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Supplementary Materials for

Schizorhodopsins: A family of rhodopsins from Asgard archaea that function as light-driven inward H^+ pumps

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Amino acid sequence of SAMEA 2622822_312577 (SzR2)

MIETLTMIILSLGTLVFFISSIVFAKMDWRQANHFNSALIVSATTAVSYAVMLAIYLGTPTTDSLSTRWLF
YIISCSLLIYHIAKVLRLNNNQRISAGYLMALIMITGFVAAEVSDLWFVLVIYLIGSAFYVLALKIIWQGTA
HTLAKLKPYLIYGWTGFPIVFLLSPAAFDLISLDSALALYLGLDIYTKIVFYKDYNQLVR

Table S1. Retinal configuration in SzR1. The composition of the retinal isomers in SzR1 determined by HPLC analysis for retinal oxime produced by the hydrolysis of retinylidene Schiff base with hydroxylamine.

Isomer	Dark	Light
all-*trans*	94.48 ± 0.5%	72.92 ± 0.2%
13-*cis*	N.O.	12.24 ± 0.6%
11-*cis*	5.51 ± 0.5%	14.83 ± 0.2%

N.O.: Not observed

Table S2. Normalization factors of SzR1 FTIR spectra shown in Fig. 5, A and B in 0% glycerol condition.

Intermediate (solvent)	Normalization factors
K (H_2O)	1
K (D_2O)	1.35
L (H_2O)	4.20
L (D_2O)	5.66
L/M (H_2O)	3.39
L/M (H_2O)	4.49
M (H_2O)	0.79
M (H_2O)	0.79

Table S3. Normalization factors of SzR1 FTIR spectra (30% glycerol) shown in Fig. 5C in 30% glycerol.

Intermediate (solvent)	Normalization factors
K (D_2O)	1
K ($D_2{}^{18}O$)	1.05
M (D_2O)	1.63
M ($D_2{}^{18}O$)	1.83

Table S4. List of primers used for site-directed mutagenesis.

Mutations	Sense primers	Anti-sense primers
SzR1 E2Q	CCGATGTAGAAAATGATTTCCTG CATATGTATATCTCCTTCTTAAA	TTTAAGAAGGAGATATACATATG CAGGAAATCATTTTCTACATCGG
SzR1 E3Q	CGCACCGATGTAGAAAATGATCT GCTCCATATGTATATCTCCTTC	GAAGGAGATATACATATGGAGC AGATCATTTTCTACATCGGTGCG
SzR1 E2Q/E3Q	CCCGCACCGATGTAGAAAATGAT CTGCTGCATATGTATATCTCCTTC TTAAA	TTTAAGAAGGAGATATACATATG CAGCAGATCATTTTCTACATCGG TGCGGG
SzR1 R67Q	CCGCATAGAACGCCCACTGGGTC CAGTAGATGCT	AGCATCTACTGGACCCAGTGGGC GTTCTATGCGG
SzR1 R67A	CGCATAGAACGCCCAAGCGGTCC AGTAGATGCTG	CAGCATCTACTGGACCGCTTGGG CGTTCTATGCG
SzR1 F70D	AGCTCACCGCATAGTCCGCCCAA CGGGTCC	GGACCCGTTGGGCGGACTATGCG GTGAGCT
SzR1 F70E	GCAGCTCACCGCATACTCCGCCC AACGGGTCCA	TGGACCCGTTGGGCGGAGTATGC GGTGAGCTGC
SzR1 F70A	GGACCCGTTGGGCGGCCTATGCG GTGAGCT	AGCTCACCGCATAGGCCGCCCAA CGGGTCC
SzR1 C75A	CAACCATCAGAAAGCTGGCGCTC ACCGCATAGAACG	CGTTCTATGCGGTGAGCGCCAGC TTTCTGATGGTTG
SzR1 C75S	GTTCTATGCGGTGAGCAGCAGCT TTCTGATGGT	ACCATCAGAAAGCTGCTGCTCAC CGCATAGAAC
SzR1 C75T	CAACCATCAGAAAGCTGGTGCTC ACCGCATAGAACG	CGTTCTATGCGGTGAGCACCAGC TTTCTGATGGTTG
SzR1 E81D	GCTCAGCAGCATGCTAATATCAA CCATCAGAAAGCTG	CAGCTTTCTGATGGTTGATATTA GCATGCTGCTGAGC
SzR1 E81Q	CAGCATGCTAATCTGAACCATCA GAAAGCTGCAG	CTGCAGCTTTCTGATGGTTCAGA TTAGCATGCTG
SzR1 E95D	CAGGCAGTTGAACACAATGATAT CCAGCTTAATGCTTTTATCG	CGATAAAAGCATTAAGCTGGATA TCATTGTGTTCAACTGCCTG
SzR1 E95Q	CCAGGCAGTTGAACACAATGATC TGCAGCTTAATGCTTTTATCGAT G	CATCGATAAAAGCATTAAGCTGC AGATCATTGTGTTCAACTGCCTG G
SzR1 D184N	AAACCTTGGTGATCAGATTCAGC ACCAGGTAAAACAG	CTGTTTTACCTGGTGCTGAATCT GATCACCAAGGTTT
SzR2 H82F	CGCAGAACCTTCGCAATGAAATA GATCAGCAGGCTGCA	TGCAGCCTGCTGATCTATTTCATT GCGAAGGTTCTGCG
SzR2 E113Q	CACAGATCGCTCACCTGCGCCGC CACGAAGC	GCTTCGTGGCGGCGCAGGTGAGC GATCTGTG
SzR2 D197E	CGCACCAGTTGATTGTACTCCTT GTAGAACACGAT	ATCGTGTTCTACAAGGAGTACAA TCAACTGGTGCG
SzR2 D197N	CAAAATCGTGTTCTACAAGAACT ACAATCAACTGGTGCG	CGCACCAGTTGATTGTAGTTCTT GTAGAACACGATTTTG

