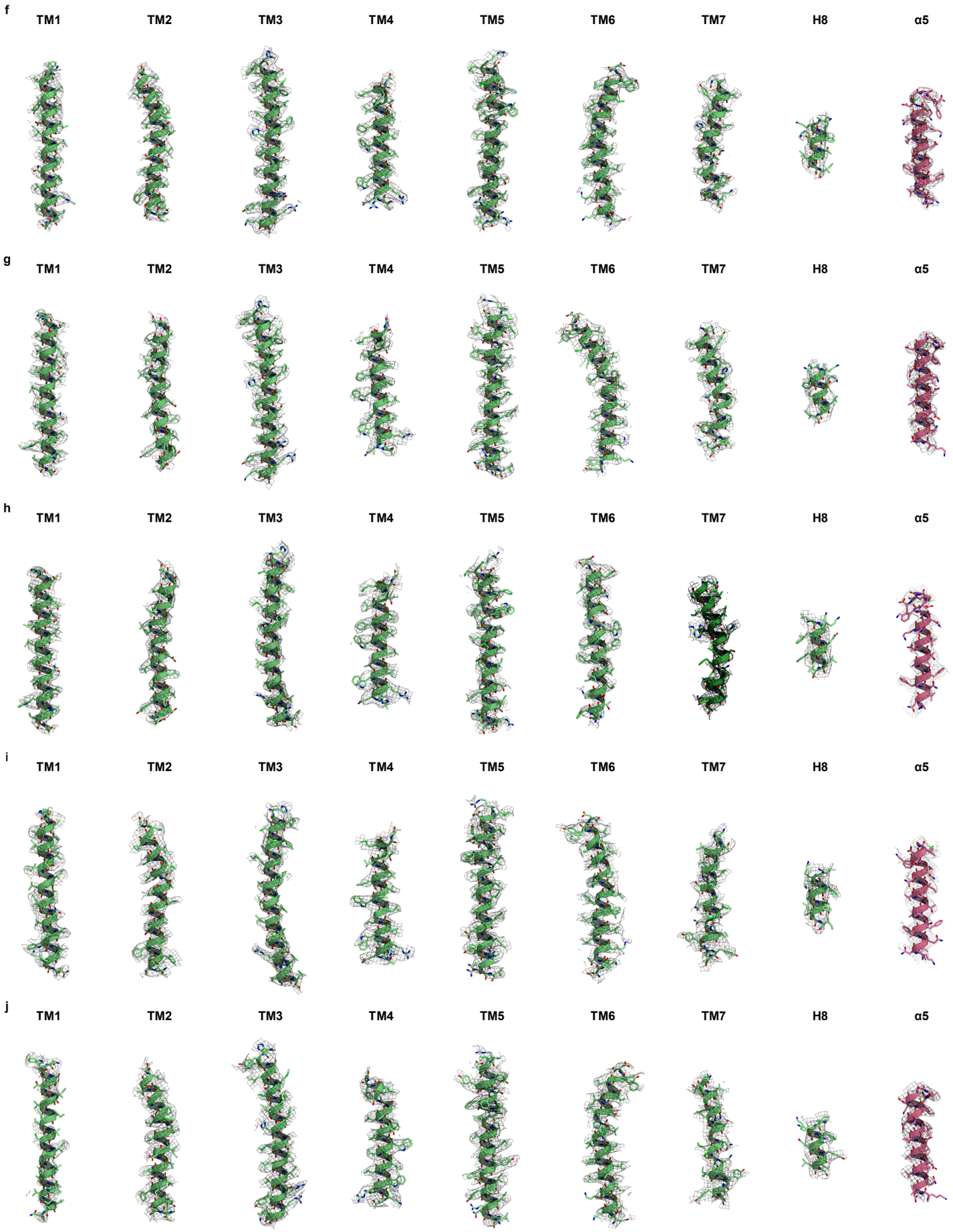





1 nm

e

20,609 movies
Motion Correction (RELION 4.0)
Patch CTF estimation
19,283 micrographs
partial micrographs
2D templates
Template Picker
15,299,681 particles
2D classification
Heterogeneous Refinement
Reference Based Motion Correction
187,814 particles
186,798 particles
GSFSC Resolution: 3.20Å
No Mask (3.6Å)
Loose (3.4Å)
Tight (3.2Å)
Corrected (3.2Å)
Local Refinement
GSFSC Resolution: 3.02Å
No Mask (3.2Å)
Loose (3.3Å)
Tight (3Å)
Corrected (3Å)
GSFSC Resolution: 3.38Å
No Mask (3.9Å)
Loose (3.5Å)
Tight (3.4Å)
Corrected (3.4Å)
186,798 particles
DC
12Å
5.8Å
3.9Å
2.9Å
2.3Å
(Å)
4.0
3.6
3.2
2.8
2.4


