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Supplemental Information

Development of “Plug and Play” Fiducial Marks for Structural Studies of GPCR Signaling Complexes by Single-Particle Cryo-EM

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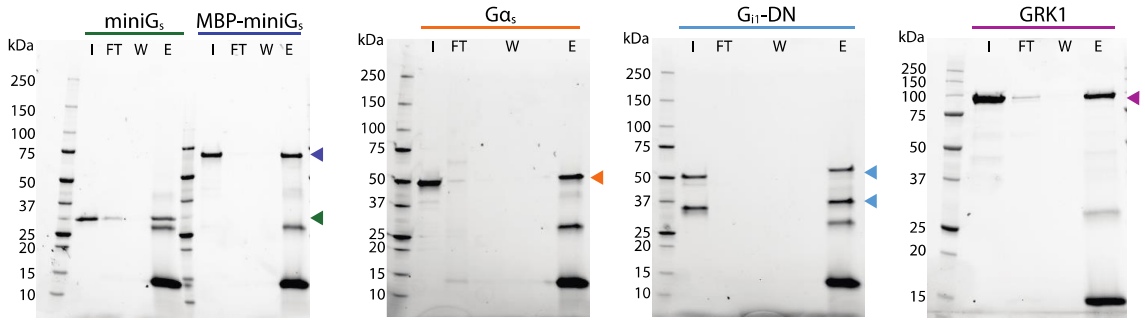
a



b

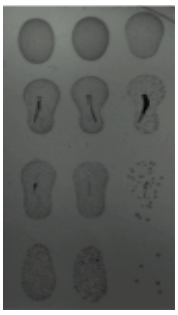
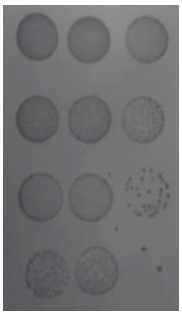
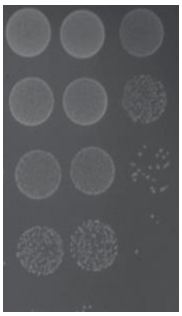
Target		mini-G_s-bt			bt-MBP-mini-G_s			bt-G_{i1}-DN			bt-$G\alpha_s$			bt-MBP-GRK1 (K46L)		
Competition		-			MBP			-			-			MBP		
Concentration (nM)		10	5	0	10	5	0	10	5	0	10	5	0	10	5	0
Dilution of phage particle	10^{-2}															
	10^{-3}															
	10^{-4}															
	10^{-5}															

Figure S1. Selection of the G-protein-specific sABs, related to Figure 1. (**a**) Labeling efficiency for mini-G_s variants, $G\alpha_s$, G_{i1}-DN and GRK1 by a pull-down assay on SA magnetic beads. **I**- input, **FT**- flow-through, **W**- wash steps, **E**- elution. Arrows indicate the target proteins. Additional two bands visible in the elution fraction at around 15 kDa and 30 kDa correlate to the SA monomer and dimer, respectively. (**b**) Enrichment of the target-specific binders after four rounds of selection.

