Supplemental Figures:

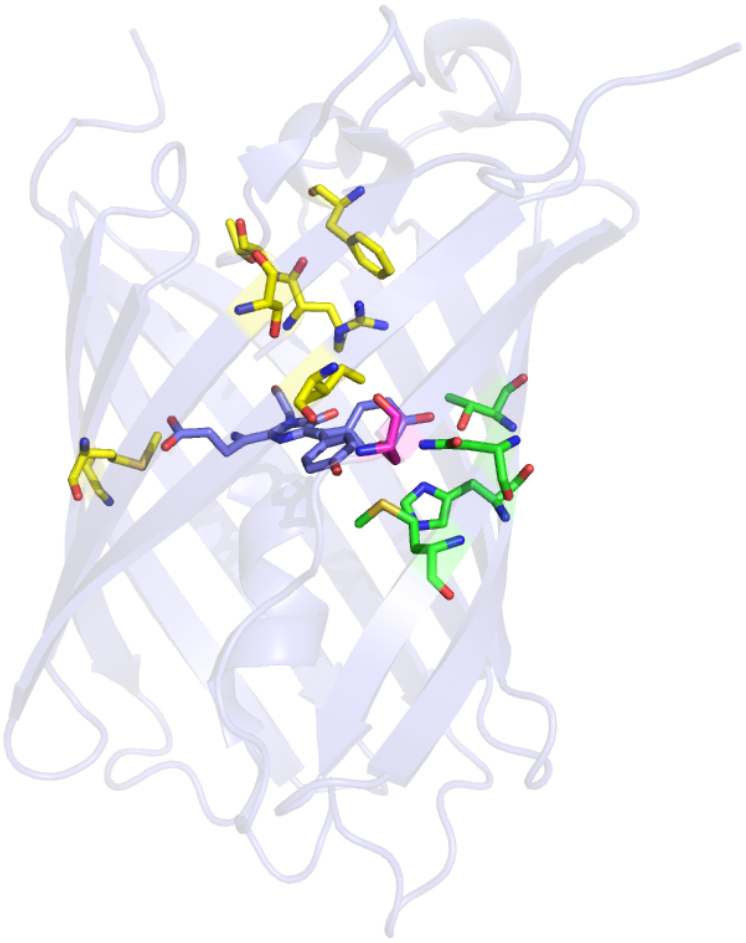

Figure S1 – The design of HcRed7. Two core libraries targeted unique structural regions of the protein core. The first region (green) surrounds the phenolate side chain of the chromophore. The second (yellow), is a very highly mutated region in RFP monomer evolution. This region holds an internal water channel, key catalytic residues, and abuts the AC interface.

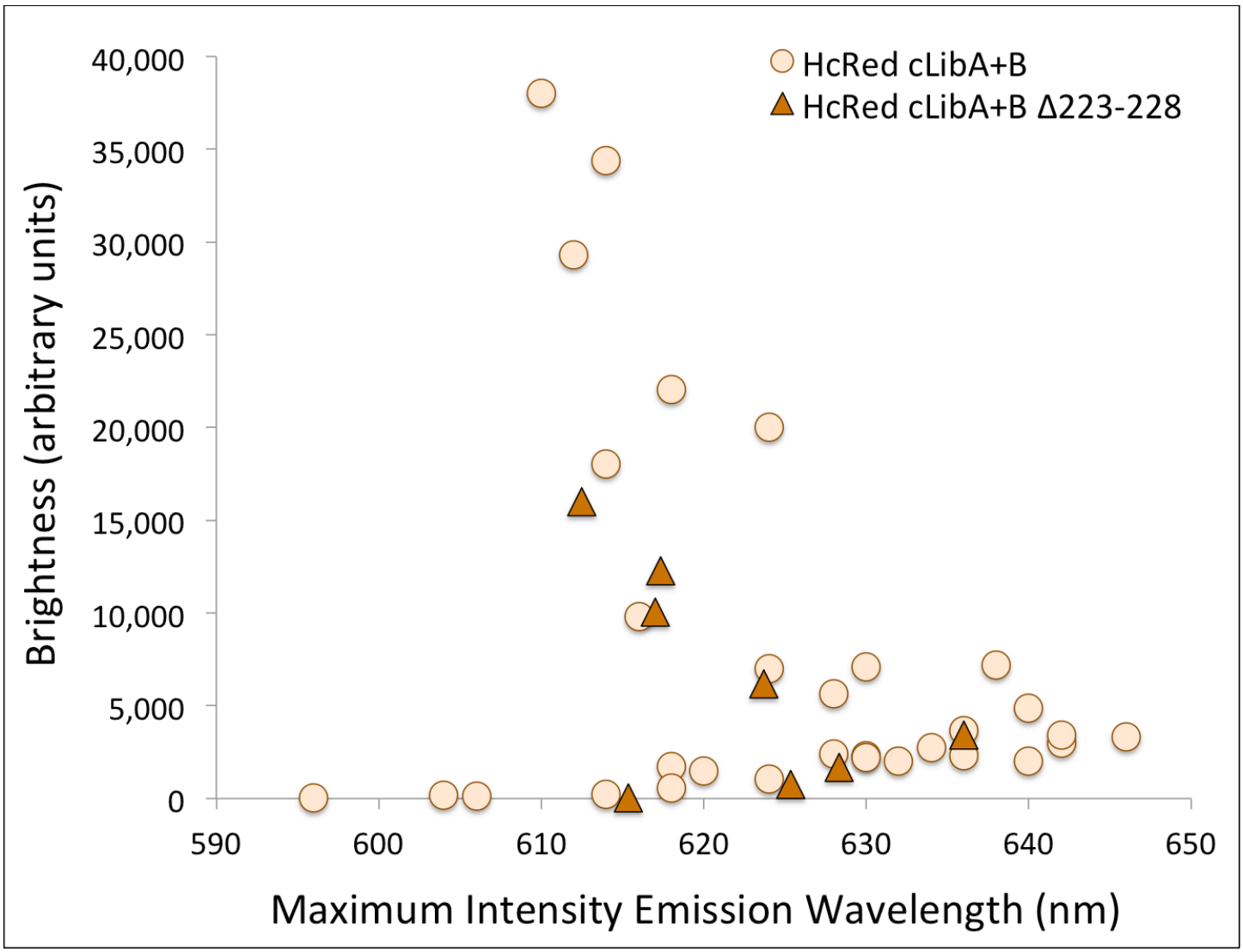

Figure S2 – The diversity of spectroscopic properties of 41 colored variants from the HcRed core libraries cLibA and cLibB. Brightness is plotted against maximum intensity emission wavelength. RFPs with emission peaks at longer wavelengths are noticeably dimmer than some of the bright, hypsochromically shifted RFPs.

