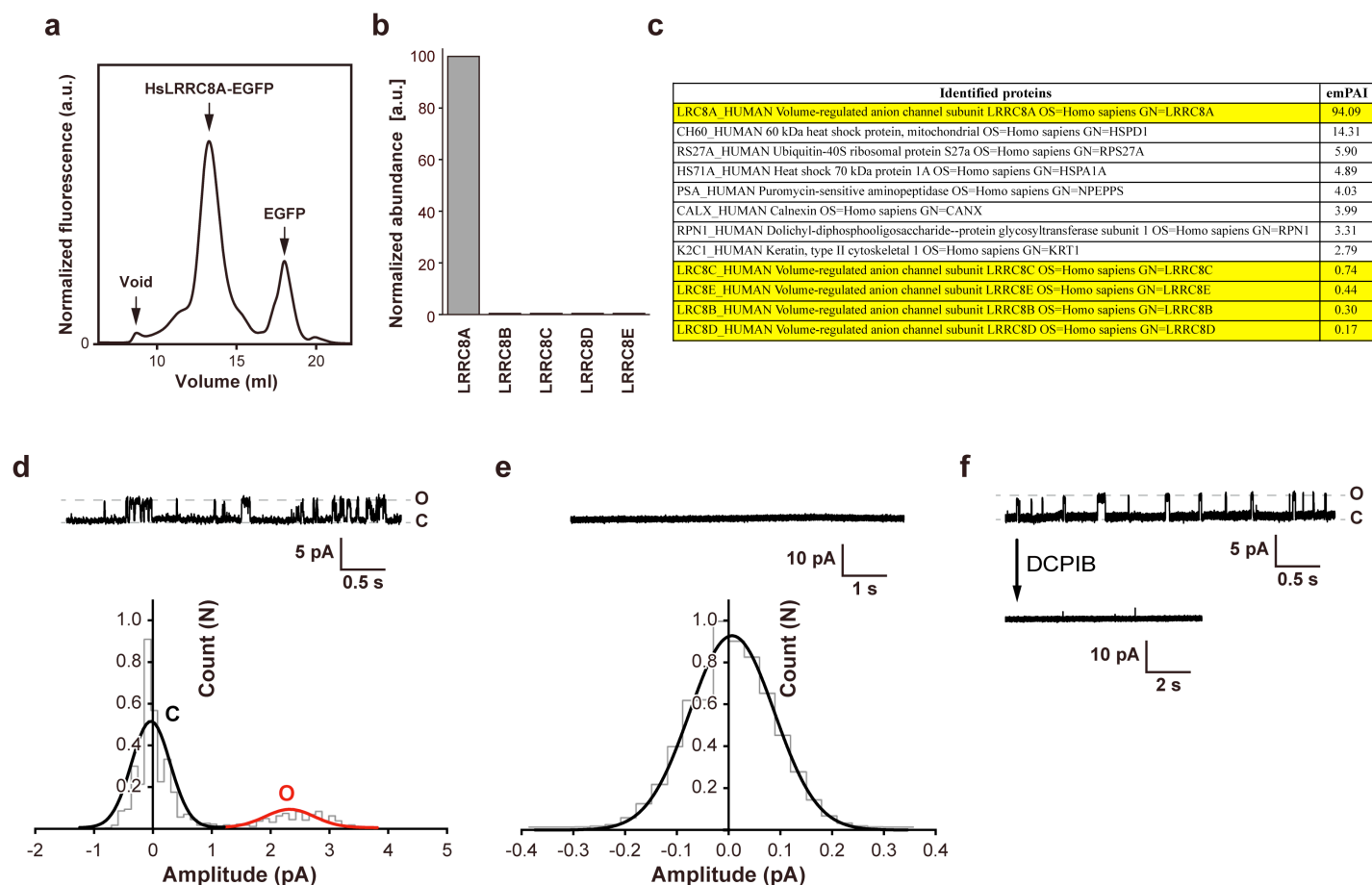


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Cryo-EM structures of the human volume-regulated anion channel LRRC8