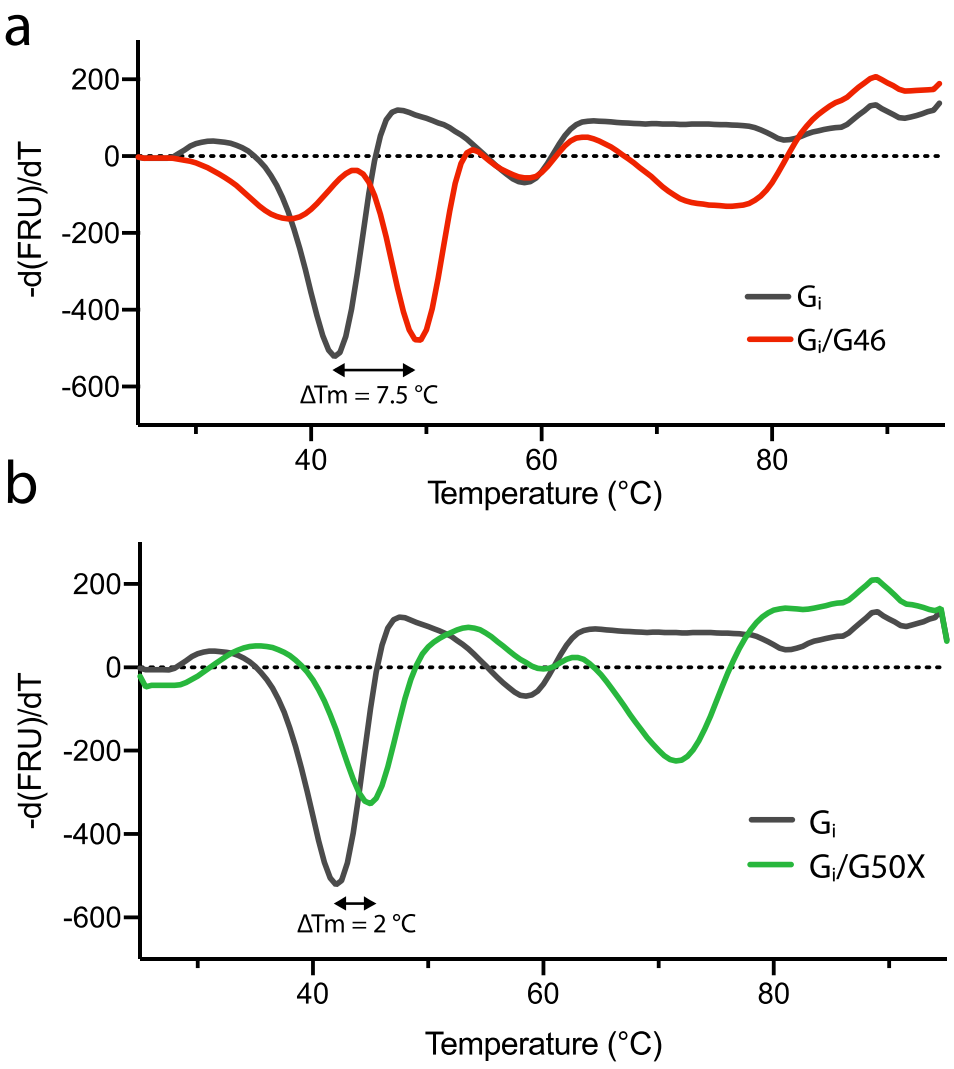


Figure S2. Thermal stabilization of the G_i by the sABs, related to Figure 2 and Figure 5. DSF melting curves of the Gi shows stabilization by selected sABs: (**a**) G46 and (**b**) G50X.

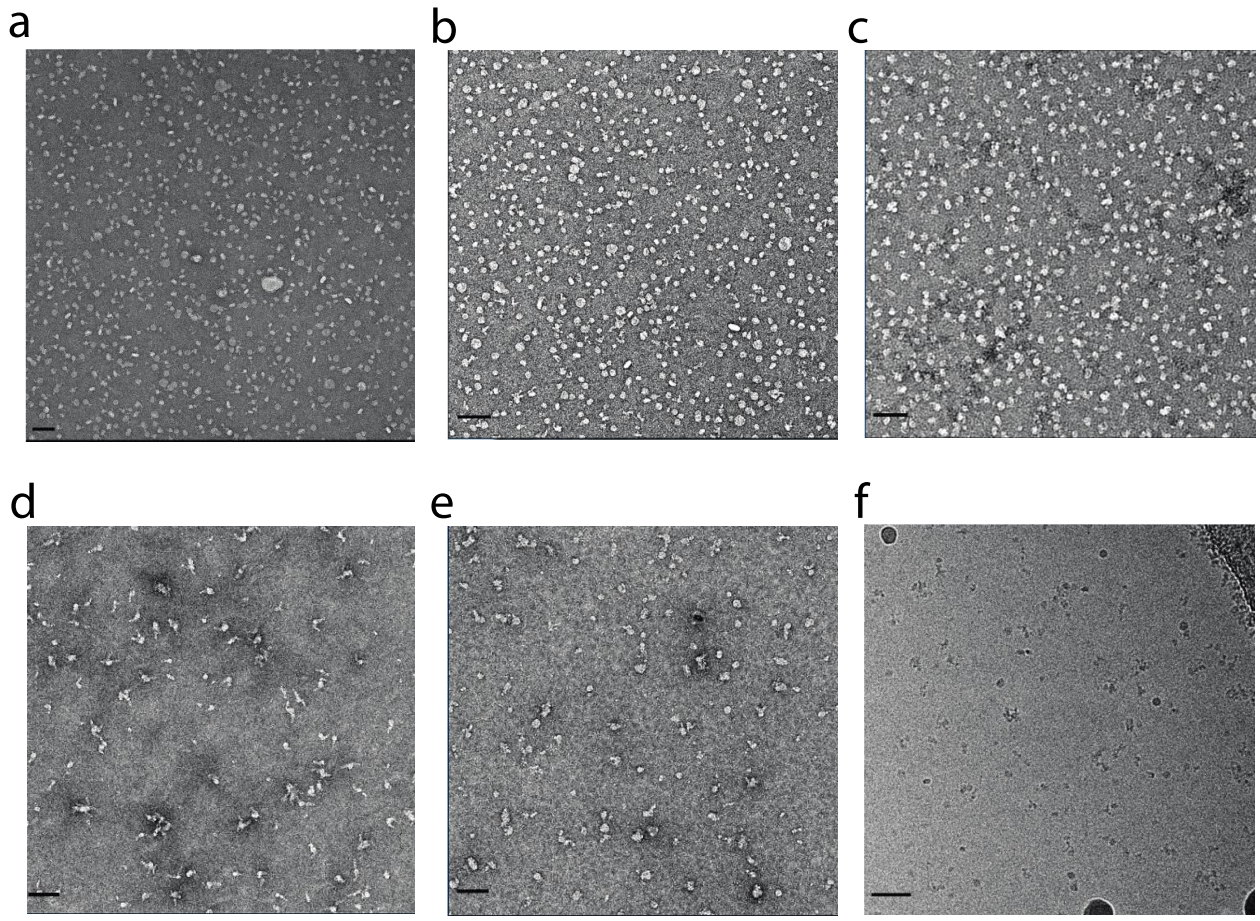
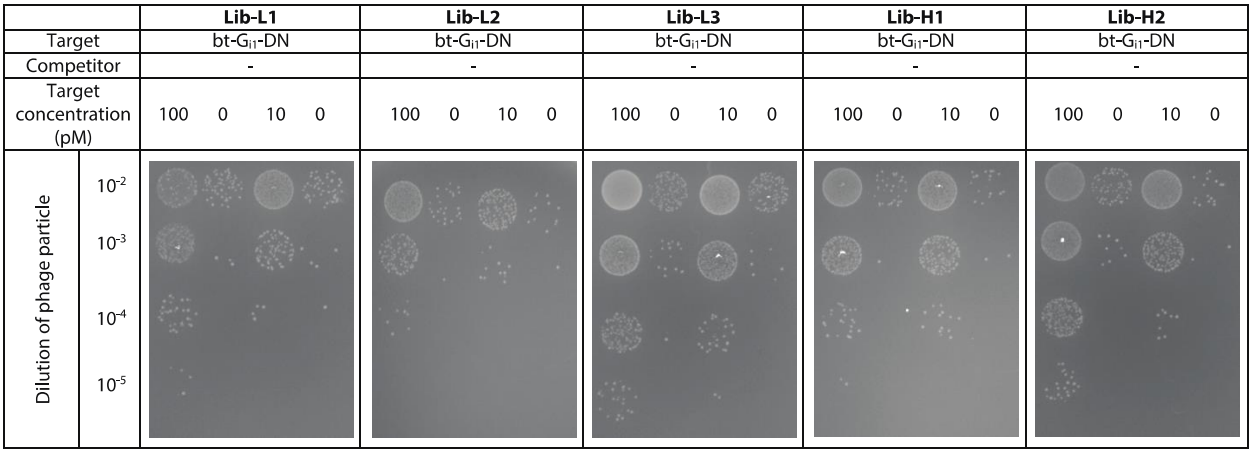