Table S5. List of primers used for subcloning into mammalian expression vector.

Gene	Sense primers	Anti-sense primers
SzR_AM_5_00977	TTCGAATTCGCCACCATGG AGCAGATCATTTTCTAC	GCTCTTTGGATCCCCTTGT TCGATGTGCTTAAAGG
SzR1	TTCGAATTCGCCACCATGG AGGAAATCATTTTCTAC	GCTCTTTGGATCCCCCTTG CTAAATTTCAGGGTGG
SzR3	TTCGAATTCGCCACCATGG AGGAAATCATTTTCTTTG	GCTCTTTGGATCCCCGTTT TTGGTATACTTAAAGG
SzR_TE_8S_00242	TTCGAATTCGCCACCATGG AGGAAATCATCTTCTAC	GCTCTTTGGATCCCCCGCT TCGGTCGCCTCTTTC
SzR_TE_S2S_00499	TTCGAATTCGCCACCATGG CGGGCGAGGAATTCATC	GCTCTTTGGATCCCCTTTA ACACGGGTATACAGG
SzR_un_Tekir_02407	TTCGAATTCGCCACCATGG AGGAAATCATTTTCTAC	GCTCTTTGGATCCCCCATG TTGGTATACTTCAGG
SzR2	TTCGAATTCGCCACCATGA TTGAGACCCTGACCATG	GCTCTTTGGATCCCCACGC ACCAGTTGATTGTAGTC
SzR_TE_S2S_00499-cMyc	TCAGAAGAGGATCTGTTCT GCTACGAGAACG	GATGAGTTTTTGTTCCTTG TACAGCTCGTC

