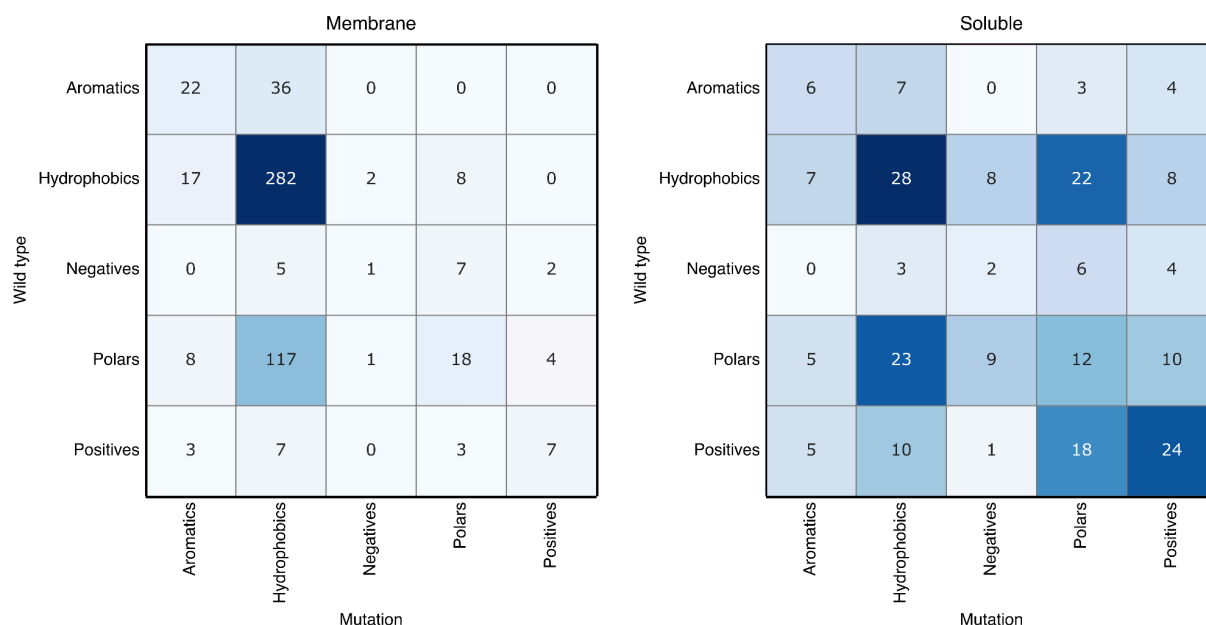
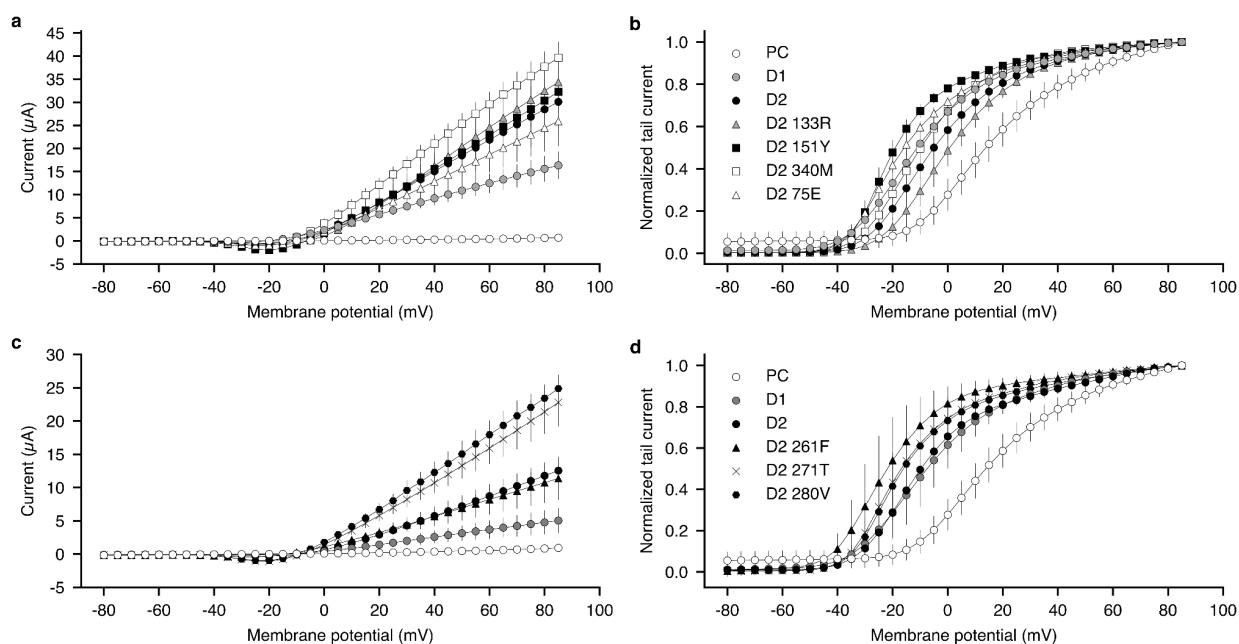


## Supplementary information



**Supplementary Figure 1. Comparing mutation types between the membrane and soluble domains.** Similar to Figure 2a, the analysis is based on a single design for each protein in the benchmark that mutates approximately 15% of the protein relative to the wild type.