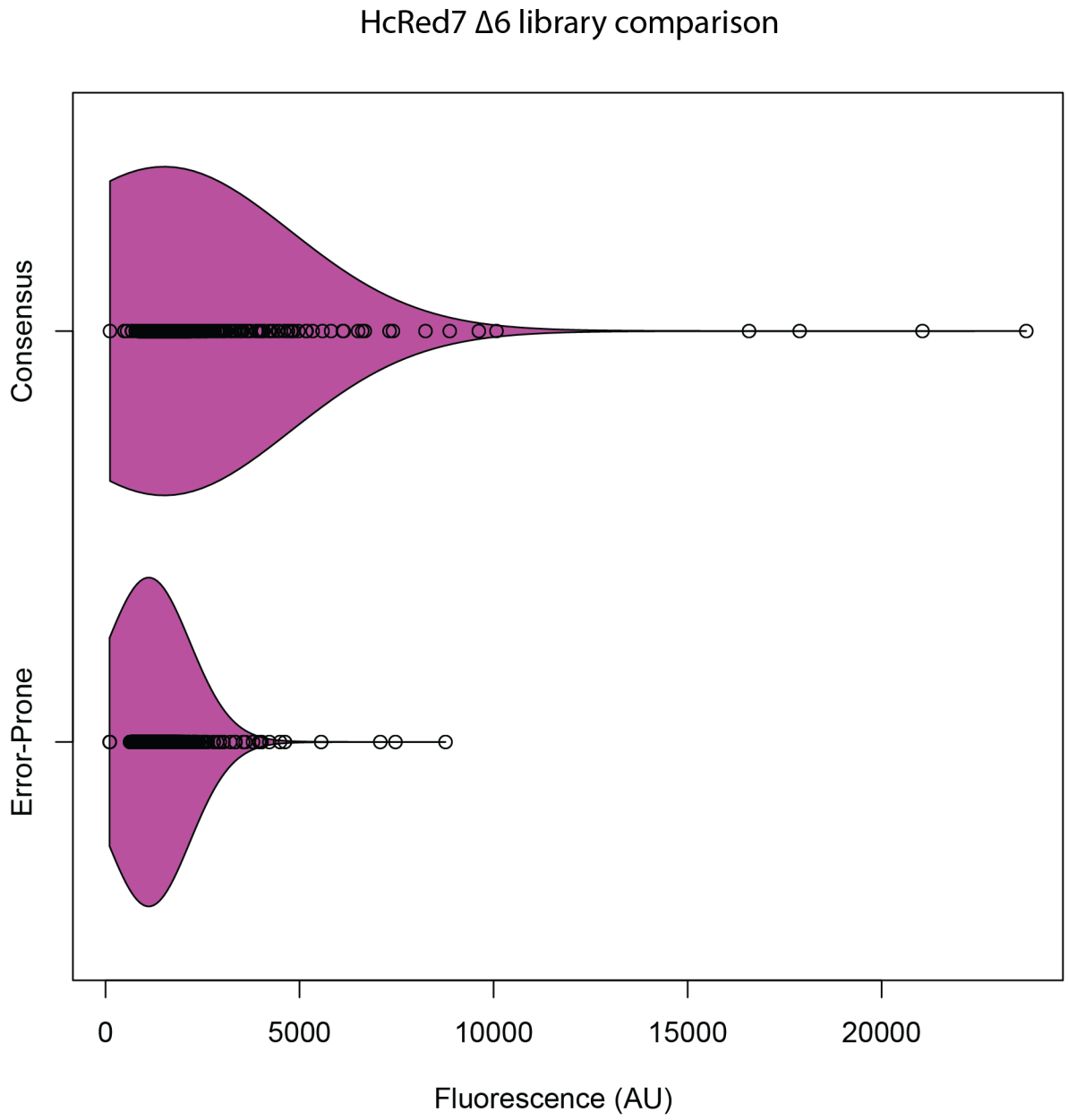

Figure S3 – Consensus design compares favorably to error-prone mutagenesis in the improvement of fluorescence in HcRed7 Δ6. The two libraries were compared by screening in 96-well plates with ~4,000 screened variants from the consensus design library and ~8,000 variants screened from the error-prone library. Both individual variants and the population from the consensus library outperform the error-prone library, although both contributed valuable variants to the engineering process.