f
TM1
TM2
TM3
TM4
TM5
TM6
TM7
H8
α5
g
TM1
TM2
TM3
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TM7
H8
α5
h
TM1
TM2
TM3
TM4
TM5
TM6
TM7
H8
α5
i
TM1
TM2
TM3
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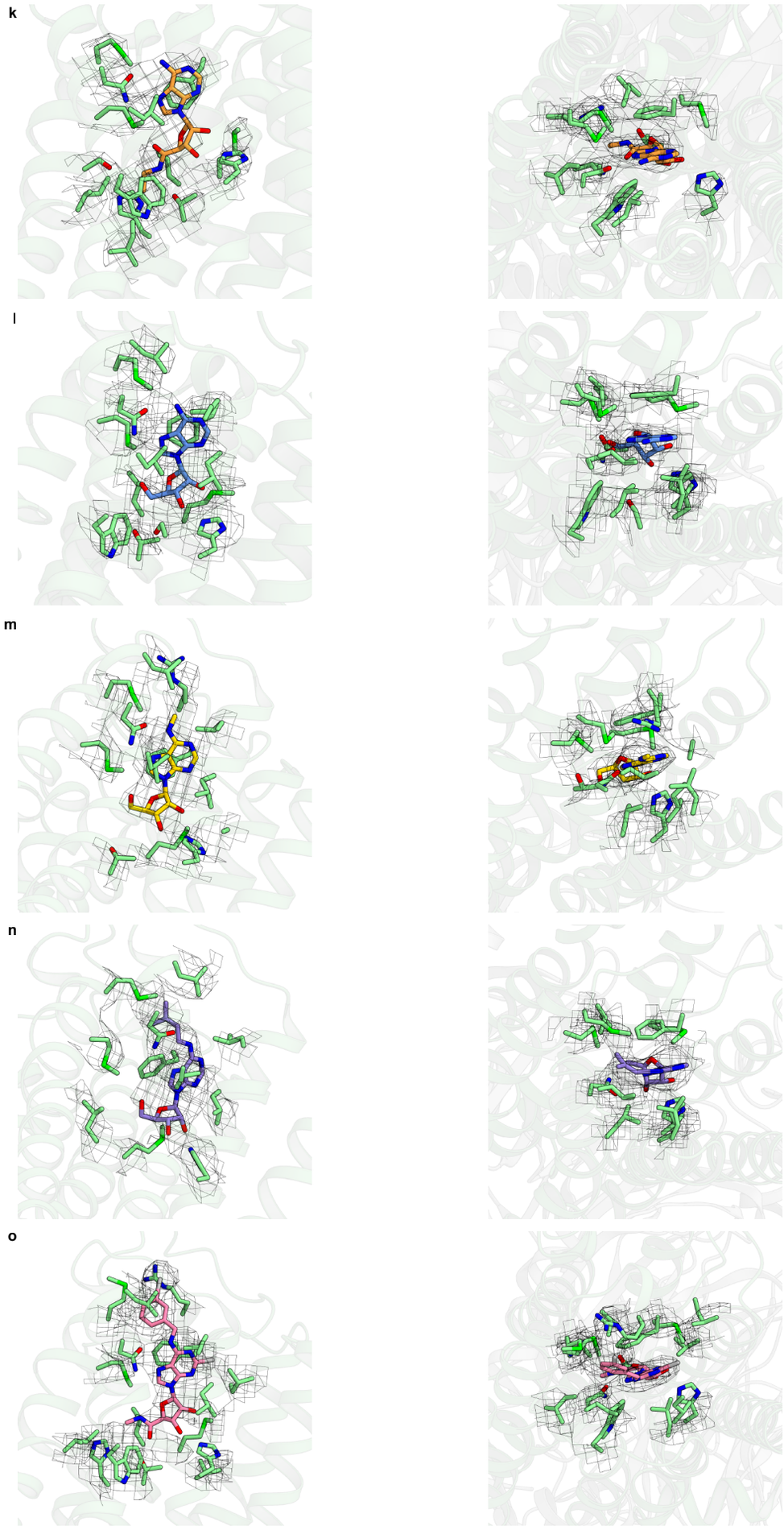
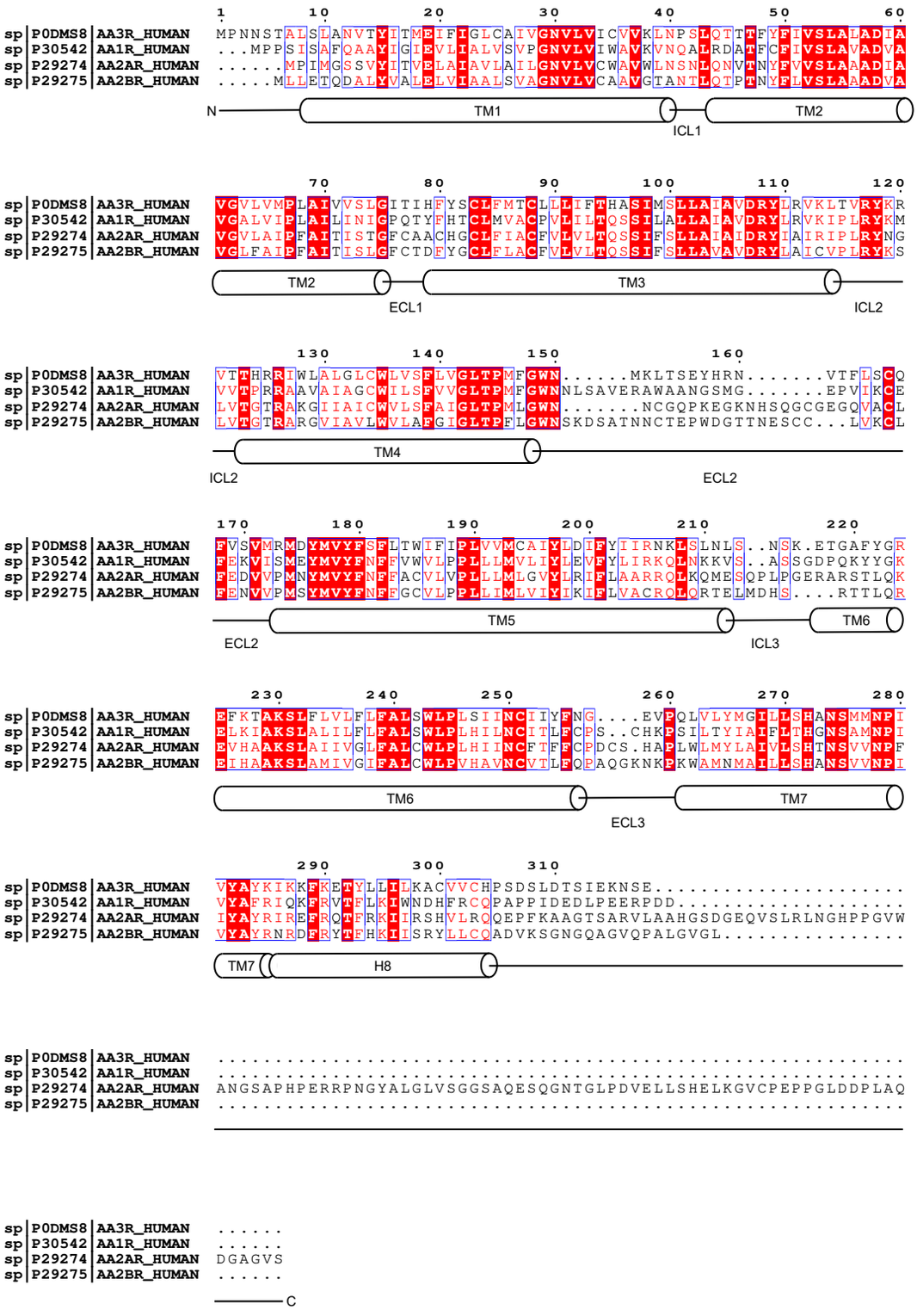
TM3
TM4
TM5
TM6
TM7
H8
α5



