



Supplementary Information for

RubyACRs, non-algal anion channelrhodopsins with highly red-shifted absorption

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Legend for Dataset S1

Other supplementary materials for this manuscript include the following:

Dataset S1

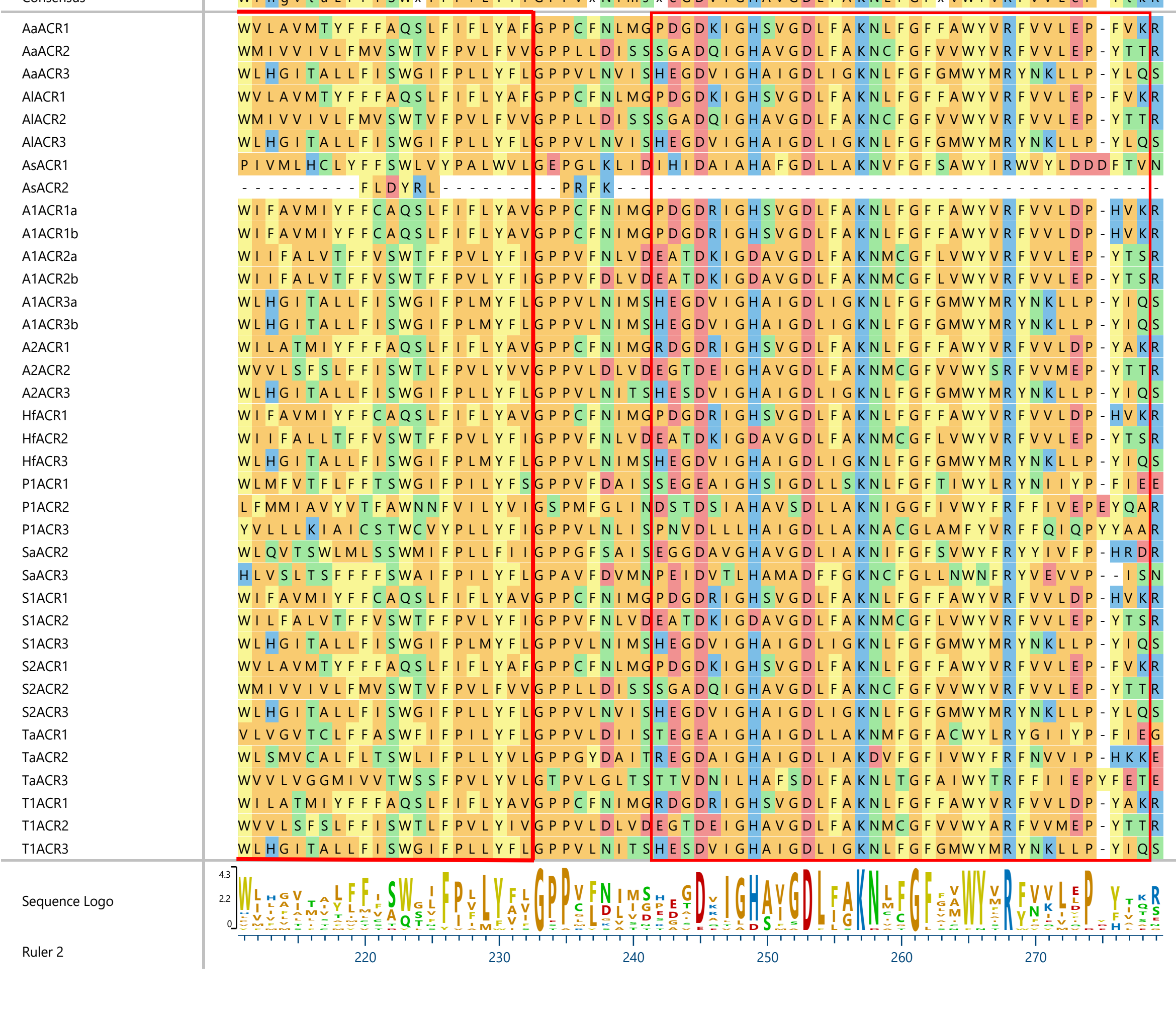
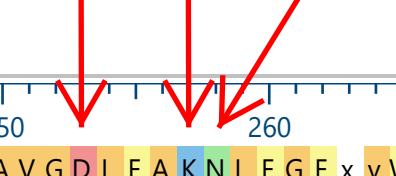
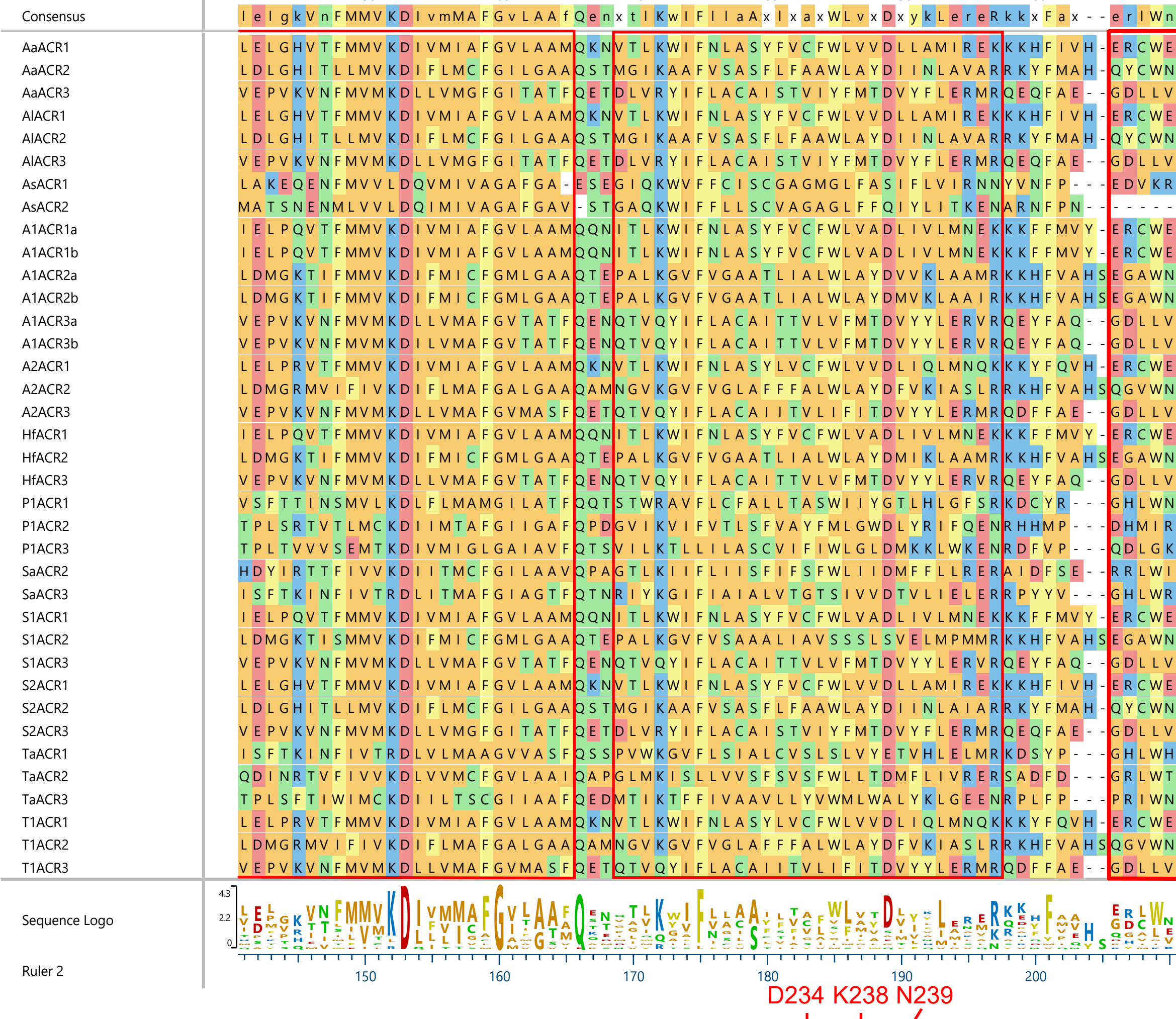
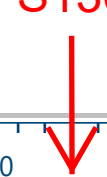
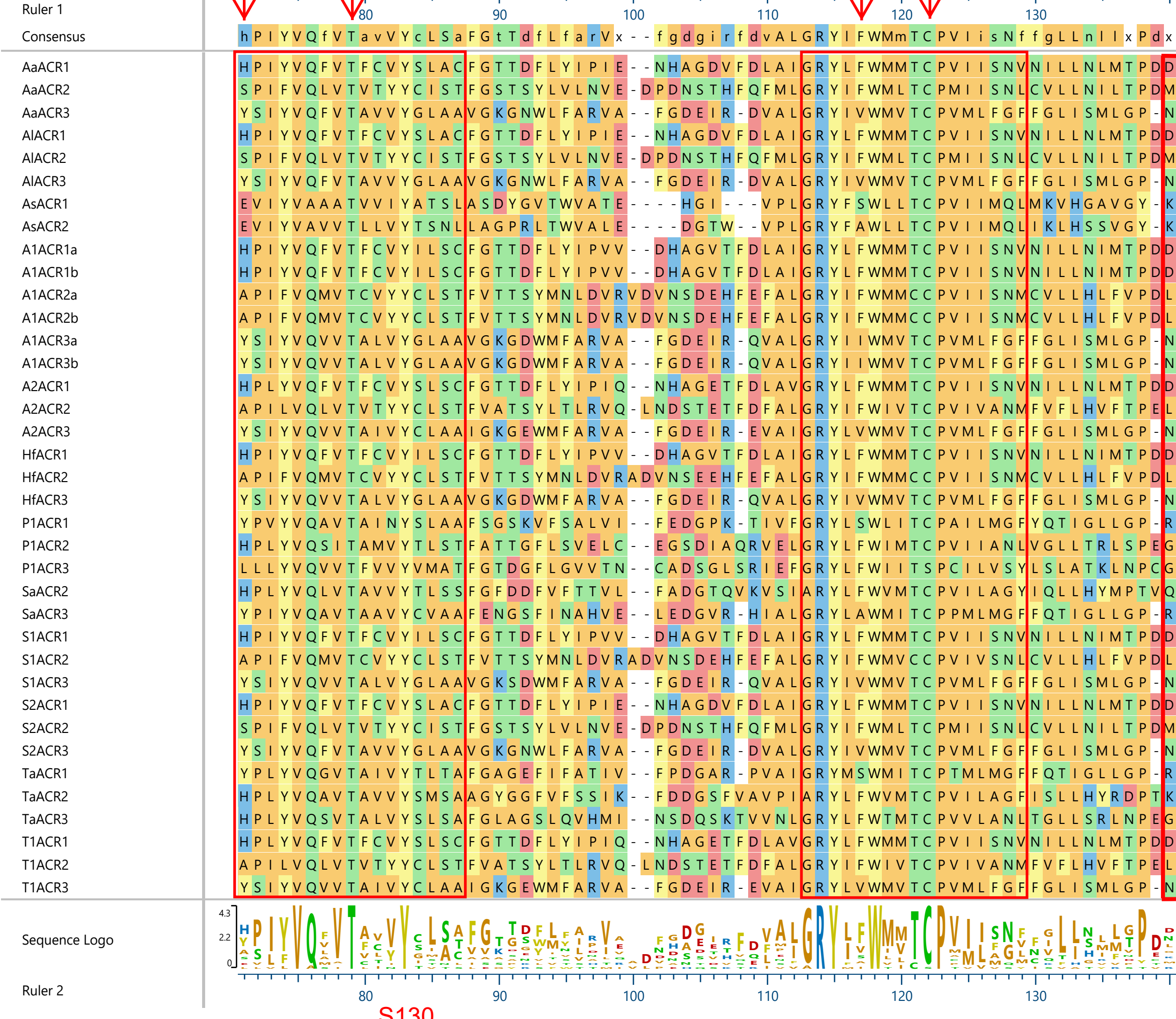
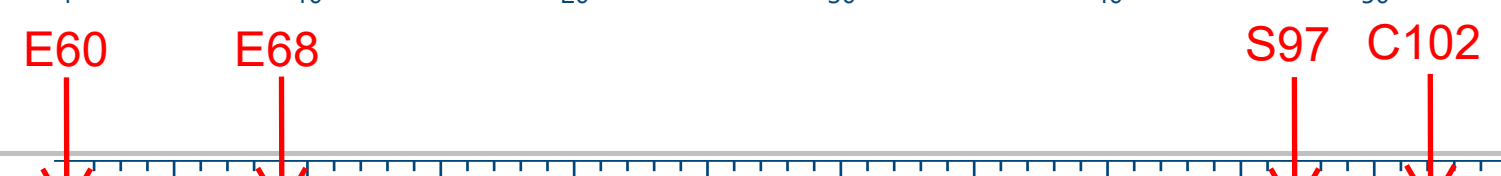
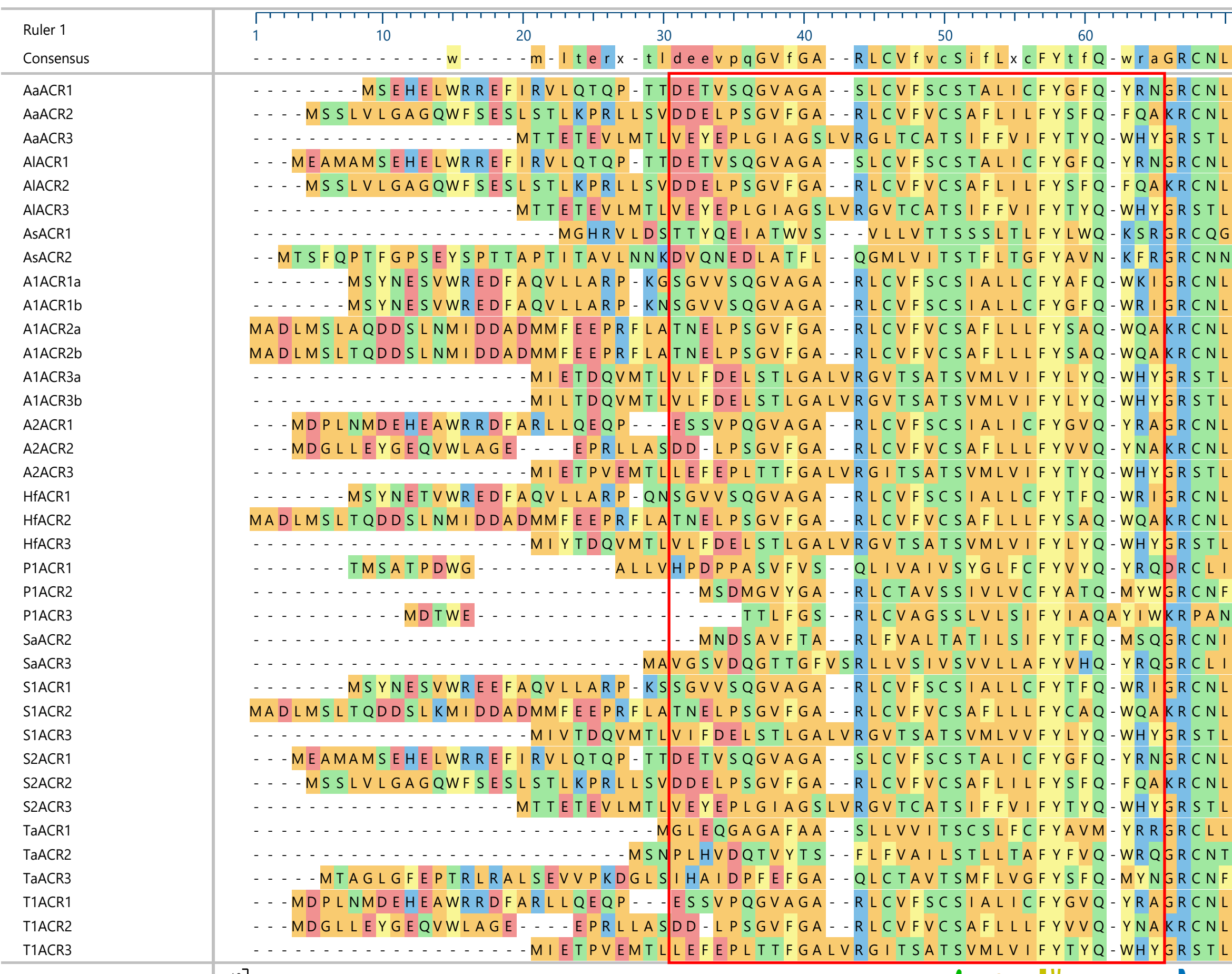