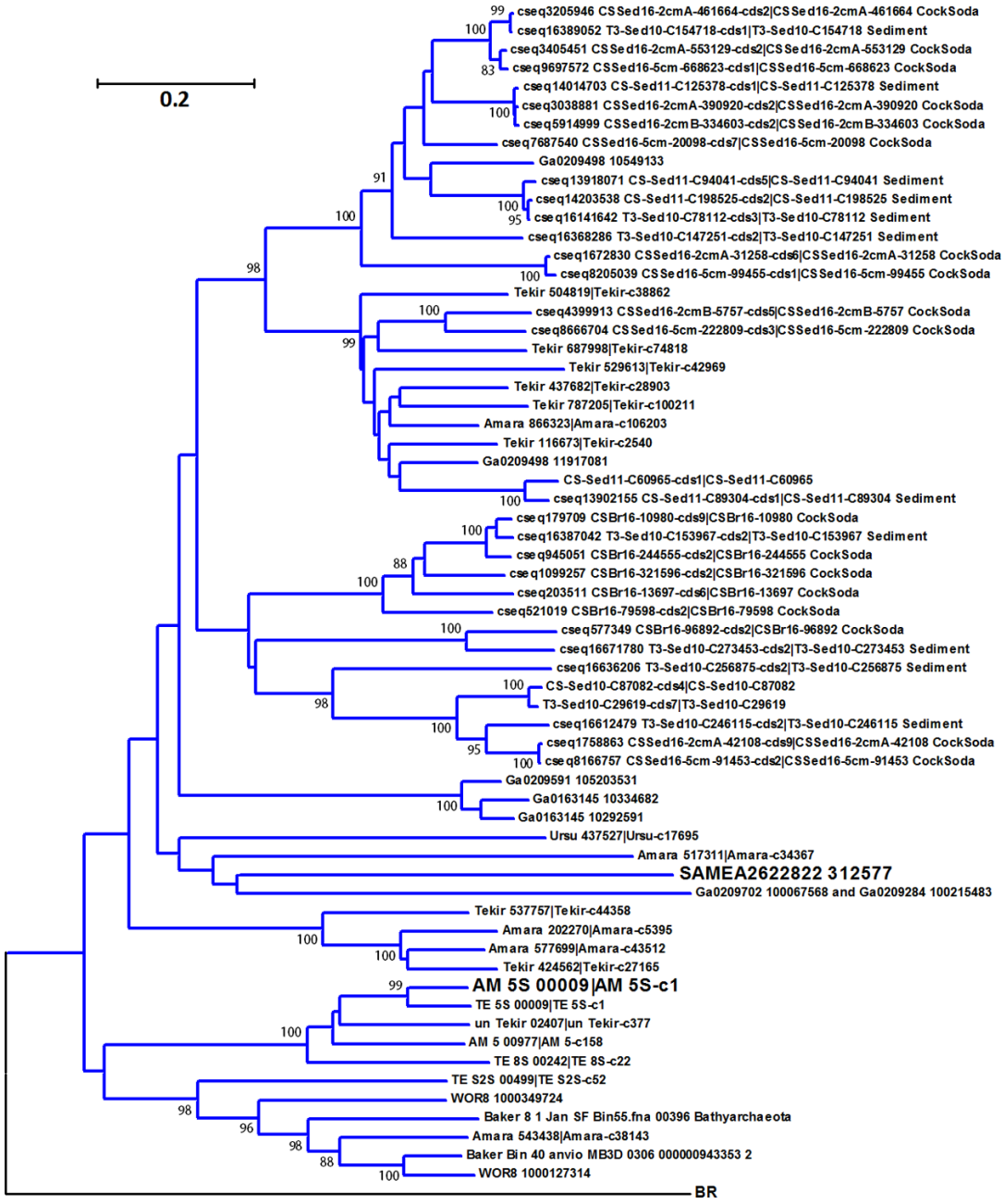

Fig. S1. Phylogenetic tree of SzRs. To investigate the phylogenetic relationship of 63 SzRs with BR as an out-group, their amino acid sequences were aligned using ClustalW (*5*). The evolutionary history was inferred using the Neighbor-Joining method (*5*). The optimal tree with the sum of branch length = 12.94306572 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (*5*). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (*5*) and are in the

units of the number of amino acid substitutions per site. The analysis involved 64 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 263 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (*5*). The percentage of replicate trees higher than 80, in which the associated taxa clustered together in the bootstrap test, are shown next to the branches.