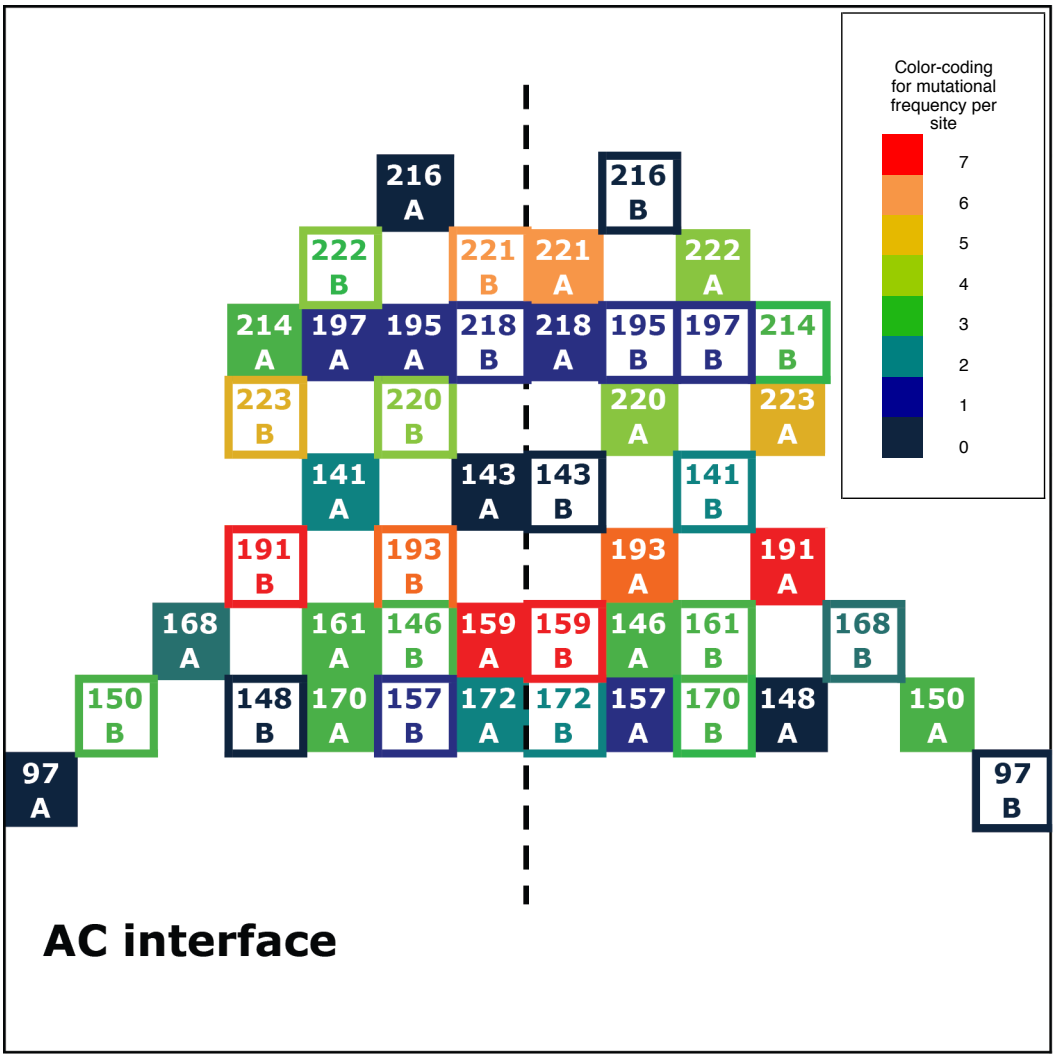

Figure S4 – A map displaying the intermolecular contacts made by each residue of HcRed in the AC interface. The map is color coded by the frequency with which the residues are mutated during past instances of fluorescent protein monomerization. Residues 146, 159, 167, 168, 170, 174, 191, 193, 197, 201, and 214 were mutated during mGinger engineering. Residues 222-223 were deleted as part of the C-terminal tail.

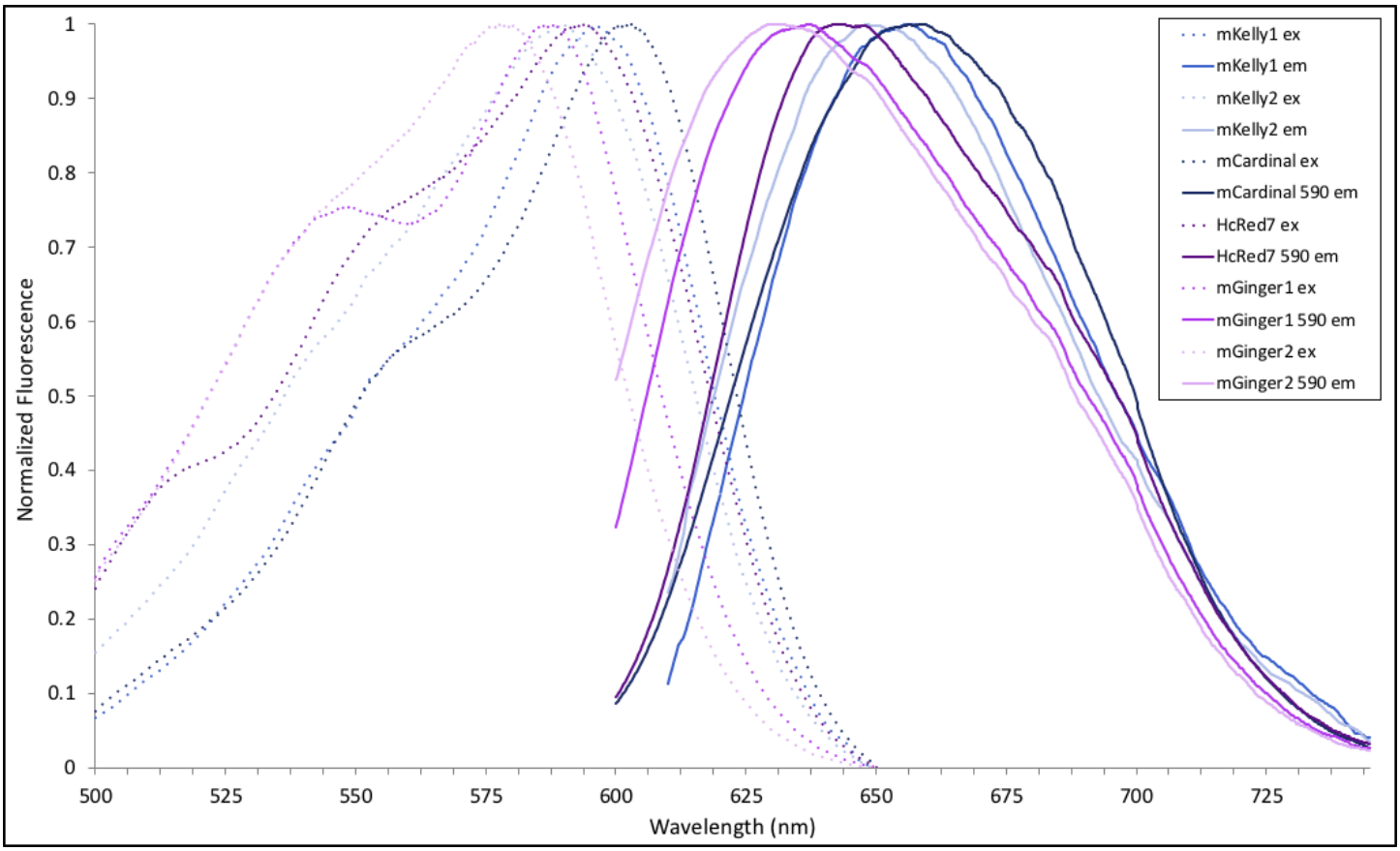

Figure S5 – Spectral scans of the monomeric fluorescent proteins mGinger1, mGinger2, mKelly1, and mKelly2, along with the dimeric proteins they were derived from.

