

Improved mutants of *Renilla* luciferase for imaging applications in living subjects

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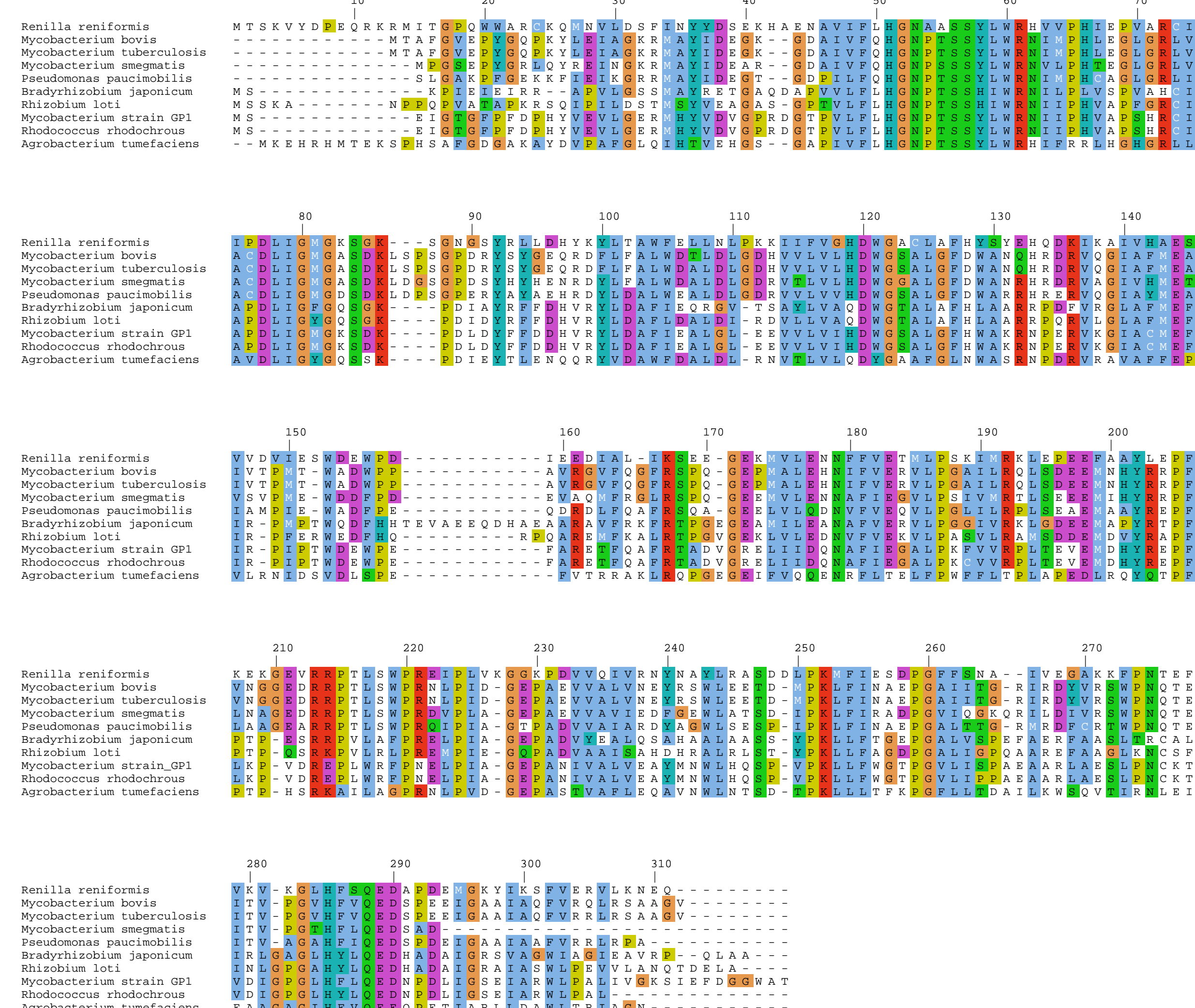
INTRODUCTION

The optical signal generated by *Renilla* Luciferase (RLuc) allows its use as a biomolecular label as well as a reporter gene. Several projects are attempting to make use of RLuc as a label by constructing fusion proteins of RLuc with engineered antibodies [1, 2] or receptor ligands [3, 4]. These RLuc labeled proteins can then be used for studying receptor targets with *in vitro* assays and *in vivo* imaging.