Figure S3. Representative raw EM images of the GPCR/effector protein/sAB complexes, related to Figure 3 and Figure 6. Negative stain images: (**a**) Rho/G_i/G46, (**b**) Rho/G_i/G50, (**c**) D1R/G_s/Gs6 (**d**) Rho/GRK/1F1, (**e**) Rho/GRK/1F1/GR6 and (**f**) cryo-EM image of Rho/GRK/1F1/GR6. Scale bar, 50 nm.

a



b

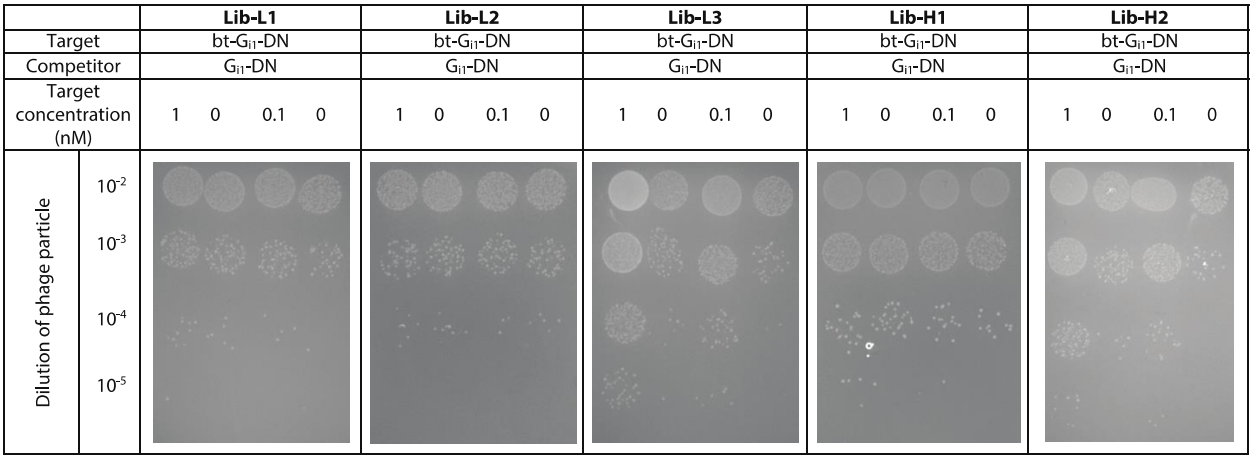


Figure S4. Phage pool enrichment after three rounds of the affinity maturation process, related to Figure 5. Affinity maturation process was performed using two different protocols: (**a**) regular selection and (**b**) off-rate selection protocol.