**Supplementary Figure 2: Current-voltage relationship of Kv1.2-Kv2.1 paddle chimera and designs compared to single point mutation revertants.** (a & c) Currents of paddle chimera and Kv1.2-Kv2.1 paddle chimera designs are plotted against varying test voltages. Each curve represents recordings from 10 oocytes, and error bars represent the standard deviation. (b & d) Normalized tail currents were recorded at -50mV as a function of the test pulse voltage (see

inset Fig. 4e). Data for paddle chimera, D1, and D2 are the same as from Fig 4. Expression was not quantified in these experiments, but equal amounts of cRNAs were injected to decrease variability.

| Index | $\Delta\Delta G$ threshold | PSSM threshold | PSSM Weight |
|-------|----------------------------|----------------|-------------|
| 1     | -4                         | 5              | 0.2         |
| 2     | -4                         | 5              | 0.2         |
| 3     | -3.5                       | 4.5            | 0.2         |
| 4     | -3                         | 4              | 0.2         |
| 5     | -3                         | 3              | 0.3         |
| 6     | -2.5                       | 3              | 0.3         |
| 7     | -2.5                       | 2              | 0.3         |
| 8     | -2                         | 2              | 0.4         |
| 9     | -2                         | 2              | 0.4         |
| 10    | -2                         | 2              | 0.4         |
| 11    | -2                         | 2              | 0.4         |
| 12    | -2                         | 1              | 0.4         |
| 13    | -2                         | 1              | 0.4         |
| 14    | -1                         | 0              | 0.5         |
| 15    | -1                         | 0              | 0.5         |
| 16    | -0.5                       | 0              | 0.5         |
| 17    | 0                          | 0              | 0.5         |
| 18    | 0.5                        | 0              | 0.5         |

**Supplementary Table 1. Weights and thresholds for the computational mutational scan.**

$\Delta\Delta G$  threshold - the threshold over which mutations are discarded.

PSSM threshold - PSSM score under which mutations are discarded.

PSSM weight - the weight used to bias ref2015\_memb energy function with the PSSM score.

| PDB entry | Protein name         | # subunits | # amino acids in structure (single chain) |
|-----------|----------------------|------------|---|
| 1FX8      | Glycerol Facilitator | Tetramer   | 254                                       |

|      |   |          |     |
|------|---|----------|-----|
| 1K4C | KcsA Potassium Channel, H+ with Fab               | Tetramer | 103 |
| 1M0L | Bacteriorhodopsin                                 | Trimer   | 222 |
| 1OTS | H+/Cl- exchange transporter                       | Dimer    | 444 |
| 1U19 | Rhodopsin (bovine outer segment)                  | Monomer  | 348 |
| 2C3E | Mitochondrial ADP/ATP Carrier                     | Monomer  | 297 |
| 2UUI | (Apo) Leukotriene Synthase                        | Trimer   | 155 |
| 2VPZ | Polysulfide Reductase                             | Dimer    | 250 |
| 2XOV | Rhomboid-Family intramembrane protease            | Monomer  | 181 |
| 3B9W | Rh50 protein                                      | Trimer   | 362 |
| 3GIA | (Apo) ApcT Na+-independent Amino Acid Transporter | Monomer  | 433 |
| 3K3F | Urea Transporter                                  | Trimer   | 332 |
| 3KLY | FocA formate transporter w/o formate              | Pentamer | 257 |
| 3M71 | SLAC1 anion channel TehA homolog                  | Trimer   | 308 |
| 3O0R | Nitric Oxide Reductase subunit B                  | Monomer  | 449 |
| 3RLB | ThiT, S component of the Thiamin Transporter      | Dimer    | 176 |
| 3V5U | Sodium Calcium Exchanger (MCX)                    | Monomer  | 297 |
| 3ZOJ | AQY1 Yeast Aquaporin                              | Tetramer | 263 |
| 4A2N | Isoprenylcysteine carboxyl methyltransferase      | Monomer  | 192 |
| 4IKV | Proton-dependent oligopeptide transporter         | Monomer  | 492 |

**Supplementary Table 2. Protein structures used in the benchmark.**

| Positions according to PDB entry 2R9R*   | Action                                | Reason                                    |
|--|---------------------------------------|---|
| 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305 | Manually restricted from design.      | The voltage-sensitive domain (S3 and S4). |
| 366, 369, 370, 372, 374, 377   | Automatically restricted from design. | Proximity to the potassium ions.          |
| 34, 36, 42, 43, 44, 45, 46, 47, 50,  | Automatically restricted              | Proximity to chain F.                     |

|   |  |  |
|---|--|--|
| 86, 89, 90, 93, 97, 99, 102, 322,<br>323, 326, 329, 330, 332, 333, 336,<br>337, 340, 341, 343, 344, 348, 355,<br>356, 357, 358, 359, 361, 363, 367,<br>370, 371, 372, 373, 374, 376, 377,<br>378, 384, 387, 388, 391, 392, 395,<br>396, 399, 400, 401, 403, 404   | from design.   |  |
| 72, 73  | Automatically restricted<br>from design.                           | Proximity to chain G.  |
| 38, 40, 41, 42, 70, 73, 75, 77, 79,<br>80, 81, 82, 83, 103, 105, 107, 108,<br>111, 177, 180, 181, 182, 184, 185,<br>186, 189, 286, 290, 293, 294, 297,<br>300, 301, 303, 307, 308, 309, 310,<br>312, 313, 324, 327, 328, 331, 362,<br>365, 369, 370, 371, 372, 373, 375,<br>398, 402, 405, 406, 409, 410, 413,<br>414 | Automatically restricted<br>from design.                           | Proximity to chain H.  |
| 32, 33, 34, 415, 416, 417   | Automatically restricted<br>from design.                           | Proximity to chain edges.  |
| 52, 84, 122, 144, 148, 172, 200,<br>203, 206, 216, 217, 227, 237, 243,<br>248, 315, 318, 351, 397   | Mutations removed<br>manually after<br>mPROSS design<br>concluded. | Positions in segments with little<br>phylogenetic data, radical<br>mutations, mutations too close<br>to the pore or the S3-S4<br>voltage-sensitive domain. |