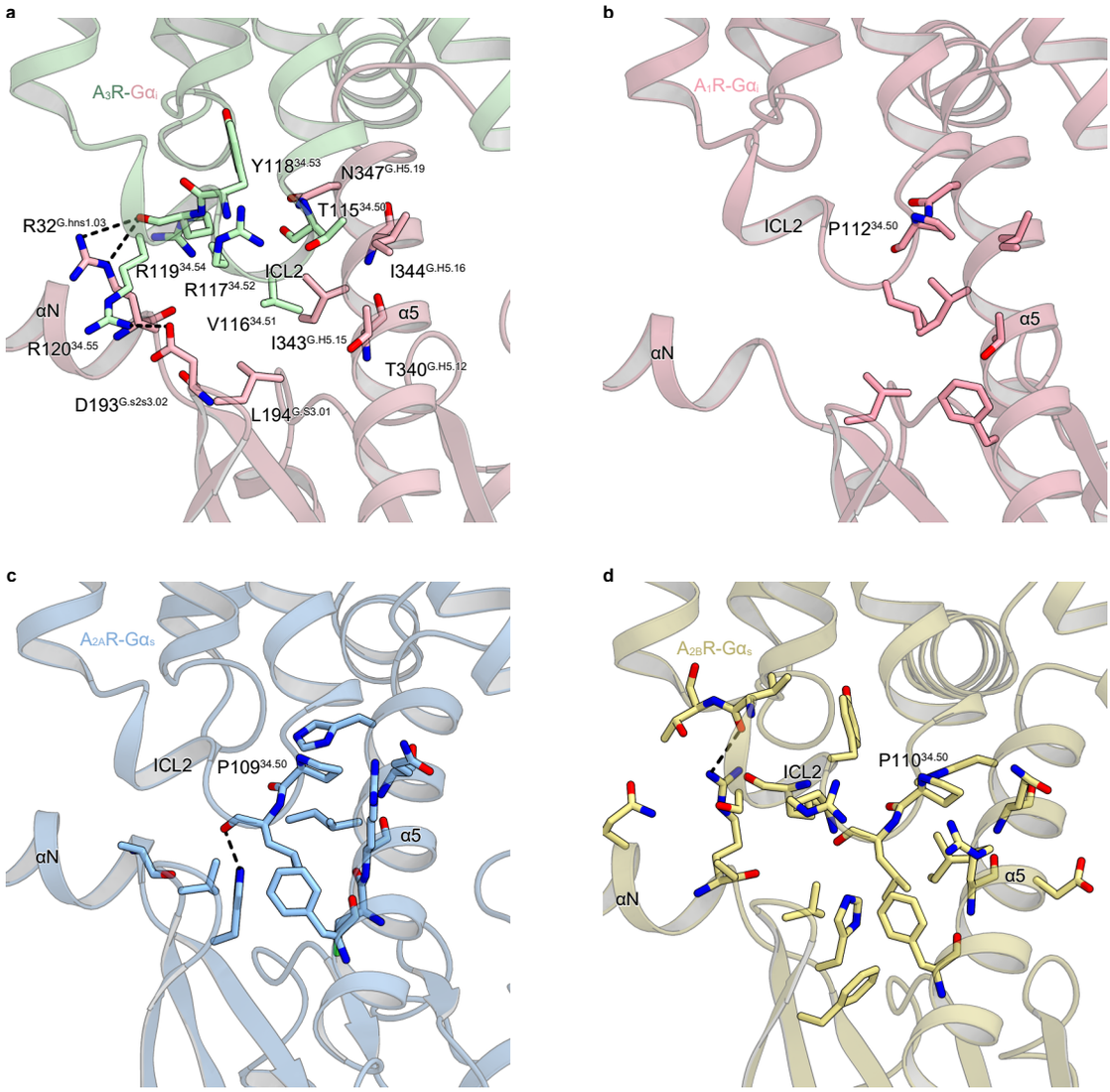
Supplementary Figure 2 | Cryo-EM workflow, maps, and model quality

(a-e) Flow charts of the cryo-EM data processing for the A_3R-G_i complexes bound to NECA (a), adenosine (b), m^6A (c), i^6A (d), and namodenoson (e). Unless otherwise noted, analyses were performed with cryoSPARC. (f-j) Agreement between the cryo-EM density and the atomic model of the A_3R-G_i complexes bound to NECA (f), adenosine (g), m^6A (h), i^6A (i), and namodenoson (j). (k-o) Agreement between the cryo-EM density and the atomic model at the binding pocket of the A_3R-G_i complexes bound to NECA (k), adenosine (l), m^6A (m), i^6A (n), and namodenoson (o).



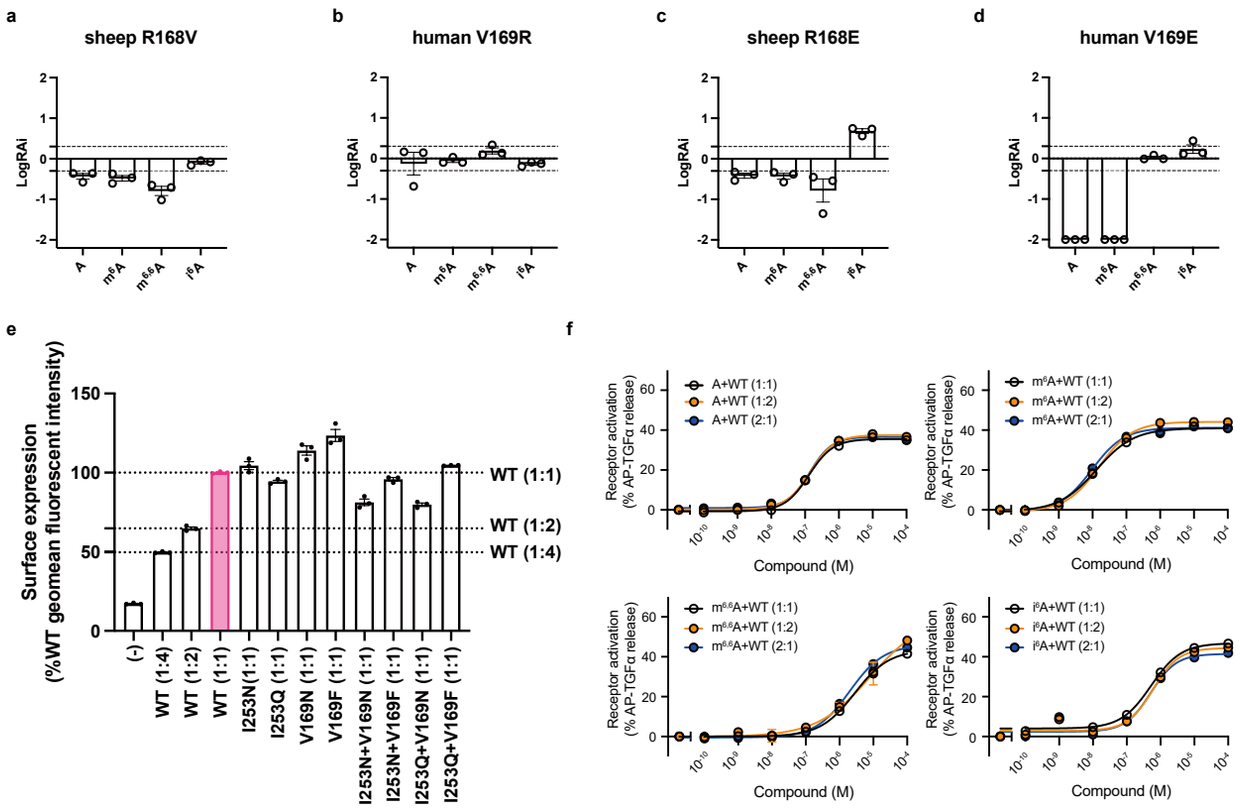
Supplementary Figure 3 | Alignment of the adenosine receptors