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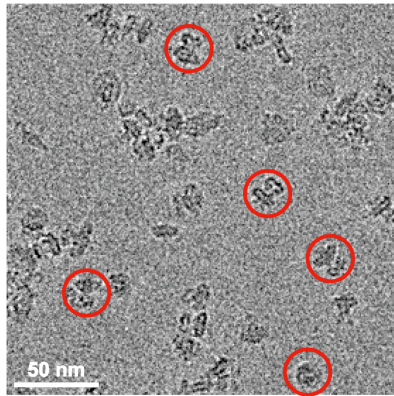
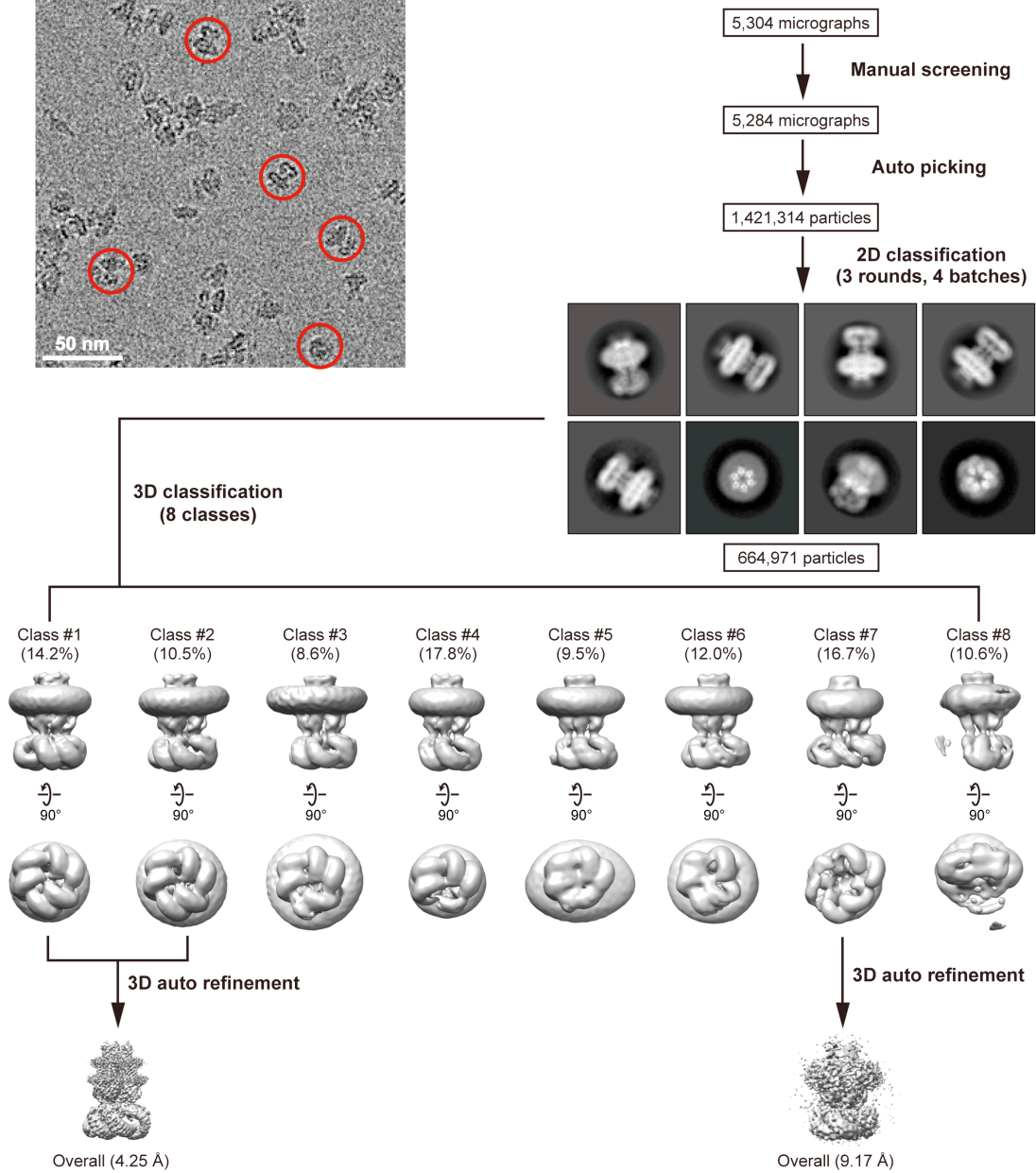
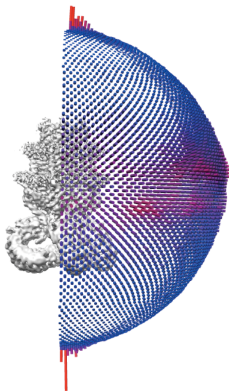
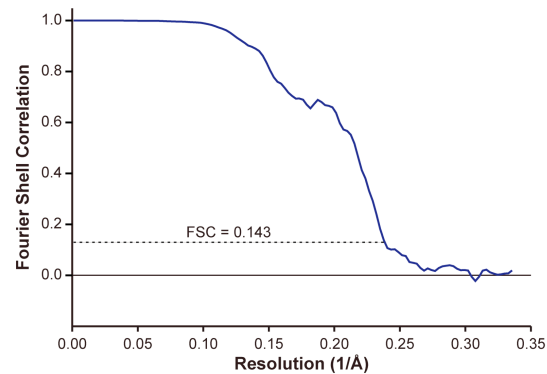
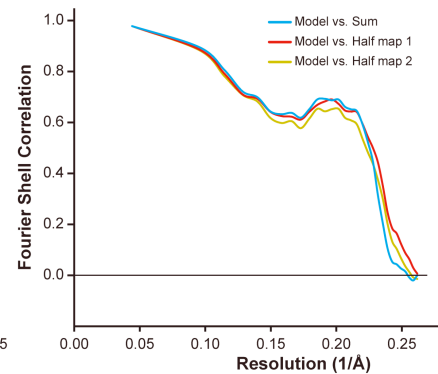
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Supplementary Figure 1

Functional properties of the HsLRRC8A protein.