### Supplementary Table 3. Positions that were restricted from design.

\* Positions are numbered the same as in the 2R9R PDB.

| Design Name       | mPROSS<br>design index | # Mutations* | % Mutations** | Rosetta energy (R.e.u.) |
|-------------------|------------------------|--------------|---------------|-------------------------|
| Paddle<br>chimera |                        |              |               | -854                    |
|                   | 1                      | 2            | 1             | -910                    |
|                   | 2                      | 2            | 1             | -905                    |
|                   | 3                      | 2            | 1             | -909                    |
|                   | 4                      | 3            | 1             | -907                    |
|                   | 5                      | 4            | 2             | -910                    |
|                   | 6                      | 9            | 4             | -922                    |
|                   | 7                      | 11           | 5             | -935                    |

|    |    |    |    |      |
|----|----|----|----|------|
|    | 8  | 14 | 6  | -945 |
|    | 9  | 14 | 6  | -943 |
|    | 10 | 14 | 6  | -945 |
|    | 11 | 14 | 6  | -943 |
| D1 | 12 | 9  | 4  | -944 |
| D2 | 16 | 15 | 7  | -971 |
| D3 | 17 | 20 | 9  | -972 |
| D4 | 18 | 26 | 12 | -980 |

**Supplementary Table 4. Design mutational load**

\* Designs not selected for testing (design index 1-11) were not manually examined, and therefore have all designed mutations.

\*\* Percent of all designable positions (223 positions, which were resolved in the 2R9R PDB structure and were not restricted from design). R.e.u. — Rosetta energy units.

| 2R9R position | Long et al. numbering* | paddle chimera | D1 | D2 |
|---------------|------------------------|----------------|----|----|
| 56            | 75                     | E              | E  | D  |
| 114           | 133                    | R              | R  | K  |
| 132           | 151                    | Y              | Y  | F  |
| 242           | 261                    | F              | F  | L  |
| 252           | 271                    | T              | R  | R  |
| 261           | 280                    | V              | V  | L  |
| 321           | 340                    | M              | L  | L  |

**Supplementary Table 5. Mutated positions of interest.**

\* Numbering according to Long et al.(28).

| Construct    | Normalized current* | Normalized $V_{1/2}$ * |
|--------------|---------------------|------------------------|
| Non-injected | 0.08±0.05           | 0.0±0.0                |
| Wild-type    | 1.0±0.21            | 1.0±0.33               |
| D1           | 5.11±1.67           | -0.52±0.22             |

|         |            |            |
|---------|------------|------------|
| D2      | 13.74±1.79 | -1.03±0.17 |
| D3      | 4.95±3.39  |            |
| D4      | 10.91±8.60 |            |
| D2 75E  | 26.98±6.52 | -0.89±0.12 |
| D2 133R | 36.91±8.15 | -0.29±0.12 |
| D2 151Y | 33.55±4.2  | -1.07±0.12 |
| D2 261F | 12.42±3.68 | -1.1±0.26  |
| D2 280V | 27.1±2.58  | -1.55±0.49 |
| D2 271T | 25.16±4.11 | -0.92±0.24 |
| D2 340M | 45.67±7.47 | -0.68±0.24 |

**Supplementary Table 6: Mean currents and V1/2 from oocytes expressing paddle chimera Kv1.2-Kv2.1 paddle chimera and various derivatives (n≥10).** To account for variability between experiments, results from separate experiments were normalized relative to the paddle chimera.

\* Normalized to measurements of the paddle chimera from the same experiment.

### Supplementary File 1. RosettaScripts scripts and flags for refinement, mutational scan, and design.

All Rosetta calculations were done using the git commit  
b210d6d5a0c21208f4f874f62b2909f926379c0f.