Use of RLuc as a label, however, requires high stability and activity in a variety of environments, including mammalian serum. To achieve this goal, we have characterized mutants of RLuc with respect to serum stability and light output, and have used the results to develop an 8 mutation form of RLuc (RLuc8). RLuc8 shows markedly improved characteristics for use as a bioluminescent label, and also shows increased light output when used as a reporter gene in mammalian cells.

ALIGNMENT

Using PredictProtein, RLuc was found to have high homology (34-56% similar) to a number of bacterial haloalkane dehalogenases; this may indicate a horizontal gene transfer event. An alignment generated using CLUSTAL W is shown below, note that the numbering is with respect to the RLuc sequence.

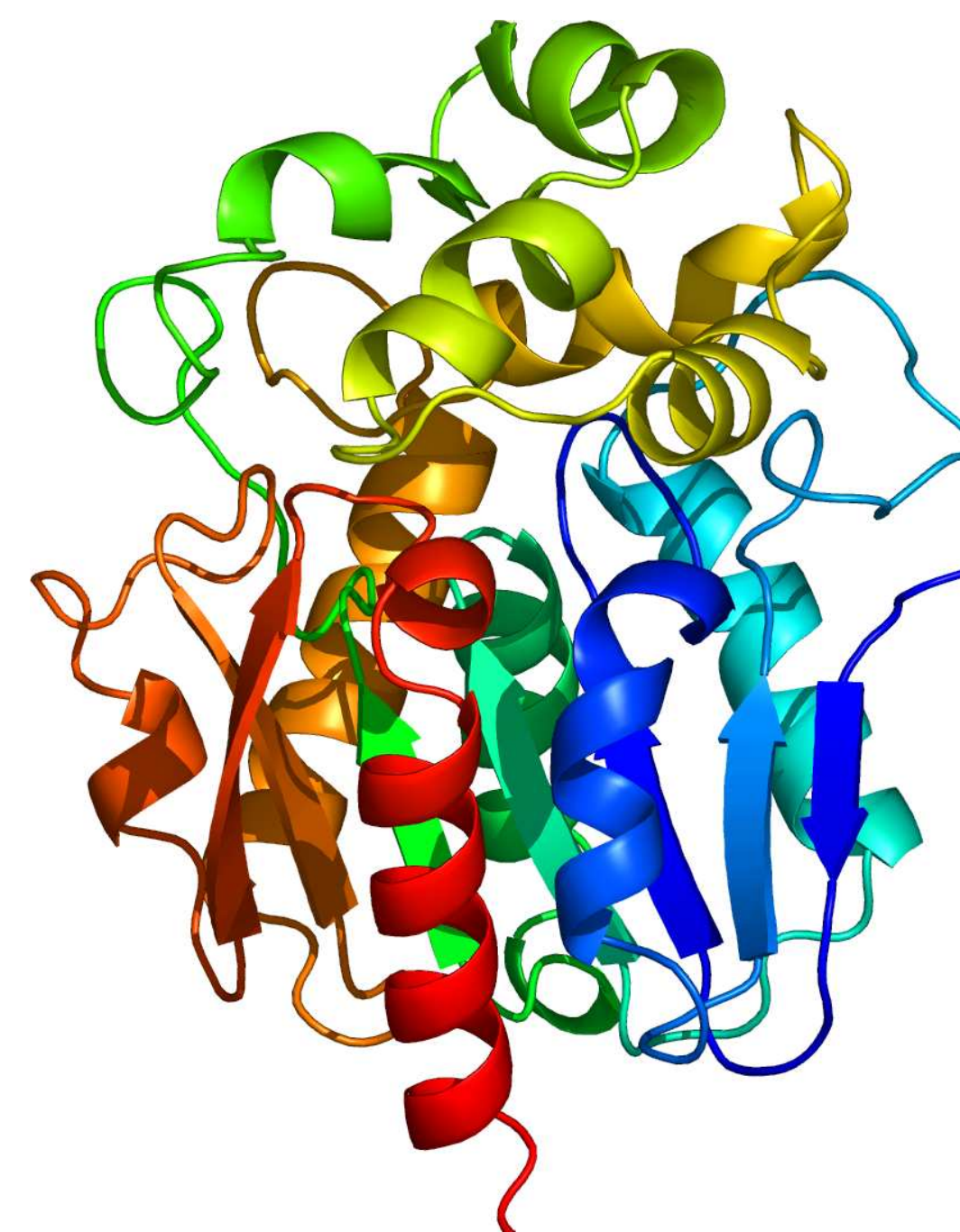


From the aligned sequences, candidate mutations were chosen at positions where RLuc clearly diverged from the consensus. This strategy was predicated upon the hypothesis that, following a horizontal gene transfer, mutations would be allowed to accumulate in the RLuc sequence that are neutral in the context of *Renilla* cytoplasm but could be deleterious in the more varied environments that a bacterial enzyme encounters.