Amino acid alignment of the adenosine receptors. Secondary structures are represented by schematic models (based on GPCRdb).



Supplementary Figure 4 | Comparison of ICL2s of adenosine receptors

(a-d) Receptor-G-protein interactions of A_1R (A; PDB 6D9H), $A_{2A}R$ (B; PDB 6GDG), $A_{2B}R$ (C; PDB 7XY7), and A_3R (D). A_3R forms extensive interactions with G_i. This may be a consequence of $T115^{34.50}$, which endows A_3R with a slightly elongated helix compared to the other adenosine receptors with $P^{34.50}$.



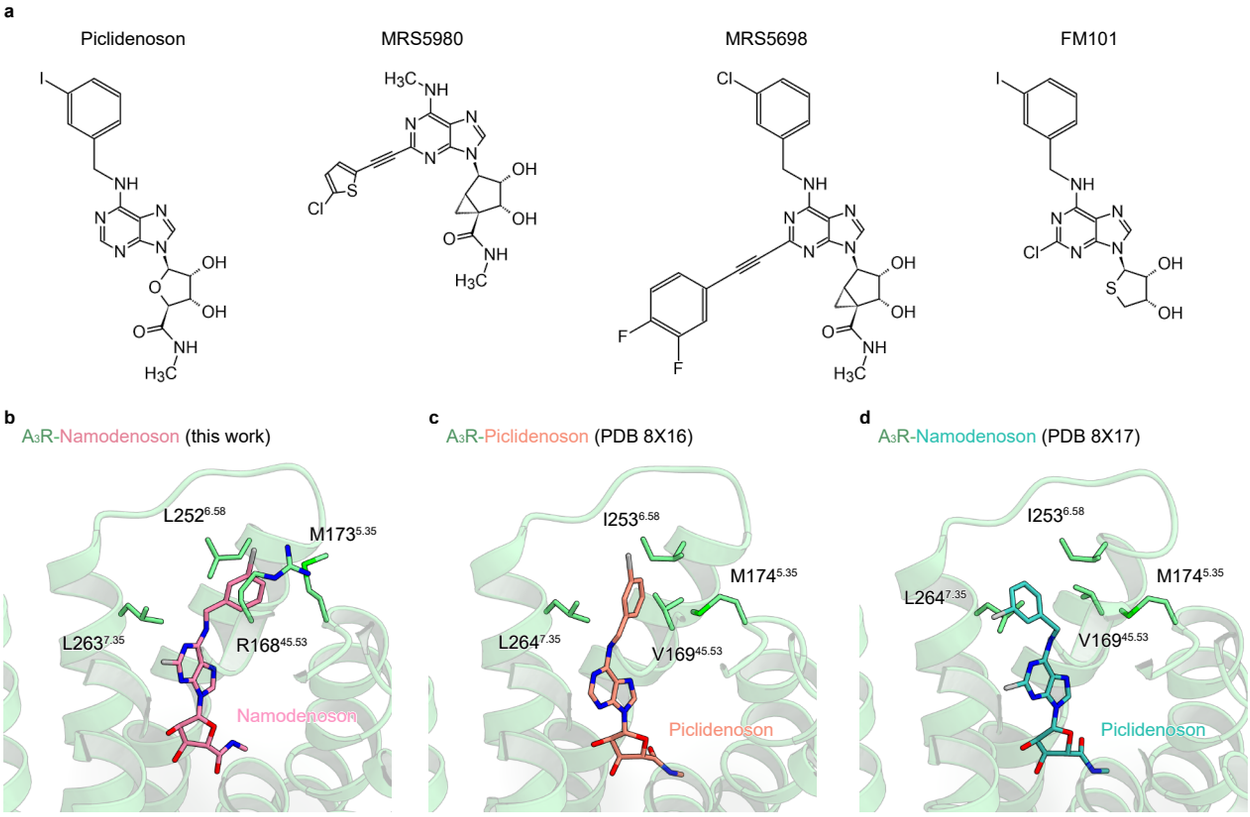
Supplementary Figure 5 | Mutagenesis studies

(a-d) Relative RAi values of unmodified and modified adenosines for A_3R mutants, as determined by the TGFα-shedding assay. Data are presented as mean values ± SEM from three independent experiments performed in triplicate (the n values are represented by circles). The LogRAi cutoff value was set to −2.

(e) Comparison of the surface-expression level of WT A_3R and representative mutants. Data are presented as mean values ± SEM (n=3; dots).

(f) TGF-α shedding assays of WT A_3R, with different ligands and various concentrations of plasmid for transfection. Symbols and error bars represent mean and SEM, respectively, of 3–12 independent experiments with each performed in triplicate.