Refinement within the virtual membrane was done using the following xml:

```
<ROSETTASCRIPTS>
<SCOREFXNS>
  <ScoreFunction name="full" weights="ref2015_memb">
    <Reweight scoretype="coordinate_constraint" weight="0.64"/>
    <Reweight scoretype="res_type_constraint" weight="0"/>
  </ScoreFunction>
  <ScoreFunction name="soft" weights="ref2015_soft">
    <Reweight scoretype="mp_res_lipo" weight="1"/>
    <Reweight scoretype="coordinate_constraint" weight="0.64"/>
    <Reweight scoretype="res_type_constraint" weight="0"/>
  </ScoreFunction>
  <ScoreFunction name="ref_no_pssm" weights="ref2015_memb">
    <Reweight scoretype="coordinate_constraint" weight="0.64"/>
  </ScoreFunction>
  <ScoreFunction name="ref_pure" weights="ref2015_memb"/>
  <ScoreFunction name="helicality" symmetric="0">
    <Reweight scoretype="mp_helicality" weight="1"/>
  </ScoreFunction>
</SCOREFXNS>
<RESIDUE_SELECTORS>
  <ResidueName name="pro" residue_name3="PRO"/>
  <ResidueName name="gly" residue_name3="GLY"/>
  <PrimarySequenceNeighborhood name="pre_pro" selector="pro" lower="1" upper="0"/>
  <Index name="ress_restrict" resnums="%%res_to_restrict%%"/>
  %%fix_prefix%%<Index name="res_to_fix" resnums="%%res_to_fix%%"/>
```

```

</RESIDUE_SELECTORS>
<TASKOPERATIONS>
<InitializeFromCommandline name="init"/>
<RestrictToRepacking name="rtr"/>
<OperateOnResidueSubset name="restrict_res" selector="ress_restrict">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
%%fix_prefix%%<OperateOnResidueSubset name="fix_res" selector="res_to_fix">
  %%fix_prefix%%<PreventRepackingRLT/>
  %%fix_prefix%%<OperateOnResidueSubset>
  %%fix_prefix%%<OperateOnResidueSubset name="not_to_cst_sc">
    %%fix_prefix%%<Not selector="res_to_fix"/>
    %%fix_prefix%%<PreventRepackingRLT/>
  %%fix_prefix%%<OperateOnResidueSubset>
</TASKOPERATIONS>
<MOVERS>
  <AddMembraneMover name="add_memb" membrane_core="10" steepness="4" span_starts="%%span_starts%%"
span_ends="%%span_ends%%" span_orientations="%%span_oris%%"/>
  %%fix_prefix%%<VirtualRoot name="virt_root" removable="true"/>
  %%fix_prefix%%<VirtualRoot name="virt_root_remover" remove="true"/>
  %%fix_prefix%%<AtomCoordinateCstMover name="fix_res_sc_cst" coord_dev="0.5" bounded="false" sidechain="true"
task_operations="not_to_cst_sc"/>
  <PackRotamersMover name="soft_repack" scorefxn="soft" task_operations="init,rtr,restrict_res%%fix_TO%%"/>
  <PackRotamersMover name="hard_repack" scorefxn="full" task_operations="init,rtr,restrict_res%%fix_TO%%"/>
  <RotamerTrialsMinMover name="RTmin" scorefxn="full" task_operations="init,rtr,restrict_res%%fix_TO%%"/>
  <MinMover name="soft_min" scorefxn="soft" chi="1" bb="1" jump="0"/>
  <MinMover name="hard_min" scorefxn="full" chi="1" bb="1" jump="0"/>
  <ConstraintSetMover name="add_CA_cst" cst_file="%%cst_full_path%%"/>
  <ParsedProtocol name="refinement_block">
    <Add mover_name="soft_repack"/>
    <Add mover_name="soft_min"/>
    <Add mover_name="soft_repack"/>
    <Add mover_name="hard_min"/>
    <Add mover_name="hard_repack"/>
    <Add mover_name="hard_min"/>
    <Add mover_name="hard_repack"/>
    <Add mover_name="RTmin"/>
    <Add mover_name="RTmin"/>
    <Add mover_name="hard_min"/>
  </ParsedProtocol>
  <LoopOver name="iter4" mover_name="refinement_block" iterations="4"/>
</MOVERS>
<FILTERS>
  <ScoreType name="stability_score_full" scorefxn="full" score_type="total_score" confidence="0" threshold="0"/>
  <ScoreType name="stability_without_pssm" scorefxn="ref_no_pssm" score_type="total_score" confidence="0" threshold="0"/>
  <ScoreType name="stability_pure" scorefxn="ref_pure" score_type="total_score" confidence="0" threshold="0"/>
  <Rmsd name="rmsd" confidence="0"/>
  <ResidueLipophilicity name="a_res_lipo" threshold="1000" confidence="0"/>
  <SpanTopologyMatchPose name="a_span_topo" confidence="0"/>
  <BuriedUnsatHbonds2 name="a_unsat" scorefxn="ref_pure" confidence="0"/>
  <TMsSpanMembrane name="a_tms_span" confidence="0" min_distance="25"/>
  <MembAccesResidueLipophilicity name="a_marl" confidence="0" verbose="0"/>
  <ScoreType name="a_helicity" scorefxn="helicity" score_type="mp_helicity" confidence="0" threshold="10"/>
  <MPSpanAngle name="a_angle_1" tm="1" ang_min="0" ang_max="50" confidence="0"/>
  <MPSpanAngle name="a_angle_2" tm="2" ang_min="0" ang_max="50" confidence="0"/>
  <Time name="timer"/>
</FILTERS>
<PROTOCOLS>
  <Add mover="add_memb"/>
  <Add filter="timer"/>
  <Add mover="add_memb"/>

```

```

<Add mover="add_CA_cst"/>
%%fix_prefix%%<Add mover="virt_root"/>
%%fix_prefix%%<Add mover="fix_res_sc_cst"/>
<Add mover="iter4"/>
%%fix_prefix%%<Add mover="virt_root_remover"/>
<Add filter="stability_score_full"/>
<Add filter="stability_without_pssm"/>
<Add filter="stability_pure"/>
<Add filter="a_res_lipo"/>
<Add filter="a_span_topo"/>
<Add filter="a_unsat"/>
<Add filter="a_tms_span"/>
<Add filter="a_marl"/>
<Add filter="a_helicity"/>
<Add filter="a_angle_1"/>
<Add filter="a_angle_2"/>
<Add filter="timer"/>
</PROTOCOLS>
<OUTPUT scorefxn="full"/>
</ROSETTASCRIPTS>

```

### With the flags:

```

-database PATH_TO_ROSETTA_DATABASE -pdb_gz -use_input_sc -extrachi_cutoff 10 -ignore_unrecognized_res
-chemical:exclude_patches LowerDNA UpperDNA Cterm_amidation SpecialRotamer VirtualBB ShoveBB VirtualDNAPhosphate
VirtualNTerm CTermConnect sc_orbitals pro_hydroxylated_case1 pro_hydroxylated_case2 ser_phosphorylated thr_phosphorylated
tyr_phosphorylated tyr_sulfated lys_dimethylated lys_monomethylated lys_trimethylated lys_acetylated glu_carboxylated
cys_acetylated tyr_diiodinated N_acetylated C_methylamidated MethylatedProteinCterm -linmem_ig 10 -ignore_zero_occupancy
false -load_PDB_components false -mp.scoring:hbond -ex1 -ex2 -s PATH_TO_PDB -parser:protocol
PATH_TO_REFINEMENT_XML -parser:script_vars res_to_fix=RESIDUES_TO_FIX fix_prefix=< fix_TO=,fix_res
res_to_restrict=RESIDUES_TO_RESTRICT cst_value=0.64 cst_full_path=PATH_TO_CONSTRAINTS_FILE
ignore_pose_profile_length_mismatch=1 scfxn=ref2015_memb
span_starts=COMA_SEPARATED_LIST_OF_SPAN_STARTING_POSITIONS
span_ends=COMA_SEPARATED_LIST_OF_SPAN_ENDING_POSITIONS
span_oris=COMA_SEPARATED_LIST_OF_SPAN_ORIENTATIONS soft_scfxn=soft_rep