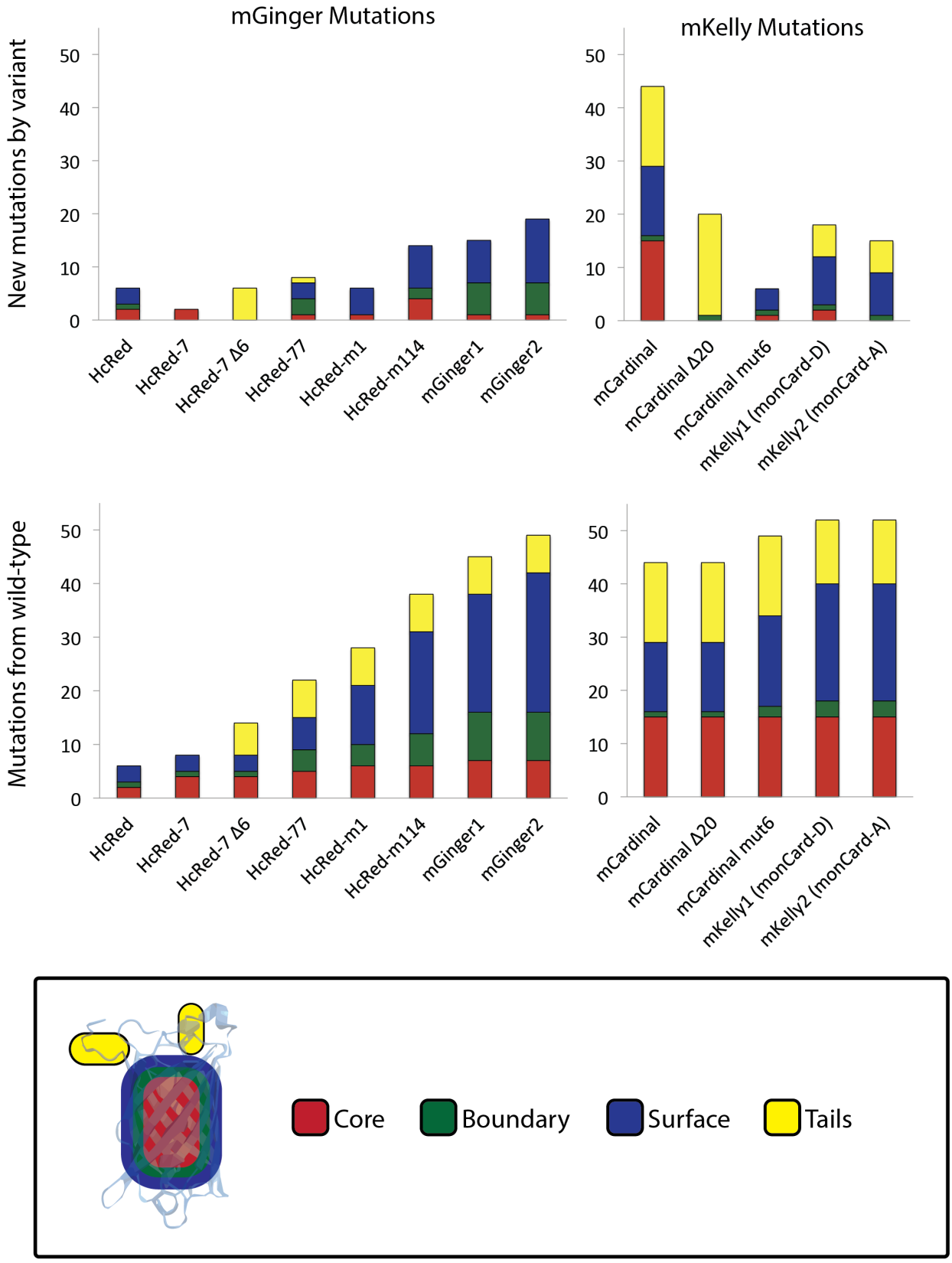

Figure S6 – Mutations during mGinger and mKelly engineering categorized by solvent-accessible surface area (SASA). (A) The number of new mutations introduced in each step of protein engineering. (B) The cumulative number of mutations that separate each variant from the wild-type progenitor (HcCP in the case of HcRed variants and eqFP578 in the case of mCardinal variants).

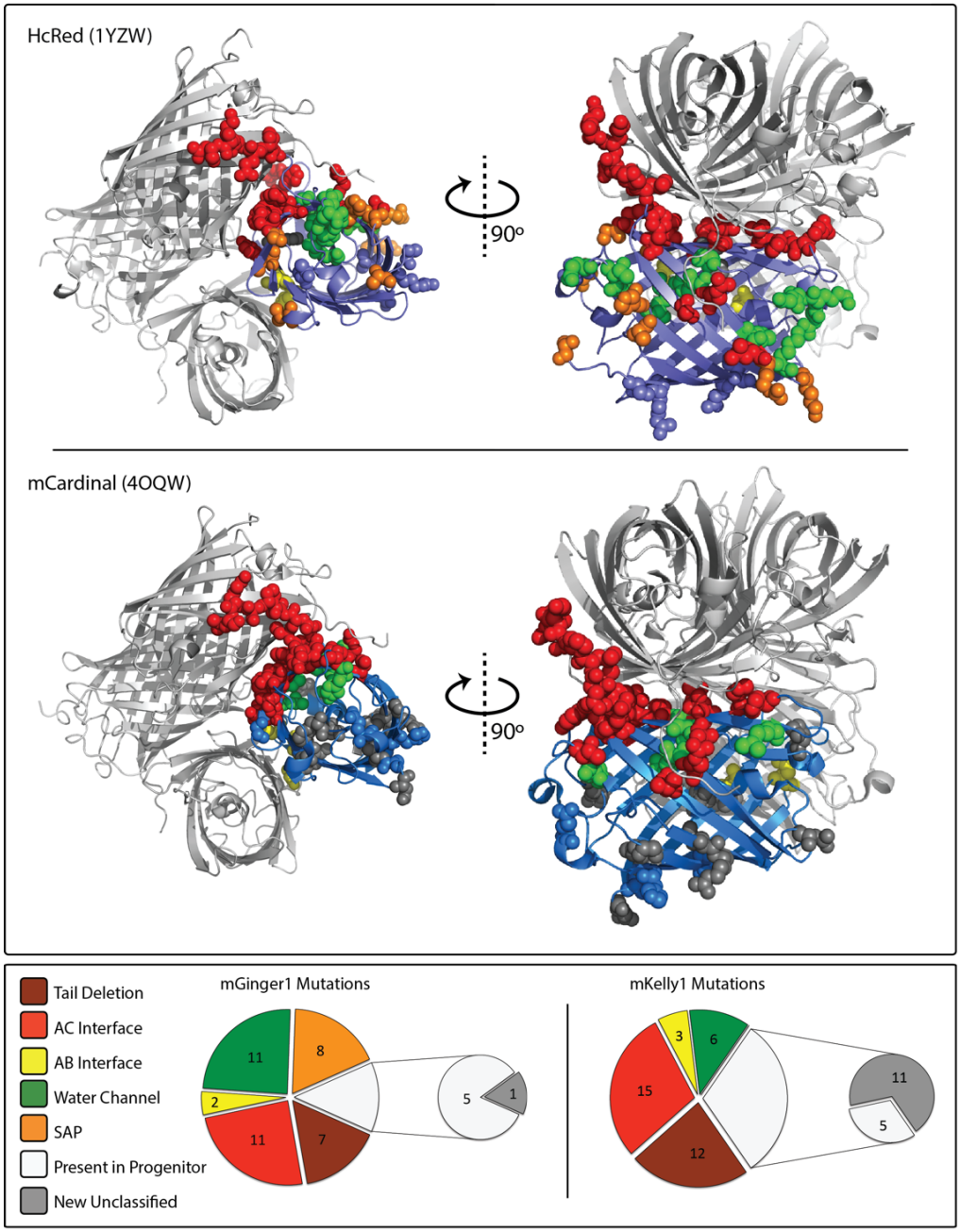

Figure S7 – Mutations during mGinger1 and mKelly1 engineering categorized by structural clustering. The tail and AC interface are shown in red in spheres, as they both participate in the AC interface, but are broken out separately in the pi-charts. SAP refers to mutations that occur near positions with high surface aggregation propensity (SAP). Many mutations cluster into more than one structural region, and in this case they are categorized hierarchically from top to bottom according to the legend of the pi-chart. So Tail Deletion residues are not included in the AC Interface count, and AC interface residues are not included in the SAP residue count, and so on.