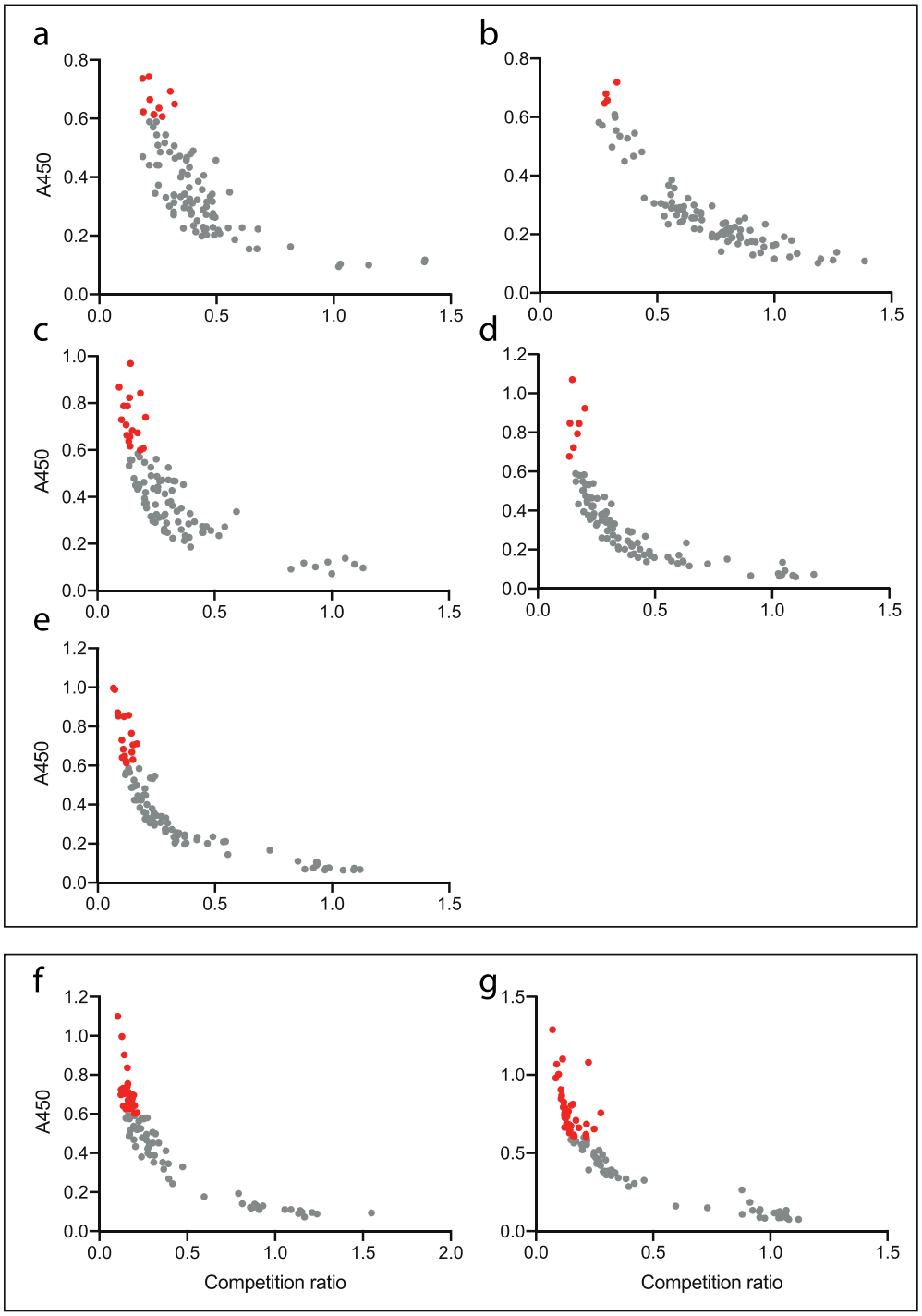


Figure S5. Initial screening of affinity matured variants of the sAB G50, related to Figure 5. Binders obtained after three rounds of affinity maturation were screened by a single-point competition phage ELISA. Clones marked in red were used for subsequent experiments. Results from regular selection protocol: (**a**) Lib-L1, (**b**) Lib-L2, (**c**) Lib-L3, (**d**) Lib-H1, (**e**) Lib-H2 and off-rate protocol: (**f**) Lib-L3 and (**g**) Lib-H2.