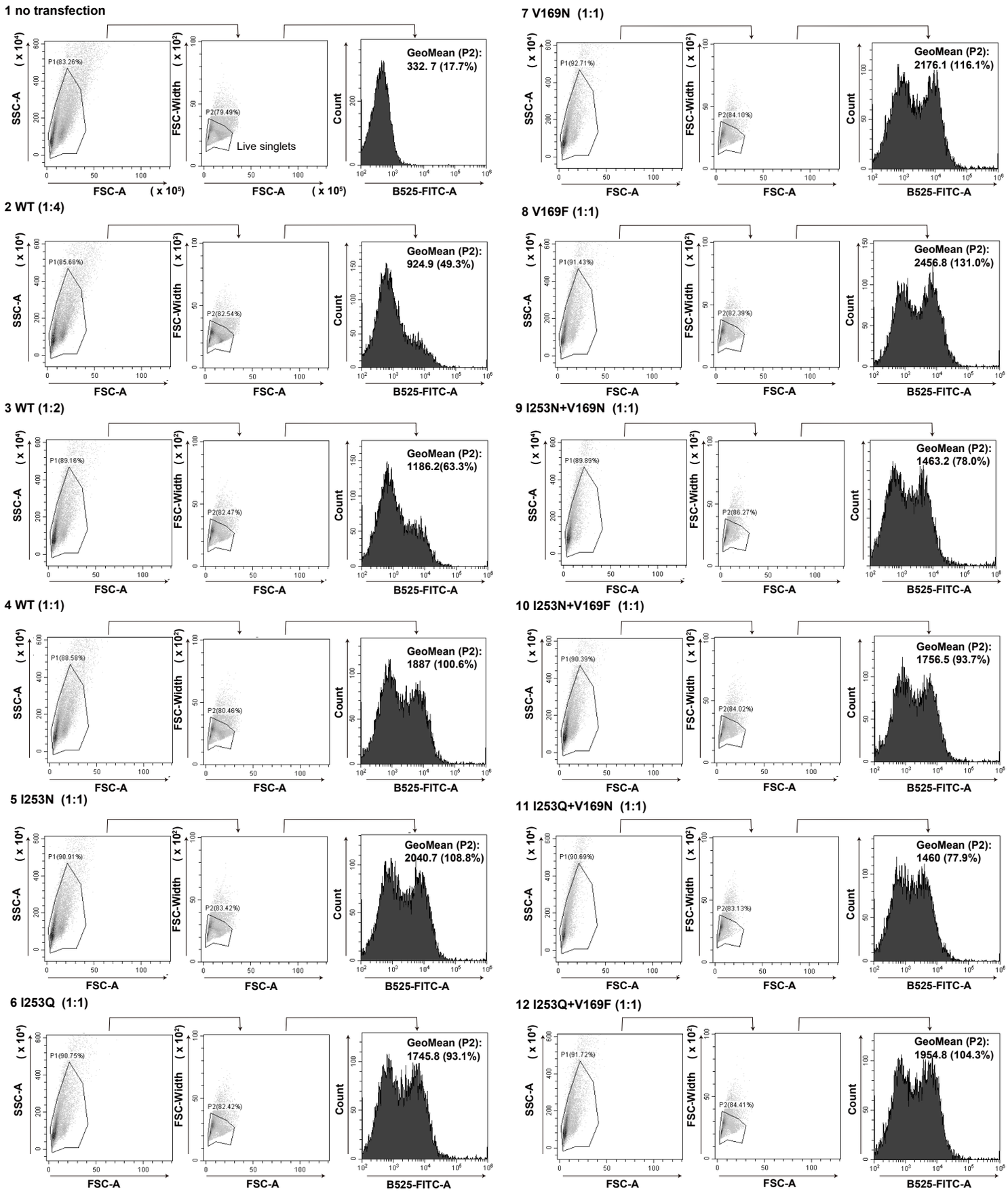
Source data are provided as a Source Data file.



Supplementary Figure 6 | A_3R-selective drugs

(a) Structure of other A_3R-selective compounds. Each drug has an hydrophobic modification at the N^6 position.

(b-d) Comparison of the binding modes of A_3R-selective drugs. (b) Namodenoson-bound A_3R (this work). (c) Piclidenoson-bound A_3R (PDB 8X16). (d) Namodenoson-bound A_3R (PDB 8X17).



Supplementary Figure 7 | Gating strategies for flow cytometory analyses.

Gating strategy for fluorescent intensity used in Supplemental Figure 5e. Representative images for each condition are shown.

Supplementary Table 1 | Comprehensive screening of modified nucleosides against human A3R

Relative GPCR activation of each nucleoside (TPA = 1)	t6A	ms2t6A	ms2i6A	m6Am	Am	m6A	m6,6A	i6A	m2,8A	N6-sucA	ms2A
hA1R +Gq/o Dabsorbance (nucleoside = 100nM)	0.00	-0.02	0.03	0.06	0.03	0.06	0.02	0.06	-0.05	-0.06	-0.06
hA2AR +Gq/s Dabsorbance (nucleoside = 1μM)	-0.01	0.01	0.00	-0.02	-0.01	0.05	0.10	0.05	-0.01	0.04	0.02
hA2BR +Gq/s Dabsorbance (nucleoside = 5μM)	0.01	0.02	0.00	0.01	0.02	0.13	0.07	0.14	0.01	0.01	-0.01
hA3R +Gq/i3 Dabsorbance (nucleoside = 100nM)	-0.04	-0.05	-0.04	0.04	-0.01	0.92	-0.02	0.19	-0.03	-0.06	-0.01

Relative GPCR activation of each nucleoside (TPA = 1)	m2A	Im	m1I	m2,2G	m2G	m1G	Gm	m7G	8OHG	ac4C	Cm
hA1R +Gq/o Dabsorbance (nucleoside = 100nM)	0.17	-0.03	0.03	-0.08	-0.01	0.00	-0.03	-0.09	-0.11	0.02	-0.04
hA2AR +Gq/s Dabsorbance (nucleoside = 1μM)	0.15	0.03	-0.01	0.00	0.03	0.01	0.03	0.01	-0.01	0.01	0.01
hA2BR +Gq/s Dabsorbance (nucleoside = 5μM)	0.24	0.02	-0.01	0.01	-0.01	-0.01	-0.01	0.01	0.00	0.03	0.01
hA3R +Gq/i3 Dabsorbance (nucleoside = 100nM)	0.34	-0.05	-0.03	-0.01	-0.01	-0.01	0.03	-0.01	-0.02	-0.03	-0.04

Relative GPCR activation of each nucleoside (TPA = 1)	m5C	f5C	m3C	m5Cm	hm5C	s2C	m4Cm	Y	acp3U	D	Um
hA1R +Gq/o Dabsorbance (nucleoside = 100nM)	-0.06	-0.05	-0.04	-0.04	-0.02	-0.01	-0.07	-0.04	-0.07	-0.08	-0.09
hA2AR +Gq/s Dabsorbance (nucleoside = 1μM)	0.02	0.02	0.02	0.00	0.02	0.05	0.05	0.03	0.01	-0.03	-0.01
hA2BR +Gq/s Dabsorbance (nucleoside = 5μM)	0.00	-0.01	0.01	0.00	0.02	-0.01	-0.02	0.00	0.00	0.01	0.01
hA3R +Gq/i3 Dabsorbance (nucleoside = 100nM)	-0.02	-0.01	-0.04	-0.01	-0.01	-0.06	-0.05	0.00	0.01	-0.03	-0.07

