

**Functional Evaluation of Key Interactions Evident in the Structure of  
the Eukaryotic Cys-loop Receptor GluCl**

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**Table S1.** Multiplicative effect of the T6'S mutation

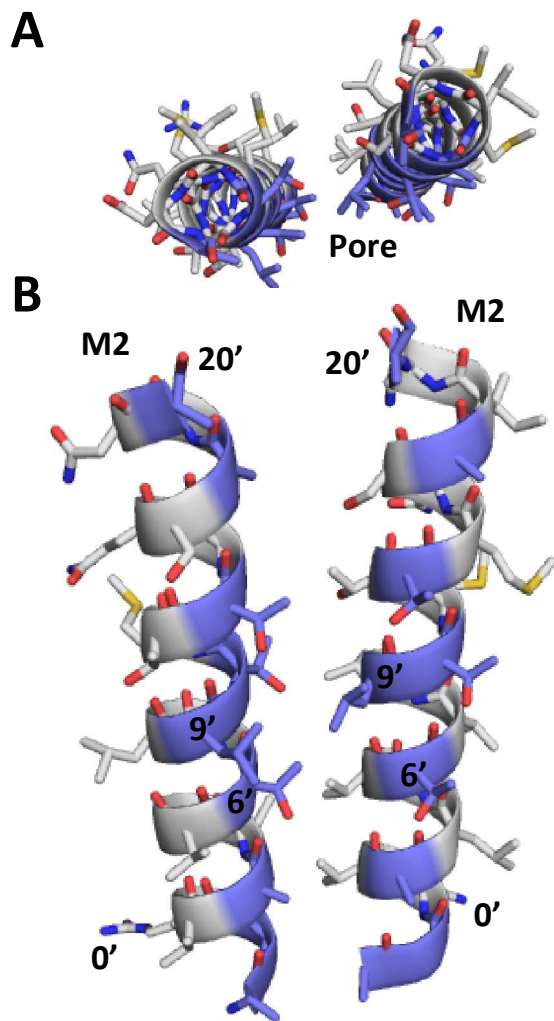
<b>Mutation</b>	<b>EC<sub>50</sub> (mM)</b>	<b>Hill</b>	<b>n</b>	<b>I<sub>max</sub> (μA)</b>	<b>Fold Shift</b>
WT	0.35 +/- 0.01	2.5 +/- 0.2	20	0.4-3.9	-
T6'S	0.0080 +/- 0.0005	2.4 +/- 0.3	27	0.06-8	-
Y206F	4.3 +/- 0.1	2.4 +/- 0.1	13	0.2-8	12.3
T6'S/Y206F	0.10 +/- 0.01	2.6 +/- 0.3	14	0.14-7	12.5

**Table S2:** Effects of receptor mutagenesis on functional expression. All mutations have the T6'S mutation unless otherwise noted.

<b>Mutation</b>	<b>EC<sub>50</sub> (μM)</b>	<b>Hill</b>	<b>n</b>	<b>I<sub>max</sub> (μA)</b>	<b>Median (μA)</b>	<b>Average (μA)</b>	<b>Fold Change Median</b>	<b>Fold Change Average</b>
WT	11 +/- 1	3.0 +/- 0.1	19	0.1-4	1.1	1.1	-	-
RRR_AAA	14 +/- 1	2.8 +/- 0.1	15	0.1-4	1.0	1.4	1	1
RSR_AAA	14 +/- 1	2.6 +/- 0.1	15	0.1-3	0.6	0.6	-2	-2
RRR/RSR_AAA	15 +/- 1	2.8 +/- 0.1	20	0.8-10	4.5	4.1	4	4
LXXLE	14 +/- 1	3.2 +/- 0.1	12	0.02-0.23	0.1	0.15	-11	-8
M3-M4 loop				No response				
α signal peptide (no T6'S)	980 +/- 20	1.9 +/- 0.1	16	3-14	8.9	8.7	9	8
α signal peptide + α1 (no T6'S)	110 +/- 10	2.6 +/- 0.2	14	0.2-6	1.0	1.4	1	1
M4-C term β/α				No response				

**Table S3.** Unnatural amino acid mutagenesis of Y206 with the α signal sequence for enhanced expression. All mutants have the T6'S background mutation.