Fig. S1. Protein sequence alignment of the rhodopsin domains of labyrinthulea channelrhodopsins. Residues are color-coded according to their chemical properties. The red rectangles show α -helical regions as detected in the *Ai*ACR1 homology model. The arrows point to the positions of the residues known to be functionally important in *Gt*ACR1 (*Gt*ACR1 numbering).

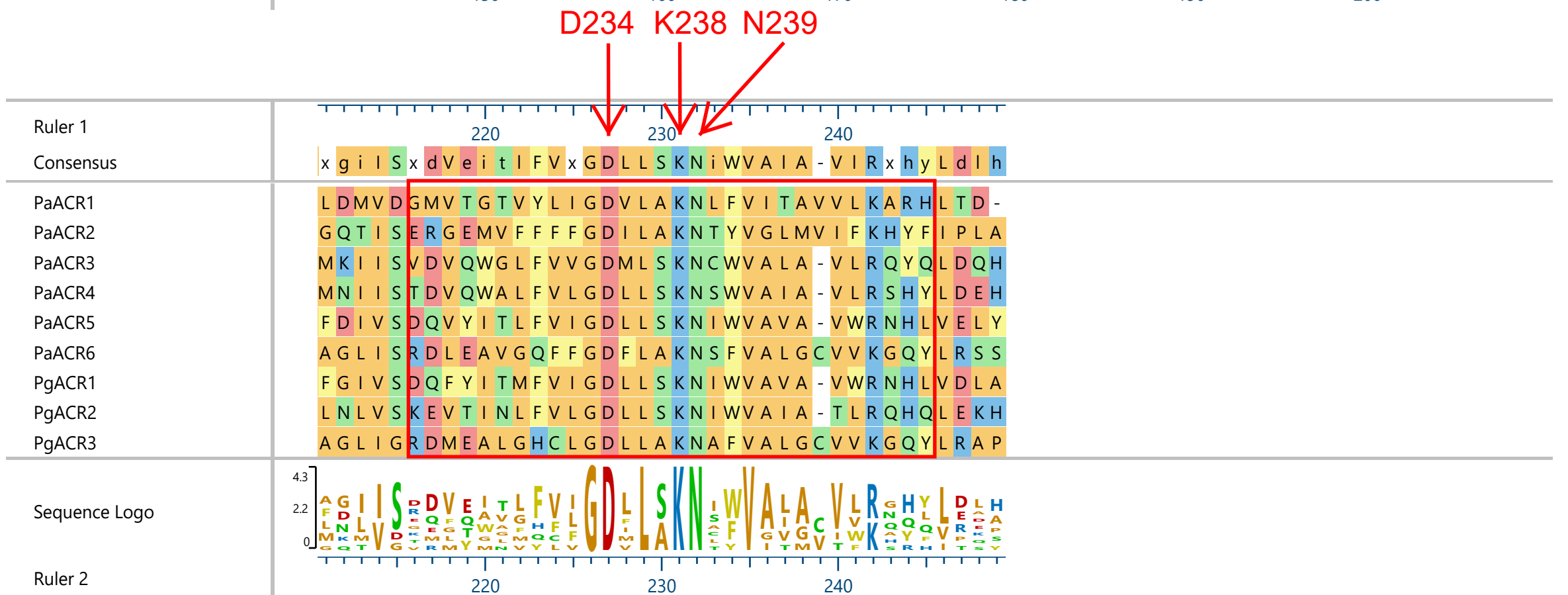
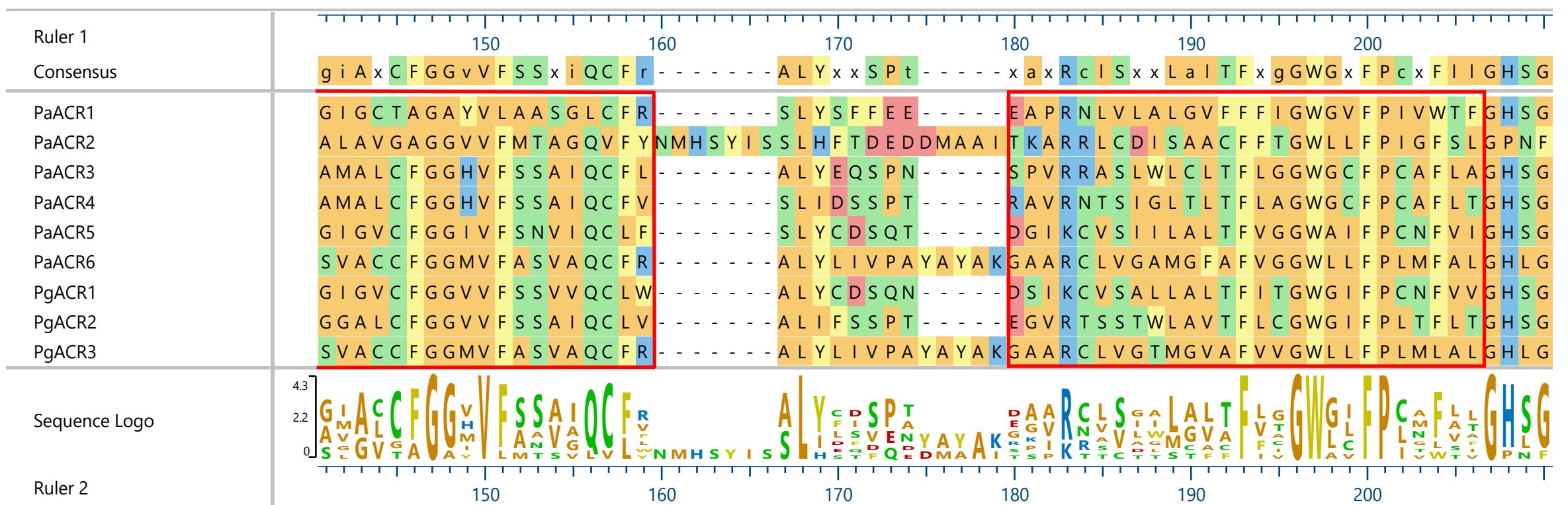
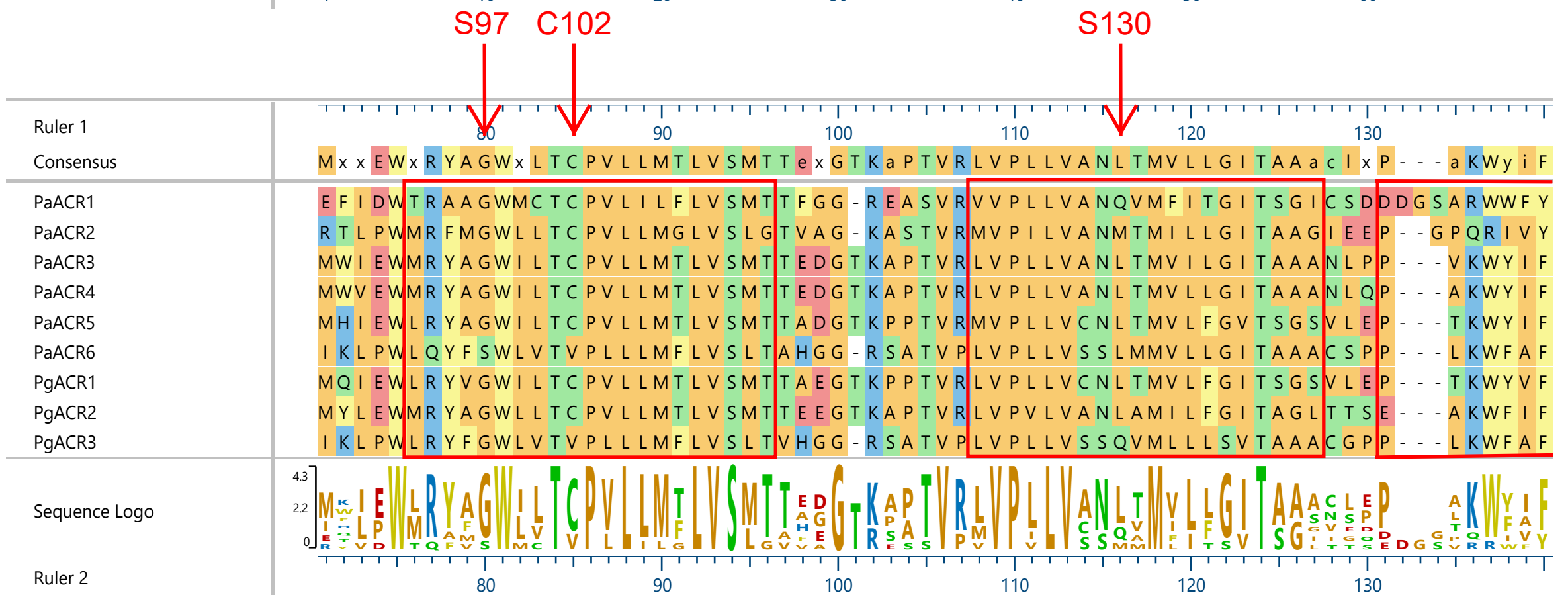
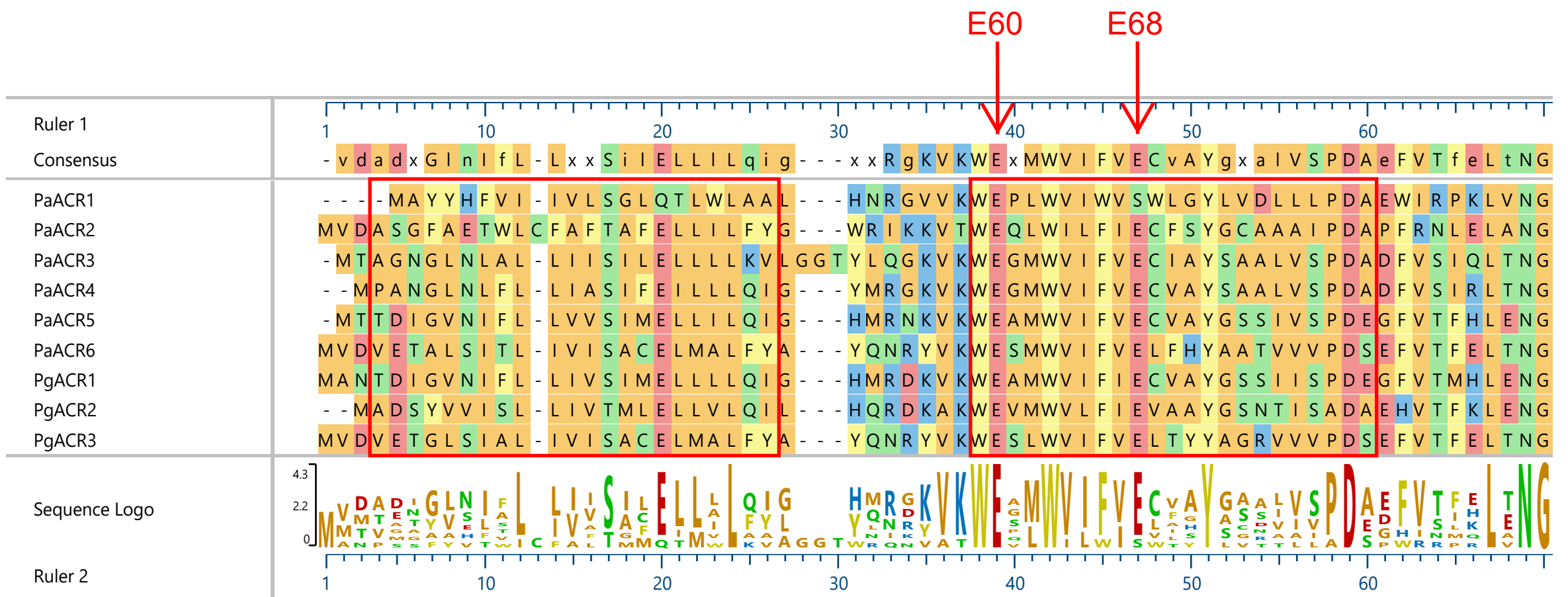