Relative GPCR activation of each nucleoside (TPA = 1)	m3U	m5U	tm5U	s2U	tm5s2U	m5Um	m1Y	s4U	ho5U
hA1R +Gq/o Dabsorbance (nucleoside = 100nM)	-0.06	-0.05	-0.06	-0.08	-0.06	0.00	0.00	-0.03	-0.01
hA2AR +Gq/s Dabsorbance (nucleoside = 1μM)	0.00	0.00	0.07	0.01	-0.02	0.01	0.03	0.01	0.00
hA2BR +Gq/s Dabsorbance (nucleoside = 5μM)	-0.01	0.01	-0.01	0.01	0.02	0.01	-0.01	0.01	0.01
hA3R +Gq/i3 Dabsorbance (nucleoside = 100nM)	-0.05	-0.04	-0.08	-0.08	-0.05	0.00	-0.02	-0.05	-0.04

A3R activation (%AP-TGFα release)

compound concentration Log10 (M)	Adenosine						m6A						m6,6A			i6A			m6A (MOCK)			m6,6A(MOCK)			i6A(MOCK)			t6A(MOCK)		
-13	2.17	-0.3	0.18	-1.6	-1.5	-2.8	-2.8	1.01	-1.2	0.46	-0.5	0.59	0.91	1.91	1.2	0.6	-1	-2.7	0.64	-0.53	-0.12	0.59	0.2	-0.79	0.19	0.25	-0.44	0	0	0
-10				3.13	-1.1	1.2				3.54	-0.5	2.78																		
-9	1.43	-5.9	-3.9	-1.6	-0.4	0.7	4.75	-2.6	3.45	5.7	-0.5	5.84	-2	3.13	2.24	-3	1.17	-1.7	-0.67	-0.18	0.23	-1.69	0.7	-0.81	0.58	-0.27	-0.92	0	0	0
-8	1.69	2.74	-0.7	2.05	5.3	3.7	18.5	18.3	24.8	25.2	22.7	25.5	-0.4	-1.5	-1.7	-2.2	-1.2	2.27	-0.25	0.27	0.5	-0.05	-0.7	-0.11	0.32	-0.16	0.06	0	0	0
-7	21.8	22	14.7	20.1	16.1	23.6	36.9	40.2	36.3	44.3	41.3	44	4.55	0.79	3.5	15.4	11.9	11	0.22	0.5	0.06	0.2	1.46	0.87	0.37	0.9	-0.56	0	0	0
-6	32.1	32.3	32.7	41.2	38.5	42.7	42	38.9	48.1	50.4	44.7	46	21.2	15.6	23.6	36.4	36.6	30.6	1.53	2.03	0.79	5.52	4.3	5.62	1.76	2.29	0.06	2.635	4.185	2.159
-5	35.5	34.6	35.9	44.6	44.9	43.8	43.3	42.2	47.2	53.9	46.4	51.2	33.4	34.8	35.2	45	46.6	42.6	2.26	2.41	2.75	12.4	11.7	9.29	6.97	6.16	6.3	0.191	0.92	-0.79
-4	38.7	33.8	36.4				46.6	40.4	45.5				44.4	45.6	47.4	49	44.4	45.4	4.09	3.76	3.91	20.3	20.2	21.3	13	12.3	10.8	0	0	0

	hA_1R				$hA_{2A}R$				$hA_{2B}R$				hA_3R			
Ligand	EC_{50} (nM)	E_{max} (%)	RAi(/A)	n	EC_{50} (μM)	E_{max} (%)	RAi(/A)	n	EC_{50} (μM)	E_{max} (%)	RAi(/A)	n	EC_{50} (nM)	E_{max} (%)	RAi(/A)	n
Adenosine	98 ± 14	15 ± 1.1	1	3	1.0 ± 0.14	19 ± 2.5	1	3	3.4 ± 0.28	60 ± 1.7	1	3	120 ± 13	38 ± 3.6	1	3
m^6A	1100 ± 440	23 ± 2.2	0.16 ± 0.042	3	68 ± 26	37 ± 5.3	0.036 ± 0.0074	3	39 ± 6.0	76 ± 4.9	0.11 ± 0.017	3	12 ± 1.8	46 ± 0.66	13 ± 3.6	3
$m^{6,6}A$	1800 ± 540	21 ± 2.0	0.10 ± 0.041	3	3.2 ± 0.46	30 ± 1.0	0.55 ± 0.13	3	22 ± 11	57 ± 15	0.20 ± 0.073	3	2256 ± 500	48 ± 5.1	0.069 ± 0.0057	3
i^6A	900 ± 310	27 ± 2.8	0.22 ± 0.069	3	6.4 ± 1.3	34 ± 3.8	0.30 ± 0.038	3	84 ± 58	99 ± 25	0.12 ± 0.045	3	280 ± 57	50 ± 1.5	0.59 ± 0.046	3
t^6A	N/A	N/A	N/A	3	N/A	N/A	N/A	3	N/A	N/A	N/A	3	N/A	N/A	N/A	3

Supplementary Table 2 | Analyses of human and sheep A_3Rs using the TGFα shedding assay

	A				m6A			
	pEC50		Emax		pEC50		Emax	
	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n
Human A3R	7.0 ± 0.079	110	41 ± 2.2	6	8.0 ± 0.023	11	47 ± 1.5	6
Sheep A3R	6.9 ± 0.043	130	43 ± 1.0	6	6.9 ± 0.043	20	49 ± 0.71	6

	m6,6A				i6A			
	pEC50		Emax		pEC50		Emax	
	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n
Human A3R	5.7 ± 0.085	1900	47 ± 0.53	3	6.6 ± 0.062	290	48 ± 1.1	3
Sheep A3R	5.6± 0.090	2600	56 ± 3.0	3	6.2 ± 0.061	640	52 ± 2.1	3

	NECA				Namodenoson			
	pEC50		Emax		pEC50		Emax	
	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n
Human A3R	8.0 ± 0.040	11	44 ± 0.62	3	8.6 ± 0.057	2.5	44 ± 0.60	3
Sheep A3R	7.8 ± 0.015	16	45 ± 0.63	3	8.5 ± 0.064	3.4	45 ± 0.58	3