Fig. S2. Multiple amino acid sequential alignments of SzRs with typical microbial rhodopsins. The amino acid sequences were aligned using ClustalW (*5*). Bacteriorhodopsin (BR), *Natronomonas pharaonis* halorhodopsin (*Np*HR), green-absorbing proteorhodopsin (GPR), *Krokinobacter* rhodopsin 2 (KR2), *Parvularcula oceani* XeR (*Po*XeR), *Chlamydomonas reinhardtii* channelrhodopsin 2 (*Cr*ChR2), and HeRs (HeR 48C12, *Thermoplasmatales* archaeon SG8521 heliorhodopsin (*Ta*HeR) and *Emiliania huxleyi* virus 156 HeR AHA55390.1) were aligned with the sequences of SzRs. Typical microbial

rhodopsins and HeRs are denoted in yellow and cyan. The residue numbers of BR and SzR1 (SzR AM_5S_00009) are shown on top of the residues, and the position of transmembrane helix of BR based on the X-ray crystallographic structure (PDB ID: 1M0L) are indicated by green rectangles.

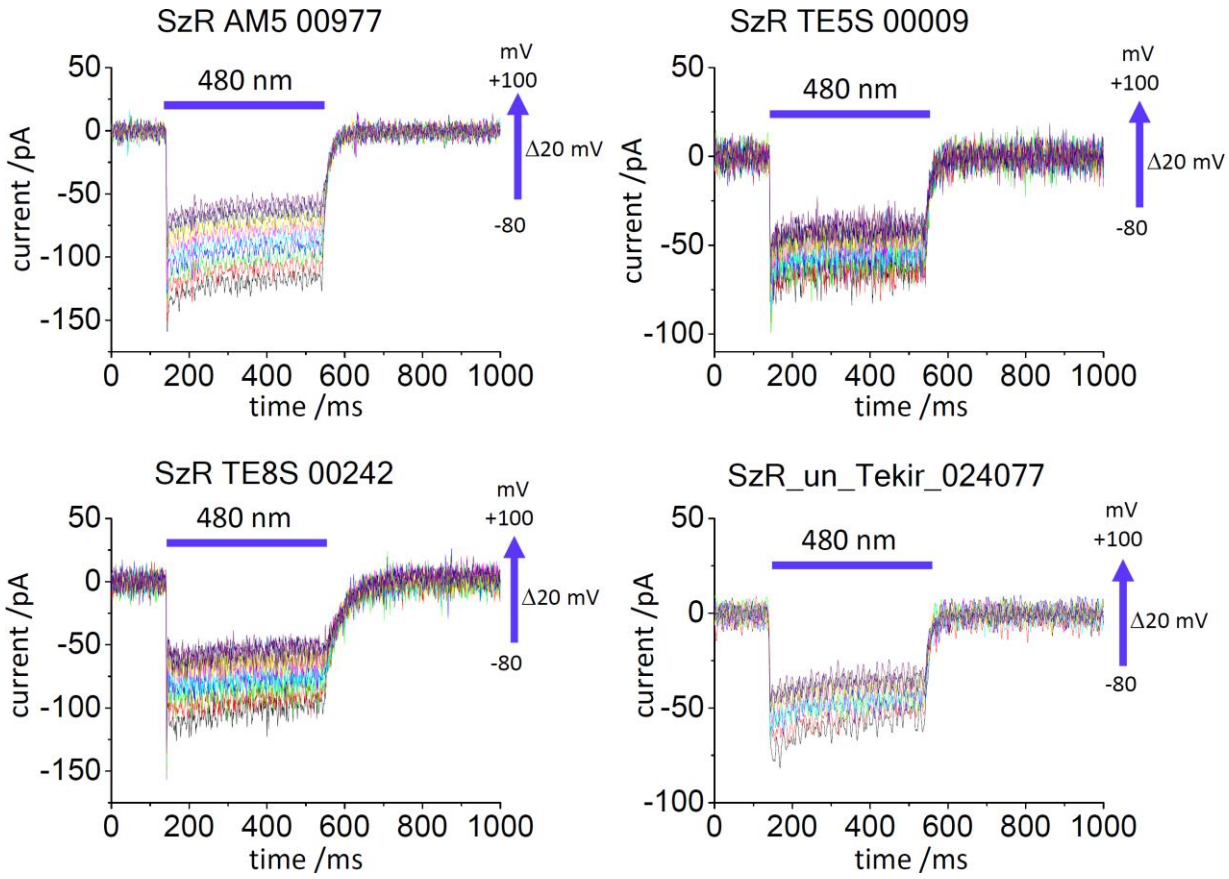

Fig. S3. Electrophysiological measurements of SzR-driven photocurrent in ND7/23 cells. The cells were illuminated with light (λ = 480 nm) during the time region shown by blue bars. The membrane voltage was scanned from –80 to +100 mV for every 20 mV.