Fig. S2. Protein sequence alignment of the rhodopsin domains of haptophyte channelrhodopsins. Residues are color-coded according to their chemical properties. The red rectangles show α -helical regions as detected in the *Pg*ACR1 homology model. The arrows point to the positions of the residues known to be functionally important in *Gt*ACR1 (*Gt*ACR1 numbering).

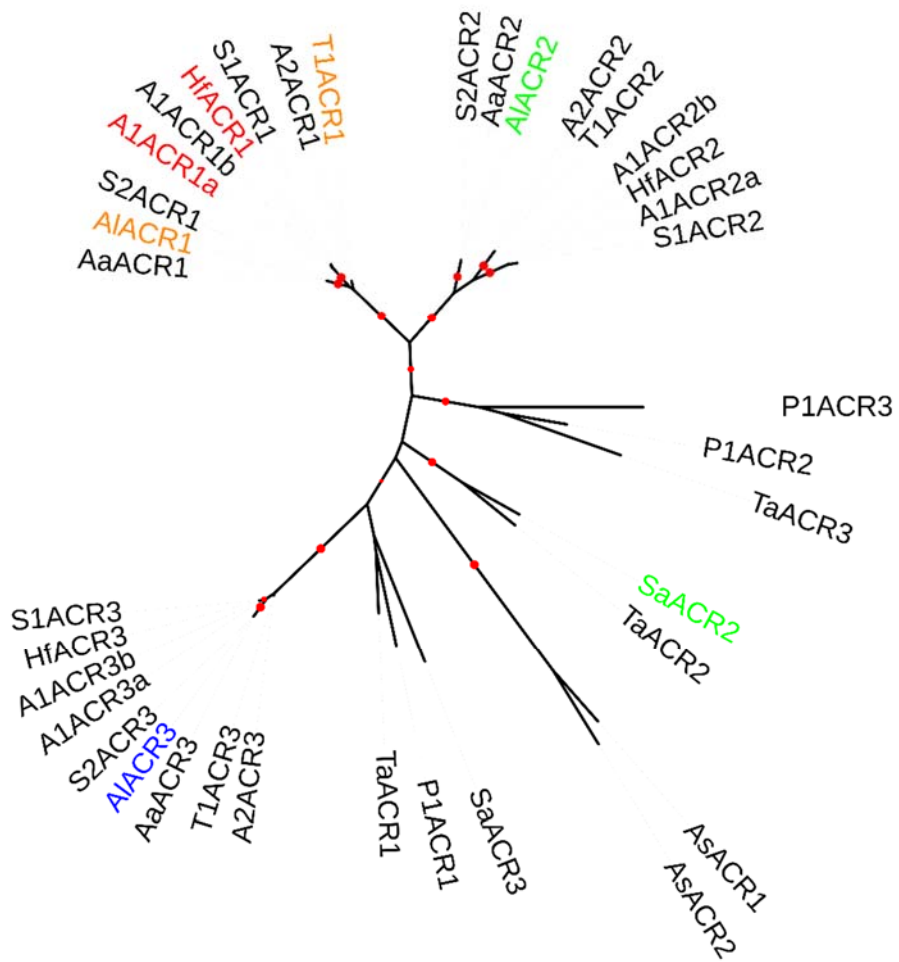


Fig. S3. An unrooted phylogenetic tree of labyrinthulea rhodopsin domains. The ultrafast bootstrap support values are shown by red circles (95-100% range). The colored leaf labels show proteins tested in this study; the color of the label corresponds to the wavelength of the maximal sensitivity.