Previous work in the literature performed targeted cysteine mutagenesis with the idea of avoiding inappropriate disulfide bond formation in a secreted version of RLuc [5]. That work showed that a C124A mutation (C152A in [5]) stabilizes RLuc, and that C73 (C101 in [5]) is required for activity. In light of the alignment data, C73 can be seen to be absolutely conserved across the enzymes, while C124 differs from the bacteria consensus.

HOMOLOGY MODEL

As no crystal structure of RLuc exists, a homology model of RLuc was constructed so that the location of mutational candidates could be identified. This model was built using SWISS-MODEL (v3.5) and crystal structures of the haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* (PDB files 1iz8, 1k63, 1k6e, 1iz7, and 1mj5). In the model below, the N-terminus is blue and the C-terminus is red. Based on data from the dehalogenases, the putative active site of RLuc is at the intersection of the red, orange, and yellow helices.

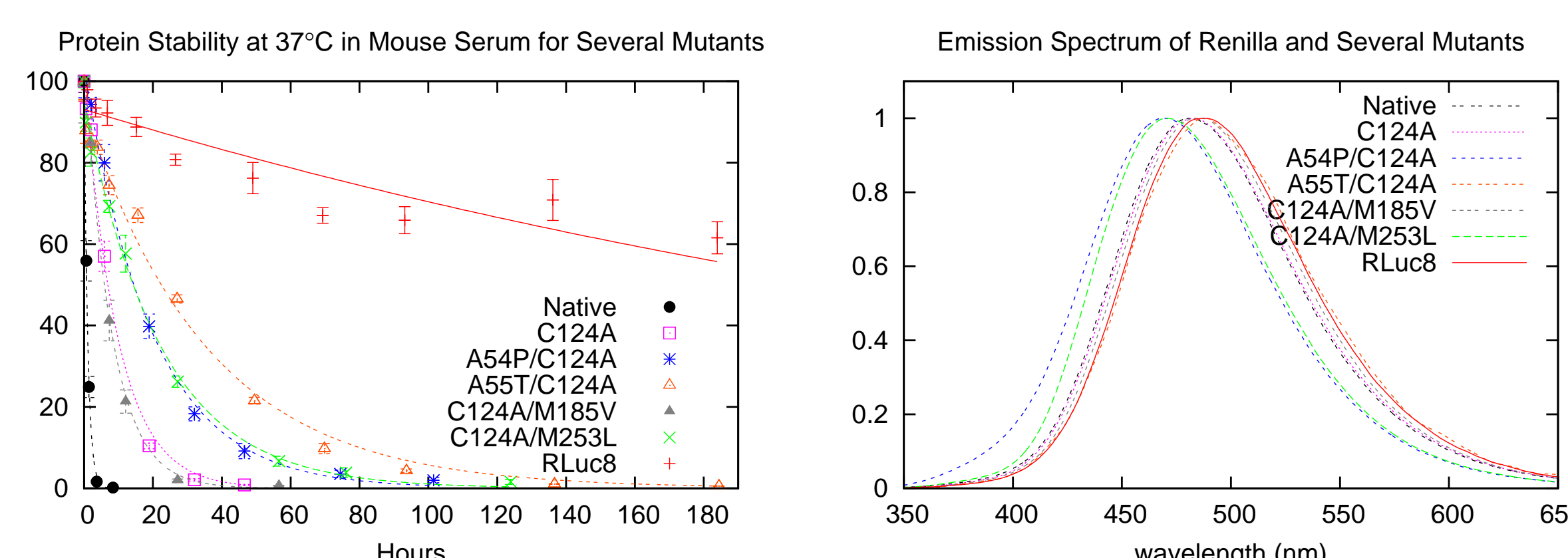


RLUC STABILIZATION

A human codon optimized version of rLuc (Promega) was cloned into the pBAD expression system which adds a Myc epitope and a 6xHis tag to the C-terminus of the construct. As part of this cloning, a pelB leader sequence was added to the N-terminus of RLuc in order to direct protein expression into the bacterial periplasm. Mutations were then introduced by site-directed mutagenesis.

For protein expression, *E. coli* LMG 194 cells were induced in log phase and grown for 12-14 hours at 32°C. The periplasmic fraction was then extracted by osmotic shock [6] and the His-tagged protein purified by nickel affinity chromatography. Following determination of protein concentration using the Bradford assay, human serum albumin was added to 1% as a carrier protein and the samples were stored at 4°C.