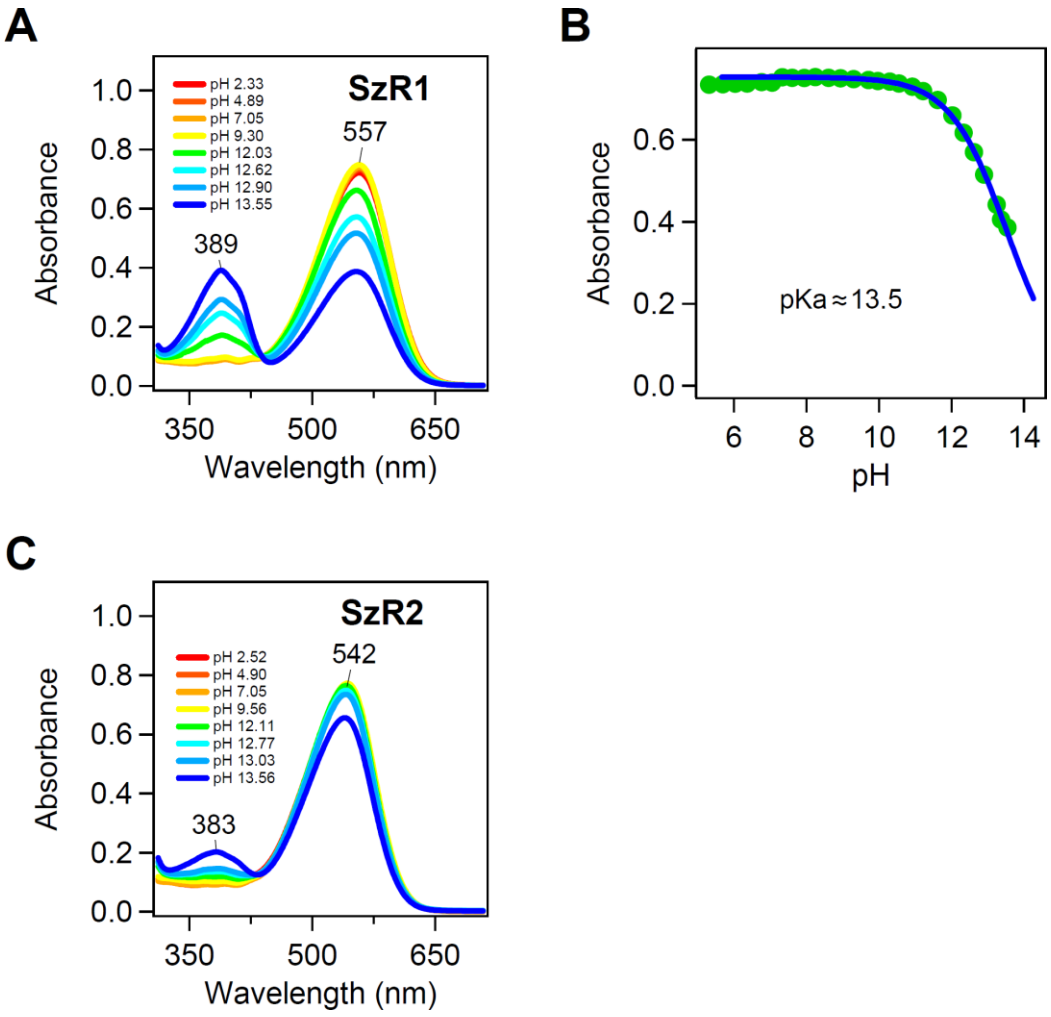

Fig. S4. pH dependence of the absorption of SzR1 and SzR2. (**A** and **B**) Absorption spectra of SzR1 (A) and the absorbance at 557 nm (B) at different pH. SzR1 showed a significant deprotonation of RSB at alkaline pH, and its pKa was estimated to be ~13.5 by fitting the absorption change at 557 nm (green circles in B) with the Henderson–Hasselbalch equation (blue solid line in B). (**C**) Absorption spectra SzR2 at different pH. The proteins were solubilized in 100 mM NaCl, 6-mix buffer (citrate, MES, HEPES, MOPS, CHES, CAPS (25 mM each)), and 0.05% DDM.

