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**Supplemental Information**

**Single-Molecule Analyses Reveal Rhomboid Proteins Are Strict and Functional Monomers in the Membrane**

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**Table S1.** Summary of photobleaching events for purified proteins in planar supported lipid bilayers. All bacterial proteins were reconstituted in 70:30 DMPE:DMPG except GlpG-Halo in PO and GlpG-Halo in *E. coli*, which were 70:30 POPE:POPG and *E. coli* polar lipid extract, respectively. DmR4 was reconstituted in yeast phospholipids.

<b>Protein</b>	<b>1-step</b>	<b>2-step</b>	<b>3-step</b>	<b>4-step</b>	<b>Total Events</b>
$\Delta$ NGlpG-HaloC	163	13	0	0	176
GlpG-HaloC	146	6	0	0	152
MsbA-HaloC	10	106	0	0	116
AqpZ-HaloC	4	4	5	81	94
GlpF-HaloC	3	3	3	44	53
LacY-HaloC	92	5	0	0	97
GST- $\Delta$ NGlpG-HaloC	11	90	0	1	102
GST-GlpG-HaloC	8	84	0	0	92
AqpZ-HaloC C20S	242	12	0	0	254
GlpF-HaloC E43A	249	6	0	0	255
HiGlpG CysC-JF549	120	0	0	0	120
NCys-JF549-HiGlpG	140	2	0	0	142
$\Delta$ N-GlpG CysC-JF549	123	4	0	0	127
NCys-JF549- $\Delta$ N-GlpG	138	4	0	0	142
GlpG CysC-JF549	168	5	0	0	173
NCys-JF549-GlpG	144	1	0	0	145
AarA-HaloC	160	8	0	0	168
NHalo-DmR4	190	2	0	0	192
NHalo-DmR4 + Calcium	156	5	0	0	161
GlpG-HaloC in PO	205	10	0	0	215
GlpG-HaloC in <i>E. coli</i>	145	6	0	0	151

**Table S2.** Summary of photobleaching events of rhomboid proteins in HEK293T cells.

<b>Protein</b>	<b>1-step</b>	<b>2-step</b>	<b>3-step</b>	<b>4-step</b>	<b>Total Events</b>
NHalo-RHBDL2	138	6	1	0	145
NHalo-RHBDL3	307	12	1	0	320
iRhom1-HaloC	238	9	1	0	248
iRhom2-HaloC	173	7	0	0	180
NHalo-EhR1	363	12	3	0	378
NHalo-TvR1	253	7	1	0	261
RHBDL2-HaloC	97	6	0	0	103
NHalo-iRhom2	135	7	0	0	142
EhR1-HaloC	130	6	0	0	136
NHalo-DmR4	98	8	0	0	106

**Table S3.** Summary of photobleaching events of proteins in 0.1% DDM detergent bound to Ni-coverslips.

<b>Protein</b>	<b>1-step</b>	<b>2-step</b>	<b>3-step</b>	<b>4-step</b>	<b>Total Events</b>
LacY-HaloC	241	8	1	0	250
MsbA-HaloC	6	250	0	0	256
AqpZ-HaloC	3	7	1	352	363
GlpF-HaloC	3	11	1	428	443
$\Delta$ NGlpG-HaloC	53	67	0	0	120
GlpG-HaloC	45	103	0	0	148
NCys-JF549-HiGlpG	102	220	0	0	322
NHalo-DmR4	94	76	0	0	170

**Table S4.** Summary of photobleaching events of proteins in 0.1% NG detergent bound to Ni-coverslips.

<b>Protein</b>	<b>1-step</b>	<b>2-step</b>	<b>3-step</b>	<b>4-step</b>	<b>Total Events</b>
LacY-HaloC	152	3	0	0	155
MsbA-HaloC	10	285	0	3	298
AqpZ-HaloC	1	5	0	165	171
GlpF-HaloC	1	4	1	120	126
$\Delta$ NGlpG-HaloC	6	278	1	0	285
GlpG-HaloC	11	191	1	1	204
NCys-JF549-HiGlpG	20	207	0	0	227
NHalo-DmR4	26	108	0	0	134