```

Virtual mutational scan within the virtual membrane was done using the following xml:

```

<ROSETTASCRIPTS>
<SCOREFXNS>
<ScoreFunction name="ref_full" weights="ref2015_memb">
  <Reweight scoretype="coordinate_constraint" weight="0.64"/>
  <Reweight scoretype="res_type_constraint" weight="%%PSSM_WEIGHT%%"/>
</ScoreFunction>
</SCOREFXNS>
<RESIDUE_SELECTORS>
<ResidueName name="pro" residue_name3="PRO"/>
<ResidueName name="gly" residue_name3="GLY"/>
<PrimarySequenceNeighborhood name="pre_pro" selector="pro" lower="1" upper="0"/>
<Index name="ress_restrict" resnums="%%res_to_restrict%%" error_on_out_of_bounds_index="0"/>
%%fix_prefix%%<Index name="ress_fix" resnums="%%res_to_fix%%"/>
</RESIDUE_SELECTORS>
<TASKOPERATIONS>
<InitializeFromCommandline name="init"/>
<OperateOnResidueSubset name="restrict_pro" selector="pro">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
<OperateOnResidueSubset name="restrict_gly" selector="gly">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
<OperateOnResidueSubset name="restrict_pre_pro" selector="pre_pro">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>

```



```

<OperateOnResidueSubset name="restrict_res" selector="ress_restrict">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
%%fix_prefix%%OperateOnResidueSubset name="fix_res" selector="ress_fix">
%%fix_prefix%%PreventRepackingRLT/>
%%fix_prefix%%OperateOnResidueSubset>
%%fix_prefix%%OperateOnResidueSubset name="not_to_cst_sc">
  %%fix_prefix%%Not selector="ress_fix"/>
  %%fix_prefix%%PreventRepackingRLT/>
%%fix_prefix%%OperateOnResidueSubset>
<DesignAround name="des_around" design_shell="0.1" resnums="%%current_res%" repack_shell="8.0"/>
  <SeqprofConsensus name="pssm_cutoff" filename="%%pssm_full_path%" min_aa_probability="%%min_aa_probability%"
probability_larger_than_current="0" ignore_pose_profile_length_mismatch="%%ignore_pose_profile_length_mismatch%"
convert_scores_to_probabilities="0" keep_native="%%keep_n%"/>
</TASKOPERATIONS>
<MOVERS>
  <AddMembraneMover name="add_memb" membrane_core="10" steepness="4" span_starts="%%span_starts%"
span_ends="%%span_ends%" span_orientations="%%span_oris%"/>
  <ConstraintSetMover name="add_CA_cst" cst_file="%%cst_full_path%"/>
  %%fix_prefix%%VirtualRoot name="virt_root" removable="true"/>
  %%fix_prefix%%VirtualRoot name="virt_root_remover" remove="true"/>
  %%fix_prefix%%AtomCoordinateCstMover name="fix_res_sc_cst" coord_dev="0.5" bounded="false" sidechain="true"
task_operations="not_to_cst_sc"/>
  <FavorSequenceProfile name="FSP" scaling="none" weight="1" pssm="%%pssm_full_path%" scorefxns="ref_full" />
  <MinMover name="min_all" scorefxn="ref_full" chi="1" bb="1" jump="0"/>
</MOVERS>
<FILTERS>
  <ScoreType name="stability_score_full" scorefxn="ref_full" score_type="total_score" threshold="0.0"/>
  <Delta name="delta_score_full" filter="stability_score_full" upper="1" lower="0" range="0.5"/>
  <Time name="timer"/>
  <FilterScan name="filter_scan" scorefxn="ref_full" relax_mover="min_all" keep_native="1"
task_operations="init,des_around,restrict_res,pssm_cutoff%%added_tasks%%%%fix_TO%%"
delta_filters="delta_score_full" delta="true" resfile_name="%%resfiles_path%%/res_%%current_res%" report_all="1"
delta_filter_thresholds="%%fs_thresholds%" score_log_file="%%scores_path%%/res_%%current_res%%_score_full.log"
dump_pdb="1" dump_pdb_name="%%pdb_path_and_name%"/>
</FILTERS>
<PROTOCOLS>
  <Add filter="timer"/>
  <Add mover="add_memb"/>
  <Add mover="add_CA_cst"/>
  %%fix_prefix%%Add mover="virt_root"/>
  %%fix_prefix%%Add mover="fix_res_sc_cst"/>
  <Add mover="FSP"/>
  %%fix_prefix%%Add mover="virt_root_remover"/>
  <Add filter="filter_scan"/>
  <Add filter="timer"/>
</PROTOCOLS>
<OUTPUT scorefxn="ref_full"/>
</ROSETTASCRIPTS>