Supplementary Table 3 | Mutational analyses using the TGFα shedding assay

	A				m6A				m6,6A				i6A			
	pEC50		Emax		pEC50		Emax		pEC50		Emax		pEC50		Emax	
	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n
A3R-WT	6.9 ± 0.039	130	43 ± 1.0	12	7.9 ± 0.034	14	49 ± 1.0	12	5.4 ± 0.022	3900	57 ± 0.65	12	6.3 ± 0.025	480	52 ± 0.60	16
V169A	7.0 ± 0.032	95	40 ± 0.82	6	7.7 ± 0.026	18	49 ± 0.95	5	5.6 ± 0.018	2400	55 ± 4.2	3	7.0 ± 0.071	98	52 ± 3.0	3
M174A	5.8 ± 0.052	1600	59 ± 1.6	3	6.6 ± 0.023	280	64 ± 1.7	3	NA	NA	NA	3	7.4 ± 0.017	36	70 ± 4.5	3
M174L	6.6 ± 0.061	260	53 ± 1.6	3	7.8 ± 0.11	16	59 ± 1.1	3	5.3 ± 0.018	5400	62 ± 1.8	3	6.2 ± 0.018	660	60 ± 3.1	3
I253A	6.5 ± 0.050	320	51 ± 1.2	3	7.0 ± 0.064	110	58 ± 1.3	3	4.8 ± 0.021	17000	70 ± 0.61	3	7.4 ± 0.063	41	64 ± 1.2	3
L264A	6.7 ± 0.11	220	50 ± 0.71	4	7.9 ± 0.078	12	57 ± 1.4	3	5.4 ± 0.040	4000	65 ± 2.3	3	7.2 ± 0.044	69	63 ± 1.2	3
V169L	7.0 ± 0.023	97	41 ± 0.66	9	7.7 ± 0.037	18	49 ± 1.6	6	5.6 ± 0.017	2300	52 ± 2.4	3	5.9 ± 0.027	1200	54 ± 3.9	3
V169F	6.7 ± 0.053	210	53 ± 1.7	3	7.0 ± 0.045	100	59 ± 2.6	3	4.5 ± 0.16	35000	80 ± 5.9	3	5.9 ± 0.044	1300	60 ± 1.8	5
M174F	6.5 ± 0.057	360	56 ± 2.4	3	7.1 ± 0.046	74	60 ± 0.66	3	4.5 ± 0.0092	35000	82 ± 1.7	3	5.4 ± 0.029	4200	64 ± 1.5	3
I253F	6.5 ± 0.16	330	51 ± 0.95	3	7.4 ± 0.18	51	56 ± 2.2	3	4.9 ± 0.11	13000	62 ± 0.57	3	7.1 ± 0.18	85	62 ± 1.6	3
I253M	6.4 ± 0.038	400	57 ± 1.5	3	7.3 ± 0.037	47	62 ± 1.6	4	4.8 ± 0.047	16000	72 ± 0.12	3	6.4 ± 0.024	430	66 ± 1.9	3
L264F	5.1 ± 0.064	8600	56 ± 1.6	3	6.0 ± 0.019	1100	64 ± 1.9	3	NA	NA	NA	3	5.5 ± 0.024	3400	70 ± 1.0	3
L264M	6.8 ± 0.042	180	43 ± 0.75	4	7.3 ± 0.060	48	51 ± 0.76	3	4.9 ± 0.034	11000	63 ± 2.2	3	6.0 ± 0.0070	1100	58 ± 1.2	3
V169T	7.0 ± 0.030	94	40 ± 1.1	5	8.0 ± 0.046	11	43 ± 1.1	4	5.6 ± 0.023	2400	52 ± 0.93	3	6.3 ± 0.047	470	50 ± 1.8	3
M174Y	5.4 ± 0.17	4400	65 ± 1.8	3	5.7 ± 0.015	1800	64 ± 2.5	3	5.1 ± 0.050	8500	41 ± 10	4	5.2 ± 0.039	6200	67 ± 3.0	3
I253N	6.8 ± 0.041	170	51 ± 1.2	5	7.2 ± 0.042	69	55 ± 2.4	4	4.8 ± 0.11	15000	70 ± 1.6	3	7.3 ± 0.040	45	60 ± 1.5	3
L264N	6.1 ± 0.081	790	58 ± 0.33	3	7.1 ± 0.051	81	62 ± 2.1	3	4.2 ± 0.26	98000	100 ± 14	3	5.8 ± 0.0043	1600	66 ± 0.91	3
V169N	6.7 ± 0.041	190	54 ± 0.61	4	7.2 ± 0.17	75	55 ± 1.1	4	5.2 ± 0.014	5600	64 ± 0.33	3	6.2 ± 0.28	1400	60 ± 1.1	4
I253Q	6.3 ± 0.058	500	57 ± 1.3	3	7.0 ± 0.085	99	62 ± 1.4	3	4.4 ± 0.11	46000	89 ± 8.7	3	7.1 ± 0.015	80	68 ± 2.3	3
L264Q	6.7 ± 0.061	230	52 ± 0.53	3	8.0 ± 0.044	9.5	57 ± 2.1	3	5.5 ± 0.069	3500	67 ± 3.1	3	6.6± 0.051	267	63 ± 1.4	4
I253N+V169N	6.9 ± 0.033	130	47 ± 0.54	3	6.8 ± 0.032	170	53 ± 0.57	3	4.8 ± 0.24	19000	58 ± 6.3	3	6.9 ± 0.075	140	59 ± 1.0	3
I253N+V169F	6.3 ± 0.011	470	51 ± 0.86	3	6.2 ± 0.018	680	55 ± 1.9	3	4.6 ± 0.29	36000	44 ± 9.2	3	6.1 ± 0.015	830	62 ± 1.3	3
I253Q+V169N	5.5 ± 0.054	3200	13 ± 0.48	3	5.4 ± 0.034	4400	17 ± 1.9	3	4.6 ± 0.13	26000	29 ± 3.1	3	5.1 ± 0.061	8400	31 ± 1.6	3
I253Q+V169F	6.1 ± 0.031	720	45 ± 0.46	3	6.0 ± 0.038	1100	53 ± 1.9	3	4.7 ± 0.082	21000	33 ± 0.34	3	5.7 ± 0.032	1800	61 ± 0.26	3