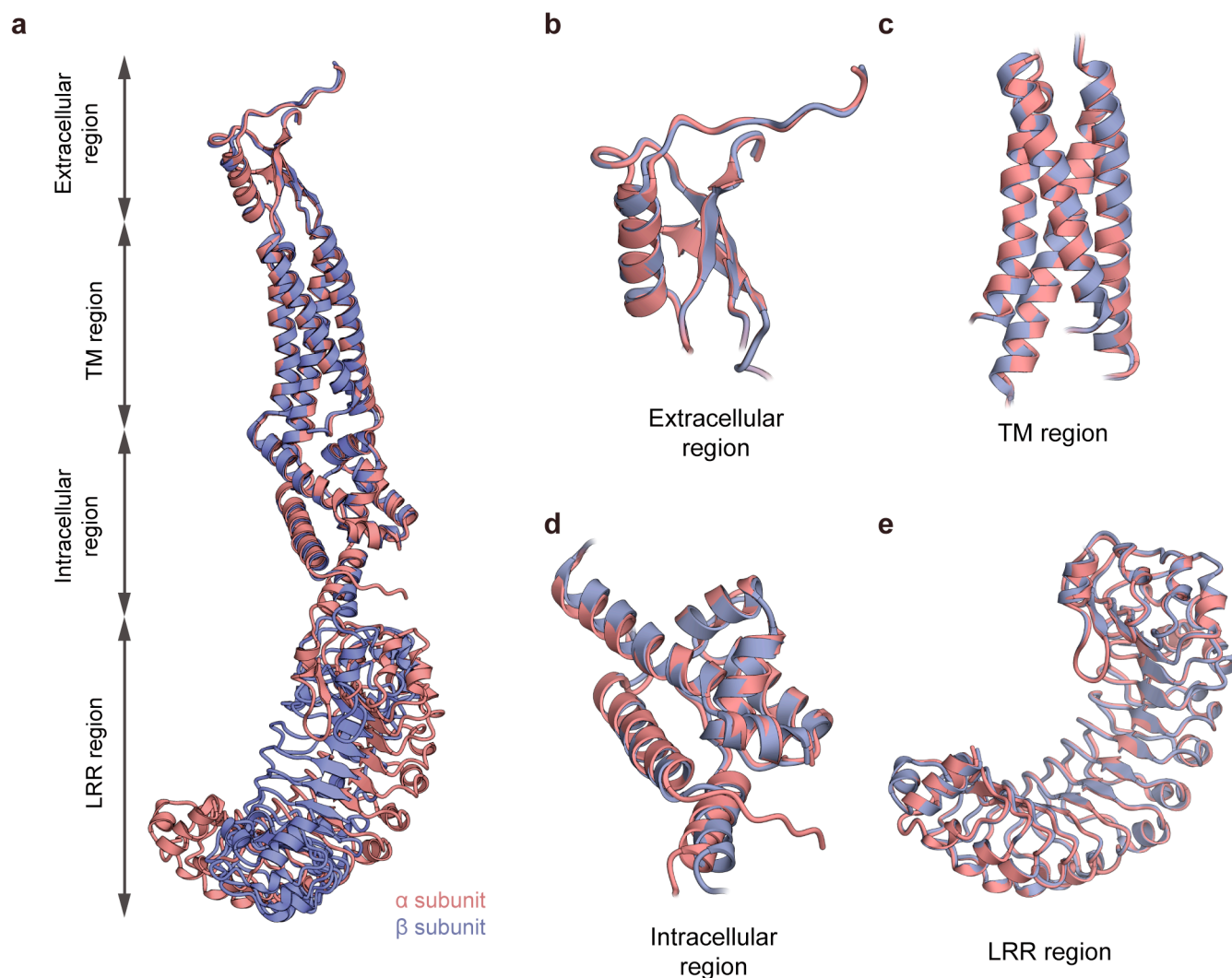
a, FSEC profile on a Superose 6 Increase 10/300 GL column (GE Healthcare) for the EGFP-fused HsLRRC8A expressed in HEK293S GnT1⁻ cells. The arrows indicate the estimated elution positions of the void volume, EGFP-fused HsLRRC8A, and free EGFP. **b**, Abundance of each LRRC8 protein normalized to the LRRC8A isoform, as estimated by mass spectrometry. **c**, List of proteins detected by mass spectrometry from the purified HsLRRC8A protein samples. According to the emPAI (The Exponentially Modified Protein Abundance Index) score, which correlates to the amount of each protein contained in the tested sample, the LRRC8 isoforms (highlighted in yellow) and other detected proteins are listed in descending order of their amounts. **d**, Single-channel current recordings of the HsLRRC8A protein after reconstitution in liposomes under asymmetric salt conditions (500 mM KCl in bath, 70 mM KCl in pipette) and normalized all-points amplitude histogram analysis of HsLRRC8A in the open and closed states at 100 mV. The gray dashed lines indicate the closed (C) and open (O) states. For the plot, the bin width is set at 0.1 pA/bin and the total count of events is normalized to 1.0. The distribution data were fit by the sum of two Gaussians, and the peaks correspond to the closed (C) and open (O) states. **e**, Single-channel current recordings of HsLRRC8A after reconstitution in liposomes under symmetric salt conditions (70 mM KCl in bath, 70 mM KCl in pipette) and normalized all-points amplitude histogram analysis of HsLRRC8A at 100 mV. **f**, HsLRRC8A-induced currents were blocked by 40 μ M DCPIB.

a**b****c****d****e**

Supplementary Figure 2

Cryo-EM analysis of HsLRRC8A.

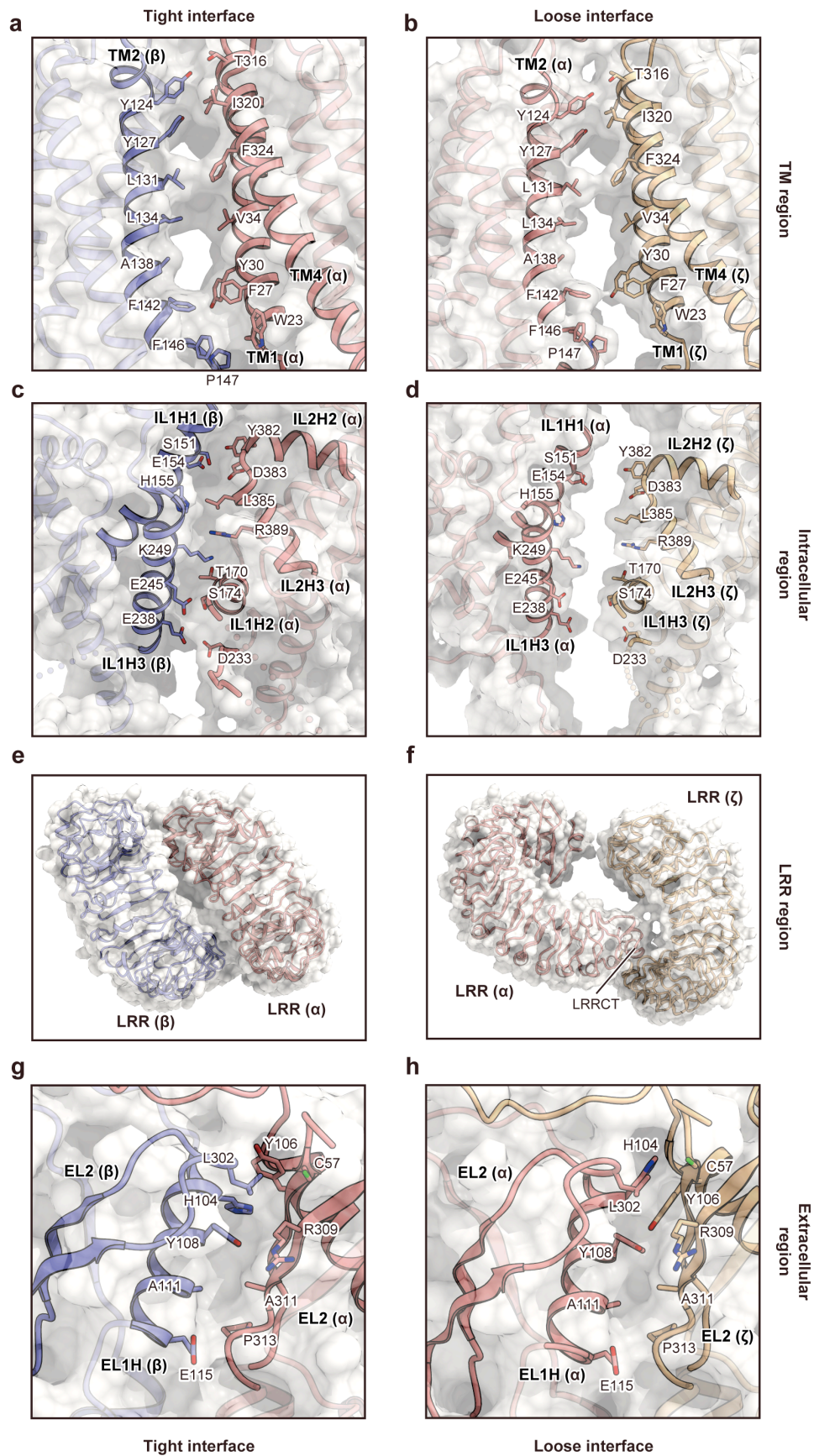
a, A representative cryo-EM micrograph of HsLRRC8A. Red circles indicate individual particles. The white scale bar represents 50 nm. **b**, Flowchart of cryo-EM data processing of the HsLRRC8A structure, including particle picking, classification, and 3D refinement. **c**, Angular distribution plot of particles included in the final 3D reconstruction of HsLRRC8A, with C3 symmetry imposed. **d**, Fourier shell correlation (FSC) curve of the final 3D reconstruction model calculated using `relion_postprocess` with masked resolution of 4.25 Å, corresponding to the gold-standard cutoff criterion of $\text{FSC} = 0.143$. **e**, Cross-validation FSC curves for the refined model versus half maps (Half maps 1 and 2) and versus the summed map.