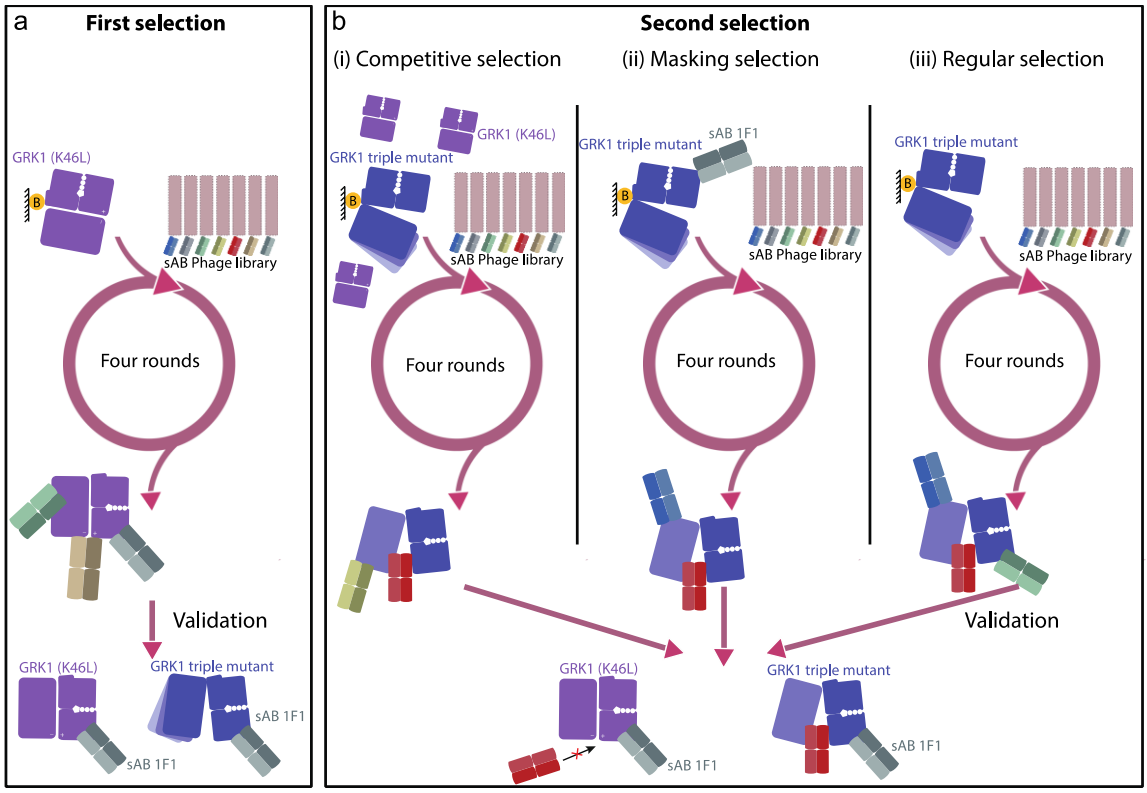
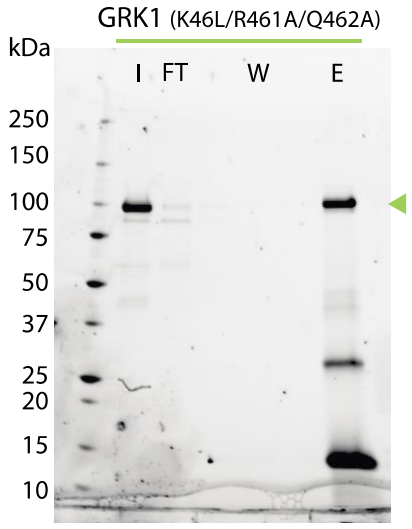


Figure S6. Selection strategy to obtain conformationally selective sABs against GRK1 triple mutant, related to STAR methods and Figure 6. (**a**) In the first selection sABs were generated against GRK1 (K46L) mutant and analyzed for binding to both variants K46L and ionic lock mutant. (**b**) Second selection aimed to develop conformationally specific antibodies against GRK1 triple mutant. Here three independent strategies were employed - (i) Competitive selection: selection pressure was employed using GRK1 (K46L) as a soluble competitor. (ii) Masking selection: conformationally insensitive sAB 1F1 was used to occlude the epitope. (iii) Regular selection: sABs were generated against GRK1 triple mutant without additional selection pressure.

a



b

Target		bt-MBP-GRK1 (K46L/R461A/Q462A)								
Competition		MBP			MBP			MBP-GRK1 (K46L)		
Masking		-			sAB 1F1			-		
Target concentration (nM)		10	5	0	10	5	0	10	5	0
Dilution of phage particle	10^{-2}									
	10^{-3}									
	10^{-4}									
	10^{-5}									

Figure S7. Selection of the conformationally specific sABs against GRK1 ionic lock mutant, related to Figure 6. (**a**) Labeling efficiency for GRK1 ionic lock mutant by a pull-down assay on SA magnetic beads. **I**- input, **FT**- flow-through, **W**- wash steps, **E**- elution. Arrow indicates the target proteins. Additional two bands visible in the elution fraction at around 15 kDa and 30 kDa correlate to the SA monomer and dimer, respectively. (**b**) Enrichment of the target-specific binders after four rounds of selection.

Table S1, related to Figure 1 and Figure 6. Sequence of CDRs of the best characterized G-proteins and GRK1 binders.