```

### With the flags:

```

-database PATH_TO_ROSETTA_DATABASE -pdb_gz -use_input_sc -extrachi_cutoff 10 -ignore_unrecognized_res
-chemical:exclude_patches LowerDNA UpperDNA Cterm_amidation SpecialRotamer VirtualBB ShoveBB VirtualDNAPhosphate
VirtualNTerm CTermConnect sc_orbitals pro_hydroxylated_case1 pro_hydroxylated_case2 ser_phosphorylated thr_phosphorylated
tyr_phosphorylated tyr_sulfated lys_dimethylated lys_monomethylated lys_trimethylated lys_acetylated glu_carboxylated
cys_acetylated tyr_diodinated N_acetylated C_methylamidated MethylatedProteinCTerm -linmem_ig 10 -ignore_zero_occupancy
false -load_PDB_components false -mp:scoring:hbond -ex1 -ex2 -s PATH_TO_REFINED_PDB -overwrite -parser:protocol
PATH_TO_FILTERSCAN_XML -use_occurrence_data -parser:script_vars res_to_fix=RESIDUES_TO_FIX fix_prefix=<
fix_TO=,fix_res res_to_restrict=RESIDUES_TO_RESTRICT cst_full_path=PATH_TO_CONSTRAINTS_FILE
ignore_pose_profile_length_mismatch=1 scfxn=ref2015_memb
span_starts=COMA_SEPARATED_LIST_OF_SPAN_STARTING_POSITIONS

```

```
span_ends=COMA_SEPARATED_LIST_OF_SPAN_ENDING_POSITIONS
span_oris=COMA_SEPARATED_LIST_OF_SPAN_ORIENTATIONS soft_scfxn=soft_rep
pssm_full_path=PATH_TO_PSSM min_aa_probability=APPROPRIATE_MIN_AA_PROB fs_thresholds=FS_THRESHOLD
added_tasks=,restrict_gly,restrict_pro,restrict_pre_pro keep_n=1 PSSM_WEIGHT=APPROPRIATE_PSSM_WEIGHT
```

Design within the virtual membrane was done using the following xml:

```
<ROSETTASCRIPTS>
<SCOREFXNS>
<ScoreFunction name="ref_full" weights="ref2015_memb">
  <Reweight scoretype="coordinate_constraint" weight="0.64"/>
  <Reweight scoretype="res_type_constraint" weight="%%pssm_weight%%"/>
</ScoreFunction>
<ScoreFunction name="soft_rep_full" weights="ref2015_soft">
  <Reweight scoretype="mp_res_lipo" weight="1"/>
  <Reweight scoretype="coordinate_constraint" weight="0.64"/>
  <Reweight scoretype="res_type_constraint" weight="%%pssm_weight%%"/>
</ScoreFunction>
<ScoreFunction name="ref_no_pssm" weights="ref2015_memb">
  <Reweight scoretype="coordinate_constraint" weight="0.64"/>
</ScoreFunction>
<ScoreFunction name="ref_pure" weights="ref2015_memb"/>
<ScoreFunction name="helicality" symmetric="0">
  <Reweight scoretype="mp_helicality" weight="1"/>
</ScoreFunction>
</SCOREFXNS>
<RESIDUE_SELECTORS>
<ResidueName name="pro" residue_name3="PRO"/>
<ResidueName name="gly" residue_name3="GLY"/>
<PrimarySequenceNeighborhood name="pre_pro" selector="pro" lower="1" upper="0"/>
<Index name="ress_restrict" resnums="%%res_to_restrict%%" error_on_out_of_bounds_index="0"/>
%%fix_prefix%%<Index name="ress_fix" resnums="%%res_to_fix%%"/>
</RESIDUE_SELECTORS>
<TASKOPERATIONS>
<InitializeFromCommandline name="init"/>
<OperateOnResidueSubset name="restrict_pro" selector="pro">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
<OperateOnResidueSubset name="restrict_res" selector="ress_restrict">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
%%fix_prefix%%<OperateOnResidueSubset name="fix_res" selector="ress_fix">
  %%fix_prefix%%<PreventRepackingRLT/>
  %%fix_prefix%%</OperateOnResidueSubset>
  %%fix_prefix%%<OperateOnResidueSubset name="not_to_cst_sc">
    %%fix_prefix%%<Not selector="ress_fix"/>
    %%fix_prefix%%<PreventRepackingRLT/>
  %%fix_prefix%%</OperateOnResidueSubset>
<OperateOnResidueSubset name="restrict_gly" selector="gly">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
<OperateOnResidueSubset name="restrict_pre_pro" selector="pre_pro">
  <RestrictToRepackingRLT/>
</OperateOnResidueSubset>
<ReadResfile name="read_resfile" filename="%%in_resfile%%"/>
<RestrictToRepacking name="rtr"/>
<SeqprofConsensus name="pssm_cutoff" filename="%%pssm_full_path%%" min_aa_probability="%%min_aa_probability%%"
  convert_scores_to_probabilities="0" probability_larger_than_current="0" keep_native="%%keep_n%%"
  ignore_pose_profile_length_mismatch="%%ignore_pose_profile_length_mismatch%%"/>
</TASKOPERATIONS>
<MOVERS>
```

```

<AddMembraneMover name="add_memb" membrane_core="10" steepness="4" span_starts="%%span_starts%%"
span_ends="%%span_ends%%" span_orientations="%%span_oris%%"/>
      <PackRotamersMover name="soft_design" scorefxn="soft_rep_full"
task_operations="init,restrict_res,read_resfile,pssm_cutoff%%added_tasks%%fix_TO%%"/>
      <PackRotamersMover name="hard_design" scorefxn="ref_full"
task_operations="init,restrict_res,read_resfile,pssm_cutoff%%added_tasks%%fix_TO%%"/>
    <RotamerTrialsMinMover name="RTmin" scorefxn="ref_full" task_operations="init,restrict_res,rtr"/>
    <MinMover name="soft_min" scorefxn="soft_rep_full" chi="1" bb="1" jump="0"/>
    <MinMover name="hard_min" scorefxn="ref_full" chi="1" bb="1" jump="0"/>
    <ConstraintSetMover name="add_CA_cst" cst_file="%%cst_full_path%%"/>
      %%fix_prefix%%AtomCoordinateCstMover name="fix_res_sc_cst" coord_dev="0.5" bounded="false" sidechain="true"
task_operations="not_to_cst_sc"/>
    %%fix_prefix%%VirtualRoot name="virt_root" removable="true"/>
    %%fix_prefix%%VirtualRoot name="virt_root_remover" remove="true"/>
    <FavorSequenceProfile name="FSP" pssm="%%pssm_full_path%%" scaling="none" scorefxns="ref_full" weight="1"/>
    <ParsedProtocol name="design_block">
      <Add mover="soft_design"/>
      <Add mover="soft_min"/>
      <Add mover="soft_design"/>
      <Add mover="hard_min"/>
      <Add mover="hard_design"/>
      <Add mover="hard_min"/>
      <Add mover="hard_design"/>
      <Add mover="RTmin"/>
      <Add mover="RTmin"/>
      <Add mover="hard_min"/>
    </ParsedProtocol>
    <LoopOver iterations="4" mover_name="design_block" name="iter4"/>
  </MOVERS>
  <FILTERS>
    <ScoreType confidence="0" name="stability_score_full" score_type="total_score" scorefxn="ref_full" threshold="0"/>
    <ScoreType confidence="0" name="stability_without_pssm" score_type="total_score" scorefxn="ref_no_pssm" threshold="0"/>
    <ScoreType confidence="0" name="stability_pure" score_type="total_score" scorefxn="ref_pure" threshold="0"/>
    <Rmsd confidence="0" name="rmsd"/>
    <Time name="timer"/>
    <ScoreType name="a_total" scorefxn="ref_full" score_type="total_score" confidence="0" threshold="0"/>
    <ResidueLipophilicity name="a_res_lipo" threshold="1000" confidence="0"/>
    <SpanTopologyMatchPose name="a_span_topo" confidence="0"/>
    <BuriedUnsatHbonds2 name="a_unsat" scorefxn="ref_full" confidence="0"/>
    <TMsSpanMembrane name="a_tms_span" confidence="0"/>
    <MembAccesResidueLipophilicity name="a_marl" confidence="0" verbose="0"/>
    <ScoreType name="a_helicity" scorefxn="helicity" score_type="total_score" confidence="0" threshold="3"/>
    <TMsAAComp name="a_tms_aa_comp" confidence="0" threshold="0"/>
  </FILTERS>
  <PROTOCOLS>
    <Add filter="timer"/>
    <Add mover="add_memb"/>
    <Add mover="add_CA_cst"/>
    %%fix_prefix%%Add mover="virt_root"/>
    %%fix_prefix%%Add mover="fix_res_sc_cst"/>
    <Add mover="FSP"/>
    <Add mover="iter4"/>
    <Add filter="stability_score_full"/>
    <Add filter="stability_without_pssm"/>
    <Add filter="stability_pure"/>
    %%fix_prefix%%Add mover="virt_root_remover"/>
    <Add filter="rmsd"/>
    <Add filter="a_total"/>
    <Add filter="a_res_lipo"/>
    <Add filter="a_span_topo"/>
    <Add filter="a_unsat"/>