Supplementary Figure 3

Structural comparison of two adjacent subunits.

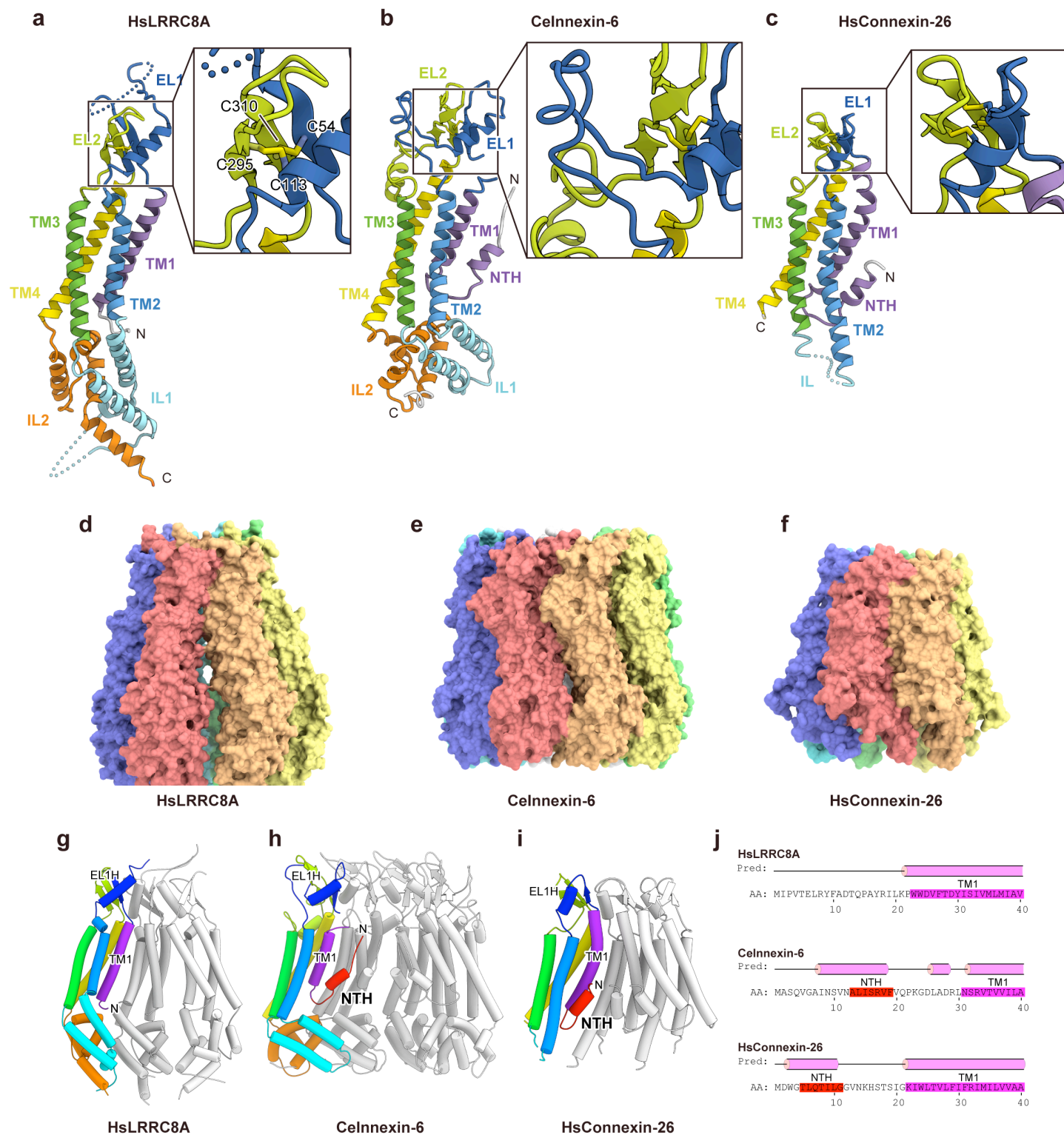
a, Two adjacent subunits (α and β subunits) of HsLRRC8A are superimposed on each other. The RMSD value for the 553 $C\alpha$ atoms from the subunits is 3.79 Å. For clarity, the overall subunits superimposed using only the TM region are presented. **b**, The extracellular regions from two adjacent subunits are superimposed on each other. The RMSD value for the 68 $C\alpha$ atoms from the two extracellular regions is 0.39 Å. **c**, The transmembrane regions from two adjacent subunits are superimposed on each other. The RMSD value for the 110 $C\alpha$ atoms from the two extracellular regions is 0.40 Å. **d**, The intracellular regions from two adjacent subunits are superimposed on each other. The RMSD value for the 119 $C\alpha$ atoms from the two extracellular regions is 0.98 Å. **e**, The LRR regions from two adjacent subunits are superimposed on each other. The RMSD value for the 395 $C\alpha$ atoms from the two extracellular regions is 0.47 Å.