	<b>EC<sub>50</sub> (mM)</b>	<b>Hill</b>	<b>n</b>	<b>I<sub>max</sub> (μA)</b>	<b>Fold Shift</b>
WT	0.93 +/- 0.02	1.8 +/- 0.1	26	2-13	-
T6'S	0.013 +/- 0.001	2.7 +/- 0.2	17	2-13	-
T6'S/Y206F	0.25 +/- 0.01	2.7 +/- 0.1	17	0.6-17	-
Phe	0.25 +/- 0.01	2.5 +/- 0.1	20	0.06-12	-
F <sub>1</sub> -Phe	1.4 +/- 0.1	2.8 +/- 0.5	15	0.4-6	6
F <sub>2</sub> -Phe	>100		20	0.1-1	
OMe-Phe	0.64 +/- 0.01	2.9 +/- 0.1	11	0.3-6	3
Br-Phe	1.5 +/- 0.1	3.1 +/- 0.1	11	1-13	6
CN-Phe	43 +/- 1	2.7 +/- 0.1	19	0.8-7	172



**Figure S1.** The M2 pore lining helix of GluCl $\beta$ . Helices from two adjacent subunits are shown. View from (A) the top of the receptor and (B) from “inside the pore” (roughly from where the word “Pore” is in part A). Residues in the ion-conducting pore are highlighted in blue and the location of the 0' arginine, 6' threonine, 9' leucine, and 20' alanine are indicated. Side chains of the GluCl $\alpha$  crystal structure were mutated to the corresponding GluCl $\beta$  side chains in PyMol.

GluCl $\alpha$  MATWIVGKLI IASLILGIQAQQARTKSQDIFEDDNDNGTTTLES LARL----TSPIHIPI  
 GluCl $\beta$  -----MTTPSSFSILLLLLLLMPVVTNG  
 \*\* \* \* \*

1  **$\alpha$ 1** 40  
 GluCl $\alpha$  EQPQTS DSKILAH LFTSGYDFRVRPPTDN---GGPVVSVNMLLR TISKIDVVNMEYSAQ  
 GluCl $\beta$  EYSMQSEQEI-LNALLKNYDMRVRPPPANSSTEGAVNVRVNIMIRMLSKIDVVNMEYSIQ  
 \* \* \* \*\* \* \* \* \* \* \* \* \* \* \* \*

59 96  
 GluCl $\alpha$  LTLRESWIDKRLSYGVKGDGQPDF--VILTVGHQI WMPDTFFPNEKQAYKHTIDKPNVLI  
 GluCl $\beta$  LTFREQWIDPRLAYENLGFYNPPAFLTVP HVKSLWIPDTFFPTEKAAHRHLIDMENMFL  
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126 Cys loop 156 174  
 GluCl $\alpha$  RIHNDGT VLYSVRISLVLSCPMYLQYYPMDVQOCSIDLAS YAYTTKDIEYLWKEHSPLQL  
 GluCl $\beta$  RIYPDGKILYSSRISLTSSCPMRLQLYPLDYQSCNFDLVS YAHTMNDIMYEWDPSTPVQL  
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202 M1  
 176 203 206  
 GluCl $\alpha$  KVG LSSSLPSFQLTNTS--TTYCTSVNTGIYSCLRTTIQLKREFSFYLLQLYIPSCMLVI  
 GluCl $\beta$  KPGVGS DLPNFILKNYTTNADCTSHTNTGSYGCLRMQLLFFKRQFSYYLVQLYAPTTMIVI  
 \*