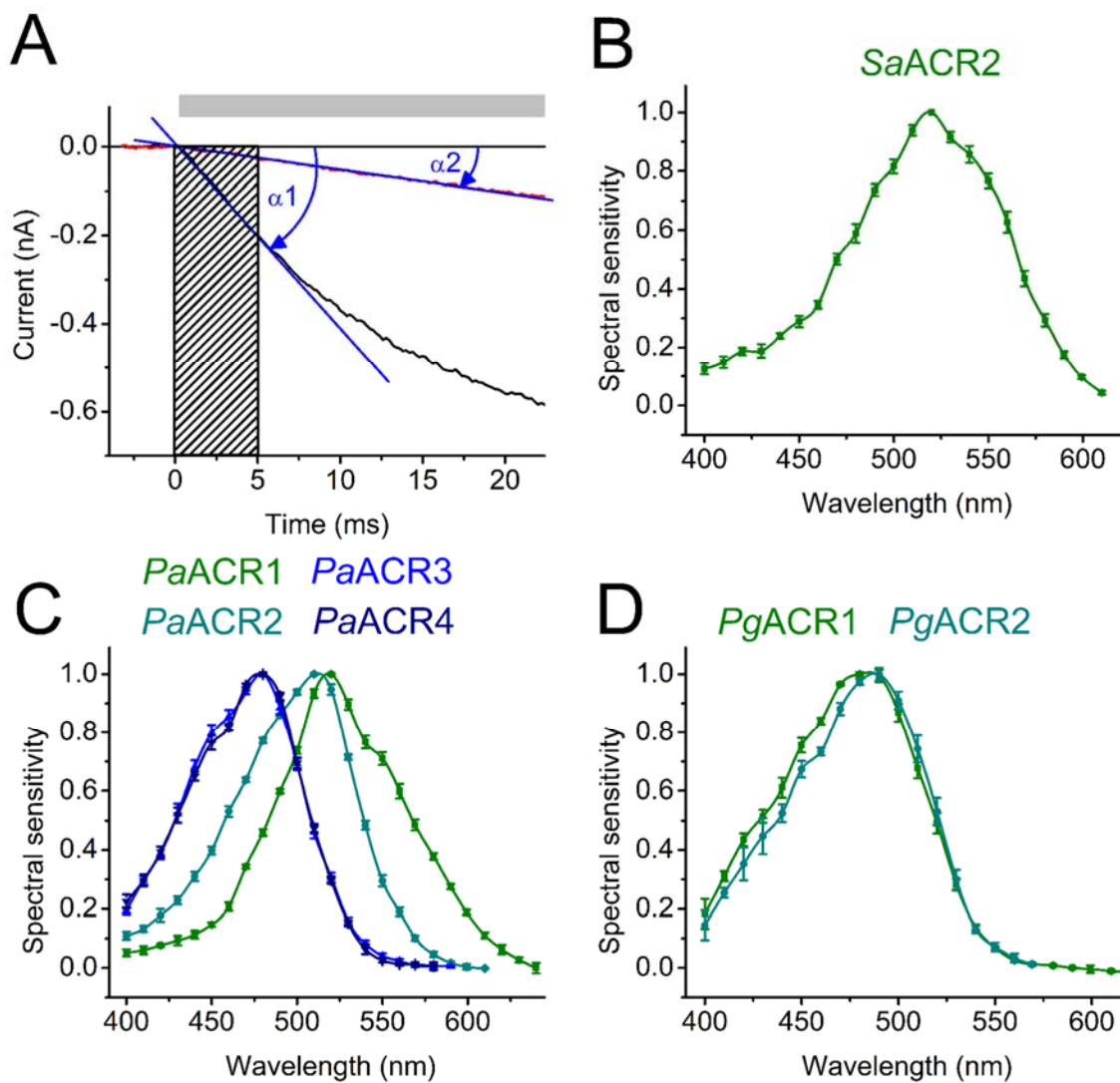


Fig. S4. (A) An example of photocurrent traces generated by *SaACR2* at two different wavelengths (black lines), linear approximation of their initial segments (blue lines) and slopes (α_1 and α_2) used for construction of the action spectra. The hatched region shows the segments of the traces used for linear approximation. (B-D) The action spectra of the indicated homologs, constructed as shown in panel A. The data points are the mean values \pm sem ($n = 6-8$ scans).

	10	20	30	40	50	60	70														
HfACR1	MSYNE	VWREDF	QVLLAR	PQNS	SGVVSQ	GVARL	CVFSCS	IALLCFY	IFQWR	IGRCNL	HPIYVQ	FVTFC	70								
A1ACR1	MSYNES	VWREDF	QVLLAR	PQNS	SGVVSQ	GVARL	CVFSCS	IALLCFY	AFQWK	IGRCNL	HPIYVQ	FVTFC	70								
	80	90	100	110	120	130	140														
HfACR1	VYILSC	FGTTF	FLYIP	VVDHAG	VTFDLA	IGRYL	FWM	MTC	CPVI	ISNVN	ILLN	IMTP	DDI	ELPQ	VTF	FMMV	KD	140			
A1ACR1	VYILSC	FGTTF	FLYIP	VVDHAG	VTFDLA	IGRYL	FWM	MTC	CPVI	ISNVN	ILLN	IMTP	DDI	ELPQ	VTF	FMMV	KD	140			
	150	160	170	180	190	200	210														
HfACR1	IVMIA	FGVLA	AMQ	QNITL	KWIF	NLAS	YFV	CFWL	VADL	IVLM	NEK	KKK	FFM	VYER	CWE	WIF	FAV	MIY	FF	CAQS	210
A1ACR1	IVMIA	FGVLA	AMQ	QNITL	KWIF	NLAS	YFV	CFWL	VADL	IVLM	NEK	KKK	FFM	VYER	CWE	WIF	FAV	MIY	FF	CAQS	210
	220	230	240	250	260	270															
HfACR1	LFIFLY	AVGPP	CFNIM	GPDG	DRIGH	SVGDL	FAKNL	EGFF	AWYV	RFV	VLD	PHV	KRIR	GARA	270						
A1ACR1	LFIFLY	AVGPP	CFNIM	GPDG	DRIGH	SVGDL	FAKNL	EGFF	AWYV	RFV	VLD	PHV	KRIR	GARA	270						

Fig. S5. An alignment of A1ACR1 and HfACR1 rhodopsin domains. Identical residues are shaded black.