Supplementary Figure 4

Subunit interfaces between two adjacent subunits.

a,b, Close-up views between two adjacent subunits at the transmembrane region, showing the tight (**a**) and loose (**b**) interactions. **c,d**, Close-up views between two adjacent subunits at the intracellular region, showing the tight (**c**) and loose (**d**) interactions. **e,f**, Close-up views between two adjacent subunits at the LRR region, showing the tight (**e**) and loose (**f**) interactions. **g,h**, Close-up views between two adjacent subunits at the extracellular region, showing the tight (**g**) and loose (**h**) interactions. A surface model with the side chains of the residues lining the subunit interfaces depicted by stick models is presented for each panel. Three adjacent subunits (α , β , and ζ subunits) of HsLRRC8A are colored according to Fig. 1d.

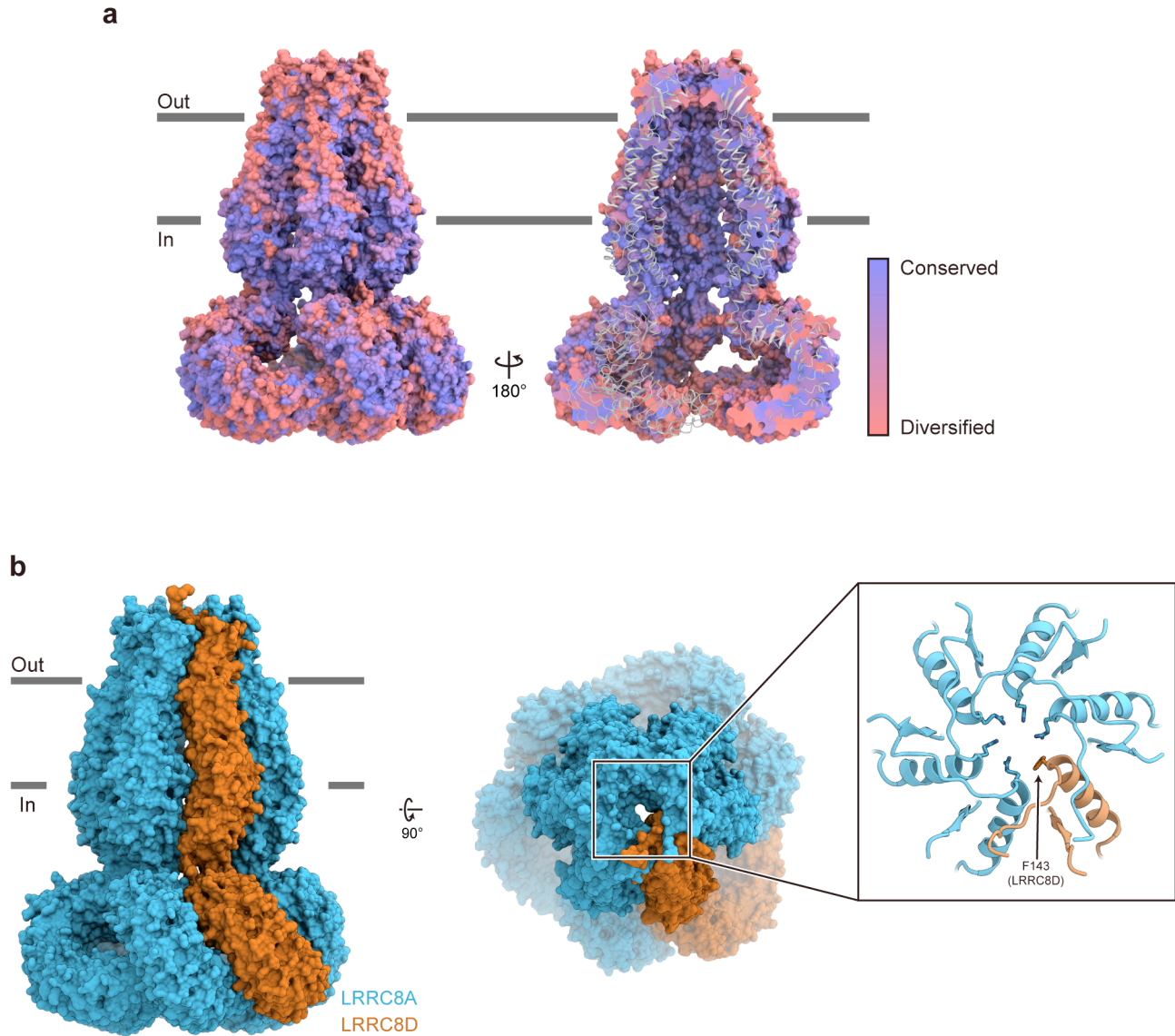


Supplementary Figure 5

Structural similarity between HsLRRC8A, innexin, and connexin.

a–c. The N-terminal halves of the HsLRRC8A subunit (**a**), the Celnnexin-6 (PDB 5H1Q) subunit (**b**), and the HsConnexin-26 (PDB 2ZW3) subunit (**c**) are presented. The subunits of Celnnexin-6 and HsConnexin-26 are colored according to HsLRRC8A coloring. The