```

```

<Add filter="a_mar1"/>
<Add filter="a_tms_span"/>
<Add filter="a_helicity"/>
<Add filter="a_tms_aa_comp"/>
<Add filter="timer"/>
</PROTOCOLS>
<OUTPUT scorefxn="ref_full"/>
</ROSETTASCRIPTS>

```

### With the flags:

```

-database PATH_TO_ROSETTA_DATABASE -pdb_gz -use_input_sc -extrachi_cutoff 10 -ignore_unrecognized_res
-chemical:exclude_patches LowerDNA UpperDNA Cterm_amidation SpecialRotamer VirtualBB ShoveBB VirtualDNAPhosphate
VirtualNterm CtermConnect sc_orbitals pro_hydroxylated_case1 pro_hydroxylated_case2 ser_phosphorylated thr_phosphorylated
tyr_phosphorylated tyr_sulfated lys_dimethylated lys_monomethylated lys_trimethylated lys_acetylated glu_carboxylated
cys_acetylated tyr_diodinated N_acetylated C_methylamidated MethylatedProteinCterm -linmem_ig 10 -ignore_zero_occupancy
false -load_PDB_components false -mp:scoring:hbond -ex1 -ex2 -s PATH_TO_REFINED_PDB -overwrite -parser:protocol
PATH_TO_DESIGN_XML -use_occurrence_data res_to_fix=RESIDUES_TO_FIX fix_prefix=<
fix_TO=,fix_res res_to_restrict=RESIDUES_TO_RESTRICT cst_full_path=PATH_TO_CONSTRAINTS_FILE
ignore_pose_profile_length_mismatch=1 scfxn=ref2015_memb
span_starts=COMA_SEPARATED_LIST_OF_SPAN_STARTING_POSITIONS
span_ends=COMA_SEPARATED_LIST_OF_SPAN_ENDING_POSITIONS
span_oris=COMA_SEPARATED_LIST_OF_SPAN_ORIENTATIONS soft_scfxn=soft_rep
pssm_full_path=PATH_TO_PSSM min_aa_probability=APPROPRIATE_MIN_AA_PROB
pssm_weight=APPROPRIATE_PSSM_WEIGHT added_tasks=,restrict_gly,restrict_pro,restrict_pre_pro keep_n=1