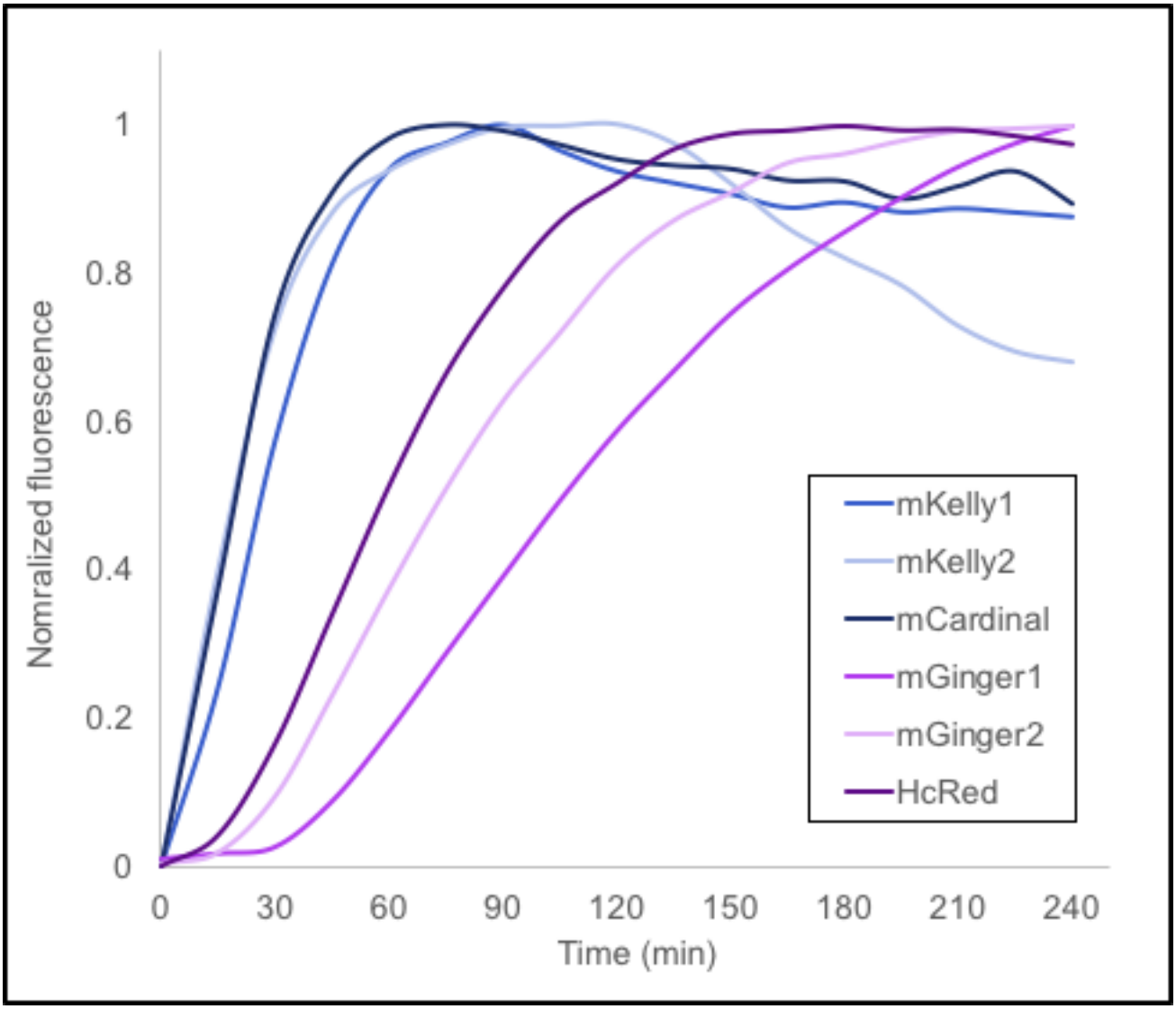

Figure S8 – Maturation kinetics of the monomeric fluorescent proteins mGinger1, mGinger2, mKelly1, and mKelly2, along with the dimeric proteins they were derived from.