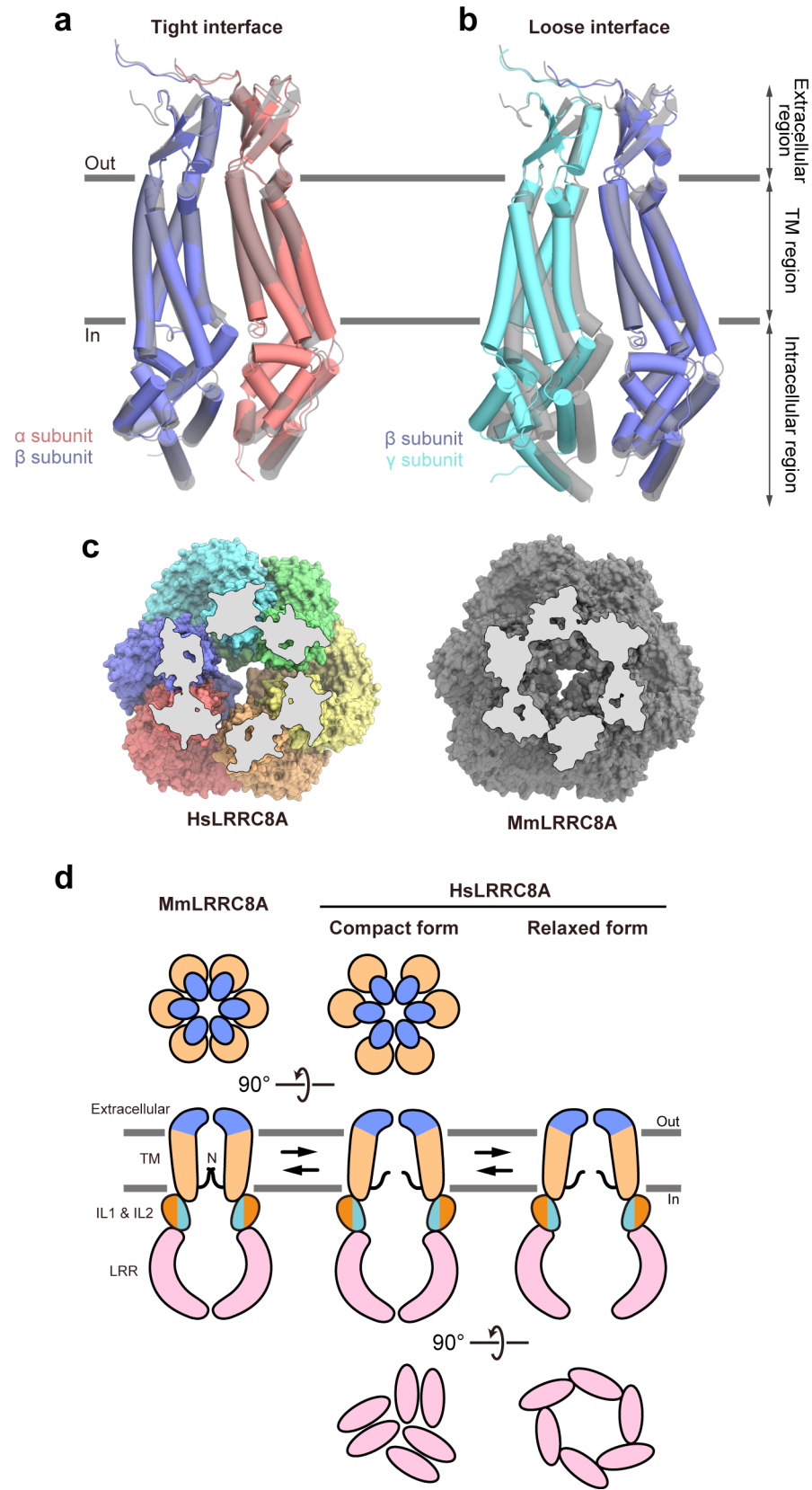
N and C termini are indicated by "N" and "C", respectively. In each structure, the cysteine residues forming disulfide bonds at the extracellular region are depicted by stick models. In each panel, a close-up view of the disulfide bonds is shown in the inset. **d–f**, Solvent-excluded molecular surfaces of HsLRRC8A (**d**), Celnnexin-6 (**e**), and HsConnexin-26 (**f**). The surfaces are colored according to subunit. For HsLRRC8A, the extracellular, TM, and intracellular regions are shown. **g–i**, Channel pore and N-terminal region of HsLRRC8A (**d**), Celnnexin-6 (**e**), and HsConnexin-26 (**f**). The protein main chains are shown as cylinder models. **j**, Secondary structure prediction of the N-terminal regions of HsLRRC8A, Celnnexin-6, and HsConnexin-26. Prediction was performed by the program PSIPRED (Buchan, D. W. A. *et al. Nucleic Acids Res.* **38**, W563–W568, 2010). The actual locations of the corresponding helices are indicated by the red and pink boxes for NTH and TM1, respectively.



Supplementary Figure 6

Model of the heteromeric LRRC8 protein.

a, Conservation score mapped on the molecular surface of HsLRRC8A. The score was calculated by the program CONSURF²⁶, based on the multiple-sequence alignment of the A–E isoforms shown in the Supplementary Note. The molecular surface is colored according to normalized conservation score, from 0.752 (pink) to –0.983 (purple). **b**, The model structure of the (LRRC8A)₅·LRRC8D heterohexamer, viewed parallel to the membrane (left) and from the extracellular side (right). The homology model of human LRRC8D was generated by the program MODELLER (Fiser, A. & Sali, A. *Methods Enzymol.* **374**, 461–491, 2003), using the alignment in the Supplementary Note, and then the α subunit of the present structure was replaced by this model. In the inset, a close-up view of the channel-pore-forming regions from the extracellular side is shown, and the amino-acid side chains of the constriction site are depicted in stick representations. The five LRRC8A subunits are colored light blue, while the LRRC8D isoform is colored light brown.



Supplementary Figure 7

Structural comparison with MmLRRC8A.

a,b, Subunit interfaces between the α and β subunits (**a**) and between the β and γ subunits(**b**). The cylinder models of MmLRRC8A (semitransparent gray) and HsLRRC8A (color-coded as in Fig. 1c) are shown. The extracellular and TM regions of the β subunits of MmLRRC8A (PDB 6G9O) and HsLRRC8A are superimposed on each other. The RMSD value for the 187 C α atoms from the subunits is 0.99 Å. **c**, Cross-sections of the TM regions of HsLRRC8A (left) and MmLRRC8A (right), viewed from the extracellular side. **d**, Schematic drawing depicting the working model for the structural transition of LRRC8, based on the currently available LRRC8A structures.

TM1 EL1β

α1 β1

1 10 20 30 40 50 60 70

HsLRRC8A MIPVTELRIFYADTQPAYRI LKPWWDVETDYISIVMLMIAVFGGTLQVTQDKMI. CLPCKWVTKDSCNDSFRGWAA.....