M2 M3  
 0' 6' 9'  
 GluCl $\alpha$  VSWVSWFDRTAIPARVTLGVTTLLTMTAQ SAGINSQLPPVSYIKAIDVWIGACMTFIFC  
 GluCl $\beta$  VSWVSWWIDLHSTAGRVALGVTTLLTMTT MQSAINAKLPPVSYVKVVDVWLGACQTFVFG  
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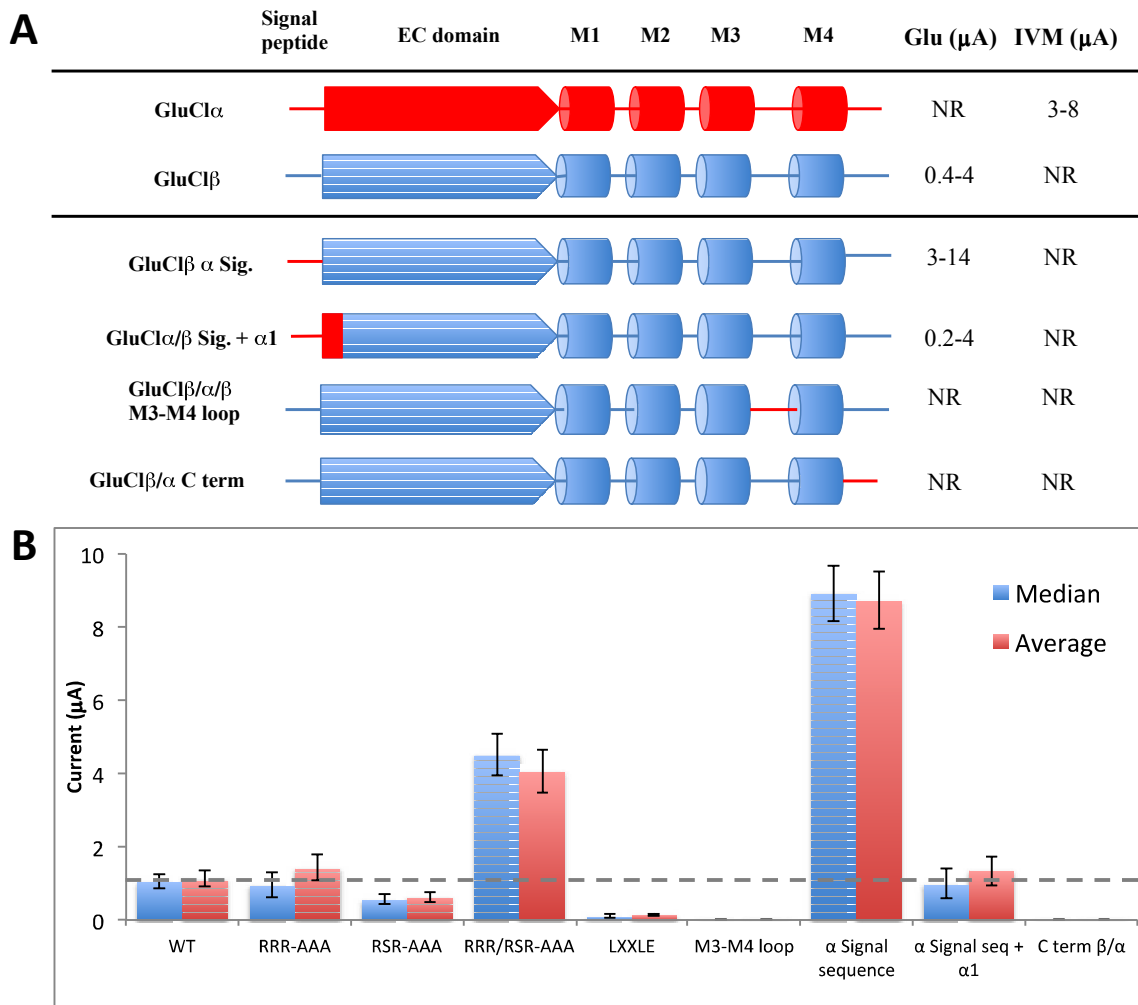
GluCl $\alpha$  ALLEFALVNHIANKQGV E R-----KARTEREKAEIPLLQNLHNDVPTKVFVNQEEKVRT  
 GluCl $\beta$  ALLEYAFVSYQDSVROND RSR EKAARKAQRREKLEMVDAEVYQPPCTCHTFEARETFRD  
 \*

M4  
 GluCl $\alpha$  VPLNRRQMNSFLNLELTKTEWNDISKRV D LISRALFPVLFFVFNILYWSRFGQQNVLF--  
 GluCl $\beta$  -----KVRRY-----FTKPDYLP AKIDFYARFV VPLAFLAFNVIYVWSCLIMSANAST  
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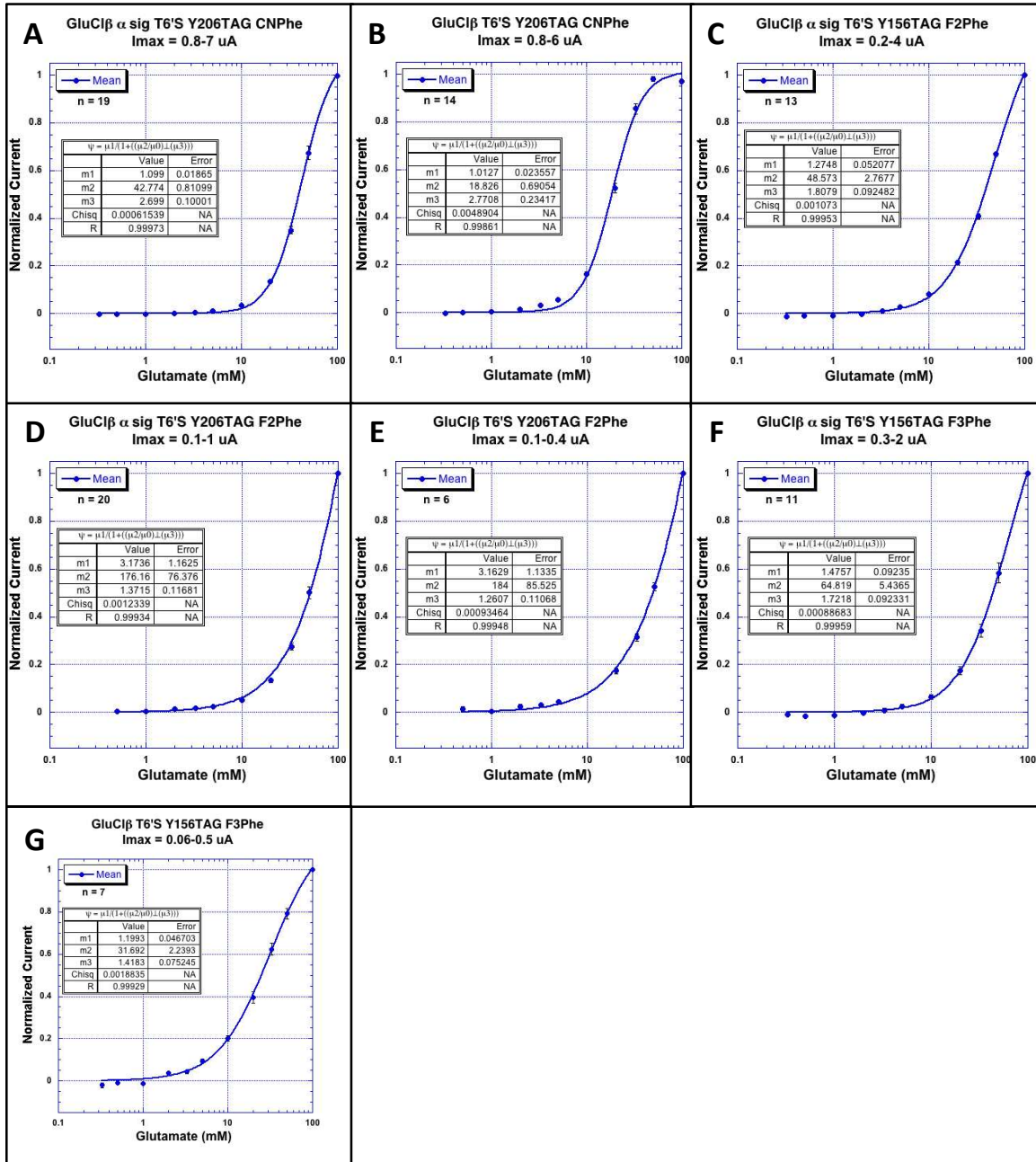
GluCl $\alpha$  -----  
 GluCl $\beta$  PESLV