Selection	sAB	Loop sequence			
		L3	H1	H2	H3
Trimeric G_i	G6	SYYVYP	VSSSSI	SIYPYYSSTS	GYFYWLRYTKSSYWGL
	G39	SSGWLV	FSSSYI	SISSYSGSTS	EISMMSTQYTYGI
	G46	SYYSPI	FSSSSI	YIYSSSGYTY	KWYYRVGSWPAM
	G50	SSSSLI	FYYSSI	SIYSYSGSTS	YPWYWWMEKPYLSLYGM
	G51	WSGTLI	VSSSYI	YISPYSGYTY	EQGGWSSYYSAI
$G\alpha_s$	GS1	MFSSKLL	IYSSSI	SIYSSYGYTS	WKQYGFYHAYHGL
	GS3	LWYRPF	LSSSSI	SISSSYGYTS	WWAGQWYGSYGI
	GS5	GYRSALV	FSSSSI	SIYPYYGYTS	WYYNFSSRYGYAGSYGM
	GS6	SSSSLI	FSSSSI	SIYPYYGYTY	WAGYYSYYMRAL
	GS13	SIYYLPI	VSYYSI	SIYSYSGYTY	TTSFGPWWEYGF
Mini-G_s	M1	ESSSRLF	FYYYSI	SISSSSGSTS	SPGPWYGPWYYFEYAM
	M4	SSSSLI	FSSSSI	SISSSSGSTS	YWGPYVYWSSYTSKSGGM
	M7	YWSSLV	FYYSSI	SIYPYSGSTY	PVYGVYSLWFGSYYSWAM
	M19	SSSSLI	FSSSSI	SISSSSGSTS	GWSEKYSIQWWGHEAI
	M20	SSSSLI	VYYSSI	SISSSYGSTY	YWSPSYWWGDSVGSYRGF
GRK1 (K46L)	1A1	SYVSSLI	VSSYSI	SIYPSSGYTS	QSYGVYYAYPWPSFHYAM
	1E4	YMYSLPI	VYYYYI	SIYPSSGSTY	DWYSAYSYYVGF
	1E12	SSSSLI	VYSSSI	SIYSSSGSTY	VWLFYIGSKVYSPFHLGF
	1F1	MYYSSLI	LSYSSI	SIYPSSGYTY	EFYSYGYYYTAF
	1H4	YWPYYPV	FSYSSI	YISPSYGSTS	LGWSYSSSFPVGWYYGM
GRK1 (K46L/R461A/ Q462A)	GR1	LQSYKLI	FSSSYI	SISPYYGSTY	HYSYPYYGAGWYNYYYGL
	GR2	QYYSLI	FSSSSI	SISSSSGSTS	QGSYKWGYIYYVSNKGL
	GR6	YWWSYPI	FYSSSI	SISSSSGSTS	WGMYYHYYSFRGF
	GR14	YYSHVLI	IYYSSI	YISPSYGYTS	RQWVGYWYWGM
	GR49	SIGYLI	VYSSSI	SIYSSYGSTY	YRSVGGWFWYSHYIGL

Table S2, related to Figure 4. Kinetic parameters for alanine scanning mutants of the sAB G50 measured by SPR.

CDR	Mutation	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)	$\Delta\Delta G_{mut-wt}$ (kcal mol^{-1})
	WT	1.2×10^{6}	1.4×10^{-3}	1.2	-
L1	S29A	5.7×10^{5}	4.4×10^{-3}	7.7	1.2
	V30A	3.4×10^{5}	1.2×10^{-2}	36.6	2.1
	S31A	4.7×10^{5}	2.3×10^{-3}	5.0	0.9
	S32A	6.5×10^{5}	2.6×10^{-3}	4.0	0.8
L2	Y50A	3.0×10^{5}	5.1×10^{-3}	17.2	1.6
	S51A	2.4×10^{5}	5.0×10^{-3}	21.4	1.8
	S53A	5.6×10^{5}	1.7×10^{-3}	3.0	0.6
	S54A	8.0×10^{5}	2.9×10^{-3}	3.7	0.7
	L55A	7.9×10^{5}	1.7×10^{-3}	2.1	0.4
	Y56A	1.9×10^{6}	2.3×10^{-3}	1.2	0.1
	S57A	6.7×10^{5}	2.5×10^{-3}	3.7	0.7
L3	S92A	6.2×10^{5}	5.1×10^{-3}	8.3	1.2
	S93A	7.8×10^{5}	1.4×10^{-3}	1.8	0.3
	S94A	3.8×10^{5}	7.5×10^{-3}	19.6	1.7
	S95A	4.1×10^{5}	1.8×10^{-3}	4.3	0.8
H1	Y33A	8.6×10^{5}	2.1×10^{-3}	2.4	0.5
	Y34A	3.2×10^{5}	7.8×10^{-3}	24.4	1.8
H2	Y57A	1.1×10^{5}	3.3×10^{-3}	29.2	1.9
	S58A	1.9×10^{6}	5.4×10^{-3}	2.9	0.6
H3	Y102A	4.2×10^{4}	3.3×10^{-3}	78.1	2.5
	P103A	9.6×10^{4}	6.9×10^{-3}	72.2	2.5
	W104A	6.9×10^{4}	2.9×10^{-3}	41.6	2.2
	Y105A	2.9×10^{4}	2.3×10^{-3}	78.8	2.5
	W106A	NB	NB	NB	-
	W107A	NB	NB	NB	-
	M108A	8.9×10^{4}	8.5×10^{-3}	94.6	2.6
	E109A	2.8×10^{6}	8.0×10^{-3}	2.9	0.6
	K110A	4.7×10^{5}	2.3×10^{-3}	4.9	0.9
	P111A	8.1×10^{4}	4.5×10^{-3}	56.0	2.3
	Y112A	9.4×10^{5}	1.4×10^{-2}	15.3	1.6
	L113A	2.8×10^{5}	3.4×10^{-3}	12.0	1.4
	S114A	2.7×10^{5}	2.4×10^{-3}	8.7	1.2
	L115A	3.2×10^{5}	3.0×10^{-3}	9.5	1.3
	Y116A	1.9×10^{4}	1.8×10^{-3}	95.4	2.6
	G117A	5.9×10^{5}	1.0×10^{-2}	17.4	1.6
	M118A	1.5×10^{5}	7.8×10^{-3}	52.0	2.3