```

### Supplementary File 2. paddle chimera design sequences.

#### paddle chimera:

```

MAHHHHHHHHHHHGLVPRGSMTVATGDPVDEAAALPGHPQDTYDPEADHESSEVVINISGLRFETQLKTL
AQFPETLLGDPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDIFSEEIRFYELGEE
AMEMFREDEGYIKEEERPLPENEFQRQVWLLFEYPPESSGPARI IAIIVSVMVILISIVSFCLETLPIFRDE
NEDMHGGGVTFHTYSQSTIGYQQSTSF TDPFFIIVETLCCIWF SFEFLVRFACPSKAGFFTNIMNIIDIV
AIIPIYYVTIFLTESNKSVLQFQNVRRVQIFRIMRILRIFKLSRHSKGLQILGQTLKASMRELGLLIFFL
FIGVILFSSAVYFAEADERDSQFPSIPDAFWWAVVSM TTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALP
VPVIVSNFNIFYHRETEGEEQAQYLQVTSSPKIPSSPDLKKSRSASTISKSDYMEIQEGVNNSNEDFREE
NLKTANSTLANTNYVNI TKMLTDV

```

#### D1:

```

MAHHHHHHHHHHHGLVPRGSMTVATGDPVDEAAALPGHPQDTYDPEADHESSEVVINISGLRFETQLKTL
AQFPETLLGDPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDIFSEEIRFYELGEE
AMEKFREDEGYIKEEERPLPENEFQRQVWLLFEYPPESSGPARI IAIISVLVILISIIIFCLETLPIFRDE
NEDMHGGGVTFHTYSQSTIGYQQSTSF TDPFFI IETLCCIWF SFEFLVRFACPSKAGFFRNIMNIIDIV
AIIPIYYVTIFLTESNKSVLQFQNVRRVQIFRIMRILRIFKLSRHSKGLQILGQTLKASMRELGLLIFFL
FIGVILFASAVYFAEAEDEPDSQFPSIPDAFWWAVVSM TTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALP
VPVIVSNFNIFYHRETEGEEQAQYLQVTSSPKIPSSPDLKKSRSASTISKSDYMEIQEGVNNSNEDFREE
NLKTANSTLANTNYVNI TKMLTDV

```

#### D2:

```

MAHHHHHHHHHHHGLVPRGSMTVATGDPVDEAAALPGHPQDTYDPEADHESSEVVINISGLRFETQLKTL
AQFPD TLLGDPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDIFSEEIKFYELGEE
AMEKFREDEGFIKEEERPLPENEFQRQVWLLFEYPPESSGPARI IAIISVLVILISIIIFCLETLPIFRDE
NEDMHGGGVTFHTYSQSTIGYQQSTSF TDPFFI IETLCCIWF SFEFLVRFACPSKAGFFRNIMNIIDIL

```

AIIPYYVTIFLTESNKSVLQFQNVRRVQIFRIMRILRIFKLSRHSKGLQILGQTLKASLRELGLLIFFL  
FIGVILFASAVYFAEAEDEPDSQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALP  
VPVIVSNFNIFYHRETEGEEQAQYLQVTSSPKIPSSPDLKKSRSASTISKSDYMEIQEGVNNNSNEDFREE  
NLKTANSTLANTNYVNI TKMLTDV

**D3:**

MAHHHHHHHHHGLVPRGSMTVATGDPVDEAAALPGHPQDTYDPEADHESSEVVINISGLRFETQLKTL  
AQFPETLLGDPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDIFLEEIKFYELGEE  
AMEKFREDEGFIKEEERPLPENYQRQVWLLFEYPESSEGPARI IAIISILVILISIIIFCLETLPIFRDE  
NEDMHGGGVTFHTYSQSTIGYYQSTSFTDPFFI IETLCI IWFTFEFLVRFLACPSKAGFFRNIMNIIDII  
AIIPYYVTIFLTESNKSVLQFQNVRRVQIFRIMRILRIFKLSRHSKGLQILGQTLKASLRELGLLIFFL  
FIGVILFASAVYFAEAEDEPDSQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALP  
VPVIVSNFNIFYHRETEGEEQAQYLQVTSSPKIPSSPDLKKSRSASTISKSDYMEIQEGVNNNSNEDFREE  
NLKTANSTLANTNYVNI TKMLTDV

**D4:**

MAHHHHHHHHHGLVPRGSMTVATGDPVDEAAALPGHPQDTYDPEADHESSEVVINISGLRFETQLKTL  
AQFPD TLLGDPKKRMRYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDIFLEEIKFYQLGEE  
AMEKFREDEGFIKEEERPLPNNEYQRQVWLLFEYPESSEGPARI IAIISILIIILISIIIFCLETLPIFRDE  
NEDMHGGGVTFHTYSQATIGYYQSTSFTDPFFI IETLCI IWFTFEFLVRFLACPSKAGFFRNIMNIIDII  
AIIPYYVTIFLTESNKSVLQFQNVRRVQIFRIMRILRIFKLSRHSKGLRILGQTLKASLRELGLLIFFL  
FIGVILFASAVYFAEAEDEPDSHFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALP  
VPVIVSNFNIFYHRETEGEEQAQYLQVTSSPKIPSSPDLKKSRSASTISKSDYMEIQEGVNNNSNEDFREE  
NLKTANSTLANTNYVNI TKMLTDV

**Author contributions**

JJW developed the algorithm, performed research, analyzed data and wrote the paper; CS designed and performed research; IK designed and performed research; AG performed research; ER designed and performed research, wrote the paper; SJF designed research and wrote the paper.

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