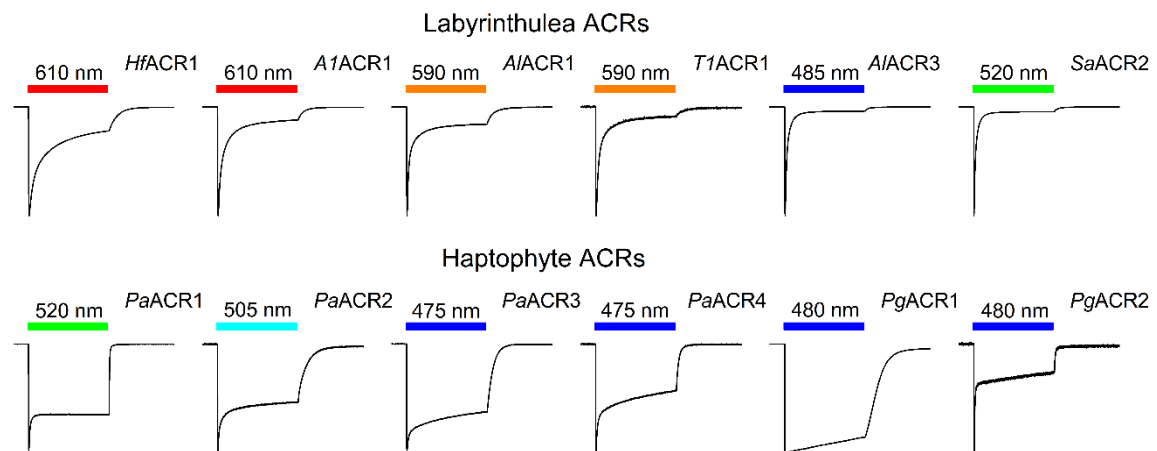


Fig. S6. Photocurrent traces recorded in response to the first 1-s light pulse at -60 mV at the amplifier output, normalized at their peak value. The duration of illumination is showed as a colored bar on top.

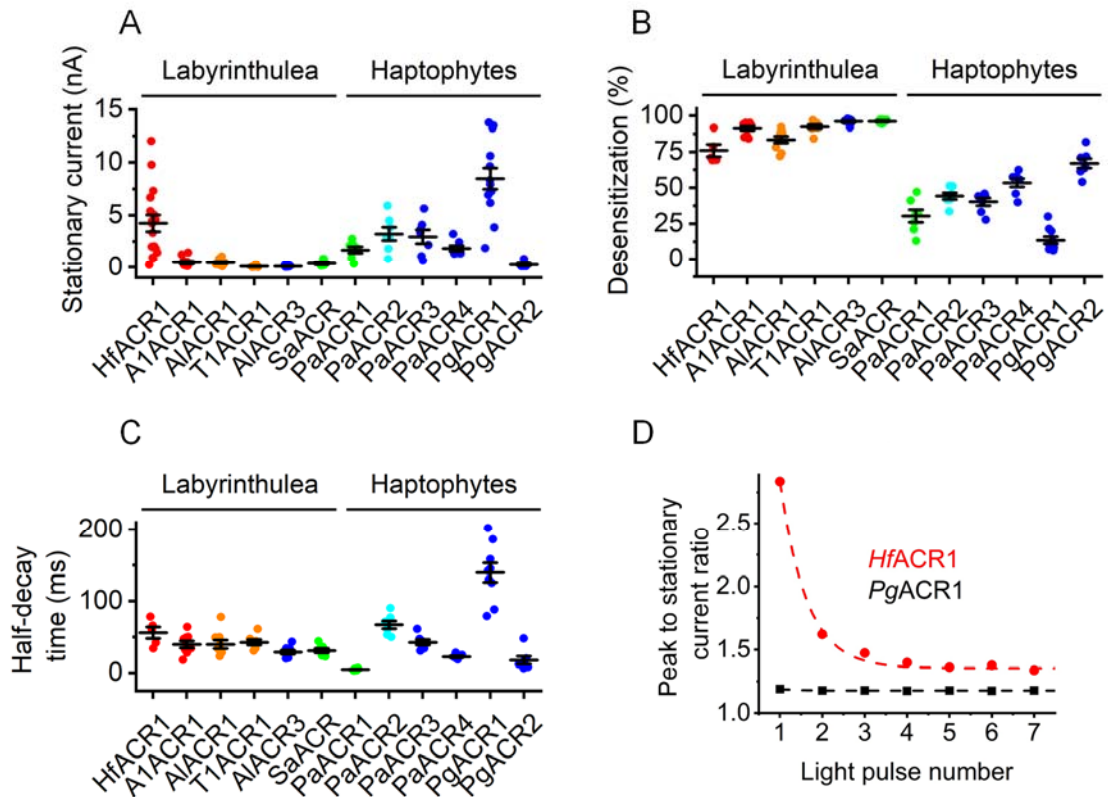


Fig. S7. (A) Stationary current measured at the end of a 1-s light pulse. (B) The magnitude of desensitization during 1-s continuous illumination. (C) Half-decay of photocurrent after switching the light off. In A-C, the black lines show the mean values and s.e.m. ($n = 5-10$ cells for each variant); colored circles, the individual data points. (D) The ratio of the peak amplitude to that of the stationary current measured in a series of 1-s pulses applied with 30-s time interval. The lines are single exponential fits.

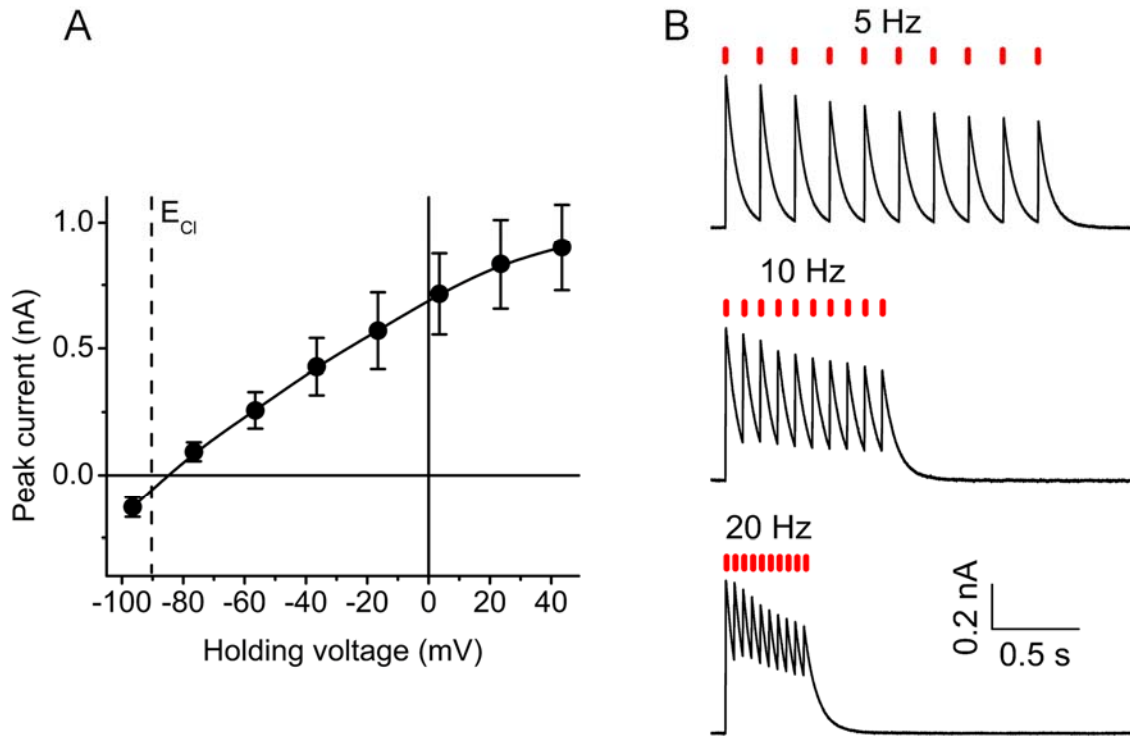


Fig. S8. Performance of *HfACR1* activated at 630 nm ($5.6 \text{ mW}\cdot\text{cm}^{-2}$) and tested under ionic conditions typical of mature neurons (for solution compositions see Methods). (A) A current-voltage relationship. The data points are the mean \pm sem ($n = 5$ cells). (B) Photocurrent traces recorded from *HfACR1* upon repetitive stimulation with 2-ms light pulses at -20 mV at the amplifier output.