All the residues that make contact with the trimeric G_i in PBD: 6CMO according to PISA were mutated to alanine.

Table S3, related to Figure 4. Kinetic parameters for alanine scanning mutants of the Gαi-AHD measured by SPR.

Mutation	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)	$\Delta\Delta G_{mut-wt}$ (kcal mol^{-1})
WT	6.3×10^5	1.5×10^{-3}	2.5	-
I85A	5.0×10^5	4.2×10^{-3}	8.5	0.7
R86A	5.7×10^5	4.6×10^{-3}	8.1	0.7
R90A	8.8×10^5	9.4×10^{-3}	10.6	0.9
D94A	6.0×10^5	6.0×10^{-3}	9.9	0.8
F95A	5.3×10^5	6.2×10^{-3}	11.5	0.9
D97A	4.5×10^5	1.8×10^{-3}	3.9	0.3
R100A	8.6×10^5	5.5×10^{-3}	6.4	0.6
D103A	8.3×10^5	1.3×10^{-3}	1.5	-0.5
Q106A	7.8×10^5	1.2×10^{-2}	15.4	1.1
L107A	6.9×10^5	1.8×10^{-3}	2.5	0.0
F108A	NB	NB	NB	-
L110A	5.3×10^5	3.2×10^{-3}	6.0	0.5
E115A	5.9×10^5	1.8×10^{-3}	3.1	0.1

All the residues that make contact with sAB G50 in PDB: 6CMO according to PISA were mutated to alanine.

Table S4, related to STAR methods and Figure 5. Design of the sub-libraries for affinity maturation

Library	Modified loop	Library design									Length	Theoretical diversity	*Actual diversity
Lib-L1	CDR-L1	S29 NNC	V30 NTT	S31 DVT	S32 NVT						4	6.9×10^3	2.8×10^{13}
Lib-L2	CDR-L2	Y50 TDK	S51 VVC	A52 RST	S53 NNC	S54 NNC	L55 NTT	Y56 NNC			7	3.5×10^6	3.5×10^{13}
Lib-L3	CDR-L3	S92 NNS	S93 NNS	S94 NNS	S95 NNS	L96 NTT	I97 NTT				6	1.6×10^7	1.6×10^{13}
Lib-H1	CDR-H1	F32 NTT	Y33 NNS	Y34 NNS	S35 NNS	S36 NNS					5	2.6×10^5	2.6×10^{13}
Lib-H2	CDR-H2	S53 RST	I54 NTT	Y55 NNS	S56 NNS	Y57 NVT	S58 NNT	G59 RST	S60 VVC	T61 VVC	9	2.5×10^8	1.8×10^{13}

* The actual diversity was determined from the cfu (colony forming unit) titer of the phage libraries.

Table S5, related to Figure 5. Kinetic parameters of the affinity matured variants of the sAB G50 measured by SPR.