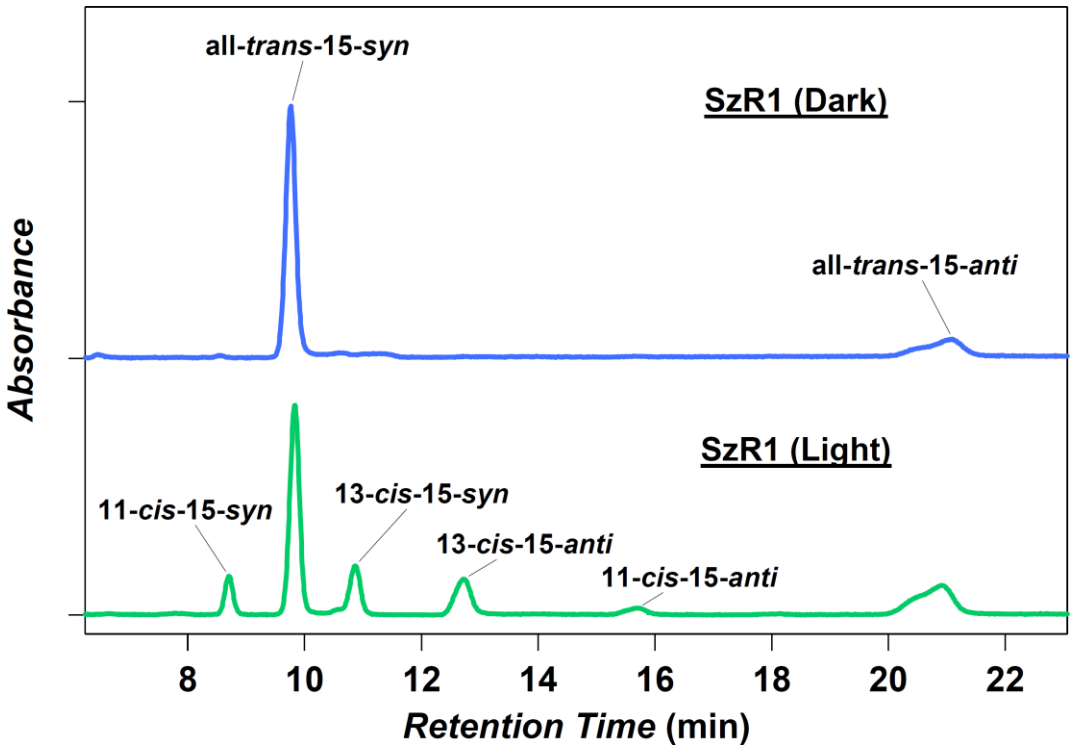

Fig. S5. HPLC analysis of the retinal configuration in SzR1. The HPLC pattern of the retinal extracted from SzR1 both in dark and light-adapted conditions. The compositions of the retinal isomers in dark and light-adapted conditions are shown in Table S1.