MmLRRC8A MIPVTELRIFYADTQPAYRI LKPWWDVETDYISIVMLMIAVFGGTLQVTQDKMI. CLPCKWVTKDSCNDSFRGWAA.....

HsLRRC8B MITLTLELKCLADAQSSYHILKPWWDVFWYITLIMLLVAVLAGALQLTQSRVLC. CLPCKVEFDNHCAVPWDILKA.....

MmLRRC8B MITLTLELKCLADAQSSYHILKPWWDVFWYITLIMLLVAVLAGALQLTQSRVLC. CLPCKVEFDNHCAVPWDILKA.....

HsLRRC8C MIPVTEFRQFSEQQPAFRVLKPWWDVFTDYLSVAMLMIGVFGCTLQVMQDKII. CLPKRVQPAQNHSLSNVSA.....

MmLRRC8C MIPVTEFRQFSEQQPAFRVLKPWWDVFTDYLSVAMLMIGVFGCTLQVMQDKII. CLPKRVQPAQNHSLSNVSA.....

HsLRRC8D MFTLAEVASLNDIQPTYRILKPWWDVFMDDYLAVMMLMVAIFAGTMQLTKDQVV. CLPVLPSPVNSKAHTPPGNAEVTNI

MmLRRC8D MFTLAEVASLNDIQPTYRILKPWWDVFMDDYLAVMMLMVAIFAGTMQLTKDQVV. CLPVLPSPVNSKAHTPPGNAEVTNI

HsLRRC8E MIPVAEFKQFTEQQPAFKV LKPWWDVLAELYLTVAMLMIGVFGCTLQVTQDKII. CLPNHELQENL.....SEA.....

MmLRRC8E MIPVAEFKQFTEQQPAFKV LKPWWDVLAELYLTVAMLMIGVFGCTLQVTQDKII. CLPSHESRENI.....SGA.....

EL1H

α2

80 90 100 110

HsLRRC8APGPEPTYPNSTILTPD.....TGPTGIKYDLD RHQYNYVDVAVCYENR L

MmLRRC8ASSPEPTYPNSTVLTPD.....TGPTGIKYDLD RHQYNYVDVAVCYENR L

HsLRRC8BSMNTSS.....NPGTPL.....PLPLRIQNDLHRQYNYVDVAVCYEKQL

MmLRRC8BSENASS.....NSGILL.....PLPLRIQNDLHRQYNYVDVAVCYEKQL

HsLRRC8CVASTTLPPEPK.....PSPANPITVEMKGLKTDLDLOQYVFNTNQMCMYERAL

MmLRRC8CVISTTLPPEPK.....PSPTNPATVEMKGLKTDLDLOQYVFNTNQMCMYERAL

HsLRRC8D PKMEAATNQDQDGR.TNDISFGTSAVTPDIPLRATYPRTDFA LPNQEAKEKKDPTGRKTNLDFQYVFNTNQMCMYERAL

MmLRRC8D PRMETATHQDQNGQT.TNDVAFGTSAVTPDIPLRATYPRTDFA LPNQEAKEKKDPTGRKTNLDFQYVFNTNQMCMYERAL

HsLRRC8EPCQQLLRGI.....P.EQIGALQEVKGLKNNL DLOQYVFNTNQMCMYERAL

MmLRRC8EPCQQLLRQGI.....S.EQMGGRLRELSGLKNNL DLOQYVFNTNQMCMYERAL

TM2 IL1H1 IL1H2

α3 α4 α5 α6

120 130 140 150 160 170 180 190

HsLRRC8A HWFAKFFPYLVLLHTLIFLACSNFWFKFPRTS SKLEHFVS IL LKCFDSPWTT RALSETVVEESDPKPAFSKMNGS.MDKK

MmLRRC8A HWFAKFFPYLVLLHTLIFLACSNFWFKFPRTS SKLEHFVS IL LKCFDSPWTT RALSETVVEESDPKPAFSKMNGS.MDKK

HsLRRC8B HWFAKFFPYLVLLHTLIFAACSNFWLHYPTSSRLIEHFVA IL LKCFDSPWTT RALSETVAESVRLPKLSKSKIL...LS

MmLRRC8B HWFAKFFPYLVLLHTLIFAACSNFWLHYPTSSRLIEHFVS IL LKCFDSPWTT RALSETVAESVRLPKLSKSKIL...LS

HsLRRC8C HWYAKFFPYLVLIHTLVFMLCSNFWFKFPGSSSKIEHFVS IL LKCFDSPWTT RALSEVSGESEEKDNRRKNMNRSGTI.

MmLRRC8C HWYAKFFPYLVLIHTLVFMLCSNFWFKFPGSSSKIEHFVS IL LKCFDSPWTT RALSEVSGESEEKDNRRKNMNRSGTI.

HsLRRC8D PWYSKFFPYLALIHITILMVSSNFWFKYPKTCSKVEHFVS IL LKCFDSPWTT RALSETACDEEENKQIRITGAQTLPKHV

MmLRRC8D PWYSKFFPYLALIHITILMVSSNFWFKYPKTCSKVEHFVS IL LKCFDSPWTT RALSETACDEEENKQIRITGAQTLPKHV

HsLRRC8E HWYTKFFPYLVLIHTLIFMVCTSFWFKFPGTS SKIEHFVS IL LKCFDSPWTT RALSEVSGENQKGPAAATERAAATVAMA

MmLRRC8E HWYTKFFPYLVLIHTLIFMVCTSFWFKFPGTS SKIEHFVS IL LKCFDSPWTT RALSEVSGENHKGPA SG.RAMVTTVT TTT

IL1H3 TM3

α7 α8

200 210 220 230 240 250 260 270

HsLRRC8A SSTVSEDVEA....TVPMLQRTKSRIEQGIVDRSETGVLDKKGEQAKALFEKVKKFRTHVEEGDILVYRIYMRQTIKVV

MmLRRC8A SSTVSEDVEA....TVPMLQRTKSRIEQGIVDRSETGVLDKKGEQAKALFEKVKKFRTHVEEGDILVYRIYMRQTIKVV

HsLRRC8B SSGCSADIDS....GKQSLPYPPQGL ESAGIESPTSSVLDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

MmLRRC8B SSGCSADIDA....SKQSLPYPPQGL ESAGIESPTSSVLDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

HsLRRC8C TQGG...EGS....LVNSQSLKSIPEKFVVDKSTAGALDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