sAB	Well ID	L1	L2	L3	H1	H2	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (pM)
wt G50		**SVSSA**	**YSASSLYS**	**SSSSLI**	**FYYSSI**	**SIYSYSGSTS**	**1.2 x 10^6**	**1.4 x 10^{-3}**	**1170**
Lib-L1		**SVSSA**							
G50.21	L1_D1	DVYRA					1.5 x10^6	3.5 x 10^{-4}	234
G50.22	L1_C2	NVYAA					1.7 x 10^6	3.3 x 10^{-4}	193
G50.23	L1_E1	RVYDA					1.5 x 10^6	3.4 x 10^{-4}	221
G50.24	L1_C1	SVYDA					1.0 x 10^6	4.1 x 10^{-4}	395
Lib-L2			**YSASSLYS**						
G50.25	L2_H2		YSAVSLFS				1.7 x 10^6	9.9 x 10^{-4}	589
Lib-L3				**SSSSLI**					
G50.09	L3_E8			SVNKFI			6.5 x 10^5	3.3 x 10^{-5}	50
G50.10	L3_F11			SLSQVI			1.1 x 10^6	1.2 x 10^{-4}	113
G50.11	L3_G6			SLRKLI			1.1 x10^6	5.5 x 10^{-6}	5.1
G50.16	L3_E8			SRSQVV			1.6 x 10^6	8.5 x 10^{-5}	51.8
G50.17	L3_F5			SKSKII			2.0 x 10^6	9.6 x 10^{-6}	47.1
G50.18	L3_F6			SFGRVV			1.4 x 10^6	3.1 x 10^{-4}	213
G50.19	L3_F7			SRGMLF			9.6 z 10^5	6.9 x 10^{-5}	71.9
Lib-H1					**FYYSSI**				
G50.27	H1_C4				FRFSSI		1.1 x 10^6	3.0 x 10^{-4}	266
G50.28	H1_G3				FGLSSI		1.2 x 10^6	8.6 x 10^{-4}	725
G50.29	H1_F3				FRLSSI		6.9 x 10^5	6.0 x 10^{-4}	866
G50.30	H1_H3				FRTSSI		5.7 x 10^5	5.4 x 10^{-4}	934
G50.31	H1_E3				FRYSSI		1.2 x 10^6	3.5 x 10^{-4}	287
Lib-H2						**SIYSYSGSTS**			
G50.01	H2_E9					GFYSYSGRGS	2.5 x 10^6	6.2 x 10^{-6}	2.4
G50.02	H2_F5					GFYAYSASDS	2.0 x 10^6	5.2 x 10^{-5}	25.5
G50.04	H2_G12					GFYSYSGRAS	1.7 x 10^6	5.7 x 10^{-5}	30.8
G50.05	H2_G7					GIYGYSGSRS	2.0 x 10^6	4.2 x 10^{-5}	21.3
G50.06	H2_H3					GFYGYSGAGS	1.9 x 10^6	9.4 x 10^{-6}	5.0
G50.12	H2_B1					GFFGYSGGTS	7.9 x 10^5	9.1 x 10^{-6}	11.5
G50.13	H2_B5					GLFGYSGNDS	1.6 x 10^6	1.3 x 10^{-5}	8.2
G50.15	H2_D10					GFFGYSGGSS	1.7 x 10^6	1.6 x 10^{-5}	9.0
G50X		RVYDA	YSASSLYS	SVNKFI	FRFSSI	GFYGYSGAGS	1.7 x 10^6	8.5 x 10^{-5}	49.3

Red ones are different from the wt sAB G50.

Table S6, related to Figure S5. Kinetic parameters for Ala scanning mutants of the affinity matured variants measured by SPR.

Clone	Mutation	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (pM)	$\Delta\Delta G_{mut-wt}$ (kcal mol^{-1})
G50.23 (Lib-L1) RVYDA	WT	1.5×10^6	3.4×10^{-4}	221	-
	R29A	9.9×10^5	6.7×10^{-4}	674	0.7
	V30A	4.6×10^5	8.0×10^{-4}	1760	1.2
	Y31A	1.1×10^6	2.1×10^{-3}	1933	1.3
	D32A	7.2 x 105	2.1×10^{-4}	295	0.2
G50.09 (Lib-L3) SVNKFI	WT	6.5×10^5	3.3×10^{-5}	50	-
	S92A	4.0×10^5	9.1×10^{-4}	2266	2.3
	V93A	9.9×10^5	8.9×10^{-5}	89.8	0.3
	N94A	3.7×10^5	2.9×10^{-4}	782	1.6
	K95A	4.0×10^5	6.6×10^{-4}	1633	2.1
	F96A	5.8×10^5	3.9×10^{-5}	66.7	0.2
	I97A	4.5×10^5	8.5×10^{-5}	187	0.8
G50.27 (Lib-H1) FRFSSI	WT	1.1×10^6	3.0×10^{-4}	266	-
	F32A	4.6×10^5	7.0×10^{-4}	1435	1.0
	R33A	8.8×10^5	1.7×10^{-3}	2020	1.2
	F34A	4.4×10^5	2.3×10^{-3}	5306	1.8
	S35A	5.1×10^5	3.7×10^{-4}	732	0.6
	S36A	6.2×10^5	1.8×10^{-3}	2897	1.4
	I37A	5.1×10^5	1.1×10^{-3}	2249	1.3
G50.12 (Lib-H2) GFFGYSGGTS	WT	7.9×10^5	9.1×10^{-6}	11.5	-
	G53A	1.7×10^6	1.7×10^{-5}	10.5	-0.1
	F54A	8.3×10^6	2.3×10^{-5}	27.7	0.5
	F55A	6.7×10^5	2.0×10^{-4}	305	1.9
	G56A	-	-	-	*
	Y57A	8.7×10^5	1.5×10^{-4}	166.0	1.6
	S58A	8.7×10^5	7.4×10^{-5}	85.0	1.2
	G59A	-	-	-	*
	G60A	1.5×10^6	2.4×10^{-4}	15.9	0.2
	T61A	-	-	-	*
	S62A	-	-	-	*

The ones in red are different from the wt sAB G50.

* Off-rate (k_{off}) can't be measured as no dissociation of the analyte was observed in the SPR runs. Thus, K_D, and $\Delta\Delta G_{mut-wt}$ were not determined.