Luciferase activity was measured by adding 1 µl of sample (diluted as necessary) to 100 µl room temperature 100 mM sodium phosphate buffer (pH 7), adding 0.5 µg substrate, and reading for 10 seconds in a Turner 20/20 luminometer. Substrates tested were coelenterazine (Native), benzyl-coelenterazine (h), coelenterazine-cp (cp), coelenterazine-n (n), and coelenterazine-400a/DeepBlueC (400a). Serum stability measurements were done by mixing 0.5 µl dilute luciferase with either 20 µl mouse serum or 50 µl rat serum, placing in a 37°C incubator, and removing aliquots for activity testing. Emission spectra at ambient temperature were measured using a Triax 320 (Horiba Jobin Yvon, Edison, NJ).



Mutation	Specific Activity (photons/sec/mole enzyme)					Serum $\tau_{1/2}$ (h)		Wavelength (nm)	
	native	h	cp	n	400a	mouse	rat	peak	mean
Native RLuc	$6.7 \pm 0.5 \times 10^{22}$	1.1×10^{23}	3.5×10^{22}	1.7×10^{22}	1.2×10^{20}	0.9	0.4	482	497
C124A	1.2 ± 0.1	0.75	0.79	0.63	0.68	7.1 ± 0.4	6.6 ± 0.5	482	498
F33R/A4M/C124A	0.15	0.15	0.16	0.12	0.20	0.3	0.3	481	497
E44G/C124A	0.94	0.78	0.74	0.66	0.98	2.6	3.3	486	502
A54G/A55G/C124A	0.12	0.10	0.06	0.15	0.19	2.4	3.0	476	492
A54P/A55T/C124A	0.21	0.15	0.11	0.38	0.22	119	129	470	483
A54P/C124A	0.05	0.04	0.05	0.08	0.06	14	13	468	482
A55T/C124A	1.7	1.2	0.58	1.4	2.4	30	29	486	504
F116L/C124A	1.3	1.0	1.3	0.88	1.8	11	9.4	486	502
C124A/S130A	1.7	1.4	1.7	1.4	2.6	18	14	482	498
C124A/K136R	2.5 ± 0.3	2.1	1.9	1.9	2.6	12	11	482	498
C124A/A143M	1.7	1.3	0.95	1.5	1.6	30	29	480	497
C124A/F180A	0.02	0.01	0.03	0.01	0.01	1.6	1.6	488	504
C124A/M185V	3.4	3.0	15	7.8	44	5.7	3.7	485	500
C124A/M191L	1.1	0.99	0.97	1.0	1.2	6.5	5.1	480	496
C124A/E195S/P196D	0.12	0.10	0.12	0.10	0.15	1.0	0.7	482	498
C124A/F199M	0.58	0.44	0.53	0.49	0.46	6.7	6.0	480	495
C124A/L203R	0.55	0.55	0.52	0.41	0.43	2.7	2.2	484	501
C124A/G229E	0.02	0.01	0.03	0.03	0.01	1.9	1.8	473	490
C124A/Q235A	1.2	1.1	1.1	1.0	1.2	3.3	3.6	473	489
C124A/M253L	1.9	1.4	1.6	1.6	1.7	15	10	471	488
C124A/S257G	1.1	0.95	1.3	1.1	3.0	1.3	1.4	477	493
C124A/F261L/F262L	0.00	0.00	0.00	0.00	0.00	N/A	N/A	N/A	N/A
C124A/F262L	0.03	0.03	0.01	0.06	0.03	5.8	6.4	478	495
C124A/S287L	3.9	2.8	3.4	5.0	9.5	28	20	478	496
C124A/M295I	1.0	0.83	0.57	0.72	0.86	5.0	4.9	480	497
C124A/K300A	1.1	1.0	1.1	1.0	1.3	3.5	3.9	481	497

The 7 mutations with the most favorable increases in specific activity and/or stability were combined along with the C124A mutation into a single protein (RLuc8). The 8 mutations present in RLuc8 are A55T, C124A, S130A, K136R, A143M, M185V, M253L, and S287L. Since the Myc epitope encoded by the expression plasmid was removed during the cloning of RLuc8, an equivalent C124A construct was made in order to facilitate a valid comparison.