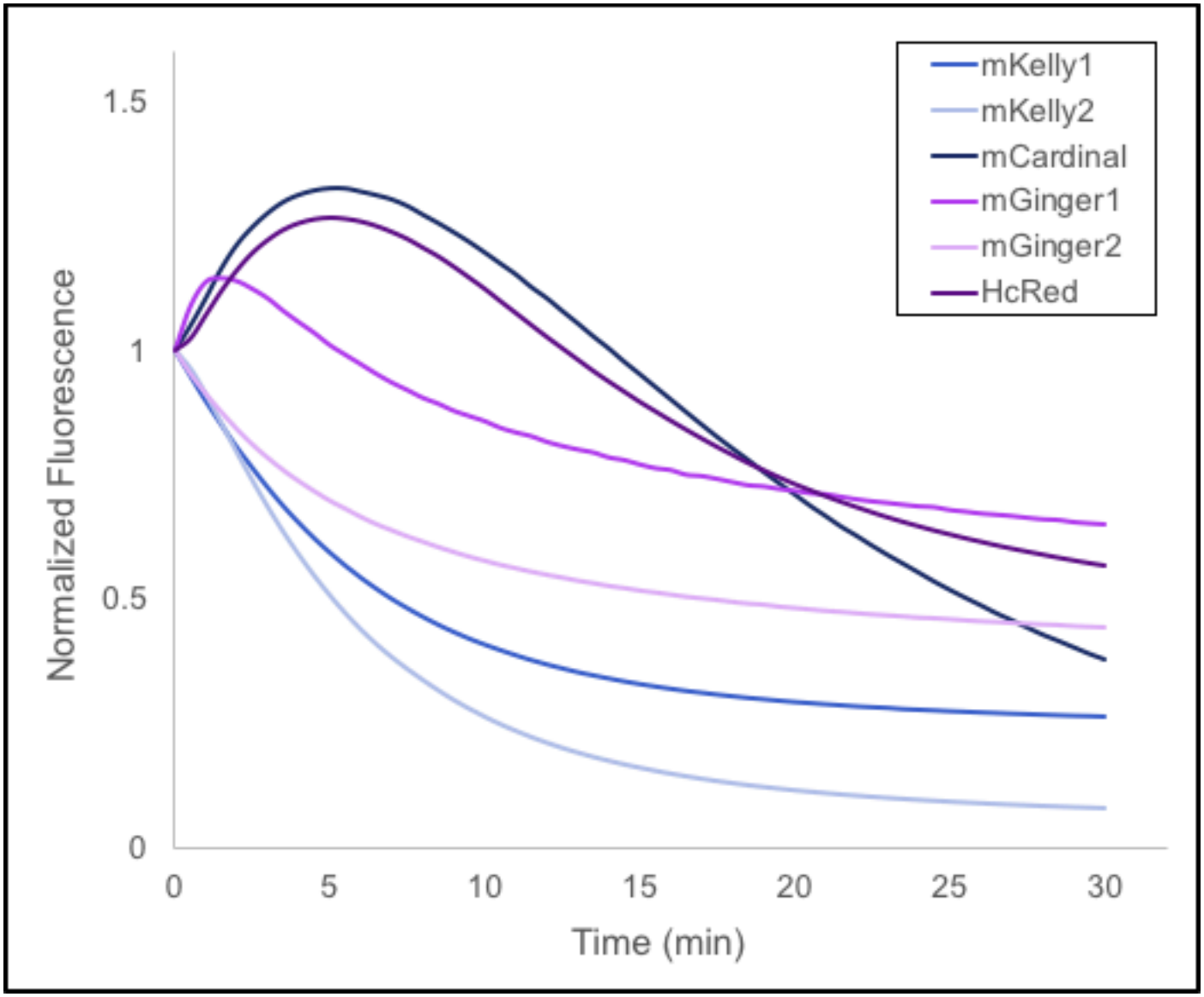

Figure S9 – Photobleaching kinetics of the monomeric fluorescent proteins mGinger1, mGinger2, mKelly1, and mKelly2, along with the dimeric proteins they were derived from.

Supplemental Tables:

Table S1. Far-red RFP alignment and the core libraries queried in HcRed.

	Library A - Chromophore phenolate pocket									A / B	Library B - Above chromophore, near structural waters + chromophore backing positions 28 and 41								
Position (DsRed)	*63*	*95*	*143*	*159*	*161*	*163*	*177*	*179*	*199*	*146*	*70*	*71*	*83*	*148*	*181*	*195*	*197*	*215*	*217*
Position (hcCP)	*60*	*92*	*140*	*156*	*158*	*160*	*173*	*175*	*198*	*143*	*67*	*68*	*80*	*145*	*177*	*194*	*196*	*213*	*215*
hcCP	**P**	**R**	**W**	**G**	**N**	**M**	**L**	**T**	**L**	**C**	**R**	**T**	**F**	**E**	**Y**	**T**	**I**	**E**	**S**
HcRed	--	--	--	--	--	--	H	--	--	S	--	--	--	--	--	--	--	--	--
HcRed7	--	--	--	--	--	--	H	--	--	S	K	--	--	--	--	--	Y	--	--
aeCP597	**P**	**R**	**W**	**G**	**S**	**M**	**L**	**T**	**I**	**C**	**K**	**T**	**F**	**E**	**Y**	**G**	**H**	**E**	**A**
AQ143	--	--	--	--	A	--	--	--	--	S	--	--	--	--	--	--	--	--	--
#20CP	**P**	**R**	**W**	**G**	**N**	**M**	**F**	**S**	**L**	**N**	**I**	**P**	**V**	**E**	**Y**	**V**	**R**	**E**	**S**
mKeima*	--	--	--	--	D	--	--	--	--	S	--	--	F	--	--	I	--	--	A
anmCP	**H**	**R**	**I**	**Q**	**C**	**I**	**F**	**L**	**I**	**T**	**P**	**F**	**A**	**T**	**F**	**V**	**V**	**E**	**T**
J-Red	--	--	--	--	A	--	--	M	T	N	--	--	--	--	--	--	I	--	--
DsRed	**P**	**R**	**W**	**G**	**I**	**K**	**F**	**S**	**L**	**S**	**K**	**V**	**K**	**E**	**Y**	**V**	**S**	**E**	**T**
DsRed.M1*	--	--	--	--	--	H	--	T	--	--	--	A	M	--	--	--	--	--	A
mRFP1*	--	--	--	--	--	M	V	T	--	--	--	A	L	--	--	T	I	--	A
mCherry*	--	--	--	--	--	Q	V	T	--	--	--	A	L	--	--	--	I	--	A
mGrape3*	--	--	--	--	V	M	V	T	--	--	--	A	M	--	--	L	Y	--	A
mPlum*	--	--	--	--	M	M	V	T	--	--	--	A	L	--	--	T	I	--	A
mRaspberry*	--	--	--	--	M	M	V	T	--	--	--	G	L	--	--	T	I	--	A
E2-Crimson	--	--	--	--	N	M	--	S	--	--	--	A	L	--	--	--	Y	--	A
mRojoA*	--	--	--	--	I	L	V	T	--	--	--	A	L	--	--	A	Y	--	C
mRouge*	--	--	--	--	M	M	V	T	--	--	--	A	L	--	--	T	T	--	N
eqFP611	**T**	**R**	**W**	**G**	**S**	**M**	**F**	**T**	**L**	**N**	**K**	**T**	**F**	**E**	**Y**	**V**	**H**	**E**	**A**
mRuby*	--	--	--	--	T	--	--	--	--	--	R	--	--	--	--	--	--	--	--
mRuby2*	--	--	--	--	T	--	--	--	--	--	R	--	--	--	--	--	--	--	--
RFP637	--	--	--	--	A	--	--	--	--	S	--	--	--	--	--	--	--	--	--
RFP639	--	--	--	--	C	--	--	--	--	S	--	--	--	--	--	--	--	--	--
eqFP578	**T**	**R**	**W**	**G**	**S**	**M**	**F**	**T**	**L**	**N**	**K**	**T**	**F**	**E**	**Y**	**V**	**H**	**E**	**A**
FusionRed*	--	--	--	--	C	--	L	--	--	S	R	--	--	--	--	--	--	--	--
TagRFP	--	--	--	--	--	--	--	--	--	--	R	--	--	--	--	--	--	--	--
Katushka	--	--	--	--	--	--	L	--	--	S	--	--	--	--	--	--	R	--	--
mKate	--	--	--	--	--	--	L	--	--	S	--	--	--	--	--	--	R	--	--
mKate2	--	--	--	--	A	--	L	--	--	S	--	--	--	--	--	--	R	--	--
Neptune	--	--	--	--	C	--	L	--	--	S	--	--	--	--	--	--	R	--	--
mNeptune	--	--	--	--	C	--	L	--	--	S	--	--	--	--	--	--	R	--	--
mNeptune2.5	--	--	--	--	C	--	L	--	--	S	--	--	--	--	--	--	R	--	--
mCardinal	--	--	--	--	C	--	L	--	--	T	--	--	--	--	--	--	R	--	--
TagRFP657	--	--	--	--	T	L	--	--	--	H	H	--	--	--	--	--	Y	--	--
eqFP650	--	--	--	--	S	--	L	--	--	S	--	--	--	--	--	--	R	--	--
eqFP670	--	--	--	--	N	--	L	--	--	--	--	--	--	--	--	--	R	--	--
Residues allowed in Library					***I***	***H***	***L***	***S***		***N***	***K***	***A***	***L***				***H***		***A***
					A	***L***	***F***				***H***	***P***	***M***				***R***		
					C	***Q***	***V***										***Y***		
					S														
					T														
					M														
	1	***1***	***1***		***7***	***4***	***4***	***2***	***1***	***2***	***3***	***3***	***3***	***1***	***1***	***1***	***4***	***1***	***2***
Library Size	***448***										***432***								