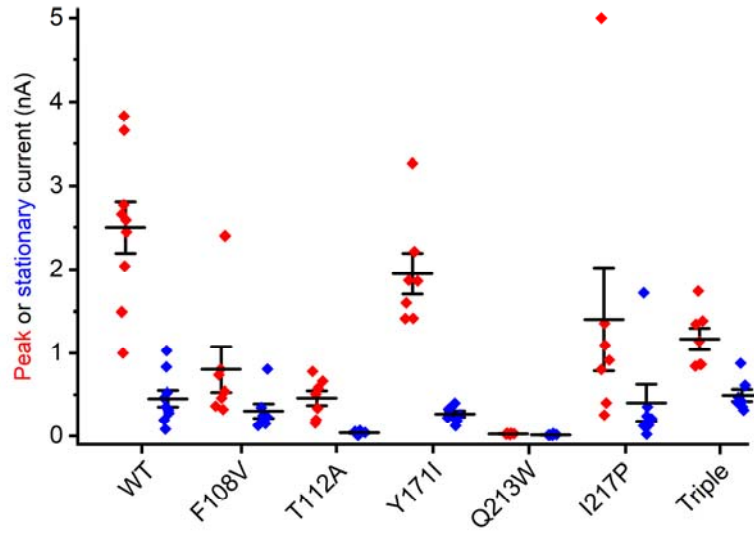


Fig. S9. Peak (red) and stationary (blue) photocurrents recorded from color-tuning mutations of *A/ACR1*. The mean \pm sem are shown as lines and whiskers ($n = 7$), data from individual cells, as diamonds. The data for wild type (WT) are taken from Fig. 1C. Triple, the F108V_Y171I_I217P mutant.

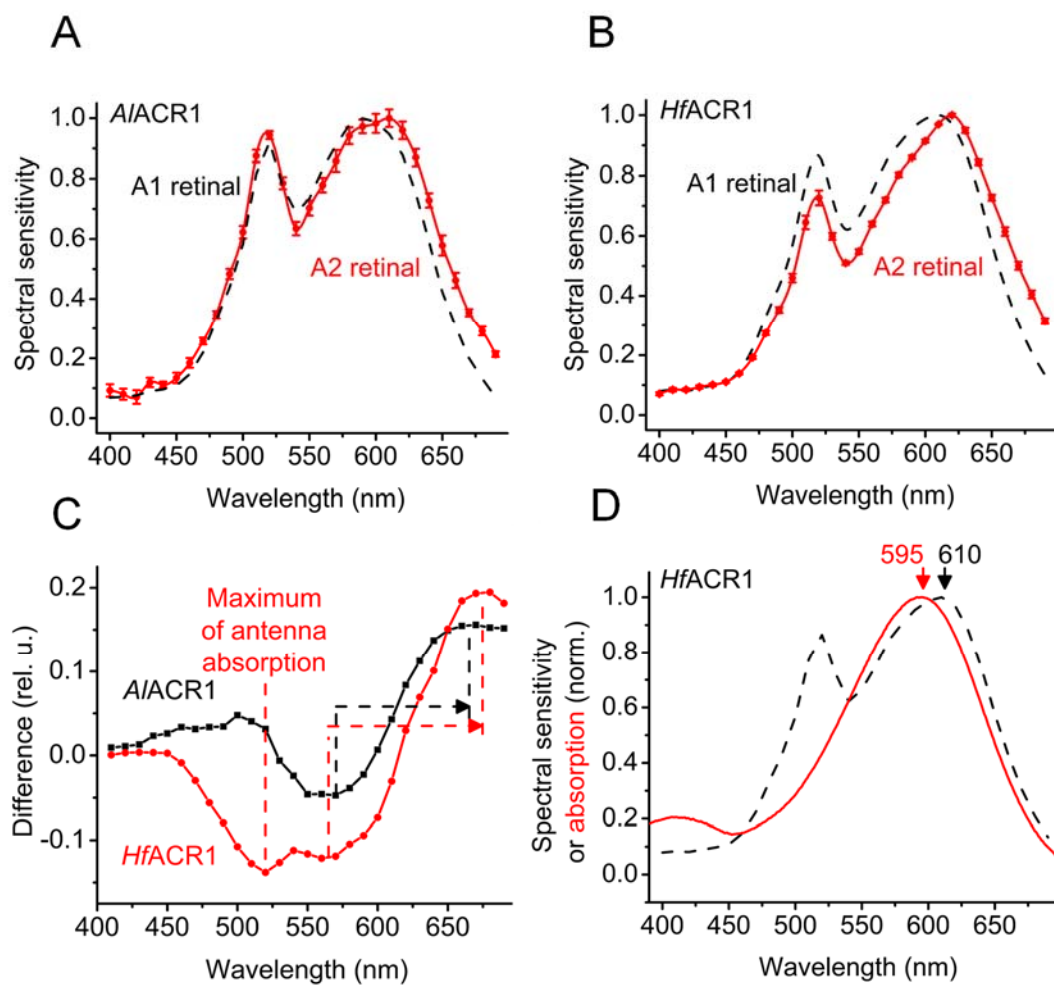


Fig. S10. (A and B) The action spectra of photocurrents generated by indicated proteins reconstituted with A1 (black) and A2 (red) retinal. (C) The difference spectra (A2-A1 retinal). (D) The absorption spectrum of *HfACR1* detergent-purified from *Pichia* (red solid line) compared to the action spectrum of photocurrents generated upon its expression in HEK293 cells from Fig. 1B (black dashed line).

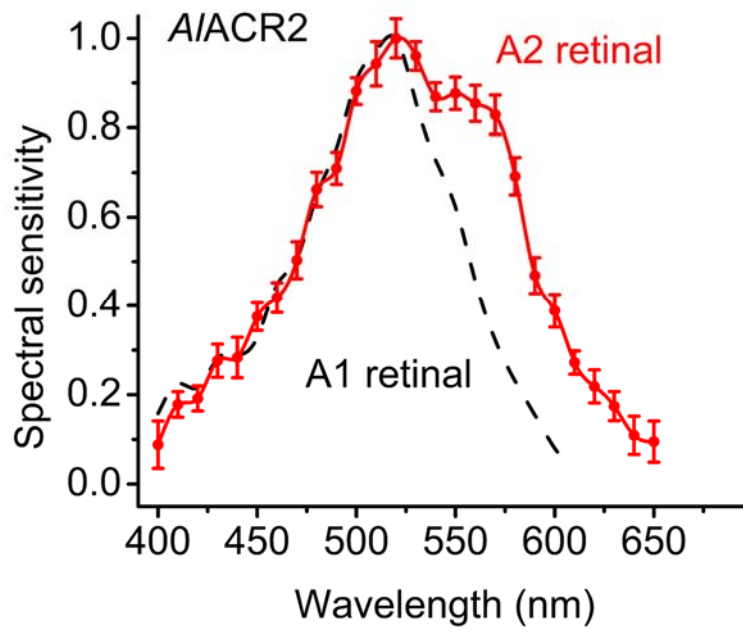


Fig. S11. The action spectrum of photocurrents generated by *A/ACR2* reconstituted with A1 (black) and A2 (red) retinal.

Table S1. A list of analyzed genomes.