Mutation	Specific Activity (relative to RLuc)					Serum $\tau_{1/2}$ (h)		Wavelength (nm)	
	native	h	cp	n	400a	mouse	rat	peak	mean
C124A-ΔMyc	1.3 ± 0.1	0.91	1.1	0.87	1.0	4.0	4.5	481	499
RLuc8	4.3 ± 0.2	3.0	5.8	8.8	59	197	94	487	503

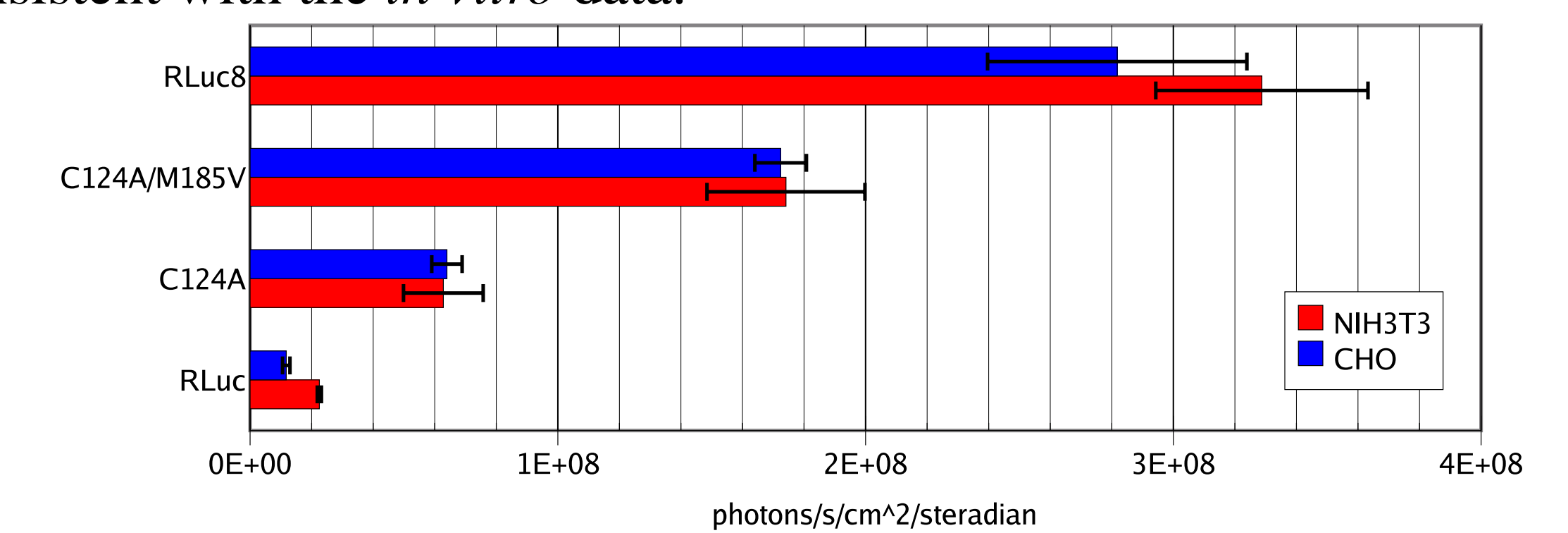
ACTIVE SITE RESIDUES

The haloalkane dehalogenases contain a conserved aspartate, glutamate, and histidine triad in their enzymatic core. Based on the alignment data, the equivalent triad in *Renilla* (D120, E144, and H285) were mutated, and shown to be critical for luciferase activity.

	Specific Activity (relative to RLuc)					Serum $\tau_{1/2}$ (h)	
	native	h	cp	n	400a	mouse	rat
RLuc8	4.3 ± 0.2	3.0	5.8	8.8	59	197	94
RLuc8-D120A	0.000	0.001	0.001	0.003	0.21	> 100	> 100
RLuc8-D120N	0.023	0.016	0.050	0.34	5.1	> 100	> 100
RLuc8-E144A	0.000	0.000	0.000	0.000	0.000	57	13
RLuc8-E144Q	0.000	0.000	0.000	0.000	0.002	> 100	> 100
RLuc8-H285A	0.023	0.020	0.046	0.028	0.20	> 100	21

MAMMALIAN REPORTER GENE

To demonstrate the applicability of these results in the context of a mammalian reporter gene, expression vectors were constructed for RLuc, C124A, C124A/M185V, and RLuc8 in a pcDNA 3.1 backbone, with the gene under the control of a constitutive promoter (CMV). These mammalian expression plasmids were then transiently transfected into 293T or CHO cells. The results 24 hours post transfection are shown below with the bars representing the standard error of the mean. For a given cell line, all differences between mutants were significant at $p \leq 0.06$ using a two-tailed *t*-test. The increased light output for the mutants was consistent with the *in vitro* data.



RLUC DESTABILIZATION

In many cases where a reporter gene is employed, the goal is to follow the dynamics of gene