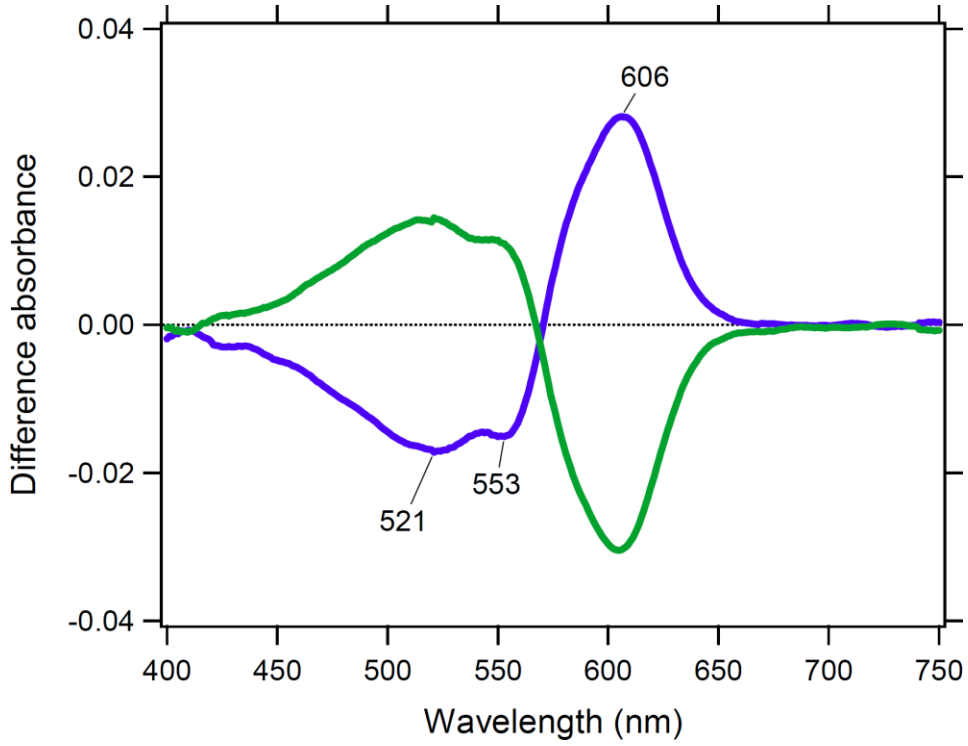

Fig. S6. Light-induced low-temperature differences in UV-visible absorption spectroscopy of SzR1. The K-minus-dark (blue) and dark-minus-K (green) light-induced low-temperature differences UV-visible absorption spectra obtained by illuminating at 520 and > 590 nm at T = 110 K.

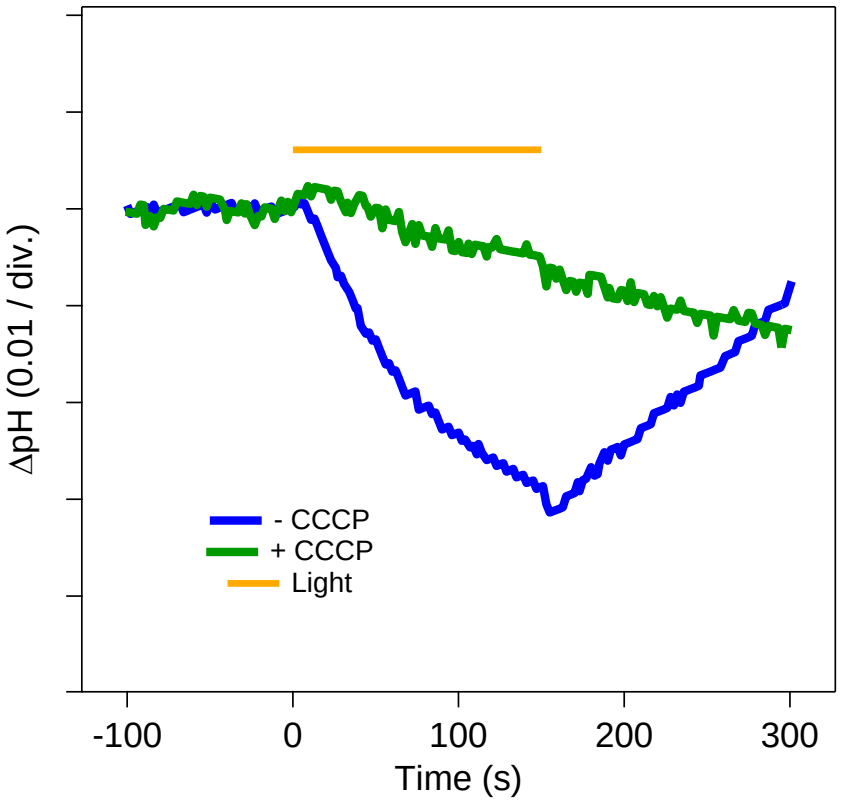

Fig. S7. Light-driven active inward H^+ transport by Heimdallarchaeia DTK rhodopsin. The signals were measured in the absence (blue) and presence (green) of CCCP in 100 mM NaCl at pH ~ 7.0. Light (λ > 500 nm) was illuminated at t = 0–150 s (indicated with a yellow bar).