MmLRRC8C TQGG...EGN....LVRSQSLKSIPEKFVVDKSTAGALDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

HsLRRC8D .STSSDEGSPSASTPMINKTGKFSAEKPVIEVPSMTILDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

MmLRRC8D .STSSDEGSPSASTPMINKTGKFSAEKPVIEVPSMTILDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

HsLRRC8E GTGP...GKA....GEGEKEKVLAEPEKVVTPEPVVTILDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

MmLRRC8E GAGS...GKV....GEGEKEKVLIEPEKVVTPEPVVTILDKKGEQAKALFEKVKKFRMHVEGQDILVYRIYMRQTIKVV

EL2β1 EL2β2 TM4

β2 β3 α9

280 290 300 310 320 330 340 350

HsLRRC8A IKFILLICYTVVYVHNKIFDVC TVDIESLTGYRTYRCAHPLATLFKI LASFYISLVIF YGLICMYELWMLLRSLKKYS

MmLRRC8A IKFALLICYTVVYVHNKIFDVC TVDIESLTGYRTYRCAHPLATLFKI LASFYISLVIF YGLICMYELWMLLRSLKKYS

HsLRRC8B ILFVLLICYTVVYFLTHITLEIDCSVDVQAF TGYKRYQCVYSLAEIFKVLASFYIVILVILYGLTSSYLWMLLRSLKKYS

MmLRRC8B ILFVLLICYTVVYFLSYITLEIDCSIDVQAF TGYKRYQCVYSLAEIFKVLASFYIVILVILYGLTSSYLWMLLRSLKKYS

HsLRRC8C IKFLIITAYNSALVSKVQFTVDCNVDIQD TGYKNFSNHTMAHLFSKLSFCYLCFVSIYGLTCLYLYWLFYRSLREYS

MmLRRC8C IKFLIITAYNSALVSKVQFTVDCNVDIQD TGYKNFSNHTMAHLFSKLSFCYLCFVSIYGLTCLYLYWLFYRSLREYS

HsLRRC8D AKFIIFLCYTANFVNAISFEHVCKPKVEHLI GYEVFECHNMA YMLKKLLISYISIIICVYGFI CLYLFWLFRIPTKEYS

MmLRRC8D AKFIIFLCYTANFVNAISFEHVCKPKVEHLI GYEVFECHNMA YMLKKLLISYISIIICVYGFI CLYLFWLFRIPTKEYS

HsLRRC8E CKFLAFLVYNYVVEKISFLVACRVETSEVTGYASFCNHTKAHLFSKLAFCYISFVCVYGITCLYLYWLFHRRPTKEYS

MmLRRC8E CKFFAFLVYNYVVEKISFLVACRVETSEVTGYASFCNHTKAHLFSKLAFCYISFVCVYGITCLYLYWLFHRRPTKEYS

IL2H1 IL2H2 IL2H3 IL2H4 LRRNT LRR1

α10 α11 α12 α13 α14 β4

360 370 380 390 400 410 420 430

HsLRRC8A FESIREESSYSDIPVKNDFAFMLHLIDQYDPLYSKRFAVFLSEVSENKLRQLNLNNEWTLDKLRQLRTKNAQDKIELHL

MmLRRC8A FESIREESSYSDIPVKNDFAFMLHLIDQYDPLYSKRFAVFLSEVSENKLRQLNLNNEWTLDKLRQLRTKNAQDKIELHL

HsLRRC8B FEALREKSNYSIDIPVKNDFAFMLHLADQYDPLYSKRFSIFLSEVSENKLRQLNLNNEWTVERKLSKLKVNQADKIELHL

MmLRRC8B FEALREKSNYSIDIPVKNDFAFMLHLADQYDPLYSKRFSIFLSEVSENKLRQLNLNNEWTVERKLSKLKVNQADKIELHL

HsLRRC8C FEYVROETGIDIPDKNDFAFMLHMDQYDPLYSKRFAVFLSEVSENKLRQLNLNNEWTDPKLRQLQTNHNNRIELPL

MmLRRC8C FEYVROETGIDIPDKNDFAFMLHMDQYDPLYSKRFAVFLSEVSENKLRQLNLNNEWTDPKLRQLQTNHNNRIELPL

HsLRRC8D FEKVREESSFSDIPVKNDFAFMLHMDQYDOLYSKRFGVFLSEVSENKLRQLNLNNEWTPEKLRQHVSRNAQDKIELHL

MmLRRC8D FEKVREESSFSDIPVKNDFAFMLHMDQYDOLYSKRFGVFLSEVSENKLRQLNLNNEWTPEKLRQHVSRNAQDKIELHL

HsLRRC8E FRSVREETGMDIPDKNDFAFMLHLIDQYDOLYSKRFAVFLSEVSESRQLQLNLNNEWTPEKLRQLQNRNMRGRIELSL

MmLRRC8E FRSVREETGMDIPDKNDFAFMLHLIDQYDOLYSKRFAVFLSEVSESRQLQLNLNNEWTPEKLRQLQNRNMRGRIELSL

Sequence alignment of LRRC8 isoforms

The amino acid sequences of LRRC8 isoforms were aligned using Clustal Omega (Sievers, F. *et al. Mol. Syst. Biol.* **7**, 2011), and are shown using ESPript3 (Robert, X. & Gouet, P. *Nucleic Acids Res.* **42**, 320–324, 2014). The secondary structure elements and the colors of the domains from HsLRRC8A are labeled above the alignments. For the sequence alignment, the human and mouse LRRC8 isoforms were used: human (HsLRRC8A, NCBI Reference sequence number: NP_062540.2; HsLRRC8B, NP_056165.1; HsLRRC8C, NP_115646.2; HsLRRC8D, NP_060573.2; HsLRRC8E, AAH70089.1) and mouse (MmLRRC8A, NP_808393.1; MmLRRC8B, NP_001028722.1; MmLRRC8C, NP_598658.1; MmLRRC8D, NP_848816.3; MmLRRC8E, NP_082451.2).