	Organism name	Genome assembly number	URL	Principal investigator /Submitter	Status
1.	<i>Aplanochytrium kerguelense</i> PBS07	1.0	https://phycocosm.jgi.doe.gov/Aplke1/Aplke1.home.html	Jackie Collier	Complete, annotated
2.	<i>Aurantiochytrium acetophilum</i> HS399	ASM433257v1	https://www.ncbi.nlm.nih.gov/genome/79056?genome_assembly_id=492055	Heliae Development	Shotgun
3.	<i>Aurantiochytrium limacinum</i> ATCC MYA-1381	Not defined	https://phycocosm.jgi.doe.gov/Aurli1/Aurli1.home.html	Jackie Collier	Complete, annotated
4.	<i>Aurantiochytrium</i> sp. KH105	Auran_KH105_1.0	https://www.ncbi.nlm.nih.gov/genome/41915?genome_assembly_id=374513	Okinawa Institute of Science and Technology	Shotgun
5.		Not defined	https://marinegenomics.oist.jp/aurantiochytrium_sp_kh105/viewer?project_id=56	Okinawa Institute of Science and Technology	Not defined, annotated
6.	<i>Aurantiochytrium</i> sp. T66	ASM146250v1	https://www.ncbi.nlm.nih.gov/genome/41915?genome_assembly_id=259575	ThraustoEng	Shotgun
7.	<i>Hondaea fermentalgiana</i> FCC1311 (<i>Aurantiochytrium</i> sp. FCC1311)	<i>Aurantiochytrium</i> _FCC1311_v1	https://www.ncbi.nlm.nih.gov/genome/72081?genome_assembly_id=400191	Fermentalg	Shotgun
8.	<i>Schizochytrium aggregatum</i> ATCC 28209	Not defined	https://mycocosm.jgi.doe.gov/Schag1/Schag1.home.html	Jackie Collier	Complete, annotated
9.	<i>Schizochytrium</i> sp. CCTCC M209059	ASM81894v1	https://www.ncbi.nlm.nih.gov/genome/35766?genome_assembly_id=217282	Nanjing Tech University	Shotgun
10.	<i>Schizochytrium</i> sp. TIO01	ASM476469v1	https://www.ncbi.nlm.nih.gov/genome/35766?genome_assembly_id=491898	Third Institute of Oceanography	Shotgun
11.	<i>Thraustochytrium aureum</i> ATCC 34304	Tau_assembly01	https://www.ncbi.nlm.nih.gov/genome/88990?genome_assembly_id=889105	Kyushu University	Shotgun
12.	<i>Thraustochytrium</i> sp. ATCC 26185	ASM215423v1	https://www.ncbi.nlm.nih.gov/genome/54615?genome_assembly_id=318815	University of Saskatchewan	Shotgun
13.	<i>Parietichytrium</i> sp. I65-24A	Pari_assembly01	https://www.ncbi.nlm.nih.gov/genome/88991?genome_assembly_id=889106	Kyushu University	Shotgun
14.	<i>Phaeocystis antarctica</i> CCMP1374	2.2	https://phycocosm.jgi.doe.gov/Phaant1/Phaant1.home.html	Kevin Arrigo	Not defined, annotated
15.	<i>Phaeocystis globosa</i> Pg-G	2.3	https://mycocosm.jgi.doe.gov/Phaglo1/Phaglo1.home.html	Andy Allen	Not defined, annotated

Table S2. A list of labyrinthulea ACR homologs (in bold – synthesized and tested by patch clamp in this study)

	GenBank accession number	Abbreviated protein name	Source organism	JGI gene model name or WGS/MMETSP sequence name	λ_{\max} (nm)
1.		<i>Aa</i> ACR1	<i>Aurantiochytrium acetophilum</i> HS399	QDJC01000532	
2.		<i>Aa</i> ACR2		QDJC01003161	
3.		<i>Aa</i> ACR3		QDJC01000037	
4.	MT002467	<i>A</i>ACR1	<i>Aurantiochytrium limacinum</i> ATCC MYA-1381	fgenes1_pg.12 # 284	590
5.	MT002473	<i>A</i>ACR2		fgenes1_pg.12 # 285	545
6.	MT002476	<i>A</i>ACR3		fgenes1_pg.1 # 498	485
7.		<i>As</i> ACR1	<i>Aplanochytrium stocchinoi</i> *	DN5182_c0_g3_i1.p1	
8.		<i>As</i> ACR2		DN9014_c0_g1_i1.p1, DN12229_c0_g1_i1.p1	
9.	MT002468	<i>A</i>1ACR1a	<i>Aurantiochytrium</i> sp. KH105	BGKB01000037	610
10.		<i>A</i> 1ACR1b		BGKB01000105	
11.		<i>A</i> 1ACR2a		BGKB01000037†	
12.		<i>A</i> 1ACR2b		BGKB01000105†	
13.		<i>A</i> 1ACR3a		BGKB01000099	
14.		<i>A</i> 1ACR3b		BGKB01000102	
15.		<i>A</i> 2ACR1		<i>Aurantiochytrium</i> sp. T66	LNGJ01004228
16.		<i>A</i> 2ACR2	LNGJ01004228†		
17.		<i>A</i> 2ACR3	LNGJ01002066		
18.	MT002469	<i>Hf</i>ACR1	<i>Hondaea fermentalgiana</i> FCC1311 (<i>Aurantiochytrium</i> sp. FCC1311)	BEYU01000006	610
19.		<i>Hf</i> ACR2		BEYU01000006†	
20.		<i>Hf</i> ACR3		BEYU01000001	
21.		<i>P</i> 1ACR1	<i>Parietichytrium</i> sp. I65-24A	BLSF01000016	
22.		<i>P</i> 1ACR2		BLSF01000022	
23.		<i>P</i> 1ACR3		BLSF01000101	
24.	MT002463	<i>Sa</i>ACR2	<i>Schizochytrium aggregatum</i> ATCC 28209	fgenes1_pg.3 # 476	520
25.		<i>Sa</i> ACR3		fgenes1_pg.3 # 475	
26.		<i>S</i> 1ACR1	<i>Schizochytrium</i> sp. CCTCC M209059	JTFK01000019	
27.		<i>S</i> 1ACR2		JTFK01000019†	
28.		<i>S</i> 1ACR3		JTFK01000324	
29.		<i>S</i> 2ACR1	<i>Schizochytrium</i> sp. TIO01	SMSO01000032	
30.		<i>S</i> 2ACR2		SMSO01000032†	
31.		<i>S</i> 2ACR3		SMSO01000014	
32.		<i>Ta</i> ACR1	<i>Thraustochytrium aureum</i> ATCC 34304	BLSG01000269	
33.		<i>Ta</i> ACR2		BLSG01000269†	
34.		<i>Ta</i> ACR3		BLSG01000370	
35.	MT002470	<i>T</i>1ACR1	<i>Thraustochytrium</i> sp. ATCC 26185	MUFY01006470	590
36.		<i>T</i> 1ACR2		MUFY01006469	
37.		<i>T</i> 1ACR3		MUFY01009420	

*This species was analyzed by Rozenberg et al. (16).

†These proteins are encoded by the complement strand of the WGS sequence.

Table S3. A list of haptophyte ACR homologs tested in this study

	GenBank accession number	Abbreviated protein name	Source organism	JGI gene model name	λ_{\max} (nm)
1.	MT002471	<i>Pa</i> ACR1	<i>Phaeocystis antarctica</i> CCMP1374	Phant.0066s0015.1	520
2.	MT002474	<i>Pa</i> ACR2		Phant.0011s0329.1	510
3.	MT002477	<i>Pa</i> ACR3		Phant.0016s0461.1, Phant.0016s0462.1, Phant.0016s0464.1	480
4.	MT002464	<i>Pa</i> ACR4		Phant.0060s0074.1	480
5.	MT002465	<i>Pa</i> ACR5		Phant.0086s0086.1	N.A.
6.	MT002466	<i>Pa</i> ACR6		Phant.0001s0932.1	N.A.
7.	MT002472	<i>Pg</i> ACR1	<i>Phaeocystis globosa</i> Pg-G	Phglo.0395s0005.1	485
8.	MT002475	<i>Pg</i> ACR2		Phglo.0149s0014.1	485
9.	MT002478	<i>Pg</i> ACR3		Phglo.0128s0040.1	N.A.

Legend for Dataset S1

Dataset S1. GenBank accession numbers, abbreviated protein names and source organism names for previously known channelrhodopsins included in the tree shown in Fig. 1A in the main text.