* = True Monomer

Table S2. Consensus design from an alignment of 741 fluorescent proteins. Shown are positions that are relevant to mCardinal or HcRed design. Highlighted residues are positions where mutations from the wild-type parent are present, and Score indicates the degree to which the residue is conserved in the alignment.

Alignment Position	Consensus	Score	HcRed numbering	HcRed Residue	mCardinal Numbering	mCardinal Residue
59	G	3.784211	28	G	32	T
110	P	3.602255	73	T	77	T
52	G	3.257101	21	G	25	N
265	G	2.812273	167	R	171	G
109	Y	2.531613	72	H	76	H
144	F	2.325096	96	Y	100	Y
306	H	2.20648	192	H	197	**Y**
136	Y	2.029933	88	F	92	F
86	G	1.931355	49	A	53	G
91	S	1.749646	54	A	58	A
234	P	1.543969	142	P	146	A
220	D	1.385952	129	D	133	N
236	S	1.363104	143	**S**	147	**T**
268	G	1.207594	167	-	172	G
99	F	1.200324	62	C	66	F
260	L	1.183173	164	V	168	L
176	G	1.154547	112	G	116	**D**
270	Y	1.125099	169	L	174	L
57	I	1.113139	26	C	30	C
330	L	1.089431	211	L	217	Q
226	K	1.065694	135	N	139	K
76	L	1.048276	43	I	47	I
108	K	1.028747	71	H	75	N
352	L	1.027895	222	L	228	L
180	F	1.021909	115	L	119	**L**
262	D	0.995714	166	D	170	G
118	Y	0.988199	79	F	83	**F**
165	I	0.97184	108	T	112	T
247	D	0.96563	151	N	155	D
328	V	0.899642	209	F	215	V
310	H	0.866656	196	I	201	**R**
225	Q	0.865105	134	K	138	Q
308	V	0.856601	194	T	199	V
228	T	0.837791	137	S	141	T
212	F	0.835726	121	V	125	**L**
97	P	0.834999	60	P	64	T
112	G	0.788785	75	E	79	G
292	V	0.786413	183	V	188	A
104	R	0.765227	67	R	71	K
276	F	0.755987	173	**H**	178	**L**
282	K	0.749343	178	R	183	R
239	K	0.744543	146	V	150	**T**
301	L	0.736413	188	M	193	M
291	P	0.731317	182	A	187	P
334	A	0.725708	215	S	221	A
313	E	0.723365	199	Q	204	E
67	E	0.715552	36	**A**	40	E
175	E	0.714952	111	E	115	Q
166	T	0.687786	109	S	113	S
137	S	0.683989	89	T	93	T

64	N	0.673053	33	N	37	K
161	A	0.664725	104	A	108	**V**
103	N	0.65018	66	S	70	S
312	I	0.629169	198	L	203	L
84	T	0.628706	47	E	51	E
141	S	0.618213	93	T	97	**V**
37	P	0.608886	10	R	14	H
32	I	0.601155	5	L	9	I
179	C	0.593207	114	C	118	C
240	M	0.591409	147	V	151	L
158	V	0.574808	101	I	105	V
321	D	0.57157	205	K	211	**N**
44	L	0.566795	13	M	17	L
294	A	0.565383	185	A	190	N
159	C	0.563901	102	L	106	L
215	V	0.546451	124	T	128	V
286	A	0.546279	179	S	184	S
219	A	0.542286	128	A	132	S
280	T	0.535297	176	S	181	T
142	M	0.534053	94	T	98	T
107	T	0.519627	70	V	74	I
79	K	0.509817	46	T	50	V
54	E	0.506783	23	Y	27	H
105	V	0.497836	68	T	72	T
177	D	0.496069	113	N	117	G
251	K	0.49169	155	C	159	**E**
249	V	0.489803	153	V	157	G
45	H	0.482752	14	Y	18	Y
184	E	0.479977	118	K	122	N
96	S	0.459784	59	A	63	A
343	S	0.452457	220	S	226	C
305	Y	0.444745	191	F	196	**V**
254	V	0.441988	158	N	162	**C**
273	R	0.43852	170	I	175	H
49	S	0.435338	18	T	22	T
38	L	0.425599	11	I	15	M
111	D	0.403056	74	A	78	Q
353	P	0.399819	223	P	229	P
66	Y	0.390898	35	F	39	Y
298	K	0.388511	187	T	192	K
253	D	0.385118	157	R	161	**R**
13	S	0.380175	2	A	1	-
261	E	0.380001	165	G	169	V
326	T	0.375741	207	E	213	T
74	M	0.375667	41	M	45	**Q**
364	A	0.368916	226	A	232	L
100	G	0.361518	63	E	67	M
77	K	0.360493	44	H	48	K
365	K	0.352152	227	N	233	G
320	K	0.348154	204	K	210	D
58	E	0.340323	27	E	31	T
316	K	0.340278	202	R	207	K
275	D	0.33596	172	H	177	**N**
246	R	0.332927	150	E	154	A
75	K	0.32791	42	R	46	**R**
62	K	0.315448	31	D	35	E