ACH4\_HUMAN : KITLCISVLLSLTVFLLLITEI  
 ACH2\_HUMAN : KITLCISVLLSLTVFLLLITEI  
 ACHD\_HUMAN : KTSVAISVLLAQSVFLLLISKR  
 ACHB\_HUMAN : KMGLSIFALLTLTVFLLL LADK  
 5HT3\_HUMAN : RVSFKITLLLGYSVFLIIVSDT  
 GAA4\_HUMAN : RTVFGITTVLTM T T L S I S A R H S  
 GAB1\_HUMAN : RVALGITT V L T M T T I S T H L R E T  
 GRA1\_HUMAN : RVGLGITT V L T M T T Q S S G S R A S  
 GluCl-alpha : RVTLGVT T L L T M T A Q S A G I N S Q  
 GluCl-beta : RVALGV T T L L T M T T M Q S A I N A K

**Figure S2.** Sequence alignment of GluCl $\alpha$  and GluCl $\beta$ . Sites for chimera synthesis and receptor mutagenesis are indicated:  $\alpha$  signaling peptide chimera spliced at red line,  $\alpha$  signaling peptide +  $\alpha$ 1 helix spliced at orange line, M3-M4 loop spliced between green lines, C terminus spliced at purple line, and putative ER retention RxR motifs and ER export LxxLE motif are highlighted in black boxes. Residues targeted for mutagenesis are highlighted in red. Sequence alignment was made using Clustal Omega. Also shown are alignments of the M2 regions of several Cys-loop receptor subunits: nicotinic acetylcholine receptor  $\alpha$ 4,  $\alpha$ 2,  $\delta$ , and  $\beta$ 1; GABA<sub>A</sub> receptor  $\alpha$ 4 and  $\beta$ 1; glycine receptor  $\alpha$ 1.



**Figure S3.** Effects of mutations and chimeras on GluCl $\beta$  functional expression. **(A)** Schematic of chimeras examined and **(B)** average and median currents from *X. laevis* oocytes expressing the specified mutant receptor.



**Figure S4.** EC<sub>50</sub> plots of mutant receptors that demonstrate a large loss-of-function. (A) Y206CNPhe with the  $\alpha$  signal sequence (B) Y206CNPhe with the WT  $\beta$  signal sequence (C) Y156F<sub>2</sub>Phe (D) Y206F<sub>2</sub>Phe with the  $\alpha$  signal sequence (E) Y206F<sub>2</sub>Phe with the WT  $\beta$  signal sequence (F) Y156F<sub>3</sub>Phe with the  $\alpha$  signal sequence (G) Y156F<sub>3</sub>Phe the WT  $\beta$  signal sequence