	A				m6A				m6,6A				i6A			
	pEC50		Emax		pEC50		Emax		pEC50		Emax		pEC50		Emax	
	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n	Mean ± SEM	EC50 (nM)	Mean ± SEM	n
Human A3R V169E	5.2 ± 0.16	6900 ± 2800	12 ± 1.7	3	5.6 ± 0.021	2500 ± 120	21 ± 1.7	3	5.7 ± 0.077	2000 ± 340	32 ± 5.7	3	6.5 ± 0.16	360 ± 140	33 ± 4.0	3
Human A3R V169R	6.9 ± 0.40	260 ± 210	22 ± 0.29	3	8.2 ± 0.033	5.9 ± 0.11	25 ± 1.7	3	5.9 ± 0.11	1500 ± 360	35 ± 2.3	3	6.4 ± 0.011	390 ± 10	34 ± 1.9	3
Human A3R I253L	6.7 ± 0.16	260 ± 100	50 ± 0.34	3	7.5 ± 0.032	35 ± 2.5	53 ± 0.83	3	5.1 ± 0.062	7300 ± 990	64 ± 2.4	3	6.1 ± 0.012	740 ± 21	59 ± 1.9	3
sheep A3R R168V	6.1 ± 0.15	920 ± 340	52 ± 2.0	3	7.2 ± 0.043	66 ± 6.8	57 ± 2.1	3	4.2 ± 0.15	71000 ± 26(	92 ± 6.0	3	5.7 ± 0.23	2900 ± 160(	60 ± 2.1	3
sheep A3R R168E	6.1 ± 0.13	920 ± 310	54 ± 2.1	3	7.2 ± 0.055	61 ± 8.2	59 ± 1.7	3	4.2 ± 0.39	150000 ± 1(	104 ± 25	3	6.4 ± 0.25	530 ± 320	62 ± 1.6	3

Supplementary Table 4 | Distance between the ligand and the residues

Distances (Å)	A3R-A	A3R-m6A	A3R-i6A	A2AR-A	A2BR-A	A1R-A
45.53	4.13	3.93	4.70	3.06	N/A	3.66
5.35	4.59	3.64	3.44	5.13	4.34	4.36
6.58	4.05	3.33	4.41	6.42	6.09	6.58
7.52	3.57	3.67	3.39	3.54	5.06	4.63
average	4.09	3.64	3.99	4.54	5.16	4.81

Supplementary Table 5 | Details of each tested nucleoside

compounds	abbreviation	source	identifier
N^6-Threonylcarbamoyladenosine	t6A	Wako	custom-synthesized
2-Methylthio-N^6-threonylcarbamoyladenosine	ms2t6A	Wako	custom-synthesized; CAS: 70333-82-3
2-Methylthio-N^6-isopentenyladenosine	ms2i6A	Wako	custom-synthesized; CAS: 20859-00-1
N^6, 2'-*O*-Dimethyladenosine	m6Am	Toronto Research Chemicals	Cat#D447415; CAS:57817-83-1
2'-*O*-Methyladenosine	Am	Tokyo Chemical Industry	Cat#M2291; CAS:2140-79-6
2'-*O*-Methylinosine	Im	Santa Cruz Biotechnology	Cat#sc-283498; CAS:3881-21-8
1-Methylinosine	m1I	Santa Cruz Biotechnology	Cat#sc-483758; CAS:2140-73-0
N^6-Methyladenosine	m6A	Abcam	Cat#ab145715; CAS:1867-73-8
N^6,N^6-Dimethyladenosine	m6,6A	MedChem Express	Cat#HY-101984; CAS:2620-62-4
N^6-Isopentenyladenosine	i6A	Wako	custom-synthesized
N^4-Acetylcytidine	ac4C	Combi-Blocks	Cat#QB-9019; CAS:3768-18-1
2'-*O*-Methylcytidine	Cm	Tokyo Chemical Industry	Cat#M2317; CAS:2140-72-9
5-Methylcytidine	m5C	Tokyo Chemical Industry	Cat#M1931; CAS:2140-61-6
5-Formylcytidine	f5C	Toronto Research Chemicals	Cat#F698965; CAS:148608-53-1
3-Methylcytidine	m3C	MedChem Express	Cat#HY-111645; CAS: 2140-64-9
5,2'-O-Dimethylcytidine	m5Cm	Glanlen	Cat#GL100726; CAS: 113886-70-7
5-Hydroxymethylcytidine	hm5C	Tokyo Chemical Industry	Cat#H1863; CAS: 19235-17-7
N^2,N^2-Dimethylguanosine	m2,2G	MedChem Express	Cat#HY-113137; CAS:2140-67-2
N^2-Methylguanosine	m2G	Biosynth Carbosynth	Cat#NM35522; CAS:2140-77-4
1-Methylguanosine	m1G	Santa Cruz Biotechnology	Cat#sc-500889; CAS:2140-65-0
2'-*O*-Methylguanosine	Gm	Tokyo Chemical Industry	Cat#M2318; CAS:2140-71-8
7-Methylguanosine	m7G	Sigma-Aldrich	Cat#M0627; CAS:20244-86-4
Pseudouridine	Ψ	Tokyo Chemical Industry	Cat#P2396; CAS:1445-07-4
3-(3-Amino-3-carboxypropyl) uridine	acp3U	Alsachim	custom-synthesized
5,6-Dihydrouridine	D	Toronto Research Chemicals	Cat#D449668; CAS:5627-05-4
2'-*O*-Methyluridine	Um	Tokyo Chemical Industry	Cat#M2290; CAS:2140-76-3
3-Methyluridine	m3U	Sigma-Aldrich	Cat#M4129; CAS:2140-69-4
5-Methyluridine	m5U	Tokyo Chemical Industry	Cat#M1405; CAS:1463-10-1
5-Taurinomethyluridine	τm5U	Wako	custom-synthesized; CAS: 497258-53-4
2-Thiouridine	s2U	Tokyo Chemical Industry	Cat#T4108; CAS: 20235-78-3
5-Taurinomethyl-2-thiouridine	τm5s2U	Wako	custom-synthesized; CAS: 497258-54-5
5,2'-O-Dimethyluridine	m5Um	Combi-Blocks	Cat#QC-7341; CAS: 55486-09-4
N1-Methylpseudouridine	m1Ψ	MedChem Express	Cat#HY-112582 ; CAS: 13860-38-3
4-Thiouridine	s4U	Tokyo Chemical Industry	Cat#T4059; CAS:13957-31-8
5-Hydroxyuridine	ho5U	Tokyo Chemical Industry	Cat#H1865; CAS: 957-77-7
2-Methyladenosine	m2A	Santa Cruz Biotechnology	Cat#sc-500888; CAS: 16526-56-0
8-Hydroxyguanosine	8OHG	Cayman Chemical	Cat#89300; CAS: 3868-31-3
2,8-Dimethyladenosine	m2,8A	MedChem Express	Cat#HY-152702; CAS: 63954-66-5
N6-Succinyl adenosine	N6-sucA	Biosynth	Cat#NS16562; CAS: 4542-23-8
2-Methylathioadenosine	ms2A	Carbosynth	Cat#NM09610; CAS: 4105-39-9
2-Thiocytidine	s2C	Santa Cruz Biotechnology	Cat#sc-283290; CAS: 13239-97-9
N4,2'-O-dimethylcytidine	m4Cm	Biosynth	Cat#NM144592; CAS: 13048-95-8