337	F	0.313271	218	R	224	**R**
335	K	0.292243	216	V	222	V
314	I	0.289447	200	M	205	R
56	D	0.28287	25	K	29	K
327	H	0.28026	208	Y	214	Y
317	D	0.279842	202	-	208	E
72	T	0.279091	40	S	44	T
229	L	0.277339	138	G	142	L
325	K	0.275445	206	D	212	E
304	P	0.264224	190	G	195	G
259	L	0.26367	163	K	167	K
319	D	0.262327	203	K	209	A
162	T	0.244603	105	H	109	T
315	T	0.231042	201	**L**	206	I
34	K	0.227888	7	E	11	E
163	N	0.220261	106	Q	110	Q
98	V	0.219453	61	C	65	**C**
363	K	0.21031	225	K	231	K
16	K	0.198648	3	-	4	K
293	A	0.184208	184	R	189	K
278	K	0.183258	174	Y	179	K
15	S	0.174135	3	-	3	S
255	N	0.170664	159	V	163	**D**
362	K	0.150924	224	E	230	S
333	H	0.144189	214	A	220	**V**
213	H	0.138919	122	**H**	126	**R**
35	D	0.13887	8	S	12	N
14	V	0.13754	3	G	2	**V**
295	D	0.127611	186	L	191	L
344	D	0.12689	221	D	227	D
18	L	0.123553	3	-	6	E
331	V	0.10229	212	Y	218	H
338	Y	0.079708	219	Y	225	Y
17	E	0.075634	3	-	5	G
369	I	0.05317	227	-	237	N
367	L	0.040043	227	-	235	K
370	G	0.037604	227	-	238	G
366	K	0.035173	227	-	234	H
373	M	0.017878	227	-	241	E
19	N	0.012455	3	-	7	**E**
371	G	0.012455	227	-	239	M
372	H	0.012455	227	-	240	D
368	S	0.008044	227	-	236	L

Table S3. Specification of the mCardinal monomerization library:

eqFP578 amino acid position	Amino acid present in mCardinal-mut6Δ19	Reason for inclusion	Allowed amino acid residues
21	N	top consensus	WT + G
28	A	top consensus	WT + G
54	A	top consensus	WT + S
72	H	top consensus	WT + Y
73	S	top consensus	WT + P
88	F	top consensus	WT + Y
96	Y	top consensus	WT + F
142	A	top consensus	WT + P
146	T	monomerization (CORE)	WT + R,Y
148	Y	monomerization (FRINGE)	WT + V
155	E	monomerization (FRINGE)	WT + V
157	R	monomerization (FRINGE)	WT + T
159	D	monomerization (CORE)	WT + T,K
171	H	monomerization (CORE)	WT + T,K
192	V	monomerization (CORE)	T,R,E,K
193	Y	top consensus	WT + H
194	F	monomerization (CORE)	Q,K,E,S
196	D	monomerization (FRINGE)	WT + T
198	R	monomerization (FRINGE)	WT + K
216	V	monomerization (FRINGE)	WT + R
218	V	monomerization (FRINGE)	WT + Y
220	R	monomerization (FRINGE)	WT + K

Table S4. Proteins included in Figure 4

	M/D/T	Tm	Φ
DsRmCh	T	98.5	0.24
DsRed	T	94.5	0.84
mCardinal	D	74	0.12
HcRed	D	69	0.05
HcRed7	D	75	0.08
HcRed7Δ5	D	70.5	0.06
HcRed77	D	67.5	0.05
HcRedm1	M	64	0.01
HcRedm13	M	65	0.03
HcRedm14	M	58	0.02
chimeraC	M	70.5	0.02
mGinger0.1	M	79	0.02
mGinger0.2	M	80	0.04
mRaspberry	M	91	0.14
mCherry	M	89.5	0.21
mPlum E16P	M	82	0.12
FusionRed	M	90	0.22

Table S5. Proteins included in Figure 5 (in addition to mKellys and mGingers)

	λ_{em}	Φ	ε	Brightness
mScarlet	594	0.70	100,000	70.0
pHTomato	580	0.68	70,500	47.9
mRuby2	600	0.38	113,000	42.9
TagRFP	584	0.41	98,000	40.2
mRuby	605	0.35	112,000	39.2
mApple	592	0.49	75,000	36.8
TagRFP-T	584	0.41	81,000	33.2
mStrawberry	596	0.29	90,000	26.1
mNectarine	578	0.45	58,000	26.1
PA-TagRFP	595	0.36	66,000	23.8
FusionRed	608	0.19	94,500	18.0
mCherry2 M66C/Q213L	580	0.40	44,000	17.6
mBeRFP	611	0.27	65,000	17.6
mCherry	610	0.22	72,000	15.8
PAmCherry2	596	0.53	24,000	12.7
mRFP1	607	0.25	50,000	12.5
mRaspberry	625	0.15	79,000	11.9
mTangerine	585	0.30	38,000	11.4
mGarnet2	671	0.09	105,000	9.1
mGarnet	670	0.09	95,000	8.6
PAmCherry1	595	0.46	18,000	8.3
mRFPmars	602	0.25	32,600	8.2
TTN.A3	619	0.13	56,000	7.3
AYC.A3	621	0.08	63,000	5.0
PAmCherry3	596	0.24	21,000	5.0
TTN.A9	623	0.11	39,000	4.3
rsTagRFP	585	0.11	36,800	4.0
mRojoB	631	0.06	61,000	3.7
AYC.A6	625	0.06	61,000	3.7
DsRed.M1	586	0.10	35,000	3.5
mKeima	620	0.24	13,400	3.2
mPlum	649	0.10	22,000	2.2
mGrape1	625	0.03	50,000	1.5
mGrape2	636	0.03	33,000	1.0
mRojoA	633	0.02	48,000	1.0
mRouge	637	0.02	43,000	0.9
TTN.H11	628	0.03	28,000	0.8
mGrape3	646	0.02	41,000	0.8
PA-mRFP1	605	0.08	10,000	0.8
rsCherryRev	608	0.01	42,300	0.2

Table S6. X-ray data reduction and crystallographic refinement statistics for HcRed7 (PDB-ID: 6DEJ)

(A) X-ray data reduction statistics	
Space group	P1 21 1
Unit cell dimensions (*a, b, c*)	54.3 Å, 122.1 Å, 108.8Å
Resolution	39.3 Å – 1.628 Å
(last shell)	1.72 Å – 1.628 Å
Total measurements (last shell)	761,418 (83,674)
Number of unique reflections (last shell)	111,329 (13,784)
Wavelength	
R-merge (last shell)	0.093 (1.554)
I/σ(I) (last shell)	11.6 (1.1)
Completeness (last shell)	0.959 (0.734)
Multiplicity (last shell)	6.8 (6.1)
(B) Crystallographic refinement statistics	
Resolution	33.69 Å – 1.628 Å
(last shell)	1.646 Å – 1.628 Å
No. of reflections (working set)	111,112
No. of reflections (test set)	5,487
R-factor (last shell)	0.178 (0.407)
R-free (last shell)	0.214 (0.419)
No. of amino acid residues	893
No. of atoms	7,154
No. of solvent molecules	655
Average *B*-factor	
Protein	30.57 $Å^2$
Solvent	44.05 $Å^2$
R.m.s.d. from ideal geometry	
Bond lengths	0.007 Å
Bond angles	1.052°